

00-048963



Organización CSA Ltda

# Industria y Comercio

SUPERINTENDENCIA

Delegatura de propiedad Industrial

División de Nuevas Creaciones

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

INVENTARIO

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SOLICITUD

## PATENTE DE INVENCION

21. EXPEDIENTE No.

00-48963

54. TÍTULO

DERIVADOS DE ERITROPORETINA

51. CLASIFICACIÓN INTERNACIONAL

A61K 38/62, 35/12

71. SOLICITANTE

F. HOFFMANN - LA ROCHE A.G.

DOMICILIO

BASICA, SUR

74. APODERADO

JOSE ANTONIO LOREDA

T.P. #10.784  
Código 231

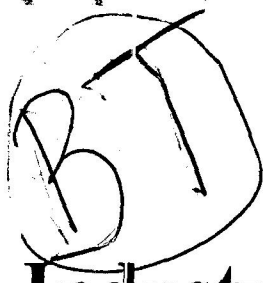
22. BOGOTÁ, D. C.,

29 JUN 2000

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**Industria y Comercio**

**SUPERINTENDENCIA**

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**SOLICITUD**

**PATENTE DE INVENCION**

**00-48963**

1. EXPEDIENTE No. \_\_\_\_\_

4. TITULO "DERIVADOS DE ERITROPOYETINA." \_\_\_\_\_

51. CLASIFICACION INTERNACIONAL A61K<sup>38</sup>/02, 35/12.

71. SOLICITANTE F. HOFFMANN-LA ROCHE A.G.

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T.P. #10.784  
CODIGO 231

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63



**DATOS DEL SOLICITANTE**

(71) Nombres y Apellidos o Razón Social F.HOFFMANN-LA ROCHE AG. sociedad existente y organizada de conformidad con las leyes de Suiza

No. de Identificación \_\_\_\_\_

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**DATOS DE LA PATENTE DE INVENCION**

(54) Título de la Invención " DERIVADOS DE ERITROPOYETINA "

\_\_\_\_\_

\_\_\_\_\_

PRIORIDAD REIVINDICADA SI ☒ NO ☐ TIPO DE PRIORIDAD ANDINA ☐ UNIONISTA ☒

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**- 2 ENE. 2001**

CLASIFICACION INTERNACIONAL

A.61k<sup>38</sup>/02

## RESUMEN CON EL OBJETO Y LA FINALIDAD DE LA INVENCION

La presente invención se refiere a conjugados de eritropoyetina con poli(etilen glicol) comprendiendo una glucoproteína eritropoyetina que presenta al menos un grupo amino libre y que presenta la actividad biológica in vivo que hace que las células de la médula ósea incrementen la producción de reticulocitos y glóbulos rojos y que es seleccionada del grupo que consiste en eritropoyetina humana y análogos de la misma que tienen la secuencia de la eritropoyetina humana modificada mediante la adición de entre 1 y 6 sitios de glicosilación o mediante una nueva disposición de al menos un sitio de glicosilación; dicha glucoproteína estando covalentemente unida a "n" grupos de poli(etilenglicol) de la fórmula  $-CO-(CH_2)_x-(OCH_2CH_2)_m-OR$  con el carbonilo de cada grupo de poli(etilenglicol) formando un enlace amida con uno de dichos grupos amino; en donde R es alquilo inferior; x es 2 ó 3; m se encuentra entre alrededor de 450 y alrededor de 900; n se encuentra entre 1 y 3; y n y m son elegidos de forma que el peso molecular del conjugado menos la glucoproteína eritropoyetina se encuentre entre 20 kilodaltons y 100 kilodaltons.

## ANEXOS DE LA SOLICITUD

|                                                                                                                                                   |                                     |
|---------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------|
| Certificado de Existencia y Representación Legal cuando el solicitante sea Persona Jurídica                                                       | <input type="checkbox"/>            |
| Los poderes debidamente otorgados, o mención de su protocolización ante la S.I.C. 15.256                                                          | <input checked="" type="checkbox"/> |
| Recibo de la tasa respectiva \$ 608.000,00                                                                                                        | <input checked="" type="checkbox"/> |
| Recibo de la tasa por concepto de excedente de palabras en la publicación                                                                         | <input type="checkbox"/>            |
| Si se reivindica prioridad Andina:                                                                                                                |                                     |
| Copia dela primera solicitud                                                                                                                      | <input type="checkbox"/>            |
| Traducción oficial                                                                                                                                | <input type="checkbox"/>            |
| Si se reivindica prioridad Unionista: <b>SI</b>                                                                                                   | <input checked="" type="checkbox"/> |
| Copia certificada de la primera solicitud:                                                                                                        |                                     |
| US 60/142.254 DE FECHA 02-07-99                                                                                                                   |                                     |
| US 60/150.225 DE FECHA 23-08-99                                                                                                                   |                                     |
| US 60/151/548 DE FECHA 31-08-99 Y                                                                                                                 |                                     |
| US 60/166.151 DE FECHA 17-11-99                                                                                                                   |                                     |
| Nota: Debe presentarse dentro de los tres (3) meses, contados a partir de la fecha de radicación de la solicitud posterior                        |                                     |
| Traducción Simple de EDUARDO CALADO NOGUERA Nos. 0272 de Fecha 15-06-00; 0271 de fecha 14-06-00; 0273 de fecha 14-06-00 y 0269 de fecha 14-06-00. | <input checked="" type="checkbox"/> |
| Capítulo descriptivo                                                                                                                              | <input checked="" type="checkbox"/> |
| Dibujos, planos necesarios                                                                                                                        | <input type="checkbox"/>            |
| Capítulo reivindicatorio                                                                                                                          | <input checked="" type="checkbox"/> |
| Tarjeta archivo propietarios según formato                                                                                                        | <input checked="" type="checkbox"/> |
| Tarjeta archivo temático según formato                                                                                                            | <input checked="" type="checkbox"/> |
| Extracto para publicación según formato                                                                                                           | <input checked="" type="checkbox"/> |
| Arte final 12 x 12 cms. Y 6x6 cms                                                                                                                 | <input type="checkbox"/>            |
| Otros Documentos                                                                                                                                  | <input type="checkbox"/>            |
| Nota: Anexo información sobre la fuente normativa de reciprocidad y petición Reconocimiento Personería                                            | <input checked="" type="checkbox"/> |

**SOLICITO LA CONCESIÓN DE LA PATENTE DE INVENCION Y DECLARO QUE LA INVENCION ES NUEVA Y DE MI PROPIEDAD**

FIRMA

NOMBRE JOSE ANTONIO LLOREDA

C.C. 17.159.681 de Bogotá TP No. 10.784



# Industria y Comercio

SUPERINTENDENCIA

SUPERINTENDENCIA Y COMERCIO

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Folios: 200

Fecha (AMM): 2000-05-29 17:02:32

Temate : 000 PATENTES Y REGISTRO (11 PRESENTA

Superendencia: 0000 DIVISION DE NUEVAS PRESENTACIONES

ESTADO DE LOS REGISTROS DE MARCA

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TOTAL

ESTADO DE LOS REGISTROS DE MARCA

### ANEXO

Atentamente manifiesto a usted que la fuente normativa de la reciprocidad de la cual se deriva el derecho del solicitante para reivindicar prioridad en el presente caso, se encuentra:

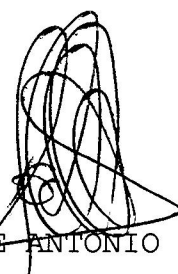
- a) En el Artículo 2 del Anexo 1C, Aspectos de los Derechos de Propiedad Intelectual relacionados con el Comercio, del Acuerdo por el cual se establece la "Organización Mundial de Comercio (OMC)" suscrito en Marruecos el 15 de abril de 1994. Este acuerdo fue aprobado por el Congreso de Colombia mediante Ley 170 de 1994 y por la Corte Constitucional Colombiana mediante Sentencia C-137-95 de 28 de marzo de 1995, y entró en vigencia en nuestro país el 30 de abril de 1995, 30 días después de su ratificación.

Dicho Artículo establece que en lo que respecta a las partes II, III y IV del Acuerdo (referidas a la existencia, alcance y ejercicio de los derechos de propiedad intelectual, entre otros) los Miembros cumplirán los artículos 1 a 12 y el Artículo 19 del Convenio de París --el Artículo que establece la prioridad de las patentes de invención es el Artículo 4--

- b) En el Artículo 4 del mencionado Anexo 1C, Aspectos de los Derechos de Propiedad Intelectual relacionados con el Comercio, del Acuerdo por el cual se establece la Organización Mundial de Comercio (OMC), el cual consagra el "Trato de la nación más favorecida" para los países Miembros del mismo.
- c) En el Artículo 4 del Convenio de París, del cual ya era parte Colombia en el momento en que se presentó la solicitud **cuyas prioridades ahora reivindico**, con lo cual se cumple la reciprocidad exigida en el Artículo 12 de la Decisión 344 del Acuerdo de Cartagena para con solicitudes provenientes de los Países Miembros del Acuerdo de Cartagena.

ANEXO

El documento que acredita la calidad REPRESENTANTES LEGALES en Bogotá de los doctores JOSE LLOREDA CAMACHO y/o ALFONSO LLOREDA CAMACHO y/o JOSE ANTONIO LLOREDA para TODOS LOS ASUNTOS RELACIONADOS CON LA PROPIEDAD INDUSTRIAL de la sociedad peticionaria, que faculta a cada uno de dichos abogados para actuar individualmente, se encuentra protocolizado ante esa División bajo el No. 15.256. En consecuencia, ruego a usted reconocer la personería de cada uno de dichos Representantes Legales para actuar dentro del presente expediente, a fin de que cualquiera de ellos pueda intervenir en cualquier tiempo respecto de la solicitud en él contenida.



JOSE ANTONIO LLOREDA

T.P. No. 10.784

CODIGO 231

# RESUMEN OBJETO Y FINALIDAD DE LA INVENCION

La presente invención se refiere a 'conjugados de eritropoyetina con poli(etilen glicol) comprendiendo una glucoproteína eritropoyetina que presenta al menos un grupo amino libre y que presenta la actividad biológica in vivo que hace que las células de la médula ósea incrementen la producción de reticulocitos y glóbulos rojos y que es seleccionada del grupo que consiste en eritropoyetina humana y análogos de la misma que tienen la secuencia de la eritropoyetina humana modificada mediante la adición de entre 1 y 6 sitios de glicosilación o mediante una nueva disposición de al menos un sitio de glicosilación; dicha glucoproteína estando covalentemente unida a "n" grupos de poli(etilenglicol) de la fórmula  $\text{-CO- (CH}_2\text{)}_x \text{- (OCH}_2\text{CH}_2\text{)}_m \text{-OR}$  con el carbonilo de cada grupo de poli(etilenglicol) formando un enlace amida con uno de dichos grupos amino; en donde R es alquilo inferior; x es 2 ó 3; m se encuentra entre alrededor de 450 y alrededor de 900; n se encuentra entre 1 y 3; y n y m son elegidos de forma que el peso molecular del conjugado menos la glucoproteína eritropoyetina se encuentre entre 20 kilodaltons y 100 kilodaltons.

## CONOCIMIENTO PREVIO DE LA INVENCION

La eritropoyesis es la producción de glóbulos rojos, que se produce con el objetivo de contrarrestar la destrucción celular. La eritropoyesis es un mecanismo fisiológico controlado, que proporciona los suficientes glóbulos rojos necesarios para una oxigenación tisular adecuada. La eritropoyetina humana producida de forma natural (hEPO), se produce en el riñón y es el factor plasmático humoral que estimula la producción de glóbulos rojos (Carnot, P and Deflandre, C (1906) C.R. Acad. Sci. 143: 432; Erslev, AJ (1953 Blood 8: 349; Reissmann, KR (1950) Blood 5: 372; Jacobson, LO, Goldwasser, E, Freid, W and Plzak, LF (1957) Nature 179: 6331-4). La EPO que se produce de forma natural estimula la división y diferenciación de los progenitores eritrocíticos específicos en la médula ósea y ejerce su actividad biológica mediante la unión a receptores en los precursores eritrocíticos (Krantz, BS (1991) Blood 77: 419).

La eritropoyetina se ha producido biosintéticamente mediante el uso de la tecnología del DNA recombinante (Egrie, JC, Strickland, TW, Lane, J et al. (1986) Immunobiol. 72: 213-224) y es el producto del gen EPO humano clonado, insertado y expresado en células de tejido de ovario de hámster chino (células CHO). La estructura primaria de la forma completamente procesada y predominante de hEPO se ilustra en SEQ ID NO:1. Hay dos puentes disulfuro entre Cys<sup>7</sup>-Cys<sup>161</sup> y Cys<sup>29</sup>-Cys<sup>33</sup>. El peso molecular de la cadena polipeptídica de EPO sin las porciones de los azúcares es



18.236 Da. En la molécula intacta de EPO, aproximadamente el 40% del peso molecular viene dado por los grupos de carbohidrato que glicosilan la proteína en los sitios de glicosilación de la proteína (Sasaki, H. Bothner, B, Dell, A and Fukuda, M (1987) J. Biol. Chem. 262: 12059).

Debido a que la eritropoyetina humana es esencial en la formación de glóbulos rojos, la hormona es útil en el tratamiento de los trastornos sanguíneos caracterizados por la baja o defectiva producción de glóbulos rojos. Clínicamente, la EPO es utilizada en el tratamiento de la anemia en pacientes con fallo renal crónico (CRF) (Eschbach, JEW, Egri, JC, Downing, MR et al. (1987) NEJM 316: 73-78; Eschbach, JEW, Abdulhadi, MH, Browne, JK et al. (1989) Ann. Intern. Med. 111: 992; Egrie, JC, Eschbach, JW, McGuire, T. Adamson, JEW (1988) Kidney Intl. 33: 262; Lim, VS, Degowin, RL, Zavala, D et al. (1989) Ann. Intern. Med. 110: 108-114) y en pacientes con SIDA y cáncer que han sido tratados con quimioterapia (Danna, RP, Rudnick, SA, Abels, RI In: MB, Garnick, ed. Erythropoietin in Clinical Applications-An International Perspective. New York, NY: Marcel Dekker; 1990: p. 301-324). Sin embargo, la disponibilidad biológica de agentes terapéuticos basados en proteínas disponibles comercialmente tales como EPO, se encuentra limitada por su corta vida media en el plasma y por su susceptibilidad a la degradación por parte de proteasas. Estos defectos impiden que se pueda alcanzar una potencia clínica máxima.

## RESUMEN de LA INVENCION

Esta invención proporciona un conjugado de eritropoyetina, dicho conjugado comprende una glucoproteína, la eritropoyetina, que presenta al menos un grupo amino libre, que presenta una actividad biológica *in vivo* con la que consigue que las células de la médula ósea incrementen la producción de reticulocitos y glóbulos rojos y que se selecciona del grupo de la eritropoyetina humana y análogos de la misma que presentan la secuencia de la eritropoyetina humana modificada mediante la adición de entre 1 y 6 sitios de glicosilación o mediante una nueva disposición de al menos un sitio de glicosilación; dicha glucoproteína se encuentra unida covalentemente a "n" grupos de poli(etilenglicol) de la fórmula  $-\text{CO}-(\text{CH}_2)_x-(\text{OCH}_2\text{CH}_2)_m-\text{OR}$ , con el  $-\text{CO}$  (por ejemplo carbonilo) de cada grupo de poli(etilenglicol) formando un enlace amida con uno de dichos grupos amino; en donde R es alquilo inferior; x es 2 ó 3; m se encuentra entre alrededor de 450 y alrededor de 900; n se encuentra entre 1 y 3; y n y m se eligen de modo que el peso molecular del conjugado menos la glucoproteína eritropoyetina se encuentre entre 20 kilodaltons y 100 kilodaltons. Esta invención también proporciona composiciones que contienen conjugados descritos aquí en las cuales el porcentaje de conjugados en la composición en la que n es 1 es al menos del noventa por ciento.

Si se comparan con las EPO no modificadas (es decir, EPO sin un PEG unido) y con los conjugados EPO-PEG convencionales, los presentes conjugados tienen tanto una vida

media de circulación como un tiempo de residencia en el plasma mayores, un menor aclaramiento, y una mayor actividad clínica *in vivo*. Los conjugados de esta invención presentan los mismos usos que EPO. En concreto, los conjugados de esta invención son útiles para tratar pacientes mediante la estimulación de la división y diferenciación de los progenitores eritrocíticos específicos en la médula ósea por la misma vía por la que se utiliza EPO para el tratamiento de los pacientes.

# DESCRIPCIÓN DETALLADA de LA INVENCION

Esta invención proporciona conjugados, dichos conjugados comprenden una glucoproteína, la eritropoyetina, que presenta al menos un grupo amino libre, que presenta una actividad biológica *in vivo* con la que consigue que las células de la médula ósea incrementen la producción de reticulocitos y glóbulos rojos y que se selecciona del grupo de la eritropoyetina humana y análogos de la misma que presentan la secuencia de la eritropoyetina humana modificada mediante la adición de entre 1 y 6 sitios de glicosilación o mediante una nueva disposición de al menos un sitio de glicosilación; dicha glucoproteína se encuentra unida covalentemente a "n" grupos de poli(etilenglicol) de la fórmula  $-CO-(CH_2)_x-(OCH_2CH_2)_m-OR$ , con el  $-CO$  (es decir, carbonilo) de cada grupo de poli(etilenglicol) formando un enlace amida con uno de dichos grupos amino; en donde R es alquilo inferior; x es 2 ó 3; m se encuentra entre alrededor de 450 y alrededor de 900; n se encuentra entre 1 y 3; y n y m se eligen de modo que el peso molecular del conjugado menos la glucoproteína eritropoyetina se encuentre entre 20 kilodaltons y 100 kilodaltons.

Se ha descubierto que los conjugados de esta invención se pueden usar del mismo modo que la EPO no modificada. Sin embargo, los conjugados de esta invención presentan tanto una vida media de circulación como tiempo de residencia en el plasma mayores, una menor aclaramiento y una mayor actividad clínica *in vivo*. Debido a la mejora de estas propiedades, los conjugados de esta invención se pueden adminis-

tratar una vez a la semana, en lugar de administrar tres veces a la semana la EPO no modificada. Se espera que esta menor frecuencia de administración dé como resultado un mejor cumplimiento del paciente lo cual mejore los resultados del tratamiento, así como la calidad de vida del paciente. Si se comparan con los conjugados convencionales de EPO unidos a poli(etilen glicol), se ha descubierto que los conjugados que presentan un peso molecular y una estructura de unión de los conjugados de esta invención tienen una potencia, estabilidad, ABC y vida media en circulación mejorados y un perfil de coste menor.

Los conjugados de acuerdo con esta invención, se pueden administrar en una cantidad terapéuticamente efectiva a pacientes del mismo modo en que se administra EPO. La cantidad terapéuticamente efectiva es la cantidad de conjugado necesaria para dar la actividad biológica *in vivo* que consigue que las células de la médula ósea incrementen la producción de reticulocitos y de glóbulos rojos. La cantidad exacta de conjugado es una cuestión de preferencia sujeta a factores tales como el tipo exacto de trastorno a tratar, el estado del paciente a tratar, así como otros ingredientes en la composición. Por ejemplo, entre 0,01 y 10 µg por kg de peso corporal, preferiblemente entre 0,1 y 1 µg por kg de peso corporal, se pueden administrar por ejemplo una vez a la semana.

Las composiciones farmacéuticas que contienen el conjugado se pueden formular con una potencia efectiva para la administración por varios medios a un paciente humano que

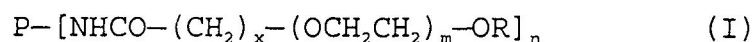
experimente trastornos sanguíneos caracterizados por la baja o defectiva producción de glóbulos rojos. La media de las cantidades efectivas terapéuticamente del conjugado pueden variar y en concreto deben estar basadas en las recomendaciones y prescripción de un médico cualificado.

Los productos de la glucoproteína eritropoyetina preparados de acuerdo con esta invención, se pueden preparar en composiciones farmacéuticas adecuadas para la inyección con un vehículo o portador farmacéuticamente aceptable mediante métodos conocidos en el campo. Por ejemplo, se han descrito composiciones adecuadas en W097/09996, W097/40850, W098/58660, y W099/07401. Entre los portadores farmacéuticamente aceptables preferidos para la formulación de los productos de la invención se encuentran la albúmina de suero humano, proteínas de plasma humano, etc. Los compuestos de la presente invención se podrían formular en tampón de fosfato de sodio/potasio 10 mM a pH 7 que contiene un agente de tonicidad, por ejemplo cloruro de sodio 132 mM. De forma opcional la composición farmacéutica puede contener un conservante. La composición farmacéutica puede contener diferentes cantidades de eritropoyetina, por ejemplo 10-1000 µg/ml, por ejemplo 50 µg or 400 µg.

El término "eritropoyetina" o "EPO" se refiere a una glucoproteína que presenta la secuencia aminoacídica expuesta en (SEQ ID NO: 1) ó (SEQ ID NO: 2) o una secuencia aminoacídica substancialmente homóloga a las mismas, cuyas propiedades biológicas se refieren a la estimulación de la producción de glóbulos rojos y a la estimulación de la di-

visión y diferenciación de los progenitores eritrocíticos específicos en la médula ósea. Tal como se emplean aquí, estos términos incluyen dichas proteínas modificadas de forma deliberada, como por ejemplo, por mutagénesis dirigida o de forma accidental, por mutaciones. Estos términos también incluyen análogos que presentan entre 1 y 6 sitios adicionales para la glicosilación, análogos que presentan al menos un aminoácido adicional en el extremo carboxilo de la glucoproteína, en donde el aminoácido adicional incluye al menos un sitio de glicosilación y análogos que presentan una secuencia aminoacídica que incluye una nueva disposición de al menos un sitio para la glicosilación. Estos términos incluyen tanto la eritropoyetina humana producida de forma natural como la producida de forma recombinante.

Los conjugados de eritropoyetina de esta invención se pueden representar mediante la Fórmula 1:



en donde x, m, n y R son tal como se han descrito anteriormente. En la Fórmula I, P es el residuo de la glucoproteína eritropoyetina descrita aquí (es decir, sin el grupo o grupos amino que forman un enlace amida con el carbonilo mostrado en la Fórmula I), con una actividad biológica in vivo que consigue que las células de la médula ósea incrementen la producción de reticulocitos y glóbulos rojos.

En una realización preferida de la presente invención, R es metilo. Preferiblemente, m se encuentra entre alrededor de 650 y alrededor de 750 y n preferiblemente es 1.

En la realización más preferida de la presente invención, R es metilo, m se encuentra entre alrededor de 650 y alrededor de 750, y n es 1, es decir el conjugado es como se ha definido anteriormente con la fórmula

$$5 \quad [\text{CH}_3\text{O}(\text{CH}_2\text{CH}_2\text{O})_m\text{CH}_2\text{CH}_2\text{CH}_2\text{CO-NH}]_n-\text{P}$$

en donde  $m$  se encuentra entre 650 y 750,  $n$  es 1 y  $P$  es como se ha definido anteriormente. Preferiblemente  $m$  presenta un valor medio de alrededor de 680.

Preferiblemente, la glucoproteína de los conjugados como se ha definido anteriormente es una eritropoyetina humana. La eritropoyetina humana y proteínas análogas como se han definido anteriormente se pueden expresar mediante una activación génica endógena. Las glucoproteínas eritropoyetinas humanas preferidas son aquéllas que presentan la SEQ ID NO:1 y SEQ ID NO:2, más preferiblemente las que presentan la SEQ ID NO:1.

Además P se puede seleccionar del grupo que consiste en residuos de eritropoyetina humana y análogos de la misma que contienen entre 1 y 6 sitios adicionales para la glicosilación. Tal como se expone en detalle a continuación, la preparación y purificación de EPO son bien conocidas en el campo. Mediante EPO se denota la proteína natural o recombinante, preferiblemente humana, así como la obtenida de cualquier fuente convencionales tales como tejidos, síntesis protéica, cultivo celular con células naturales o recombinantes. Se incluye cualquier proteína que presente actividad de EPO, tal como muteínas o proteínas modificadas de otra manera. La EPO recombinante se puede preparar me-

25 sis protéica, cultivo celular con células naturales o re-  
combinantes. Se incluye cualquier proteína que presente ac-  
tividad de EPO, tal como muteínas o proteínas modificadas  
de otra manera. La EPO recombinante se puede preparar me-



diante su expresión en líneas celulares CHO-, BHK- o HeLa mediante la tecnología del DNA recombinante o mediante activación génica endógena. La expresión de proteínas, incluyendo EPO, mediante activación génica endógena es bien conocida en el campo y se revela, por ejemplo en las Patentes Estadounidenses Nos. 5.733.761, 5.641.670 y 5.733.746 y en la publicación de patente internacional Nos. WO 93/09222, WO 94/12650, WO 95/31560, WO 90/11354, WO 91/06667 y WO 91/09955, cuyos contenidos se incorporan aquí por referencia. Las especies de EPO preferidas para la preparación de productos de glucoproteína eritropoyetina son especies de EPO humanas. La especie EPO más preferida es la EPO humana que presenta la secuencia aminoacídica expuesta en SEQ ID NO: 1 ó SEQ ID NO:2, más preferiblemente la secuencia aminoacídica SEQ ID NO:1.

En una realización, P puede ser el residuo de un análogo de glucoproteína que tiene entre 1 y 6 sitios adicionales para la glicosilación. La glicosilación de una proteína, con uno o más grupos de oligosacárido, se produce en localizaciones específicas a lo largo de la cadena polipeptídica y afecta en gran medida a las propiedades físicas de la proteína tales como estabilidad, secreción, localización subcelular y actividad biológica de la proteína. Normalmente la glicosilación es de dos tipos. Los oligosacáridos que presentan unión O se encuentran unidos a residuos de serina o de treonina y los que presentan unión N se encuentran unidos a residuos de asparagina. Un tipo de oligosacárido hallado tanto en oligosacáridos con unión N como con unión

O es ácido N-acetilneuramínico (ácido siálico), el cual es una familia de amino azúcares que contienen 9 o más átomos de carbono. El ácido siálico normalmente es el residuo terminal tanto de oligosacáridos con unión N como con unión O y debido a que lleva carga negativa, confiere propiedades acídicas a la glucoproteína. La eritropoyetina humana, que tiene 165 aminoácidos, contiene tres cadenas de oligosacáridos N-unidos y una de oligosacárido con unión O, que comprenden alrededor del 40% del peso molecular total de la glucoproteína. La glicosilación con unión N se produce en los residuos de asparagina localizados en las posiciones 24, 38, y 83 y la glicosilación con unión O se produce en un residuo de serina localizado en la posición 126. Las cadenas de oligosacáridas se encuentran modificadas con residuos terminales de ácido siálico. La separación enzimática de todos los residuos de ácido siálico de la eritropoyetina glicosilada provoca la pérdida de actividad *in vivo* pero no de actividad *in vitro* porque la sialilación de la eritropoyetina evita su unión, y la posterior aclaramiento, por la proteína de unión hepática.

Las glucoproteínas de la presente invención incluyen análogos de la eritropoyetina humana con uno o más cambios en la secuencia aminoacídica de la eritropoyetina humana, que resultan en un incremento en el número de sitios de unión del ácido siálico. Estos análogos de glucoproteína se pueden generar por mutagénesis dirigida con adiciones, deleciones o sbstituciones de residuos aminoacídicos que incrementan o alteran sitios que están disponibles para la

glicosilación. Los análogos de glucoproteína con mayores niveles de ácido siálico que los hallados en la eritropoyetina humana, se generan mediante la adición de sitios de glicosilación que no perturban la conformación secundaria o terciaria necesaria para la actividad biológica. Las glucoproteínas de la presente invención también incluyen análogos con mayores niveles de unión a carbohidrato en un sitio de glicosilación que normalmente involucra la substitución de uno o más aminoácidos cercanos a un sitio con unión N o con unión O. Las glucoproteínas de la presente invención también incluyen análogos que tienen uno o más aminoácidos que se prolongan a partir del extremo carboxilo de la eritropoyetina y que proporcionan al menos un sitio de unión a carbohidrato adicional. Las glucoproteínas de la presente invención también incluyen análogos que tienen una secuencia aminoacídica que incluye una nueva disposición de al menos un sitio para la glicosilación. Dicha nueva disposición del sitio de glicosilación involucra la delección de uno o más sitios de glicosilación en la eritropoyetina humana y la adición de uno o más sitios de glicosilación que no se dan de forma natural. El aumento del número de cadenas de carbohidrato en la eritropoyetina y por lo tanto, el número de ácido siálico por molécula de eritropoyetina, puede conferir propiedades beneficiosas tales como una mayor solubilidad, una mayor resistencia a la proteólisis, una menor inmunogenicidad, una mayor vida media en suero y una mayor actividad biológica. Los análogos de eritropoyetina con sitios de glicosilación adicionales se revelan con

más detalle en la Aplicación de Patente Europea 640 619, de Elliot publicada el 1 de Marzo de 1995.

En una realización preferida, las glucoproteínas de la presente invención comprenden una secuencia aminoacídica que incluye al menos un sitio adicional para la glicosilación tales como, pero no limitadas a, eritropoyetinas que comprenden la secuencia de la eritropoyetina humana modificada por una modificación seleccionada de las siguientes:

Asn<sup>30</sup>Thr<sup>32</sup>;  
10 Asn<sup>51</sup>Thr<sup>53</sup>;  
Asn<sup>57</sup>Thr<sup>59</sup>;  
Asn<sup>69</sup>;  
Asn<sup>69</sup>Thr<sup>71</sup>;  
Ser<sup>68</sup>Asn<sup>69</sup>Thr<sup>71</sup>;  
15 Val<sup>87</sup>Asn<sup>88</sup>Thr<sup>90</sup>;  
Ser<sup>87</sup>Asn<sup>88</sup>Thr<sup>90</sup>;  
Ser<sup>87</sup>Asn<sup>88</sup>Gly<sup>89</sup>Thr<sup>90</sup>;  
Ser<sup>87</sup>Asn<sup>88</sup>Thr<sup>90</sup>Thr<sup>92</sup>;  
Ser<sup>87</sup>Asn<sup>88</sup>Thr<sup>90</sup>Ala<sup>162</sup>;  
20 Asn<sup>69</sup>Thr<sup>71</sup>Ser<sup>87</sup>Asn<sup>88</sup>Thr<sup>90</sup>;  
Asn<sup>30</sup>Thr<sup>32</sup>Val<sup>87</sup>Asn<sup>88</sup>Thr<sup>90</sup>;  
Asn<sup>89</sup>Ile<sup>90</sup>Thr<sup>91</sup>;  
Ser<sup>87</sup>Asn<sup>89</sup>Ile<sup>90</sup>Thr<sup>91</sup>;  
Asn<sup>136</sup>Thr<sup>138</sup>;  
25 Asn<sup>138</sup>Thr<sup>140</sup>;  
Thr<sup>125</sup>; y  
Pro<sup>124</sup>Thr<sup>125</sup>.

La notación que se ha utilizado para la modificación de la secuencia aminoacídica indica que la posición de la correspondiente proteína no modificada (por ejemplo hEPO de SEQ ID NO:1 ó SEQ ID NO:2) indicada por el número en super-  
 5 índice se sustituye por el aminoácido que precede inmediatamente al número en superíndice.

La glucoproteína también puede ser un análogo con al menos un aminoácido adicional en el extremo carboxilo de la glucoproteína, en donde el aminoácido adicional incluye al  
 10 menos un sitio de glicosilación, es decir, el conjugado tal como se ha definido anteriormente también se refiere a un compuesto en donde la glucoproteína tiene una secuencia que comprende la secuencia de eritropoyetina humana y una segunda secuencia en el extremo carboxilo de la secuencia de  
 15 la eritropoyetina humana, en donde la segunda secuencia contiene al menos un sitio de glicosilación. El aminoácido adicional puede comprender un fragmento peptídico derivado del extremo carboxilo de la gonadotropina coriónica. Preferiblemente, la glucoproteína es un análogo seleccionado del  
 20 grupo que consiste en (a) eritropoyetina humana que tiene la secuencia aminoacídica Ser Ser Ser Ser Lys Ala Pro Pro Pro Ser Leu Pro Ser Pro Ser Arg Leu Pro Gly Pro Ser Asp Thr Pro Ile Leu Pro Gln (SEQ ID NO:3), prolongándose desde el extremo carboxilo; (b) el análogo en (a) también comprende  
 25 EPO Ser<sup>87</sup> Asn<sup>88</sup> Thr<sup>90</sup>; y (c) el análogo en (a) también comprende EPO Asn<sup>30</sup> Thr<sup>32</sup> Val<sup>87</sup> Asn<sup>88</sup> Thr<sup>90</sup>.

con objeto de conjugarse al extremo de la gonadotropina coriónica

La glucoproteína también puede ser un análogo con una secuencia aminoacídica que incluya una nueva disposición de

al menos un sitio para la glicosilación. La nueva disposición puede comprender una delección de cualquiera de los sitios de unión N a carbohidrato en la eritropoyetina humana y una adición de un sitio de unión N de carbohidrato en la  
5 posición 88 de la secuencia aminoacídica de la eritropoyetina humana. Preferiblemente, la glucoproteína es un análogo seleccionado del grupo que consiste en EPO Gln<sup>24</sup> Ser<sup>87</sup> Asn<sup>88</sup> Thr<sup>90</sup>; EPO Gln<sup>38</sup> Ser<sup>87</sup> Asn<sup>88</sup> Thr<sup>90</sup>; y EPO Gln<sup>83</sup> Ser<sup>87</sup> Asn<sup>88</sup> Thr<sup>90</sup>.

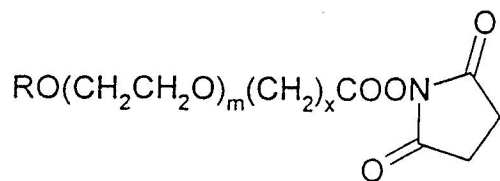
10 Tal como se ha empleado aquí, el término "alquilo inferior" significa un grupo alquilo lineal o ramificado que presenta entre uno y seis átomos de carbono. Ejemplos de grupos alquilo inferior incluyen metilo, etilo e isopropilo. De acuerdo con esta invención, R es cualquier alquilo  
15 inferior. Se prefieren los conjugados en los que R es metilo.

El símbolo "m" representa el número de residuos de óxido de etileno (OCH<sub>2</sub>CH<sub>2</sub>) en el grupo de poli(etileno óxido). Una única subunidad de PEG de óxido de etileno tiene  
20 un peso molecular de alrededor de 44 daltons. Así, el peso molecular del conjugado (excluyendo el peso molecular de la EPO) depende del número "m". En los conjugados de esta invención, "m" se encuentra entre alrededor de 450 y alrededor de 900 (correspondiendo al peso molecular de entre 20  
25 kDa y alrededor de 40 kDa), preferiblemente entre alrededor de 650 y alrededor de 750 (correspondiendo al peso molecular de alrededor de 30 kDa). El número m se selecciona de forma que el conjugado resultante de esta invención tenga

una actividad fisiológica comparable a la EPO no modificada, cuya actividad puede representar igual o más que una fracción de la correspondiente actividad de la EPO no modificada. Un peso molecular de "alrededor" de un cierto número, significa que está dentro de un rango razonable de dicho número determinado mediante técnicas analíticas convencionales. El número "m" se selecciona de forma que el peso molecular de cada grupo poli(etilenglicol) unido de forma covalente a la glucoproteína eritropoyetina se encuentre entre alrededor de 20 kDa a alrededor de 40 kDa, y se encuentre preferiblemente entre alrededor de 30 kDa.

En los conjugados de esta invención, el número "n" es el número de grupos de polietilenglicol unidos covalentemente a grupos amino libres (incluyendo grupos ε-amino de un aminoácido de lisina y/o el grupo amino del extremo amino) de la proteína eritropoyetina mediante uniones amida. Un conjugado de esta invención puede tener uno, dos o tres grupos PEG por molécula de EPO. "n" es un entero que se encuentra entre 1 y 3, preferiblemente "n" es 1 ó 2, y más preferiblemente "n" es 1.

El compuesto de la Fórmula I se puede preparar a partir de material polimérico conocido:



(II)

en el cual R y m son como se han descrito anteriormente, por condensación del compuesto de Fórmula II con la glucoproteína eritropoyetina. Los compuestos de Fórmula II en los cuales x es 3 son alfa-alcoxilo inferior, ácido butírico succinimidílicos ésteres de poli(etilenglicol) (alcoxilo inferior-PEG-SBA). Los compuestos de Fórmula II en los cuales x es 2 son alfa-alcoxilo inferior, ácido propiónico ésteres succinimidílicos de poli(etilenglicol) (alcoxilo inferior-PEG-SPA). Se puede utilizar cualquier método convencional para hacer reaccionar un éster activado con una amina para formar una amida. En la reacción descrita anteriormente, el éster succinimidílico ejemplificado es un grupo saliente causante de la formación de la amida. El uso de los ésteres succinimidílicos tales como los compuestos de la fórmula II para producir conjugados con proteínas se revelan en la Patente Estadounidense No. 5.672.662, publicada el 30 de Septiembre de 1997 (Harris, et al.).

La EPO humana contiene nueve grupos amino libres, el grupo amino del extremo amino más los grupos  $\epsilon$ -amino de 8 residuos de lisina. Cuando el reactivo de pegilación se combinó con un compuesto SBA de Fórmula II, se halló que a pH 7,5, una relación proteína: PEG de 1:3 y una temperatura de reacción de alrededor de 20-25 °C, se obtuvo una mezcla de especies mono-, di-tripegiladas y pequeñas cantidades de especies tri-pegiladas. Cuando el reactivo de pegilación era un compuesto SPA de la Fórmula II, en condiciones similares excepto que la relación de proteína:PEG era de 1:2, se produjeron principalmente las especies mono-pegiladas.



La EPO pegilada se puede administrar en forma de mezcla, o como diferentes especies pegiladas separadas a través de cromatografía de intercambio catiónico. Mediante la manipulación de las condiciones de reacción (por ejemplo la relación de reactivos, pH, temperatura, concentración de la proteína, tiempo de reacción, etc.) se pueden variar las cantidades relativas de las diferentes especies pegiladas.

La eritropoyetina humana (EPO) es una glucoproteína que estimula la formación de eritrocitos. Su preparación y aplicación terapéutica están descritas en detalle por ejemplo en la Patente Estadounidense Nos. 5.547.933 y 5.621.080, EP-B 0 148 605, Huang, S.L., Proc. Natl. Acad. Sci. USA (1984) 2708-2712, EP-B 0 205 564, EP-B 0 209 539 y EPB 0 411 678 así como por Lai, P.H. et al., J. Biol. Chem. 261 (1986) 3116-3121, y Sasaki, H. et al., J. Biol. Chem. 262 (1987) 12059-12076. La eritropoyetina para usos terapéuticos se puede producir de forma recombinante (EP-B 0 148 605, EP-B 0 209 539 y Egrie, J.C., Strickland, T.W., Lane, J. et al. (1986) Immunobiol. 72: 213-224).

Los métodos para la expresión y preparación de la eritropoyetina en medio sin suero están descritas por ejemplo en WO 96/35718, to Burg publicado el 14 de Noviembre de 1996, y en European Patent Publication No. 513 738, to Koch publicado el 12 de Junio de 1992. En adición a las publicaciones mencionadas anteriormente, se sabe que se puede llevar a cabo una fermentación libre de suero de células CHO recombinantes que contengan un gen EPO. Tales métodos están descritos por ejemplo en EP-A 0 513 738, EP-A 0 267 678 y

de forma general por Kawamoto, T. et al., Analytical Biochem. 130 (1983) 445-453, EP-A 0 248 656, Kowar, J. y Franek, F., Methods in Enzymology 421 (1986) 277-292, Bavis-  
ter, B., Expcology 271 (1981) 45-51, EP-A 0 481 791, EP-A 0  
5 307 247, EP-A 0 343 635, WO 88/00967.

En EP-A 0 267 678 se describe una cromatografía de intercambio iónico en S-Sefarosa, un HPLC en fase reversa preparativo en una columna C<sub>8</sub> y una cromatografía de filtración en gel para la purificación de EPO producida en cultivo libre de suero tras realizar una diálisis. En esta relación, el paso de cromatografía de filtración en gel se puede sustituir por cromatografía de intercambio iónico en S-Sefarosa fast flow. También se propone realizar una cromatografía con pigmento en una columna de Azul de Trisacrilo  
10 previamente a la cromatografía de intercambio iónico.  
15

Se describe un proceso para la purificación de la EPO recombinante en Nobuo, I. et al., J. Biochem. 107 (1990) 352-359. En este proceso, la EPO se trata con una solución de Tween® 20, fluoruro de fenilmetilsulfonilo, etilmaleimida, pestatina A, sulfato de cobre y ácido oxámico de forma  
20 previa a los pasos de purificación. Las publicaciones, incluyendo WO 96/35718, a Burg publicado el 14 de Noviembre de 1996, revela un proceso para la preparación de eritropoyetina en un proceso de fermentación libre de suero  
25 (EPOsf).

La actividad específica de EPO o conjugados de EPO de acuerdo con esta invención, se puede determinar mediante varios ensayos conocidos en el campo. La actividad biológica

ca de las proteínas EPO purificadas de esta invención es tal que la administración de la proteína EPO por inyección a pacientes humanos consigue que las células de la médula ósea incrementen la producción de reticulocitos y glóbulos rojos comparado con los sujetos no inyectados o grupos control. La actividad biológica de las proteínas EPO, o fragmentos de las mismas, obtenidos y purificados de acuerdo con esta invención se pueden determinar por métodos de acuerdo con Annable, et al., Bull. Wld. Hlth. Org. (1972) 47: 99-112 y Pharm. Europa Spec. Issue Erythropoietin BRP Bio 1997(2). Otro ensayo biológico para determinar la actividad de la proteína EPO, el ensayo normocitémico de ratón, se describe en el Ejemplo 4.

Esta invención proporciona una composición formada por conjugados tal como se ha descrito anteriormente. Se puede preparar una composición que contiene al menos el noventa por ciento de conjugados mono-PEG, es decir en la cual n es 1, tal como se muestra en el Ejemplo 5. Normalmente se prefieren los conjugados mono-PEG de glucoproteínas eritropoyetinas porque tienden a tener una mayor actividad que los conjugados di-PEG. El porcentaje de conjugados mono-PEG así como la relación de especies mono y di-PEG se pueden controlar mediante la combinación de las fracciones anchas del pico de elución para disminuir el porcentaje de mono-PEG, o de las fracciones estrechas para incrementar el porcentaje de mono-PEG en la composición. Alrededor del noventa por ciento de conjugados mono-PEG es un buen balance de rendimiento y actividad. Se pueden preferir algunas composicio-

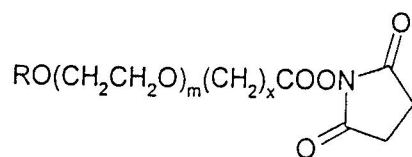
nes en las cuales, por ejemplo, al menos un noventa y dos por ciento o al menos noventa y seis por ciento de los conjugados sean especies mono-PEG ( $n$  igual a 1). En una realización de esta invención, el porcentaje de conjugados en donde  $n$  es 1 se encuentra entre el noventa por ciento y el noventa y seis por ciento.

La invención también se refiere a las correspondientes composiciones farmacéuticas que comprenden un conjugado o una composición tal como se ha descrito anteriormente y un excipiente farmacéuticamente aceptable.

Los conjugados y composiciones de la presente invención son especialmente útiles para la preparación de medicamentos para el tratamiento o profilaxis de enfermedades relacionadas con la anemia en pacientes con fallo renal crónico (CRF), y para el tratamiento de pacientes con SIDA y cáncer que han sido sometidos a quimioterapia.

Una realización adicional de la presente invención se refiere a un método para el tratamiento profiláctico y/o terapéutico de trastornos relacionados con la anemia en pacientes con fallo renal crónico (CRF), y pacientes con SIDA y cáncer que han sido sometidos a quimioterapia, comprende el paso de la administración a un paciente de una composición tal como se ha descrito anteriormente.

Además, la invención se refiere a un proceso para la preparación de compuestos como se ha descrito anteriormente, cuyo proceso comprende la condensación del compuesto de Fórmula II



(II)

5

con la glucoproteína eritropoyetina y en la cual R, m y x son como se han definido anteriormente.

La invención se refiere también a compuestos como se han definido anteriormente para el tratamiento de enfermedades las cuales están asociadas con anemia en pacientes  
10 con fallo renal crónico (CRF), y pacientes con SIDA y cáncer que han sido sometidos a quimioterapia.

La invención se entenderá mejor con los siguientes ejemplos que ilustran, pero no por ello limitan, la invención  
15 descrita hasta aquí.

EJEMPLOS**EJEMPLO 1: Fermentación y Purificación de EPO Humana**

## a) Preparación del inóculo y Fermentación

Se toma un vial del Working Cell Bank, originada a  
5 partir de una línea celular CHO que produce EPO (se puede  
usar ATCC CRL8695, revelada en EP 411 678 (Genetics Insti-  
tute)) a partir de la fase gas del depósito de almacena-  
miento de nitrógeno líquido. Las células se transfirieron  
en tubos de centrifuga de vidrio y se cultivaron en un me-  
10 dio tamponado de hidrógeno carbonato en un incubador humi-  
dificado de CO<sub>2</sub>. Los medios sin suero típicos usados para la  
preparación del inóculo y la fermentación se revelan en la  
Aplicación de Patente Europea 513 738, de Koch publicado el  
12 Junio de 1992, o WO 96/35718, de Burg publicado el 14 de  
15 Noviembre de 1996, por ejemplo contienen como medio  
DMEM/F12 (por ejemplo JRH Biosciences/Hazleton Biologics,  
Denver, US, No. 57736) y de modo adicional hidrógenocarbo-  
nato sódico, L+glutamina, D+glucosa, insulina recombinan-  
te, selenita sódica, diaminobutano, hidrocortisona, sulfato  
20 de hierro (II), asparagina, ácido aspártico, serina y un  
estabilizante para células de mamífero tales como por ejem-  
plo alcohol de polivinilo, metil celulosa, polidextrano,  
polietilenglicol, Pluronic F68, expansor de plasma polige-  
lina (HEMACCEL®) o polivinil pirrolidona (WO 96/35718).

25 Los cultivos se comprobaron microscópicamente para de-  
tectar la ausencia de microorganismos contaminantes y se  
determinaron las densidades celulares. Estos ensayos se  
realizaron en cada paso de fraccionamiento.

Tras el período de crecimiento inicial, el cultivo celular se diluyó con medio fresco hasta conseguir la densidad celular inicial y comenzar otro ciclo celular. Este procedimiento se repitió hasta que se obtuvo un volumen de cultivo de aproximadamente 2 litros por tubo de centrífuga de vidrio. Tras aproximadamente 12 duplicaciones, se usó de 1 a 5 litros de este cultivo como inóculo para la fermentación de 10 litros.

Tras entre 3 y 5 días, el cultivo del fermentador de 10 litros se puede usar como inóculo para el fermentador de inóculo de 100 litros.

Tras otros 3-5 días de cultivo, el cultivo en los 100 litros de fermentación se puede usar como inóculo para el fermentador de inóculo de 1000 litros.

#### 15 b) Recogida y Separación Celular

Se usó un proceso de realimentación en lote, es decir cuando se alcanza la densidad celular deseada, aproximadamente el 80% del cultivo se recoge. El cultivo remanente es rellenado con medio de cultivo fresco y se hace crecer hasta la siguiente recogida. Una ronda de producción consiste en un máximo de 10 ciclos de recogida: 9 ciclos de recogidas parciales y 1 ciclo de recogida completa al final de la fermentación. El ciclo de recogida se da al cabo de entre 3 y 4 días.

25 El volumen de cultivo recogido es transferido a un recipiente enfriado. Las células son separadas por centrifugación o filtración y eliminadas. La EPO que contiene el sobrenadante del paso de la centrifugación se filtró en lí-

nea y se recogió en un segundo recipiente enfriado. Cada cultivo recogido se procesa de modo separado durante la purificación.

Un proceso típico para la purificación de la proteína EPO se revela en WO 96/35718, de Burg publicado el 14 de Noviembre de 1996. El proceso de purificación se explica a continuación.

a) Cromatografía de Sefarosa Azul

La Sefarosa azul (Pharmacia) consiste en bolitas de Sefarosa, en la superficie de las cuales el colorante Cibacron azul se encuentra unido covalentemente. Como la EPO se une con más fuerza a la Sefarosa Azul que muchos de los contaminantes no protéicos, algunas impurezas protéicas y pVA, la EPO se puede enriquecer en este paso. La elución de la columna de Sefarosa Azul se lleva a cabo incrementando la concentración de sales, así como el pH.

La columna se empaqueta con entre 80-100 litros de Sefarosa Azul, se regenera con NaOH y se equilibra con tampón de equilibración (cloruro de sodio/calcio y acetato sódico). Se carga el sobrenadante de la fermentación acidificado y filtrado. Tras finalizar la carga, la columna se lava primero con un tampón similar al tampón de equilibrado, que contiene una mayor concentración de cloruro de sodio y posteriormente con un tampón Tris-base. El producto se eluye con un tampón Tris-base y se recoge en una fracción única de acuerdo con el perfil de elución patrón.



b) Cromatografía de Butil Toyopearl

El Butil Toyopearl 650 C (Toso Haas) es una matriz basada en poliestireno a la cual están acoplados residuos alifáticos de butilo. Como la EPO se une con más fuerza a este gel que muchas de las impurezas y PVA, se eluye con un  
5 tampón que contiene isopropanol.

La columna se empaqueta con entre 30-40 litros de Butil toyopearl 650 C, se regenera con NaOH, se lava con un tampón Tris-base y se equilibra con un tampón Tris-base que  
10 contiene isopropanol.

El eluido de Sefarosa Azul se ajusta a la concentración de isopropanol en el tampón de quilibración de la columna y se carga en la columna. Entonces la columna se lava con el tampón de equilibración siguiendo una concentración  
15 creciente de isopropanol. El producto se eluye con el tampón de elución (tampón Tris-base con un mayor contenido de isopropanol) y se recoge en una fracción única de acuerdo con el perfil de elución patrón.

c) Cromatografía Ultrogel Hidroxiapatita

El Ultrogel Hidroxiapatita (Biosepra) consiste en Hidroxiapatita que se incorpora en una matriz de agarosa para mejorar las propiedades mecánicas. La EPO tiene una menor afinidad por la Hidroxiapatita y se puede eluir por tanto a concentraciones menores de fosfato que las impurezas pro-  
25 téicas.

La columna se rellena con entre 30-40 litros de Ultrogel Hidroxiapatita y se regenera con un tampón de fosfato potásico/cloruro cálcico y NaOH seguido por un tampón Tris-

base. Entonces se equilibra con un tampón Tris-base que contiene una cantidad menor de isopropanol y cloruro sódico.

La EPO contenida en el eluido de la cromatografía Butil Toyopearl se introduce en la columna. Posteriormente la columna se lava con tampón de equilibrado y un tampón Tris-base sin isopropanol y cloruro sódico. El producto se eluye con un tampón Tris-base que contiene una menor concentración de fosfato potásico y se recoge en una fracción única de acuerdo con el perfil de elución patrón.

d) HPLC de Fase Reversa en Vydac C4

El material RP-HPLC Vydac C4 (Vydac) consiste en partículas de gel de sílice, las superficies de las cuales llevan cadenas C4-alquilo. La separación de EPO de las impurezas protéicas se basa en las diferencias en cuanto a la fuerza de las interacciones hidrofóbicas. La elución se lleva a cabo con un gradiente de acetonitrilo en ácido trifluoroacético diluido.

El HPLC preparativo se realiza mediante una columna de acero inoxidable (rellenada con entre 2,8 y 3,2 litros de gel de sílice Vydac C4). El eluido Hidroxiapatita Ultrogel se acidificó mediante la adición de ácido trifluoroacético y se introdujo en la columna Vydac C4. Para el lavado y la elución se utilizó un gradiente de acetonitrilo en ácido trifluoroacético. Las fracciones se recogieron e inmediatamente se neutralizaron con tampón fosfato. Las fracciones EPO que se encontraron dentro de los límites IPC se combinaron.

## e) Cromatografía Sefarosa DEAE

El material de Sefarosa DEAE (Pharmacia) consiste en grupos dietilaminoetil (DEAE) que se encuentran covalentemente unidos a la superficie de bolas de Sefarosa. La unión de EPO a los grupos DEAE está mediada por interacciones iónicas. El acetonitrilo y el ácido trifluoroacético pasan a través de la columna sin ser retenidos. Después de que estas sustancias han sido lavadas, se eliminan las trazas de impurezas mediante el lavado de la columna con tampón acetato a bajo pH. Entonces la columna se lavó con tampón fosfato neutro y la EPO se eluyó con un tampón con fuerza iónica creciente.

La columna se empaquetó con Sefarosa DEAE fast flow. El volumen de la columna se ajustó para asegurar una carga de EPO en el rango de 3-10 mg EP/ml gel. La columna se lavó con agua y tampón de equilibrado (fosfato sódico/potásico). Las fracciones combinadas del eluido de HPLC se cargaron y la columna se lavó con tampón de equilibración. Entonces la columna se lavó con tampón de lavado (tampón de acetato sódico) y posteriormente con tampón de equilibrado. Posteriormente, la EPO se eluyó de la columna con tampón de elución (cloruro sódico, fosfato sódico/potásico) y se recogió en un fracción única de acuerdo con el perfil de elución patrón.

El eluido de la columna Sefarosa DEAE se ajustó a la conductividad especificada. La sustancia resultante se filtró para quedar estéril en botellas de Teflon y se conservó a  $-70^{\circ}\text{C}$ .

**EJEMPLO 2: Pegilación de EPO con mPEG-SBA**

La EPO purificada de acuerdo con el procedimiento sin suero del Ejemplo 1 (EPOsf) fue homogéneo tal como se determinó mediante métodos analíticos y mostró el patrón típico isoforma consistente en 8 isoformas. Tuvo una actividad biológica específica de 190.000 IU/mg, tal como se determinó mediante el ensayo normocitémico de ratón. El reactivo de pegilación usado fue metoxi-PEG-SBA, el cual es un compuesto de Fórmula II en el cual R es metilo; x es 3; y m se encuentra entre 650 y 750 (valor medio entre alrededor de 680, correspondiente a una peso molecular medio de alrededor de 30 kDa).

**Reactivación de Pegilación**

A cien miligramos de EPOsf (9,71 ml de una solución madre de EPOsf de 10,3 mg/ml, 5,48  $\mu$ mol) se añadieron 10 ml de un tampón de fosfato de potasio, pH, 7,5, conteniendo 506 mg de metoxi-PEG-SBA (16,5  $\mu$ mol) (obtenido a partir de Shearwater Polymers, Inc., Huntsville, Alabama) y se mezcló durante 2 horas a temperatura ambiente (20-23 °C). La concentración de proteína final fue de 5 mg/ml y la relación proteína: reactivo PEG fue de 1:3. Tras dos horas, la reacción se paró mediante el ajuste del pH a 4,5 con ácido acético glacial y se conservó a -20 °C, hasta el momento de la purificación.

**Purificación.**

1. **Mecla del conjugado:** Aproximadamente 28 ml de SP-SEPHAROSE FF (resina de intercambio catiónico de sulfopropilo) se empaquetaron en una columna de vidrio AMICON

(2,2 x 7,5 cm) y se equilibró con tampón de acetato 20 mM, pH 4,5 a un flujo de 150 ml/h. Seis mililitros de la mezcla de reacción que contenían 30 mg de proteína se diluyeron 5 veces con el tampón de equilibración y se aplicaron a la columna. Los materiales no adsorbidos se lavaron con el tampón y la mezcla del conjugado con PEG adsorbida se eluyó de la columna con NaCl 0,175 M en el tampón de equilibrado. La EPOsf no modificada aún remanente en la columna se eluyó con NaCl 750 mM. La columna se volvió a equilibrar en el tampón de inicio. Las muestras se analizaron mediante SDS-PAGE y se determinó su nivel de pegilación. Se halló que el eluido de NaCl 0,175 M contenía especies mono- así como di-pegiladas y pequeñas cantidades de especies tri-pegiladas, mientras que el eluido de NaCl 750 mM contenía la EPOsf sin modificar.

2. Di-PEG y Mono-PEG-EPOsf: La mezcla del conjugado purificada eluída de la columna en el paso anterior se diluyó 4 veces con el tampón, se volvió a aplicar en la columna y se lavó como se ha descrito anteriormente. La Di-PEG-EPOsf y mono-PEG-EPOsf se eluyeron de modo separado de la columna con NaCl 0,1 M y NaCl 0,175 M respectivamente. La elución también se realizó con NaCl 750 mM con la finalidad de eluir cualquier EPOsf remanente no modificado.

Por otro lado, la mezcla de reacción se diluyó 5 veces con el tampón de acetato y se aplicó en la columna de SP-Sefarosa (~0.5 mg proteína/ml gel). La columna se lavó y las mono-PEG-EPOsf, di-PEG-EPOsf adsorbidas y EPOsf no mo-

dificada se eluyeron como se ha descrito en la sección anterior.

### Resultados

Se sintetizó PEG-EPOsf mediante la conjugación química de una molécula de PEG lineal con un valor de peso molecular medio de 30 KDa. La EPO-EPOsf se derivó de la reacción entre los grupos amino primarios de EPOsf y el éster de succinimidilo derivado de un ácido PEG-butírico de 30 kDa, resultando en un enlace amida.

Los resultados se resumen en la Tabla 2. La mezcla de conjugado purificada estaba formada por mono y di-PEG-EPOsf y estaba libre de EPOsf no modificada como se determinó mediante análisis por SDS-PAGE. La mezcla del conjugado resultó en 23,4 mg o 78% del material de partida. La separación cromatográfica por intercambio catiónico de mono y di-PEG-EPOsf indicó que la relación mono a di-PEG en la mezcla de conjugado fue de casi 1:1. Tras la finalización de la reacción, la relación de los componentes individuales de Mono:Di: No modificada fueron de 40:38:20 (%). El rendimiento global fue casi cuantitativo.

Tabla 1. Resumen de los resultados de pegilación de EPOsf

| Muestra                   | Proteína (mg) | Rendimiento (%) |
|---------------------------|---------------|-----------------|
| Mezcla de reacción        | 30            | 100             |
| Mono-                     | 12,0          | 40              |
| Di-                       | 11,4          | 38              |
| No modificada             | 6,0           | 20              |
| Mezcla del conjuga-<br>do | 23,4          | 78              |

5 EJEMPLO 3: Pegilación de EPO con mPEG-SPA

Una alícuota diferente de EPOsf utilizada en el Ejem-  
plo 2 se hizo reaccionar con metoxi-PEG-SPA de 30 kDa  
(Shearwater Polymers, Inc., Huntsville, Alabama). La reac-  
ción se llevó a cabo a una relación de proteína: reactivo  
10 de 1: 2 y las técnicas de purificación utilizadas están de  
acuerdo con el Ejemplo 2. Se produjeron principalmente las  
especies mono-pegiladas.

EJEMPLO 4: Actividad in vivo de EPO pegilada determinada  
15 mediante el ensayo normocitémico de ratón.

El bioensayo normocitémico de ratón es conocido en el  
campo (Pharm. Europa Spec. Issue Erythropoietin BRP Bio  
1997(2)) y se describe un método en la monografía de eri-  
tropoyetina de Ph. Eur. BRP. Las muestras se diluyeron con  
20 BSA-PBS. A ratones sanos normales, de 7-15 semanas de edad,  
se les administraron s.c. 0,2 ml de la fracción de EPO que

contenía EPO no pegilada o EPO tri-, di- o mono-pegilada del ejemplo 2 ó 3. Durante un período de 6 días, se les extrajo sangre por punción de la vena de la cola y se diluyó de forma que 1  $\mu$ l de sangre se encontraba presente en 1 ml de una solución de tinción naranja de acridina 0,15  $\mu$ mol. El tiempo de tinción fue de entre 3 y 10 minutos. Los conteos de reticulocito se llevaron a cabo de forma microfluorométrica en un citómetro de flujo mediante análisis del histograma de fluorescencia rojo. Los conteos de reticulocito se dieron en términos de figuras absolutas (por 30.000 células sanguíneas analizadas). Para los datos presentados, cada grupo consistió en 5 ratones por día, y los ratones fueron sangrados sólo una vez.

En experimentos separados, se administraron a ratones una única dosis de EPO no modificada (25 ng de EPO), la mezcla de PEG(SBA)-EPO del ejemplo 2 (10 ng de conjugado), EPOs mono- y di-pegiladas del Ejemplo 2 (10 ng del conjugado), el PEG(SPA)-EPO del Ejemplo 3 (10 ng del conjugado) y solución tampón. Los resultados se muestran en la Tabla 2. Los resultados muestran una mayor actividad y una vida media prolongada de las especies de EPO pegiladas, lo cual viene indicado por las cantidades significativamente incrementadas de reticulocitos y el cambio del máximo número de reticulocitos usando la misma dosis por ratón (10 ng), comparado con una dosis de 25 ng para EPO no modificado.



TABLA 2

|       | EPO<br><sup>25ng</sup><br>(No modi-<br>ficado) | SPA<br>PEG 30<br>kDa | Mono SBA<br>de 30 K | Di<br>SBA<br>30 K | Mezcla Con-<br>jugado PEG-<br><sup>10ng</sup><br>EPO SBA | Tampón<br>Con-<br>trol |
|-------|------------------------------------------------|----------------------|---------------------|-------------------|----------------------------------------------------------|------------------------|
|       |                                                |                      |                     |                   |                                                          |                        |
| 72 h  | 1000                                           | 1393                 | 1411                | 994               | 1328                                                     | 857                    |
| 96 h  | 500                                            | 1406                 | 1501                | 926               | 1338                                                     | 697                    |
| 120 h | ~200                                           | 1100                 | 1182                | 791               | 944                                                      | 701                    |
| 144 h | ~0                                             | 535                  | 607                 | 665               | 660                                                      | 708                    |
|       |                                                |                      |                     |                   |                                                          |                        |

EJEMPLO 5: Preparación de mono-PEG-EPO predominante

5 Reacción de Pegilación

Se inició con 100 mg (5,48 µmol) de EPOsf en tampón de fosfato de potasio pH 7,5, preparado de acuerdo con el Ejemplo 1, se anadió 329 mg (10,96 µmol) de reactivo de PEG-SBA 30 kDa disueltos en 30 ml de HCl 1mM. Se añadió el suficiente tampón de fosfato de potasio 100 mM para llevar el volumen de la mezcla de reacción a 20 ml. La concentración de proteína final fue de 5 mg/ml y la relación de proteína: reactivo de PEG fue de 1:2. La mezcla de reacción se mezcló durante 2 h a temperatura ambiente -(20-2 °C). Tras 2h, la reacción se paró mediante el ajuste del pH a 4,5 con ácido acético glacial y se conservó congelada a -20°C hasta su purificación.

### Purificación

La mezcla de reacción del paso anterior se diluyó 1:5 con acetato de sodio 10 mM, pH 4,5 y se aplicó a 300 ml de SP-Sefarosa FF (resina de intercambio catiónico de sulfopropilo) empaquetada en una columna de 4,2 x 19 cm. La columna se equilibró previamente con el mismo tampón. Se hizo un seguimiento de los efluyentes de la columna a 280 nm con un monitor UV Gilson y se grabó con un grabador Kipp y Zonnen. La columna se lavó con 300 ml o un volumen de residencia de tampón de equilibrado para eliminar los reactivos en exceso, los productos colaterales de reacción y PEG-EPO oligoméricos. Esto fue seguido por un lavado con 2 volúmenes de residencia de NaCl 100 mM para eliminar di-PEG-EPO. El Mono-PEG-EPO fue entonces eluído con NaCl 200 mM. Durante la elución de mono-PEG-EPO, los primeros 50 ml del pico de proteína fueron eliminados y el mono-PEG-EPO fue recogido como una fracción de 150 ml. Las EPOsf no modificadas que permanecieron en la columna fueron eluídas con NaCl 750 mM. Todos los tampones de elución se elaboraron con tampón de equilibrado. Todas las muestras eluídas se analizaron mediante SDS-PAGE y mediante Cromatografía de Exclusión por tamaño de alta resolución (SEC). El conjunto de mono-PEG-EPO obtenido de la fracción de 150 ml, que no presentaba EPOsf no modificado detectable, fue entonces concentrado a entre ~4.5 - 7,5 mg/ml y diafiltrado en tampón de conservación, fosfato de potasio 10 mM, NaCl 100 mM, pH 7,6. La concentración/ Diafiltración se llevó a cabo con el Milli-

pore Labscale™ TFF System con una membrana de tamaño de poro de 50 kDa off Millipore Pellicon XL Biomax 50 a temperatura ambiente. El mono-PEG-EPO concentrado se filtró para quedar estéril y se conservó a -20 °C.

- 5       Aproximadamente el 75% de las EPOsf se pegilaron. Tras la purificación, el rendimiento total fue de ~30% de mono-PEG-EPO con EPOsf no modificada no detectable y alrededor del 25% de di-PEG-EPO. Oligómeros y EPOsf no pegilada quedaron en la proteína remanente. El mono-PEG-EPO obtenido
- 10   de la fracción de 150 ml contuvo aproximadamente 90% de mono-PEG-EPO y aproximadamente el 10% de di-PEG-EPO.

LISTADO DE SECUENCIAS

(1) INFORMACIÓN GENERAL:

(i) SOLICITANTE:

5 (A) NOMBRE: F.Hoffmann-La Roche AG  
(B) CALLE: 124 Grenzacherstrasse  
(C) CIUDAD: Basilea  
(E) PAÍS: Suiza  
(F) CÓDIGO POSTAL (ZIP): CH-4070  
10 (G) TELÉFONO: (61) 688 11 11  
(H) FAX: (61) 688 13 95  
(I) TELEX: 962 292 hlr ch

(ii) TÍTULO DE LA INVENCIÓN: Conjugados de Eritropoye-  
15 tina

(iii) NÚMERO DE SECUENCIAS: 3

(iv) FORMA LEGIBLE POR COMPUTADOR:

(A) TIPO DE SOPORTE: Disco flexible  
(B) ORDENADOR: IBM PC compatible  
20 (C) SISTEMA DE OPERACIÓN: WORD  
(D) SOFTWARE: PatentIn Release 2.0

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| 5  | Ala | Pro | Pro | Arg | Leu | Ile | Cys | Asp | Ser | Arg | Val | Leu | Glu |
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|    | Arg | Tyr | Leu | Leu | Glu | Ala | Lys | Glu | Ala | Glu | Asn | Ile | Thr |
|    |     | 15  |     |     |     |     | 20  |     |     |     |     | 25  |     |
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| 10 |     |     | 30  |     |     |     |     | 35  |     |     |     |     |     |
|    | Thr | Val | Pro | Asp | Thr | Lys | Val | Asn | Phe | Tyr | Ala | Trp | Lys |
|    | 40  |     |     |     |     | 45  |     |     |     |     | 50  |     |     |
|    | Arg | Met | Glu | Val | Gly | Gln | Gln | Ala | Val | Glu | Val | Trp | Gln |
|    |     |     | 55  |     |     |     |     | 60  |     |     |     | 65  |     |
| 15 | Gly | Leu | Ala | Leu | Leu | Ser | Glu | Ala | Val | Leu | Arg | Gly | Gln |
|    |     |     |     |     | 70  |     |     |     |     |     | 75  |     |     |
|    | Ala | Leu | Leu | Val | Asn | Ser | Ser | Gln | Pro | Trp | Glu | Pro | Leu |
|    |     | 80  |     |     |     |     | 85  |     |     |     |     | 90  |     |
|    | Gln | Leu | His | Val | Asp | Lys | Ala | Val | Ser | Gly | Leu | Arg | Ser |
| 20 |     |     | 95  |     |     |     |     |     | 100 |     |     |     |     |
|    | Leu | Thr | Thr | Leu | Leu | Arg | Ala | Leu | Gly | Ala | Gln | Lys | Glu |
|    | 105 |     |     |     |     | 110 |     |     |     |     | 115 |     |     |
|    | Ala | Ile | Ser | Pro | Pro | Asp | Ala | Ala | Ser | Ala | Ala | Pro | Leu |
|    |     |     | 120 |     |     |     |     | 125 |     |     |     | 130 |     |
| 25 | Arg | Thr | Ile | Thr | Ala | Asp | Thr | Phe | Arg | Lys | Leu | Phe | Arg |
|    |     |     |     |     | 135 |     |     |     |     |     | 140 |     |     |
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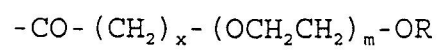
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25

Pro Gln

REIVINDICACIONES

1. Un conjugado de eritropoyetina, dicho conjugado comprende una glucoproteína, la eritropoyetina, que presenta al menos un grupo amino libre, (que presenta una actividad biológica *in vivo* con la que consigue que las células de la médula ósea incrementen la producción de reticulocitos y glóbulos rojos y que se selecciona del grupo de la eritropoyetina humana y análogos de la misma que presenten la secuencia de la eritropoyetina humana modificada mediante la adición de entre 1 y 6 sitios de glicosilación o mediante una nueva disposición de al menos un sitio de glicosilación; dicha glucoproteína se encuentra unida covalentemente a "n" grupos de poli(etilenglicol) de la fórmula



con el -CO de cada grupo de poli(etilenglicol) formando un enlace amida con uno de dichos grupos amino; en donde

R es alquilo inferior;

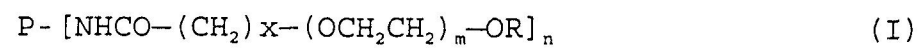
x es 2 ó 3;

m se encuentra entre alrededor de 450 y alrededor de 900;

n se encuentra entre 1 y 3; y

n y m se eligen de modo que el peso molecular del conjugado menos la glucoproteína eritropoyetina se encuentre entre 20 kilodaltons y 100 kilodaltons.

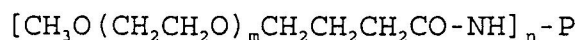
2. El conjugado de la reivindicación 1, de la fórmula:





en donde x, m, n y R son como se han definido en la reivindicación 1, y P es el residuo de la glucoproteína sin el grupo o grupos n amino que forman el enlace o enlaces con el grupo o grupos de poli(etilen glicol).

- 5 3. El conjugado de cualquier reivindicación precedente, en donde R es metilo.
4. El conjugado de cualquier reivindicación precedente, en donde m está comprendido entre 650 y 750.
5. El conjugado de cualquier reivindicación precedente, en  
10 donde n es 1.
6. El conjugado de cualquier reivindicación precedente, en donde R es metilo; m está comprendido entre 650 y 750; y n es 1.
7. El conjugado de cualquier reivindicación precedente que  
15 presente la fórmula



en donde m se encuentra entre 650 y 750, n es 1 y P es como se ha definido en la reivindicación 1.

8. El conjugado de cualquier reivindicación precedente, en  
20 donde la glicoproteína es una eritropoyetina humana.
9. El conjugado de acuerdo con cualquiera de las reivindicaciones 1 a 7, en donde la glucoproteína eritropoyetina humana es expresada mediante activación génica endógena.
10. El conjugado de acuerdo con cualquiera de las reivindi-  
25 caciones 1 a 9, en donde la glucoproteína presenta la secuencia SEQ ID NO:1.
11. El conjugado de acuerdo con cualquiera de las reivindicaciones 1 a 8, en donde la glucoproteína tiene la se-

cuencia de la eritropoyetina humana modificada mediante la adición de entre 1 y 6 sitios de glicosilación.

12. El conjugado de acuerdo con cualquiera de las reivindicaciones 1 a 11, en donde la glucoproteína tiene la secuencia de la eritropoyetina humana modificada mediante una modificación seleccionada del grupo que consiste en:

Asn<sup>30</sup>Thr<sup>32</sup>;

Asn<sup>51</sup>Thr<sup>53</sup>;

Asn<sup>57</sup>Thr<sup>59</sup>;

10 Asn<sup>69</sup>;

Asn<sup>69</sup>Thr<sup>71</sup>;

Ser<sup>68</sup>Asn<sup>69</sup>Thr<sup>71</sup>;

Val<sup>87</sup>Asn<sup>88</sup>Thr<sup>90</sup>;

Ser<sup>87</sup>Asn<sup>88</sup>Thr<sup>90</sup>;

15 Ser<sup>87</sup>Asn<sup>88</sup>Gly<sup>89</sup>Thr<sup>90</sup>;

Ser<sup>87</sup>Asn<sup>88</sup>Thr<sup>90</sup>Thr<sup>92</sup>;

Ser<sup>87</sup>Asn<sup>88</sup>Thr<sup>90</sup>Ala<sup>162</sup>;

Asn<sup>69</sup>Thr<sup>71</sup>Ser<sup>87</sup>Asn<sup>88</sup>Thr<sup>90</sup>;

Asn<sup>30</sup>Thr<sup>32</sup>Val<sup>87</sup>Asn<sup>88</sup>Thr<sup>90</sup>;

20 Asn<sup>89</sup>Ile<sup>90</sup>Thr<sup>91</sup>;

Ser<sup>87</sup>Asn<sup>89</sup>Ile<sup>90</sup>Thr<sup>91</sup>;

Asn<sup>136</sup>Thr<sup>138</sup>;

Asn<sup>138</sup>Thr<sup>140</sup>;

Thr<sup>125</sup>; y

25 Pro<sup>124</sup>Thr<sup>125</sup>.

13. El conjugado de acuerdo con cualquiera de las reivindicaciones 1 a 12, en donde la glucoproteína presenta una secuencia que comprende la secuencia de la eritropoyeti-

na humana y una segunda secuencia en el extremo carboxi-  
lo de la secuencia de la eritropoyetina humana, en donde  
la segunda secuencia contiene al menos un sitio de gli-  
cosilación.

5 14. El conjugado de la reivindicación 13, en donde la se-  
gunda secuencia comprende una secuencia derivada de la  
secuencia del extremo carboxilo de la gonadotropina co-  
riónica humana.

10 15. El conjugado de la reivindicación 13, en donde la glu-  
coproteína presenta una secuencia seleccionada del gru-  
po que consiste en:

(a) la secuencia de la eritropoyetina humana y la  
secuencia SEQ ID NO:3 en el extremo carboxilo de  
la secuencia de la eritropoyetina humana;

15 (b) la secuencia en (a) modificada por Ser<sup>87</sup> Asn<sup>88</sup>  
Thr<sup>90</sup>; y

(c) la secuencia en (a) modificada por Asn<sup>30</sup> Thr<sup>32</sup>  
Val<sup>87</sup> Asn<sup>88</sup> Thr<sup>90</sup>.

20 16. El conjugado de acuerdo con cualquiera de las reivindi-  
caciones 1 a 7, en donde la glucoproteína tiene la se-  
cuencia de la eritropoyetina humana modificada mediante  
la nueva disposición de al menos un sitio de glicosila-  
ción.

25 17. El conjugado de la reivindicación 16, en donde la nueva  
disposición comprende la delección de cualquiera de los  
sitios de glicosilación con unión N en la eritropoyetina  
humana y la adición de un sitio de glicosilación con

unión N en la posición 88 de la secuencia de la eritropoyetina humana.

18. El conjugado de la reivindicación 17, en donde la glucoproteína tiene la secuencia de la eritropoyetina humana modificada mediante una modificación seleccionada del grupo que consiste en:

Gln<sup>24</sup> Ser<sup>87</sup> Asn<sup>88</sup> Thr<sup>90</sup>;

Gln<sup>38</sup> Ser<sup>87</sup> Asn<sup>88</sup> Thr<sup>90</sup>; y

Gln<sup>83</sup> Ser<sup>87</sup> Asn<sup>88</sup> Thr<sup>90</sup>.

19. Una composición que comprende conjugados, cada uno de dichos conjugados comprendiendo una glucoproteína eritropoyetina que tiene al menos un grupo amino libre y que presenta una actividad biológica *in vivo* que hace que las células de la médula ósea incrementen la producción de reticulocitos y glóbulos rojos y que se seleccionan del grupo que consiste en eritropoyetina humana y análogos de la misma que tengan la secuencia de la eritropoyetina humana modificada mediante la adición de entre 1 y 6 sitios de glicosilación o una nueva disposición de al menos un sitio de glicosilación; la glucoproteína en cada uno de dichos conjugados está covalentemente unida a "n" grupos de poli(etilen glicol) de la fórmula  $-CO-(CH_2)_x(OCH_2CH_2)_m-OR$  con el  $-CO$  de cada grupo poli(etilen glicol) formando un enlace amida con uno de dichos grupos amino; en donde en cada uno de dichos conjugados R es alquilo inferior; x es 2 ó 3; m se encuentra entre 450 y alrededor de 900; n se encuentra entre 1 y 3; n y m son seleccionados de forma que el peso mole-

cular de cada conjugado menos la glucoproteína eritropoyetina se encuentre entre 20 kilodaltons y 100 kilodaltons; el porcentaje de conjugados donde n es 1 es al menos el noventa por ciento.

20. La composición que comprende conjugados tal como se define en cualquiera de las reivindicaciones 1 a 18, en donde el porcentaje de conjugados donde n es 1 es al menos el noventa por ciento.

21. La composición de acuerdo con las reivindicaciones 19 ó 20, en donde el porcentaje de conjugados donde n es 1 es al menos el noventa y dos por ciento.

22. La composición de la reivindicación 21 donde el porcentaje de conjugados donde n es 1 es al menos el noventa y seis por ciento.

23. La composición de la reivindicación 19 ó 20 en donde el porcentaje de conjugados donde n es 1 es entre el noventa por ciento y el noventa y seis por ciento.

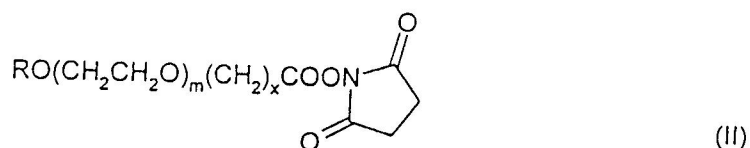
24. Una composición farmacéutica que comprende un conjugado o una composición de acuerdo con cualquiera de las reivindicaciones 1 a 23 y un excipiente farmacéuticamente aceptable.

25. Un conjugado o una composición de acuerdo con cualquiera de las reivindicaciones 1 a 23 para ser usado en la preparación de medicamentos para el tratamiento o profilaxis de enfermedades relacionadas con la anemia en pacientes con fallo renal crónico

empagado  
com.

(CRF), SIDA y para el tratamiento de pacientes de cáncer que han sido tratados con quimioterapia.

26. Un proceso para la preparación de compuestos de acuerdo con cualquiera de las reivindicaciones 1 a 23, cuyo proceso comprende la condensación del compuesto de la Fórmula II.



con la glucoproteína eritropoyetina y en la cual R, m y x son como se han definido en cualquiera de las reivindicaciones 1 a 6.

27. Compuestos de acuerdo con cualquiera de las reivindicaciones 1 a 3, siempre que se preparen mediante el proceso de la reivindicación 26.

28. Compuestos de acuerdo con cualquiera de las reivindicaciones 1 a 23 para el tratamiento de enfermedades asociadas con anemia en pacientes con fallo renal crónico (CRF), SIDA y pacientes con cáncer que han sido tratados con quimioterapia.

(21) No. SOLICITUD:

(51) INT. CL.:

(22) FECHA DE  
PRESENTACIÓN:

29-06-00

(71) SOLICITANTE: **F.HOFFMANN-LA ROCHE AG.**

(30) PRIORIDAD:

60/142.254  
60/150.225

(31) No. DE SOLICITUD:

60/151.548 y  
60/166.151

(72) INVENTOR/ES: **Pascal Sebastian Bailon**

(32) FECHA DE  
PRESENTACIÓN:

02-07-99, 23-08-99  
31-08-99 Y  
17-11-99

(33) PAÍS:

US, US, US y US

(74) APODERADO: **JOSE ANTONIO LLOREDA**

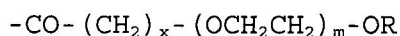
(54) TÍTULO:

**"DERIVADOS DE ERITROPOYETINA".**

(57) RESUMEN:

**Reivindicación número 1.-**

Un conjugado de eritropoyetina, dicho conjugado comprende una glucoproteína, la eritropoyetina, que presenta al menos un grupo amino libre, que presenta una actividad biológica *in vivo* con la que consigue que las células de la médula ósea incrementen la producción de reticulitos y glóbulos rojos y que se selecciona del grupo de la eritropoyetina humana y análogos de la misma que presenten la secuencia de la eritropoyetina humana modificada mediante la adición de entre 1 y 6 sitios de glicosilación o mediante una nueva disposición de al menos un sitio de glicosilación; dicha glucoproteína se encuentra unida covalentemente a "n" grupos de poli(etilenglicol) de la fórmula



con el -CO de cada grupo de poli(etilenglicol) formando un enlace amida con uno de dichos grupos amino; en donde

R es alquilo inferior;

X es 2 ó 3;

m se encuentra entre alrededor de 450 y alrededor de 900;

n se encuentra entre 1 y 3; y

n y m se eligen de modo que el peso molecular del conjugado menos la glucoproteína eritropoyetina se encuentre entre 20 kilodaltons y 100 kilodaltons.

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REPUBLICA DE COLOMBIA  
MINISTERIO DE RELACIONES EXTERIORES

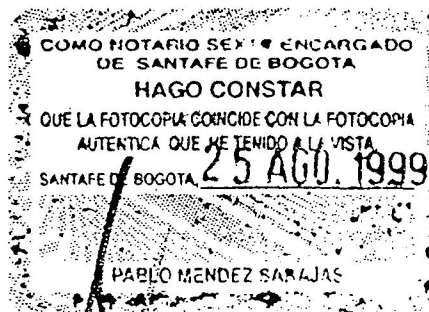
EL SUSCRITO JEFE DEL AREA DE  
LEGALIZACIONES DEL MINISTERIO DE  
RELACIONES EXTERIORES

CERTIFICA

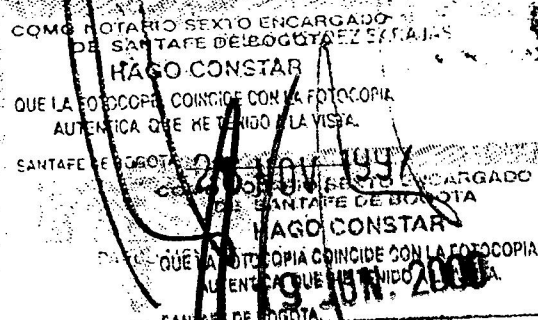
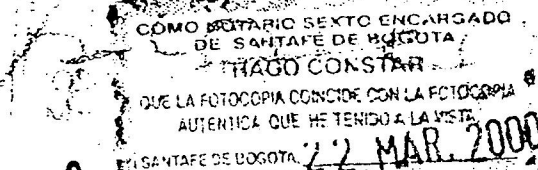
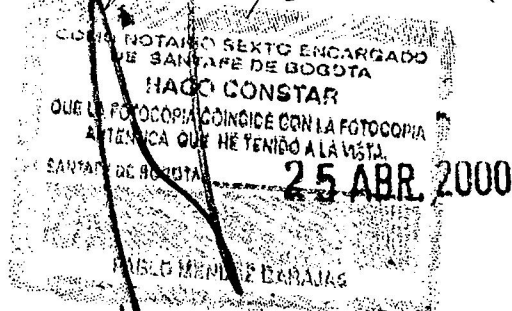
Que revisados los registros que se llevan en la oficina de Legalizaciones aparece la firma del señor **EDUARDO CALADO NOGUERA**, como traductor e intérprete oficial para los Idiomas INGLES- ESPAÑOL, ESPAÑOL- INGLES, según resolución 541 del 27 de febrero de 1976.

La anterior certificación se expide a solicitud del interesado quien se identificó con cédula de ciudadanía 17.177.588 expedida en Bogotá.

Dada en Santa Fe de Bogotá, D.C., a los seis (6) de noviembre de 1997.



*Blanchard*  
**BLANCA ACOSTA DE GARCIA**  
Jefe Area de Legalizaciones (e)





República de Colombia



Ministerio de Justicia y del Derecho

EL SECRETARIO GENERAL (E) DEL MINISTERIO DE  
JUSTICIA Y DEL DERECHO

CERTIFICA:

Que por Resolución 541 del 27 de febrero de 1976, le fue expedida licencia para ejercer funciones de Traductor e Intérprete Oficial en los idiomas INGLÉS y ESPAÑOL al señor EDUARDO CALADO NOGUERA, identificado con la cédula de ciudadanía No. 17.177.588 expedida en Bogotá.

Que dicha Resolución se encuentra vigente en la fecha.

Se expide la presente certificación a solicitud del interesado.

Dada en Santa Fe de Bogotá, D.C., a

14 OCT. 1997

ALVARO NAMEN VARGAS

ICM/mmc

COMO NOTARIO SEXTO ENCARGADO DE SANTAFE DE BOGOTA  
HAGO CONSTAR  
QUE LA FOTOCOPIA COINCIDE CON LA FOTOCOPIA AUTENTICA QUE HE TENIDO A LA VISTA.  
SANTAFE DE BOGOTA, 25 ABR. 2000  
PABLO MENDEZ BARRAJAS

COMO NOTARIO SEXTO ENCARGADO DE SANTAFE DE BOGOTA  
HAGO CONSTAR  
QUE LA FOTOCOPIA COINCIDE CON LA FOTOCOPIA AUTENTICA QUE HE TENIDO A LA VISTA.  
SANTAFE DE BOGOTA, 26 NOV. 1997

COMO NOTARIO SEXTO ENCARGADO DE SANTAFE DE BOGOTA  
HAGO CONSTAR  
QUE LA FOTOCOPIA COINCIDE CON LA FOTOCOPIA AUTENTICA QUE HE TENIDO A LA VISTA.  
SANTAFE DE BOGOTA, 2 MAR. 2000

COMO NOTARIO SEXTO ENCARGADO DE SANTAFE DE BOGOTA  
HAGO CONSTAR  
QUE LA FOTOCOPIA COINCIDE CON LA FOTOCOPIA AUTENTICA QUE HE TENIDO A LA VISTA.  
SANTAFE DE BOGOTA, 19 JUN. 2000  
PABLO MENDEZ BARRAJAS

Junio 15, 2000  
Traducción Oficial No.0272  
REF:F. HOFFMANN-LA ROCHE AG  
Patente No.60/142,254

F. Hoffmann  
Caso-20431

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DEPARTAMENTO DE COMERCIO DE LOS ESTADOS UNIDOS  
OFICINA DE MARCAS Y PATENTES

Mayo 01, 2000

Por medio del presente documento se certifica que adjunta se encuentra una fiel copia de los registros de la Oficina de Marcas y Patentes de los Estados Unidos, correspondientes a los documentos para la solicitud tal como se presentaron, con el fin de asignarle una fecha de registro bajo la norma del código 35 USC 111.

Número de Solicitud: 60/142,254

Fecha de Registro: Julio 02, 254

Autorizado por:

COMISIONADO DE MARCAS Y PATENTES

Firma: T Lawrence

Funcionario Encargado de Certificaciones

Hay sello realizado de la Oficina de Marcas y Patentes de color dorado.

*Edw. Lawrence*  
-16-1000-  
Res. 511

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CERTIFICACION BAJO EL CODIGO 37 Codigo de Regulaciones  
federales 1.10

Yo por medio del presente documento certifico que éste ha sido enviado por Correo Postal - "CORREO POSTAL AL DESTINATARIO" de los Estados Unidos el 31 de Agosto de 1999, en un sobre rotulado al "Assistant Commissioner of Patents", con el número de código EL275412751US Washington, DC., 20231.

Nombre del depositante: PAMELA JOHNSTON

Firma: PAMELA JOHNSTON

HOJA DE SOLICITUD PROVISIONAL PARA PATENTE

Esta es una petición para registrar una SOLICITUD PROVISIONAL bajo el código 37 CFR § 1.53 (b)(2):

Número de Caso: 1097P

INVENTOR(ES) / SOLICITANT(ES)

NOMBRE: Bailon, Pascal Sebastian

RESIDENCIA: Florham Park, Morris County, new Jersey

OFICINA CORREO POSTAL: 21 Woodbine Road

Florham Park, New Jersey 07932

*Edwardo Calzado*  
Firma  
Res 541

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☐ Los inventores adicionales se encuentran mencionados en una hoja adjunta a la presente.

TITULO DE LA INVENCION  
**CONJUGADOS DE ERYTHROPOIETIN**

---

DIRECCION PARA CORRESPONDENCIA

George W. Jonhston

Hoffmann-La Roche Inc.

340 Kingsland Street

Nutley, NJ 07110

PARTES DE LA SOLICITUD INCLUIDAS

(Verificar que todas apliquen)

|                                                          |                                                        |
|----------------------------------------------------------|--------------------------------------------------------|
| <input checked="" type="checkbox"/> ESPECIFICACIONES     | <input type="checkbox"/> [20] Número de Páginas        |
| <input checked="" type="checkbox"/> REIVINDICACIONES     | No. de Reivindicaciones 28 (4 páginas)                 |
| <input checked="" type="checkbox"/> LISTADO DE SECUENCIA | <input type="checkbox"/> [2] Número de Páginas         |
| <input checked="" type="checkbox"/> Declaración          | <input type="checkbox"/> [4] Número de Páginas         |
| <input type="checkbox"/> GRAFICA (S)                     | <input type="checkbox"/> [ ] Hojas de Gráficas         |
| <input checked="" type="checkbox"/> Otros                | <input type="checkbox"/> [ ] <u>Resumen (1 página)</u> |

FORMA DE DE PAGO DE HONORARIOS PARA LA SOLICITUD PROVISIONAL DE  
ESTA PATENTE (Señalar uno)

*Edwardo Calado*

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☒ El comisionado por la presente se encuentra autorizado a cobrar honorarios de registro y crédito al número de Cuenta Bancaria **08-2525**

Honorarios provisionales para registro = \$150.000

La invencion fué realizada por una agencia del Gobierno de los Estados Unidos o bajo contrato con una agencia del Gobierno de los Estados Unidos.

☒ No

☐ Sí, el nombre de la Agencia del Gobierno de los Estados Unidos y número de contrato son los siguientes:

Presentado atentamente,

FIRMA: Lewis J. Kreisler

No. de Registro: 38522

Telefono: (973) 235-4387

Telefax: (973) 235-2363

Fecha: Agosto 33, 1999

*Eduardo Calado*  
EDUARDO CALADO  
ABOGADO  
CALLE 511 TORRE 10

## REIVINDICACIONES :

1. Un conjugado, dicho conjugado consta de glicoproteína eritropoietin el cual tiene por lo menos un grupo de amino ácidos, el cual tiene una actividad biológica *in vivo* la cual produce en las células en la médula del hueso un incremento en la producción de reticulocitos y célula y glóbulos rojos, seleccionados de un grupo que consiste de eritropoietinas y análogos del mismo los cuales tienen una secuencia de eritropoietinas humanas modificadas mediante la adición desde 1 hasta 6 sitios de glicosilaciones o un reordenamiento de por lo menos un sitio de glicocilación; dicha glicoproteína siendo enlazada de covalentemente a grupos de “n” poli(etileno glicol) de la formula  $-CO-(CH_2)_x-(OCH_2CH_2)_m-$  o OR con el  $-CO$  de cada grupo de poli(etileno glicol) formando un enlace de amida con uno de los grupos mencionados grupos de amino; en el cual R es bajo alquilo; x es 2 o 3; m es aproximadamente desde 450 hasta alrededor de 450 hasta aproximadamente 900; n es desde 1 hasta 3; y n y m son escogidos para que el peso molecular del de negativo conjugado de la glicoproteína eritropoietina sea de 20 kilodaltones hasta 100 kilodaltones.

Edwards Paladea

2. El conjugado de acuerdo a la reivindicación 1, de la formula:



en el cual m, n y R son como anteriormente se definieron, y P es un residuo de la glicoproteína sin el grupo(s) n de amino, cuya forma de amidos enlaza con el grupo de poli(etileno glicol).

3. El conjugado de acuerdo a la reivindicación 2, en el cual la glicoproteína es un erithropoietina humana.

4. El conjugado de acuerdo a la reivindicación 3, en el cual la glicoproteina tiene una secuencia SEQ ID NO:1.

5. El conjugado de acuerdo a la reivindicación 4, en el cual la R es metilo.

6. El conjugado de acuerdo a la reivindicación 4, en el cual m es desde aproximadamente 650 hasta alrededor de 750.

Edvarado Calado

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7. El conjugado de acuerdo a la reivindicación 4, en la cual la n es 1.
8. El conjugado de acuerdo a la reivindicación 4, en la cual R es metilo; m es aproximadamente desde 650 hasta 750; y n es 1.
9. El conjugado de acuerdo a la reivindicación 2, en la cual la glicoproteína tiene una secuencia de eritropoietina humana modificada por la adición de aproximadamente desde 1 hasta 6 sitios de glicosilación.
10. El conjugado de acuerdo a la reivindicación 9, en la cual la glicoproteína tiene una secuencia de eritropoietina humana modificada mediante una modificación seleccionada de un grupo que consiste de:

Asn30 Thr32;

Asn51 Thr53;

Asn57 Thr59;

Asn69;

Asn69 Thr71

*Edwards Calado*



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Ser68Asn69 Thr71

Val87 Asn88 Thr90;

Ser87Asn88 Thr90

Ser87 Asn88 Gly89 Thr90;

Ser87 Asn88 Thr90 Thr92;

Ser87 Asn88 Thr90 Ala162;

Asn69 Thr71 Ser87 Asn88 Thr90;

Asn30 Thr32 Val87 Asn88 Thr90;

Asn89 Ile90 Thr91;

Ser87 Asn89 Ile90 Thr91;

Asn136 Thr138;

Asn138 Thr138;

Asn138 Thr140;

Thr125; y

Pro124Thr125.

11. El conjugado de acuerdo a la reivindicación 10, en el cual R es metilo.

12. El conjugado de acuerdo a la reivindicación 10, en el cual m es aproximadamente desde 650 hasta alrededor de 750.

*Eduardo Calabok*  
Res

13. El conjugado de acuerdo a la reivindicación 10, en el cual n es 1.

14. El conjugado de acuerdo a la reivindicación 10, en el cual R es metilo; m es desde aproximadamente 650 hasta alrededor de 750; y n es 1.

15. El conjugado de acuerdo a la reivindicación 9, en el cual la glicoproteína tiene una secuencia que consiste de una eritropoietina humana y una segunda secuencia en el terminus de carboxi de una secuencia de una eritropoietina humana, en la cual la segunda secuencia contiene por lo menos un sitio de glicosilación.

16. El conjugado de acuerdo a la reivindicación 15, en el cual la segunda secuencia consiste de una secuencia que se deriva de la secuencia terminal de carboxi de una gonadotropina corionica humana.

17. El conjugado de acuerdo a la reivindicación 16, en el cual la glicoproteína tiene una secuencia seleccionada desde un grupo que consiste de:

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- (a) la secuencia de erithrpoietina humana y la secuencia SEQ ID NO:3 en el terminus de carboxi de la secuencia arithropoietina humana;
- (b) la secuencia en (a) modificada por Ser87 Asn88 Thr90; y
- (c) la secuencia en (a) modificada por Asn30 Thr32 Val87 Asn88 Thr90.

18. El conjugado de acuerdo a la reivindicación 17, en el cual R es metilo.

19. El conjugado de acuerdo a la reivindicación 17, en el cual la m es aproximadamente desde 650 hasta alrededor de 750.

20. El conjugado de acuerdo a la reivindicación 17, en el cual n es 1.

21. El conjugado de acuerdo a la reivindicación 17, en el cual R es metilo; m es desde aproximadamente 650 hasta alrededor de 750; y n es 1.

*Eduardo Celis*

22. El conjugado de acuerdo a la reivindicación 2, en el cual la glicoproteína tiene la secuencia humana de la eritropoietina modificada para reordenamiento de por lo menos un sitio de glicosilación.

23. El conjugado de acuerdo a la reivindicación 22, en el cual la reordenación consiste en la anulación de cualquier sitio de enlace N de glicosilación en eritropoietina humana de un sitio de glicosilación en la posición 88 en la secuencia de eritropoietina humana.

24. El conjugado de acuerdo a la reivindicación 23, en el cual la glicoproteína tiene una secuencia de eritropoietina humana modificada por una modificación seleccionada de un grupo que consiste de:

Gln24 Ser87 Asn88 Thr90;

Gln38 Ser87 Asn88 Thr90; y

Gln83 Ser87 Asn88 Thr90.

25. El conjugado de acuerdo a la reivindicación 24, en el cual R es metilo.

*Eduardo Calado*  
11/12/2004

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26. El conjugado de acuerdo a la reivindicación 24, en el cual m es desde aproximadamente 650 hasta alrededor de 750.

27. El conjugado de acuerdo a la reivindicación 24, en el cual n es 1.

28. El conjugado de acuerdo a la reivindicación 24, en el cual la R es metilo; m es aproximadamente desde 650 hasta alrededor de 750; y n es 1.

*Eduardo Salado*

Caso:No1097P

DECLARACION Y PODER  
PARA LA SOLICITUD DE PATENTES

Yo en mi calidad de abajo mencionado inventor, por la presente  
declaro:

Mi residencia, dirección de Oficina Postal y ciudadanía son mencionados a  
continuación como también mi nombre.

Yo creo ser el único y primer inventor (si solo se encuentra un nombre en  
la lista) o original, primero y co-inventor del tópico en cuestión, el cual se  
reivindica pidiendo la solicitud de patente de invención con el título:

TITULO DE LA INVENCION (280 Caracteres máximo)

CONJUGADOS DE ERITHROPOIETINA

cuya especificaciones:

(Indique uno)

☒ se encuentra adjunto.

☐ Fue registrado en \_\_\_\_\_ como

No Serial de la Solicitud \_\_\_\_\_

y fue revisado en \_\_\_\_\_

*Eduardo Calado*  
Eduardo Calado  
12/10/1996

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(si aplica)

Yo por medio del presente documento declaro que he revisado y entiendo el contenido de las especificaciones anteriormente mencionadas, incluyendo las reivindicaciones.

Yo tengo conocimiento sobre mi obligación de revelar toda información que sea parte del material patentable tal como lo define el Artículo 37 C.F.R. §1.56.

Yo por la presente reivindico beneficios de prioridad bajo el Título 35, del Código de los Estados Unidos, Sección §119 (a) -(d) de cualquiera de la solicitud(es) extranjera para patente (s) o certificado como inventor o § 365 (a) de cualquier Solicitud para Patente Internacional (PCT), para la cual se ha nombrado por lo menos a un país además de los Estados Unidos, como es anteriormente mencionado e identificado, Indicando con una indicación en las cajillas , de cualquier solicitud extranjera para Patente certificado como inventor, o solicitud Internacional PCT con una fecha de registro anterior de aquella solicitud que aparece en la cual se reivindica prioridad.

Solicitudes extranjeras con una fecha de registro anterior (es)

Prioridades Reivindicadas

| _____    | _____  | _____                   | [ ] | [ ] |
|----------|--------|-------------------------|-----|-----|
| (Nombre) | (País) | Día/Mes/año de registro | Si  | No  |

*Eduardo Calado*

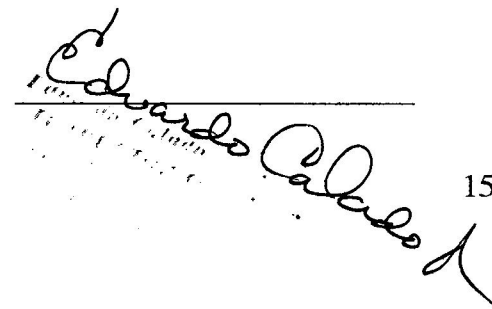
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|           |        |                         |     |     |
|-----------|--------|-------------------------|-----|-----|
| _____     | _____  | _____                   | [ ] | [ ] |
| (Nombre)  | (País) | Día/Mes/año de registro | Si  | No  |
| _____     | _____  | _____                   | [ ] | [ ] |
| (Nombre ) | (País) | Día/Mes/año de registro | Si  | No  |

Yo por medio de la presente reivindico los beneficios bajo 35 U.S.C. § 119 (e) sobre solicitudes anteriores de los Estados Unidos mencionadas a continuación:

|                 |                     |                |                     |
|-----------------|---------------------|----------------|---------------------|
| _____           | _____               | _____          | _____               |
| (No Solicitud ) | (Fecha de Registro) | (No Solicitud) | (Fecha de registro) |

Yo por medio de la presente reivindico beneficios bajo el Código 35 USC § 120 de solicitud de Patente registrada en los Estados Unidos, o §365(c) de cualquier solicitud Internacional de patentes PCT en la manera requeridas tal como se menciona anteriormente y respecto al tópico en cuestión de cada una de las reivindicaciones de esta solicitud la cual no se haya revelada con anterioridad en solicitudes anteriores en los Estados Unidos de acuerdo a lo establecido por el primer párrafo de la norma del Código 35CFR. § 112, tengo conocimiento de mi obligación de revelar toda información que se considere parte del material patentable a la Oficina de Marcas y Patentes tal como lo define la norma del Código 37 C.F.R. §1.56 el cual se hizo efectiva entre las fechas de registro de la solicitud anterior y la fecha de registro Internacional PCT de esta solicitud,

|       |       |                                                                                      |
|-------|-------|--------------------------------------------------------------------------------------|
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|-------|-------|--------------------------------------------------------------------------------------|



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(No Serial Solicitud) (Fecha de Registro) (estatus)(patentado,pendiente,  
abandonado)

\_\_\_\_\_  
(No Serial Solicitud) (Fecha de Registro) (Estatus)(patentado,pendiente,  
abandonado)

Yo, por la presente afirmo que toda declaración presentada es de mi entero conocimiento, fiel y verdadera y tengo presente que toda falsa declaración es castigada mediante una multa o el encarcelamiento, o ambos, bajo la Sección 1001 del Título 18 del Código de los Estados Unidos, y que cualquier declaración puede comprometer la validez de esta solicitud o de cualquier patente expedida inmediatamente después.

**PODER :**

Como Inventor, yo por la presente nombro al (los) siguiente (s) abogados y /o representante (s) para procesar esta solicitud y llevar a cabo toda transacción de negocios en la Oficina de Marcas y Patentes relacionadas con la presente. *(listado de nombres y números de registro)*

*Edwards*  
16

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No. de Registro

|                             |         |       |
|-----------------------------|---------|-------|
| GEORGE W. JOHNSTON          | Reg No. | 28090 |
| WILLIAM H. EPSTEIN          | Reg No. | 20008 |
| LEWIS J. KREISLER           | Reg No. | 38522 |
| DENNIS P. TRAMALONI         | Reg No. | 28542 |
| PATRICIA S. ROCHA-TRAMALONI | Reg No. | 31054 |
| JOHN P. PARISE              | Reg No. | 34403 |

ENVIAR LA CORRESPONDENCIA A:

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Ciudadanía:Estados Unidos de America

Oficina Postal:21 Woodbine Road, Florham Park, New Jersey 07932

Fecha: 7/2/99

*Eduardo Palacios*  
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De acuerdo al Título 37, del Código de Regulaciones Federales, § 1.56, tengo conocimiento sobre mi obligación de proteger y revelar información de material patentable el cual especifica que cada persona asociada con el registro y la continuación de la solicitud de patente tiene un deber de cumplimiento y buena fe en llevar a cabo todas las negociaciones con la Oficina de Patentes, la cual se debe proteger y revelar toda información de material patentable tal como lo define ésta sección. El deber de revelar toda información respecto a cada reivindicación pendiente hasta que la reivindicación sea cancelada o retirada o la solicitud sea abandonada.

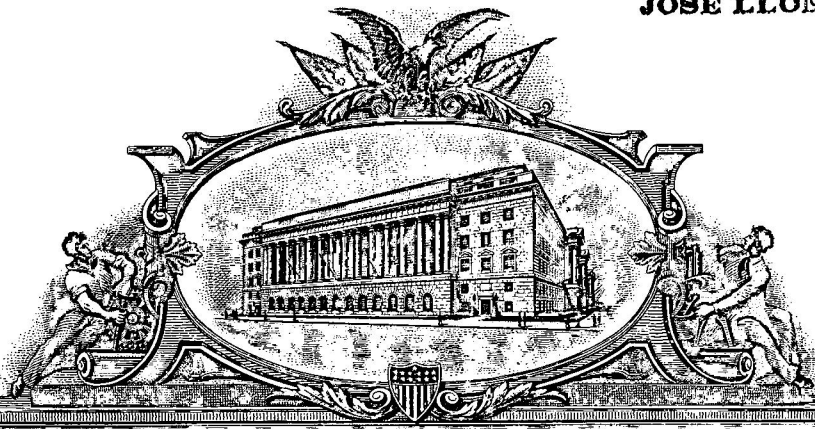
Bajo esta sección la información es material patentable hasta cuando no es cumulativo sobre información que aparezca en los registros o sean parte de los registros en la solicitud, y

- (1) Reconoce que por si sola o conjuntamente con otra información o pruebas suficientes de la patentabilidad de una reivindicación; o
- (2) Si refuta o es inconsistente con la posición de un solicitante:
  - (i) Oponerse a un argumento de no patentabilidad propuesto por la Oficina, o
  - (ii) Afirmación de un argumento sobre patentabilidad.

*Edw. Calcedo*

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PA 241706



# THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE

United States Patent and Trademark Office

May 01, 2000

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A FILING DATE UNDER 35 USC 111.

APPLICATION NUMBER: 60/142,254

FILING DATE: July 02, 1999



By Authority of the  
COMMISSIONER OF PATENTS AND TRADEMARKS

T. LAWRENCE  
Certifying Officer

CERTIFICATION UNDER 37 CFR 1.10

I hereby certify that this New Application Transmittal and the documents referred to as enclosed therein are being deposited with the United States Postal Service on this date July 2, 1999 in an envelope as "Express Mail Post Office to Addressee" Mailing Label Number EL275412751U Addressed to the: Assistant Commissioner for Patents, Washington, D.C. 20231.

Pamela Johnston  
(Print Name)

(Signature)

jc520 U.S. PTO  
60/142254  
07/02/99

PROVISIONAL APPLICATION COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION under 37 CFR 1.53(b)(2).

Docket No. 1097P

INVENTOR(s)/APPLICANT(s)

Name: Bailon, Pascal Sebastian  
Residence: Florham Park, Morris County, New Jersey  
Citizenship: U.S.A.  
Post Office Address: 21 Woodbine Road, Florham Park, New Jersey 07932

☐ Additional inventors are being named on sheets attached hereto

TITLE OF THE INVENTION

ERYTHROPOIETIN CONJUGATES

CORRESPONDENCE ADDRESS

George W. Johnston  
Hoffmann-La Roche Inc.  
340 Kingsland Street  
Nutley, NJ 07110

ENCLOSED APPLICATION PARTS (check all that apply)

|                                                      |                            |                                                 |                          |
|------------------------------------------------------|----------------------------|-------------------------------------------------|--------------------------|
| <input checked="" type="checkbox"/> Specification    | No. of Pages 20            | <input checked="" type="checkbox"/> Declaration | No. of Pages 4           |
| <input checked="" type="checkbox"/> Claims           | No. of Claims 28 (4 pages) | <input type="checkbox"/> Drawings               | Sheets of Drawings       |
| <input checked="" type="checkbox"/> Sequence Listing | No. of Pages 2             | <input checked="" type="checkbox"/> Other       | <u>Abstract (1 page)</u> |

METHOD OF PAYMENT

☒ The Commissioner is hereby authorized to charge any fees or credit any overpayment to Deposit Account Number: 08-2525

Provisional  
Filing Fee  
Amount \$150.00

DEPOSIT ACCOUNT  
NO. 08-2525  
OUR ORDER NO. 2618

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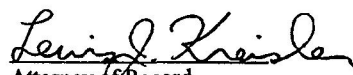
The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☒ No

☐ Yes, the name of the U.S. Government agency and the Government contract number are: \_\_\_\_\_

\_\_\_\_\_  
\_\_\_\_\_  
\_\_\_\_\_

Respectfully submitted,

  
Attorney of Record

Lewis J. Kreisler

(Reg. No. 38522)

Telephone: (973) 235-4387

Telefax: (973) 235-2363

Date: July 2, 1999

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## ERYTHROPOIETIN CONJUGATES

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### BACKGROUND OF THE INVENTION

Erythropoiesis is the production of red blood cells, which occurs to offset cell destruction. Erythropoiesis is a controlled physiological mechanism that enables sufficient red blood cells to be available for proper tissue oxygenation. Naturally occurring human erythropoietin (hEPO) is produced in the kidney and is the humoral plasma factor which stimulates red blood cell production (Carnot, P and Deflandre, C (1906) C.R. Acad. Sci. 143: 432; Erslev, AJ (1953 Blood 8: 349; Reissmann, KR (1950) Blood 5: 372; Jacobson, LO, Goldwasser, E, Freid, W and Plzak, LF (1957) Nature 179: 6331-4). Naturally occurring EPO stimulates the division and differentiation of committed erythroid progenitors in the bone marrow and exerts its biological activity by binding to receptors on erythroid precursors (Krantz, BS (1991) Blood 77: 419).

Erythropoietin has been manufactured biosynthetically using recombinant DNA technology (Egrie, JC, Strickland, TW, Lane, J et al. (1986) Immunobiol. 72: 213-224) and is the product of a cloned human EPO gene inserted into and expressed in the ovarian tissue cells of the chinese hamster (CHO cells). The primary structure of the predominant, fully processed form of hEPO is illustrated in SEQ ID NO:1. There are two disulfide bridges between Cys<sup>7</sup>-Cys<sup>161</sup> and Cys<sup>29</sup>-Cys<sup>33</sup>. The molecular weight of the polypeptide chain of EPO without the sugar moieties is 18,236 Da. In the intact EPO molecule, approximately 40% of the molecular weight are accounted for by the carbohydrate groups that glycosylate the protein at glycosylation sites on the protein (Sasaki, H, Bothner, B, Dell, A and Fukuda, M (1987) J. Biol. Chem. 262: 12059).

Because human erythropoietin is essential in red blood cell formation, the hormone is useful in the treatment of blood disorders characterized by low or defective red blood cell production. Clinically, EPO is used in the treatment of anemia in chronic

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renal failure patients (CRF) (Eschbach, JW, Egri, JC, Downing, MR et al. (1987) NEJM 316: 73-78; Eschbach, JW, Abdulhadi, MH, Browne, JK et al. (1989) Ann. Intern. Med. 111: 992; Egrie, JC, Eschbach, JW, McGuire, T, Adamson, JW (1988) Kidney Intl. 33: 262; Lim, VS, Degowin, RL, Zavala, D et al. (1989) Ann. Intern. Med. 110: 108-114)  
5 and in AIDS and cancer patients undergoing chemotherapy (Danna, RP, Rudnick, SA, Abels, RI In: MB, Garnick, ed. Erythropoietin in Clinical Applications-An International Perspective. New York, NY: Marcel Dekker; 1990: p. 301-324). However, the bioavailability of commercially available protein therapeutics such as EPO is limited by their short plasma half-life and susceptibility to protease degradation. These  
10 shortcomings prevent them from attaining maximum clinical potency.

### SUMMARY OF THE INVENTION

This invention provides an erythropoietin conjugate, said conjugate comprising  
15 an erythropoietin glycoprotein having at least one free amino group and having the *in vivo* biological activity of causing bone marrow cells to increase production of reticulocytes and red blood cells and selected from the group consisting of human erythropoietin and analogs thereof which have sequence of human erythropoietin modified by the addition of from 1 to 6 glycosylation sites or a rearrangement of at least  
20 one glycosylation site; said glycoprotein being covalently linked to "n" poly(ethylene glycol) groups of the formula  $-CO-(CH_2)_x-(OCH_2CH_2)_m-OR$  with the  $-CO$  (i.e. carbonyl) of each poly(ethylene glycol) group forming an amide bond with one of said amino groups; wherein R is lower alkyl; x is 2 or 3; m is from about 450 to about 900; n is from 1 to 3; and n and m are chosen so that the molecular weight of the conjugate  
25 minus the erythropoietin glycoprotein is from 20 kilodaltons to 100 kilodaltons.

Compared to unmodified EPO (i.e., EPO without a PEG attached) and conventional PEG-EPO conjugates, the present conjugates have an increased circulating half-life and plasma residence time, decreased clearance, and increased clinical activity *in*  
30 *vivo*. The conjugates of this invention have the same uses as EPO. In particular, the conjugates of this invention are useful to treat patients by stimulating the division and



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differentiation of committed erythroid progenitors in the bone marrow in the same way EPO is used to treat patients.

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## DETAILED DESCRIPTION OF THE INVENTION

This invention provides conjugates, said conjugates comprising an erythropoietin glycoprotein having at least one free amino group and having the *in vivo* biological activity of causing bone marrow cells to increase production of reticulocytes and red blood cells and selected from the group consisting of human erythropoietin and  
10 analogs thereof which have sequence of human erythropoietin modified by the addition of from 1 to 6 glycosylation sites or a rearrangement of at least one glycosylation site; said glycoprotein being covalently linked to "n" poly(ethylene glycol) groups of the formula  $-\text{CO}-(\text{CH}_2)_x-(\text{OCH}_2\text{CH}_2)_m-\text{OR}$  with the  $-\text{CO}$  (i.e. carbonyl) of each  
15 poly(ethylene glycol) group forming an amide bond with one of said amino groups; wherein R is lower alkyl; x is 2 or 3; m is from about 450 to about 900; n is from 1 to 3; and n and m are chosen so that the molecular weight of the conjugate minus the erythropoietin glycoprotein is from 20 kilodaltons to 100 kilodaltons.

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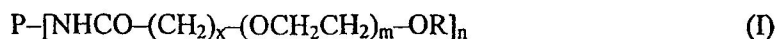
It has been found that the conjugates of this invention can be used in the same manner as unmodified EPO. However, the conjugates of this invention have an increased circulating half-life and plasma residence time, decreased clearance, and increased clinical activity *in vivo*. Because of these improved properties, the conjugates of this invention can be administered once weekly instead of the three times weekly for  
25 unmodified EPO. Decreased frequency of administration is expected to result in improved patient compliance leading to improved treatment outcomes, as well as improved patient quality of life. Compared to conventional conjugates of EPO linked to poly(ethylene glycol) it has been found that conjugates having the molecular weight and linker structure of the conjugates of this invention have an improved potency, stability,  
30 AUC, circulating half-life, and cost of goods profile.

The conjugates in accordance of this invention can be administered in a therapeutically effective amount to patients in the same way EPO is administered. The therapeutically effective amount is that amount of conjugate necessary for the *in vivo* biological activity of causing bone marrow cells to increase production of reticulocytes and red blood cells. The exact amount of conjugate is a matter of preference subject to such factors as the exact type of condition being treated, the condition of the patient being treated, as well as the other ingredients in the composition. The pharmaceutical compositions containing the conjugate may be formulated at a strength effective for administration by various means to a human patient experiencing blood disorders characterized by low or defective red blood cell production. Average therapeutically effective amounts of the conjugate may vary and in particular should be based upon the recommendations and prescription of a qualified physician.

The erythropoietin glycoprotein products prepared in accordance with this invention may be prepared in pharmaceutical compositions suitable for injection with a pharmaceutically acceptable carrier or vehicle by methods known in the art. Among the preferred pharmaceutically acceptable carriers for formulating the products of the invention are human serum albumin, human plasma proteins, etc.

The term "erythropoietin" or "EPO" refers to a glycoprotein, having the amino acid sequence set out in (SEQ ID NO: 1) or (SEQ ID NO: 2) or an amino acid sequence substantially homologous thereto, whose biological properties relate to the stimulation of red blood cell production and the stimulation of the division and differentiation of committed erythroid progenitors in the bone marrow. As used herein, these terms include such proteins modified deliberately, as for example, by site directed mutagenesis or accidentally through mutations. These terms also include analogs having from 1 to 6 additional sites for glycosylation, analogs having at least one additional amino acid at the carboxy terminal end of the glycoprotein, wherein the additional amino acid includes at least one glycosylation site, and analogs having an amino acid sequence which includes a rearrangement of at least one site for glycosylation. These terms include both natural and recombinantly produced human erythropoietin.

The erythropoietin conjugates of this invention can be represented by Formula 1:



5 wherein x, m, n and R are as above. In Formula I, P is the residue of an erythropoietin glycoprotein described herein, (i.e. without the amino group or amino groups which form an amide linkage with the carbonyl shown in Formula I), having the *in vivo* biological activity of causing bone marrow cells to increase production of reticulocytes and red blood cells. P may be selected from the group consisting of residues of human  
10 erythropoietin and analogs thereof having from 1 to 6 additional sites for glycosylation. As set out in detail below, the preparation and purification of EPO are well known in the art. By EPO is meant the natural or recombinant protein, preferably human, as obtained from any conventional source such as tissues, protein synthesis, cell culture with natural or recombinant cells. Any protein having the activity of EPO, such as muteins or  
15 otherwise modified proteins, is encompassed. Recombinant EPO may be prepared via expression in CHO-, BHK- or HeLa cell lines, by recombinant DNA technology or by endogenous gene activation. The preferred EPO species for the preparation of erythropoietin glycoprotein products are human EPO species. More preferably, the EPO species is the human EPO having the amino acid sequence set out in SEQ ID NO:1 or  
20 SEQ ID NO:2, more preferably the amino acid sequence SEQ ID NO:1.

In an embodiment, P may be the residue of a glycoprotein analog having from 1 to 6 additional sites for glycosylation. Glycosylation of a protein, with one or more oligosaccharide groups, occurs at specific locations along a polypeptide backbone and  
25 greatly affects the physical properties of the protein such as protein stability, secretion, subcellular localization, and biological activity. Glycosylation is usually of two types. O-linked oligosaccharides are attached to serine or threonine residues and N-linked oligosaccharides are attached to asparagine residues. One type of oligosaccharide found on both N-linked and O-linked oligosaccharides is N-acetylneuraminic acid (sialic acid),  
30 which is a family of amino sugars containing 9 or more carbon atoms. Sialic acid is usually the terminal residue on both N-linked and O-linked oligosaccharides and, because

it bears a negative charge, confers acidic properties to the glycoprotein. Human erythropoietin, having 165 amino acids, contains three N-linked and one O-linked oligosaccharide chains which comprise about 40% of the total molecular weight of the glycoprotein. N-linked glycosylation occurs at asparagine residues located at positions 24, 38, and 83 and O-linked glycosylation occurs at a serine residue located at position 126. The oligosaccharide chains are modified with terminal sialic acid residues. Enzymatic removal of all sialic acid residues from the glycosylated erythropoietin results in loss of *in vivo* activity but not *in vitro* activity because sialylation of erythropoietin prevents its binding, and subsequent clearance, by hepatic binding protein.

The glycoproteins of the present invention include analogs of human erythropoietin with one or more changes in the amino acid sequence of human erythropoietin which result in an increase in the number of sites for sialic acid attachment. These glycoprotein analogs may be generated by site-directed mutagenesis having additions, deletions, or substitutions of amino acid residues that increase or alter sites that are available for glycosylation. Glycoprotein analogs having levels of sialic acid greater than those found in human erythropoietin are generated by adding glycosylation sites which do not perturb the secondary or tertiary conformation required for biological activity. The glycoproteins of the present invention also include analogs having increased levels of carbohydrate attachment at a glycosylation site which usually involve the substitution of one or more amino acids in close proximity to an N-linked or O-linked site. The glycoproteins of the present invention also include analogs having one or more amino acids extending from the carboxy terminal end of erythropoietin and providing at least one additional carbohydrate site. The glycoproteins of the present invention also include analogs having an amino acid sequence which includes a rearrangement of at least one site for glycosylation. Such a rearrangement of glycosylation site involves the deletion of one or more glycosylation sites in human erythropoietin and the addition of one or more non-naturally occurring glycosylation sites. Increasing the number of carbohydrate chains on erythropoietin, and therefore the number of sialic acids per erythropoietin molecules may confer advantageous properties such as increased solubility, greater resistance to proteolysis, reduced immunogenicity, increased serum half-life, and increased biological

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activity. Erythropoietin analogs with additional glycosylation sites are disclosed in more detail in European Patent Application 640 619, to Elliot published March 1, 1995.

In a preferred embodiment, the glycoproteins of the present invention comprise an amino acid sequence which includes at least one additional site for glycosylation such as, but not limited to, erythropoietins comprising the sequence of human erythropoietin modified by a modification selected from the following:

- Asn<sup>30</sup>Thr<sup>32</sup>;  
Asn<sup>51</sup>Thr<sup>53</sup>;  
10 Asn<sup>57</sup>Thr<sup>59</sup>;  
Asn<sup>69</sup>;  
Asn<sup>69</sup>Thr<sup>71</sup>;  
Ser<sup>68</sup>Asn<sup>69</sup>Thr<sup>71</sup>;  
Val<sup>87</sup>Asn<sup>88</sup>Thr<sup>90</sup>;  
15 Ser<sup>87</sup>Asn<sup>88</sup>Thr<sup>90</sup>;  
Ser<sup>87</sup>Asn<sup>88</sup>Gly<sup>89</sup>Thr<sup>90</sup>;  
Ser<sup>87</sup>Asn<sup>88</sup>Thr<sup>90</sup>Thr<sup>92</sup>;  
Ser<sup>87</sup>Asn<sup>88</sup>Thr<sup>90</sup>Ala<sup>162</sup>;  
Asn<sup>69</sup>Thr<sup>71</sup>Ser<sup>87</sup>Asn<sup>88</sup>Thr<sup>90</sup>;  
20 Asn<sup>30</sup>Thr<sup>32</sup>Val<sup>87</sup>Asn<sup>88</sup>Thr<sup>90</sup>;  
Asn<sup>89</sup>Ile<sup>90</sup>Thr<sup>91</sup>;  
Ser<sup>87</sup>Asn<sup>89</sup>Ile<sup>90</sup>Thr<sup>91</sup>;  
Asn<sup>136</sup>Thr<sup>138</sup>;  
Asn<sup>138</sup>Thr<sup>140</sup>;  
25 Thr<sup>125</sup>; and  
Pro<sup>124</sup>Thr<sup>125</sup>.

The notation used herein for modification of amino acid sequence means that the position(s) of the corresponding unmodified protein (e.g. hEPO of SEQ ID NO:1 or SEQ ID NO:2) indicated by the superscripted number(s) is changed to the amino acid(s) that immediately precede the respective superscripted number(s).

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The glycoprotein may also be an analog having at least one additional amino acid at the carboxy terminal end of the glycoprotein, wherein the additional amino acid includes at least one glycosylation site. The additional amino acid may comprise a peptide fragment derived from the carboxy terminal end of human chorionic gonadotropin. Preferably, the glycoprotein is an analog selected from the group consisting of (a) human erythropoietin having the amino acid sequence, Ser Ser Ser Ser Lys Ala Pro Pro Pro Ser Leu Pro Ser Pro Ser Arg Leu Pro Gly Pro Ser Asp Thr Pro Ile Leu Pro Gln (SEQ ID NO:3), extending from the carboxy terminus; (b) the analog in (a) further comprising Ser<sup>87</sup> Asn<sup>88</sup> Thr<sup>90</sup> EPO; and (c) the analog in (a) further comprising Asn<sup>30</sup> Thr<sup>32</sup> Val<sup>87</sup> Asn<sup>88</sup> Thr<sup>90</sup> EPO.

The glycoprotein may also be an analog having an amino acid sequence which includes a rearrangement of at least one site for glycosylation. The rearrangement may comprise a deletion of any of the N-linked carbohydrate sites in human erythropoietin and an addition of an N-linked carbohydrate site at position 88 of the amino acid sequence of human erythropoietin. Preferably, the glycoprotein is an analog selected from the group consisting of Gln<sup>24</sup> Ser<sup>87</sup> Asn<sup>88</sup> Thr<sup>90</sup> EPO; Gln<sup>38</sup> Ser<sup>87</sup> Asn<sup>88</sup> Thr<sup>90</sup> EPO; and Gln<sup>83</sup> Ser<sup>87</sup> Asn<sup>88</sup> Thr<sup>90</sup> EPO.

As used herein, "lower alkyl" means a linear or branched alkyl group having from one to six carbon atoms. Examples of lower alkyl groups include methyl, ethyl and isopropyl. In accordance with this invention, R is any lower alkyl. Conjugates in which R is methyl are preferred.

The symbol "m" represents the number of ethylene oxide residues (OCH<sub>2</sub>CH<sub>2</sub>) in the poly(ethylene oxide) group. A single PEG subunit of ethylene oxide has a molecular weight of about 44 daltons. Thus, the molecular weight of the conjugate (excluding the molecular weight of the EPO) depends on the number "m". In the conjugates of this invention "m" is from about 450 to about 900 (corresponding to a molecular weight of about 20 kDa to about 40 kDa), preferably from about 650 to about 750 (corresponding to a molecular weight of about 30 kDa). The number m is selected such that the resulting

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conjugate of this invention has a physiological activity comparable to unmodified EPO, which activity may represent the same as, more than, or a fraction of the corresponding activity of unmodified EPO. A molecular weight of "about" a certain number means that it is within a reasonable range of that number as determined by conventional analytical techniques. The number "m" is selected so that the molecular weight of each poly(ethylene glycol) group covalently linked to the erythropoietin glycoprotein is from about 20kDa to about 40kDa, and is preferably about 30kDa.

In the conjugates of this invention, the number "n" is the number of polyethylene glycol groups covalently bound to free amino groups (including  $\epsilon$ -amino groups of a lysine amino acid and/or the amino-terminal amino group) of an erythropoietin protein via amide linkage(s). A conjugate of this invention may have one, two, or three PEG groups per molecule of EPO. "n" is an integer ranging from 1 to 3, preferably "n" is 1 or 2, and more preferably "n" is 1.

The compound of Formula I can be prepared from the known polymeric material in which R and m are as described above, by condensing the compound of Formula II with the erythropoietin glycoprotein. Compounds of Formula II in which x is 3 are alpha-lower alkoxy, butyric acid succinimidyl esters of poly(ethylene glycol) (lower alkoxy-PEG-SBA). Compounds of Formula II in which x is 2 are alpha-lower alkoxy, propionic acid succinimidyl esters of poly(ethylene glycol) (lower alkoxy-PEG-SPA). Any conventional method of reacting an activated ester with an amine to form an amide can be utilized. In the reaction illustrated above, the exemplified succinimidyl ester is a leaving group causing the amide formation. The use of succinimidyl esters such as the compounds of formula II to produce conjugates with proteins are disclosed in U.S. Patent No. 5,672,662, issued September 30, 1997 (Harris, et al.).

Human EPO contains nine free amino groups, the amino-terminal amino group plus the  $\epsilon$ -amino groups of 8 lysine residues. When the pegylation reagent was combined with a SBA compound of Formula II, it has been found that at pH 7.5, a protein:PEG ratio of 1:3, and a reaction temperature of from 20-25°C, a mixture of mono-, di-, and

trace amounts of the tri-pegylated species were produced. When the pegylation reagent was a SPA compound of Formula II, at similar conditions except that the protein:PEG ratio was 1:2, primarily the mono-pegylated species is produced. The pegylated EPO can be administered as a mixture, or as the cation exchange chromatography separated  
5 different pegylated species. By manipulating the reaction conditions (e.g., ratio of reagents, pH, temperature, protein concentration, time of reaction etc.), the relative amounts of the different pegylated species can be varied.

Human erythropoietin (EPO) is a human glycoprotein which stimulates the  
10 formation of erythrocytes. Its preparation and therapeutic application are described in detail for example in U.S. Patent Nos. 5,547,933 and 5,621,080, EP-B 0 148 605, Huang, S.L., Proc. Natl. Acad. Sci. USA (1984) 2708-2712, EP-B 0 205 564, EP-B 0 209 539 and EP-B 0 411 678 as well as Lai, P.H. et al., J. Biol. Chem. 261 (1986) 3116-3121, an Sasaki, H. et al., J. Biol. Chem. 262 (1987) 12059-12076. Erythropoietin for therapeutic  
15 uses may be produced by recombinant means (EP-B 0 148 605, EP-B 0 209 539 and Egrie, J.C., Strickland, T.W., Lane, J. et al. (1986) Immunobiol. 72: 213-224).

Methods for the expression and preparation of erythropoietin in serum free medium are described for example in WO 96/35718, to Burg published 14 November 1996, and in  
20 European Patent Publication No. 513 738, to Koch published 12 June 1992. In addition to the publications mentioned above, it is known that a serum-free fermentation of recombinant CHO cells which contain an EPO gene can be carried out. Such methods are described for example in EP-A 0 513 738, EP-A 0 267 678 and in a general form by Kawamoto, T. et al., Analytical Biochem. 130 (1983) 445-453, EP-A 0 248 656, Kowar,  
25 J. and Franek, F., Methods in Enzymology 421 (1986) 277-292, Bavister, B., Expcology 271 (1981) 45-51, EP-A 0 481 791, EP-A 0 307 247, EP-A 0 343 635, WO 88/00967.

In EP-A 0 267 678 an ion exchange chromatography on S-Sepharose, a preparative reverse phase HPLC on a Cg column and a gel filtration chromatography are described for the purification of EPO produced in serum-free culture after dialysis. In this  
30 connection the gel filtration chromatography step can be replaced by ion exchange



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chromatography on S-Sepharose fast flow. It is also proposed that a dye chromatography on a Blue Trisacryl column be carried out before the ion exchange chromatography.

5 A process for the purification of recombinant EPO is described by Nobuo, I. et al., J. Biochem. 107 (1990) 352-359. In this process EPO is treated however with a solution of Tween<sup>®</sup> 20, phenylmethylsulfonyl fluoride, ethylmaleimide, pepstatin A, copper sulfate and oxamic acid prior to the purification steps. Publications, including WO 96/35718, to Burg published 14 November 1996, discloses a process for preparing erythropoietin in a serum free fermentation process (EPOsf).

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The specific activity of EPO or EPO conjugates in accordance with this invention can be determined by various assays known in the art. The biological activity of the purified EPO proteins of this invention are such that administration of the EPO protein by injection to human patients results in bone marrow cells increasing production of reticulocytes and red blood cells compared to non-injected or control groups of subjects. 15 The biological activity of the EPO proteins, or fragments thereof, obtained and purified in accordance with this invention can be tested by methods according to Annable, et al., Bull. Wld. Hlth. Org. (1972) 47: 99-112 and Pharm. Europa Spec. Issue Erythropoietin BRP Bio 1997(2). Another biological assay for determining the activity of EPO protein, 20 the normocythaemic mouse assay, is described in Example 4.

The invention will be better understood by reference to the following examples which illustrate but do not limit the invention described herein.

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## EXAMPLE 1

### Fermentation And Purification Of Human EPO

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#### a) Inoculum Preparation and Fermentation

One vial of the Working Cell Bank, originating from an EPO-producing CHO cell line (ATCC CRL8695, disclosed in EP 411 678 (Genetics Institute) can be used) is taken from the gas phase of the liquid nitrogen storage tank. The cells are transferred into glass spinner flasks and cultivated in a hydrogen carbonate-buffered medium in a humidified CO<sub>2</sub> incubator. Typical serum free media used for the inoculum preparation and fermentation are disclosed in European Patent Application 513 738, to Koch published 12 June 1992, or WO 96/35718, to Burg published 14 November 1996, for example contain as medium DMEM/F12 (e.g. JRH Biosciences/Hazleton Biologics, Denver, US, order No. 57-736) and additionally sodium hydrogencarbonate, L+glutamine, D+glucose, recombinant insulin, sodium selenite, diaminobutane, hydrocortisone, iron(II) sulfate, asparagine, aspartic acid, serine and a stabilizer for mammalian cells such as e.g. polyvinyl alcohol, methyl cellulose, polydextran, polyethylene glycol, Pluronic F68, plasma expander polygelin (HEMACCEL<sup>®</sup>) or polyvinyl pyrrolidone (WO 96/35718).

The cultures are microscopically checked for the absence of contaminating microorganisms, and the cell densities are determined. These tests are performed at each splitting step.

After the initial growth period, the cell culture is diluted with fresh medium to the starting cell density and undergoes another growth cycle. This procedure is repeated until a culture volume of approximately 2 l per glass spinner flask has been obtained. After approx. 12 doublings 1 to 5 liter of this culture is available which then is used as inoculum for the 10 l inoculum fermenter.

After 3 - 5 days, the culture in the 10 l fermenter can be used as inoculum for the 100 l inoculum fermenter.

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After additional 3 - 5 days of cultivation, the culture in the 100 l fermenter can be used as inoculum for the 1000 l production fermenter.

5 b) Harvesting and Cell Separation

A batch refeed process is used, i.e. when the desired cell density is reached, approx. 80 % of the culture is harvested. The remaining culture is replenished with fresh culture medium and cultivated until the next harvest. One production run consists of a maximum  
10 of 10 subsequent harvests: 9 partial harvests and 1 overall harvest at the end of fermentation. Harvesting takes place every 3 - 4 days.

The determined harvest volume is transferred into a cooled vessel. The cells are removed by centrifugation or filtration and discarded. The EPO containing supernatant  
15 of the centrifugation step is in-line filtered and collected in a second cooled vessel. Each harvest is processed separately during purification.

A typical process for the purification of EPO-protein is disclosed in WO 96/35718,  
20 to Burg published 14 November 1996. The purification process is explained in the following.

a) Blue Sepharose Chromatography

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Blue Sepharose (Pharmacia) consists of Sepharose beads to the surface of which the Cibacron blue dye is covalently bound. Since EPO binds more strongly to Blue Sepharose than most non-proteinaceous contaminants, some proteinaceous impurities and PVA, EPO can be enriched in this step. The elution of the Blue Sepharose column is  
30 performed by increasing the salt concentration as well as the pH.

affinity to hydroxyapatite and can therefore be eluted at lower phosphate concentrations than protein impurities.

5 The column is filled with 30 - 40 l of Hydroxyapatite Ultrogel and regenerated with a potassium phosphate/ calcium chloride buffer and NaOH followed by a Tris-base buffer. Then it is equilibrated with a Tris-base buffer containing a low amount of isopropanol and sodium chloride.

10 The EPO containing eluate of the Butyl Toyopearl chromatography is loaded onto the column. Subsequently the column is washed with equilibration buffer and a Tris-base buffer without isopropanol and sodium chloride. The product is eluted with a Tris-base buffer containing a low concentration of potassium phosphate and collected in a single fraction in accordance with the master elution profile.

15 d) Reversed Phase HPLC on Vydac C4

The RP-HPLC material Vydac C4 (Vydac) consists of silica gel particles, the surfaces of which carry C4-alkyl chains. The separation of EPO from the proteinaceous impurities is based on differences in the strength of hydrophobic interactions. Elution is performed with an acetonitrile gradient in diluted trifluoroacetic acid.

25 Preparative HPLC is performed using a stainless steel column (filled with 2.8 to 3.2 liter of Vydac C4 silicagel). The Hydroxyapatite Ultrogel eluate is acidified by adding trifluoro-acetic acid and loaded onto the Vydac C4 column. For washing and elution an acetonitrile gradient in diluted trifluoroacetic acid is used. Fractions are collected and immediately neutralized with phosphate buffer. The EPO fractions which are within the IPC limits are pooled.

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#### e) DEAE Sepharose Chromatography

The DEAE Sepharose (Pharmacia) material consists of diethylaminoethyl (DEAE) - groups which are covalently bound to the surface of Sepharose beads. The binding of EPO to the DEAE groups is mediated by ionic interactions. Acetonitrile and trifluoroacetic acid pass through the column without being retained. After these substances have been washed off, trace impurities are removed by washing the column with acetate buffer at a low pH. Then the column is washed with neutral phosphate buffer and EPO is eluted with a buffer with increased ionic strength.

The column is packed with DEAE Sepharose fast flow. The column volume is adjusted to assure an EPO load in the range of 3 - 10 mg EPO/ml gel. The column is washed with water and equilibration buffer (sodium/ potassium phosphate). The pooled fractions of the HPLC eluate are loaded and the column is washed with equilibration buffer. Then the column is washed with washing buffer (sodium acetate buffer) followed by washing with equilibration buffer. Subsequently, EPO is eluted from the column with elution buffer (sodium chloride, sodium/ potassium phosphate) and collected in a single fraction in accordance with the master elution profile.

The eluate of the DEAE Sepharose column is adjusted to the specified conductivity. The resulting drug substance is sterile filtered into Teflon bottles and stored at -70 °C.

#### EXAMPLE 2

##### Pegylation of EPO with mPEG-SBA

EPO purified in accordance with the serum free procedure of Example 1 (EPOsf) was homogeneous as determined by analytical methods and showed the typical isoform pattern consisting of 8 isoforms. It had a specific biological activity of 190,000 IU/mg as determined by the normocythaemic mouse assay. The pegylation reagent used was a methoxy-PEG-SBA, which is a compound of Formula II in which R is methyl; x is 3; and

m is from 650 to 750 (avg. about 680, corresponding to an average molecular weight of about 30 kDa).

### Pegylation Reaction

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To one hundred milligrams of EPOsf (9.71 ml of a 10.3 mg/ml EPOsf stock , 5.48  $\mu$ mol) 10 ml of 0.1 M potassium phosphate buffer, pH, 7.5 containing 506 mg of 30kDa methoxy-PEG-SBA (16.5  $\mu$  mol) (obtained from Shearwater Polymers, Inc., Huntsville, Alabama) was added and mixed for 2h at room temperature (20-23 °C). The final protein concentration was 5 mg/ml and the protein:PEG reagent ratio was 1:3. After two hours, the reaction was stopped by adjusting the pH to 4.5 with glacial acetic acid and stored at -20°C, until ready for purification.

### Purification

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1. **Conjugate Mixture:** Approximately 28 ml of SP-SEPHAROSE FF (sulfo-propyl cation exchange resin) was packed into an AMICON glass column (2.2 x 7.5 cm) and equilibrated with 20 mM acetate buffer pH, 4.5 at a flowrate of 150 ml/h. Six milliliters of the reaction mixture containing 30 mg protein was diluted 5-fold with the equilibration buffer and applied onto the column. Unadsorbed materials were washed away with the buffer and the adsorbed PEG conjugate mixture was eluted from the column with 0.175 M NaCl in the equilibration buffer. Unmodified EPOsf still remaining on the column was eluted with 750 mM NaCl. Column was reequilibrated in the starting buffer. Samples were analyzed by SDS-PAGE and their degree of pegylation were determined. It was found that the 0.175M NaCl eluate contained, mono- as well as di- and trace amounts of the tri-pegylated species, whereas the 750 mM NaCl eluate contained unmodified EPOsf.

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2. **Di-PEG and Mono-PEG-EPOsf:** The purified conjugate mixture eluted from the column in the previous step was diluted 4-fold with the buffer and reapplied onto the column and washed as described. Di-PEG-EPOsf and mono-PEG-EPOsf were

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separately eluted from the column with 0.1M NaCl and 0.175 M NaCl, respectively. Elution was also performed with 750mM NaCl to elute any remaining unmodified EPOsf.

- 5 Alternatively, the reaction mixture was diluted 5-fold with the acetate buffer and applied onto the SP-Sepharose column (~0.5 mg protein/ml gel). Column was washed and adsorbed mono-PEG-EPOsf, di-PEG-EPOsf and unmodified EPOsf were eluted as described in the previous section.

## 10 Results

- PEG-EPOsf was synthesized by chemically conjugating a linear PEG molecule with a number average molecular weight of 30 kDa. PEG-EPOsf was derived from the reaction between the primary amino groups of EPOsf and the succinimidyl ester derivative of a 30  
15 kDa PEG-butyric acid, resulting in an amide bond.

- Results are summarized in Table1. Purified conjugate mixture comprised of mono- and di-PEG-EPOsf and was free of unmodified EPOsf as determined by SDS-PAGE analysis. Conjugate mixture accounted for 23.4 mg or 78% of the starting material. Cation  
20 exchange chromatographic separation of mono- and di-PEG-EPOsf indicated that mono- to di-PEG ratio in the conjugate mixture was almost 1:1. After completion of the reaction, ratio of the individual components of Mono: Di: Unmodified were 40: 38: 20 (%). Overall yield was almost quantitative.

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**Table 1. Summary of results of EPOsf pegylation**

|    | <u>Sample</u> | <u>Protein (mg)</u> | <u>Yield (%)</u> |
|----|---------------|---------------------|------------------|
| 5  | Rxn. Mix.     | 30                  | 100              |
|    | Mono-         | 12.0                | 40               |
|    | Di-           | 11.4                | 38               |
| 10 | Unmod.        | 6.0                 | 20               |
|    | Conju. Mix.   | 23.4                | 78               |

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### **EXAMPLE 3**

#### **Pegylation of EPO with mPEG-SPA**

20 A different aliquot of the EPOsf used in Example 2 was reacted with 30 kDa methoxy-PEG-SPA (Shearwater Polymers, Inc., Huntsville, Alabama). Reaction was performed at a protein:reagent ratio of 1:2 and purification techniques were in accordance with Example 2. Primarily the mono-pegylated species was produced.

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### **EXAMPLE 4**

#### **In-vivo activity of pegylated EPO determined by the normocythaemic mouse assay**

30 The normocythaemic mouse bioassay is known in the art (Pharm. Europa Spec. Issue Erythropoietin BRP Bio 1997(2)) and a method in the monography of erythropoietin of Ph. Eur. BRP. The samples were diluted with BSA-PBS. Normal healthy mice, 7-15 weeks old, were administered s.c. 0.2 ml of the EPO-fraction containing un-pegylated EPO or tri-, di- or mono-pegylated EPO from Example 2 or 3. Over a period of 6 days,  
35 blood was drawn by puncture of the tail vein and diluted such that 1 µl of blood was



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present in 1 ml of an 0.15  $\mu$ mol acridine orange staining solution. The staining time was 3 to 10 minutes. The reticulocyte counts were carried out microfluorometrically in a flow cytometer by analysis of the red fluorescence histogram. The reticulocyte counts were given in terms of absolute figures (per 30,000 blood cells analyzed). For the data presented, each group consisted of 5 mice per day, and the mice were bled only once.

In separate experiments, a single dose of unmodified EPO (25 ng of EPO), the PEG(SBA)-EPO mixture from Example 2 (10 ng of conjugate), mono- and di- pegylated EPOs from Example 2 (10 ng of conjugate), the PEG(SPA)-EPO from Example 3 (10 ng of conjugate), and buffer solution were administered to mice. The results are shown in Table 2. The results show the superior activity and the prolonged half life of the pegylated EPO species indicated by the significantly increased amounts of reticulocytes and the shift of the reticulocytes count maximum using the same dose per mouse (10 ng), compared to a dose of 25 ng for unmodified EPO.

**TABLE 2**

|             | <b>EPO<br/>(Unmodified)</b> | <b>30 kDa SPA<br/>PEG</b> | <b>Mono 30K<br/>SBA</b> | <b>Di 30K<br/>SBA</b> | <b>PEG-EPO<br/>SBA Conjugate<br/>Mixture</b> | <b>Control<br/>Buffer</b> |
|-------------|-----------------------------|---------------------------|-------------------------|-----------------------|----------------------------------------------|---------------------------|
| <b>72h</b>  | 1000                        | 1393                      | 1411                    | 994                   | 1328                                         | 857                       |
| <b>96h</b>  | 500                         | 1406                      | 1501                    | 926                   | 1338                                         | 697                       |
| <b>120h</b> | ~200                        | 1100                      | 1182                    | 791                   | 944                                          | 701                       |
| <b>144h</b> | ~0                          | 535                       | 607                     | 665                   | 660                                          | 708                       |
|             |                             |                           |                         |                       |                                              |                           |

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| Ala | Pro | Pro | Arg | Leu | Ile | Cys | Asp | Ser | Arg | Val | Leu | Glu | Arg | Tyr | Leu |
| 1   |     |     |     | 5   |     |     |     |     | 10  |     |     |     |     | 15  |     |
| Leu | Glu | Ala | Lys | Glu | Ala | Glu | Asn | Ile | Thr | Thr | Gly | Cys | Ala | Glu | His |
|     |     |     | 20  |     |     |     |     | 25  |     |     |     |     | 30  |     |     |
| Cys | Ser | Leu | Asn | Glu | Asn | Ile | Thr | Val | Pro | Asp | Thr | Lys | Val | Asn | Phe |
|     |     | 35  |     |     |     |     | 40  |     |     |     |     | 45  |     |     |     |
| Tyr | Ala | Trp | Lys | Arg | Met | Glu | Val | Gly | Gln | Gln | Ala | Val | Glu | Val | Trp |
|     | 50  |     |     |     |     | 55  |     |     |     |     | 60  |     |     |     |     |
| Gln | Gly | Leu | Ala | Leu | Leu | Ser | Glu | Ala | Val | Leu | Arg | Gly | Gln | Ala | Leu |
| 65  |     |     |     | 70  |     |     |     |     |     | 75  |     |     |     | 80  |     |
| Leu | Val | Asn | Ser | Ser | Gln | Pro | Trp | Glu | Pro | Leu | Gln | Leu | His | Val | Asp |
|     |     |     |     | 85  |     |     |     |     | 90  |     |     |     |     | 95  |     |
| Lys | Ala | Val | Ser | Gly | Leu | Arg | Ser | Leu | Thr | Thr | Leu | Leu | Arg | Ala | Leu |
|     |     |     | 100 |     |     |     |     | 105 |     |     |     |     | 110 |     |     |
| Gly | Ala | Gln | Lys | Glu | Ala | Ile | Ser | Pro | Pro | Asp | Ala | Ala | Ser | Ala | Ala |
|     | 115 |     |     |     |     |     | 120 |     |     |     |     | 125 |     |     |     |
| Pro | Leu | Arg | Thr | Ile | Thr | Ala | Asp | Thr | Phe | Arg | Lys | Leu | Phe | Arg | Val |
|     | 130 |     |     |     |     | 135 |     |     |     |     | 140 |     |     |     |     |
| Tyr | Ser | Asn | Phe | Leu | Arg | Gly | Lys | Leu | Lys | Leu | Tyr | Thr | Gly | Glu | Ala |
| 145 |     |     |     | 150 |     |     |     |     |     | 155 |     |     |     | 160 |     |
| Cys | Arg | Thr | Gly | Asp |     |     |     |     |     |     |     |     |     |     |     |
|     |     |     |     | 165 |     |     |     |     |     |     |     |     |     |     |     |

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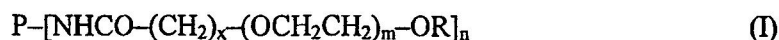
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What is claimed is:

1. A conjugate, said conjugate comprising an erythropoietin glycoprotein having at least one free amino group and having the *in vivo* biological activity of causing bone marrow cells to increase production of reticulocytes and red blood cells and selected from the group consisting of human erythropoietin and analogs thereof which have sequence of human erythropoietin modified by the addition of from 1 to 6 glycosylation sites or a rearrangement of at least one glycosylation site; said glycoprotein being covalently linked to "n" poly(ethylene glycol) groups of the formula
- 10  $-\text{CO}-(\text{CH}_2)_x-(\text{OCH}_2\text{CH}_2)_m-\text{OR}$  with the  $-\text{CO}$  of each poly(ethylene glycol) group forming an amide bond with one of said amino groups; wherein R is lower alkyl; x is 2 or 3; m is from about 450 to about 900; n is from 1 to 3; and n and m are chosen so that the molecular weight of the conjugate minus the erythropoietin glycoprotein is from 20 kilodaltons to 100 kilodaltons.

- 15 2. The conjugate of claim 1, of the formula:



- 20 wherein m, n and R are as above, and P is the residue of the glycoprotein without the n amino group(s) which form amide linkage(s) with the poly(ethylene glycol) group(s).

3. The conjugate of claim 2, wherein the glycoprotein is a human erythropoietin.
- 25 4. The conjugate of claim 3, wherein the glycoprotein has the sequence SEQ ID NO:1.
5. The conjugate of claim 4, wherein R is methyl.
- 30 6. The conjugate of claim 4, wherein m is from about 650 to about 750.

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7. The conjugate of claim 4, wherein n is 1.

8. The conjugate of claim 4, wherein R is methyl; m is from about 650 to about 750; and n is 1.

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9. The conjugate of claim 2, wherein the glycoprotein has the sequence of human erythropoietin modified by the addition of from 1 to 6 glycosylation sites.

10. The conjugate of claim 9, wherein the glycoprotein has the sequence of human

10 erythropoietin modified by a modification selected from the group consisting of:

Asn<sup>30</sup>Thr<sup>32</sup>;

Asn<sup>51</sup>Thr<sup>53</sup>;

Asn<sup>57</sup>Thr<sup>59</sup>;

Asn<sup>69</sup>;

15 Asn<sup>69</sup>Thr<sup>71</sup>;

Ser<sup>68</sup>Asn<sup>69</sup>Thr<sup>71</sup>;

Val<sup>87</sup>Asn<sup>88</sup>Thr<sup>90</sup>;

Ser<sup>87</sup>Asn<sup>88</sup>Thr<sup>90</sup>;

Ser<sup>87</sup>Asn<sup>88</sup>Gly<sup>89</sup>Thr<sup>90</sup>;

20 Ser<sup>87</sup>Asn<sup>88</sup>Thr<sup>90</sup>Thr<sup>92</sup>;

Ser<sup>87</sup>Asn<sup>88</sup>Thr<sup>90</sup>Ala<sup>162</sup>;

Asn<sup>69</sup>Thr<sup>71</sup>Ser<sup>87</sup>Asn<sup>88</sup>Thr<sup>90</sup>;

Asn<sup>30</sup>Thr<sup>32</sup>Val<sup>87</sup>Asn<sup>88</sup>Thr<sup>90</sup>;

Asn<sup>89</sup>Ile<sup>90</sup>Thr<sup>91</sup>;

25 Ser<sup>87</sup>Asn<sup>89</sup>Ile<sup>90</sup>Thr<sup>91</sup>;

Asn<sup>136</sup>Thr<sup>138</sup>;

Asn<sup>138</sup>Thr<sup>140</sup>;

Thr<sup>125</sup>; and

Pro<sup>124</sup>Thr<sup>125</sup>.

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11. The conjugate of claim 10, wherein R is methyl.

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12. The conjugate of claim 10, wherein m is from about 650 to about 750.

13. The conjugate of claim 10, wherein n is 1.

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14. The conjugate of claim 10, wherein R is methyl; m is from about 650 to about 750; and n is 1.

10 15. The conjugate of claim 9, wherein the glycoprotein has a sequence comprising the sequence of human erythropoietin and a second sequence at the carboxy terminus of the human erythropoietin sequence, wherein the second sequence contains at least one glycosylation site.

15 16. The conjugate of claim 15, wherein the second sequences comprises a sequence derived from the carboxy terminal sequence of human chorionic gonadotropin.

17. The conjugate of claim 16, wherein the glycoprotein has a sequence selected from the group consisting of:

20 (a) the sequence of human erythropoietin and the sequence SEQ ID NO:3 at the carboxy terminus of the human erythropoietin sequence;

(b) the sequence in (a) modified by Ser<sup>87</sup> Asn<sup>88</sup> Thr<sup>90</sup>; and

(c) the sequence in (a) modified by Asn<sup>30</sup> Thr<sup>32</sup> Val<sup>87</sup> Asn<sup>88</sup> Thr<sup>90</sup>.

25 18. The conjugate of claim 17, wherein R is methyl.

19. The conjugate of claim 17, wherein m is from about 650 to about 750.

20. The conjugate of claim 17, wherein n is 1.

30 21. The conjugate of claim 17, wherein R is methyl; m is from about 650 to about 750; and n is 1.

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22. The conjugate of claim 2, wherein the glycoprotein has the sequence of human erythropoietin modified by a rearrangement of at least one glycosylation site.

23. The conjugate of claim 22, wherein the rearrangement comprises deletion of any  
5 of the N-linked glycosylation sites in human erythropoietin and addition of an N-linked glycosylation site at position 88 of the sequence of human erythropoietin.

24. The conjugate of claim 23, wherein the glycoprotein has the sequence of human erythropoietin modified by a modification selected from the group consisting of:

10 Gln<sup>24</sup> Ser<sup>87</sup> Asn<sup>88</sup> Thr<sup>90</sup>;  
Gln<sup>38</sup> Ser<sup>87</sup> Asn<sup>88</sup> Thr<sup>90</sup>; and  
Gln<sup>83</sup> Ser<sup>87</sup> Asn<sup>88</sup> Thr<sup>90</sup>.

25. The conjugate of claim 24, wherein R is methyl.

26. The conjugate of claim 24, wherein m is from about 650 to about 750.

27. The conjugate of claim 24, wherein n is 1.

20 28. The conjugate of claim 24, wherein R is methyl; m is from about 650 to about 750; and n is 1.

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## ABSTRACT OF THE INVENTION

Conjugates of erythropoietin with poly(ethylene glycol) comprise an erythropoietin glycoprotein having at least one free amino group and having the *in vivo* biological activity of causing bone marrow cells to increase production of reticulocytes and red blood cells and selected from the group consisting of human erythropoietin and analogs thereof which have sequence of human erythropoietin modified by the addition of from 1 to 6 glycosylation sites or a rearrangement of at least one glycosylation site; said glycoprotein being covalently linked to "n" poly(ethylene glycol) groups of the formula  $-\text{CO}-(\text{CH}_2)_x(\text{OCH}_2\text{CH}_2)_m-\text{OR}$  with the carbonyl of each poly(ethylene glycol) group forming an amide bond with one of said amino groups; wherein R is lower alkyl; x is 2 or 3; m is about 450 to about 900; n is from 1 to 3; and n and m are chosen so that the molecular weight of the conjugate minus the erythropoietin glycoprotein is from 20 kilodaltons to 100 kilodaltons.

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# Declaration and Power of Attorney for Patent Application

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

## ERYTHROPOIETIN CONJUGATES

the specification of which

(check one)

☒ [ X ] is attached hereto.

☐ [ ] was filed on \_\_\_\_\_ as

Application Serial No. \_\_\_\_\_

and was amended on \_\_\_\_\_  
(if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR § 1.56.

I hereby claim foreign priority benefits under 35 U.S.C. § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or § 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or PCT International application having a filing date before that of the application on which priority is claimed.

### Prior Foreign Application(s)

### Priority Claimed

|                   |                    |                                 |                                     |                                    |
|-------------------|--------------------|---------------------------------|-------------------------------------|------------------------------------|
| _____<br>(Number) | _____<br>(Country) | _____<br>(Day/Month/Year Filed) | <input type="checkbox"/> [ ]<br>Yes | <input type="checkbox"/> [ ]<br>No |
| _____<br>(Number) | _____<br>(Country) | _____<br>(Day/Month/Year Filed) | <input type="checkbox"/> [ ]<br>Yes | <input type="checkbox"/> [ ]<br>No |
| _____<br>(Number) | _____<br>(Country) | _____<br>(Day/Month/Year Filed) | <input type="checkbox"/> [ ]<br>Yes | <input type="checkbox"/> [ ]<br>No |

I hereby claim the benefit under 35 U.S.C. § 119(e) of any United States provisional application(s) listed below.

|                            |                        |                            |                        |
|----------------------------|------------------------|----------------------------|------------------------|
| _____<br>(Application No.) | _____<br>(Filing Date) | _____<br>(Application No.) | _____<br>(Filing Date) |
|----------------------------|------------------------|----------------------------|------------------------|

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I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s), or § 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR § 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

|                                 |                      |                                                |
|---------------------------------|----------------------|------------------------------------------------|
| <u>(Application Serial No.)</u> | <u>(Filing Date)</u> | <u>(Status) (patented, pending, abandoned)</u> |
|---------------------------------|----------------------|------------------------------------------------|

|                                 |                      |                                                |
|---------------------------------|----------------------|------------------------------------------------|
| <u>(Application Serial No.)</u> | <u>(Filing Date)</u> | <u>(Status) (patented, pending, abandoned)</u> |
|---------------------------------|----------------------|------------------------------------------------|

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. *(list name and registration number)*

|                                     |                                              |
|-------------------------------------|----------------------------------------------|
| George W. Johnston (Reg. No. 28090) | Dennis P. Tramaloni (Reg. No. 28542)         |
| William H. Epstein (Reg. No. 20008) | Patricia S. Rocha-Tramaloni (Reg. No. 31054) |
| Lewis J. Kreisler (Reg. No. 38522)  | John P. Parise (Reg. No. 34403)              |

Send Correspondence to:

George W. Johnston, Esq., Hoffmann-La Roche Inc., 340 Kingsland Street,  
Nutley, New Jersey 07110-1199

Direct Telephone Calls to: (name and telephone number)

Lewis J. Kreisler (973) 235-4387

Pascal Sebastian Bailon

Full name of sole or first inventor

Pascal Sebastian Bailon

Inventor's signature

7/2/99  
Date

Florham Park, Morris County, New Jersey

Residence

U.S.A.

Citizenship

21 Woodbine Road, Florham Park, New Jersey 07932

Post Office Address

108  
106

Full name of second joint inventor, if any

Second Inventor's signature

Date

Residence

Citizenship

Post Office Address

Full name of third joint inventor, if any

Third Inventor's signature

Date

Residence

Citizenship

Post Office Address

Full name of fourth joint inventor, if any

Fourth Inventor's signature

Date

Residence

Citizenship

Post Office Address

109  
107

Full name of fifth joint inventor, if any

Fifth Inventor's signature

Date

Residence

Citizenship

Post Office Address

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107

Title 37, Code of Federal Regulations, §1.56, duty to disclose information material to patentability provides, in part, that each individual associated with the filing and prosecution of a patent application has a duty of candor and good faith in dealing with the Office, which includes a duty to disclose to the Office all information known to that individual to be material to patentability as defined in this section. The duty to disclose information exists with respect to each pending claim until the claim is cancelled or withdrawn from consideration, or the application becomes abandoned.

Under this section, information is material to patentability when it is not cumulative to information already of record or being made of record in the application, and

- (1) It establishes, by itself or in combination with other information, a prima facie case of unpatentability of a claim; or
- (2) It refutes, or is inconsistent with, a position the applicant takes in:
  - (i) Opposing an argument of unpatentability relied on by the Office, or
  - (ii) Asserting an argument of patentability.

Junio 14, 2000  
Traducción Oficial No.0271  
REF:F. HOFFMANN-LA ROCHE AG  
Patente No.60/150,225

F. Hoffmann  
Caso-20431

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DEPARTAMENTO DE COMERCIO DE LOS ESTADOS UNIDOS

OFICINA DE MARCAS Y PATENTES

Abril 10, 2000

Por medio del presente documento se certifica que adjunta se encuentra una fiel copia de los registros de la Oficina de Marcas y Patentes de los Estados Unidos, correspondientes a los documentos para la solicitud tal como se presentaron, con el fin de asignarle una fecha de registro bajo la norma del código 35 USC 111.

Número de Solicitud: 60/150,225

Fecha de Registro: Agosto 23, 1999

Autorizado por:

COMISIONADO DE MARCAS Y PATENTES

Firma: P. Swain

Funcionario Encargado de Certificaciones

Hay sello realizado de la Oficina de Marcas y Patentes de color dorado.



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CERTIFICACION BAJO EL CODIGO 37 Codigo de Regulaciones  
federales 1.10

Yo por medio del presente documento certifico que éste ha sido enviado por Correo Postal - "CORREO POSTAL AL DESTINATARIO" de los Estados Unidos el 31 de Agosto de 1999, en un sobre rotulado al "Assistant Commissioner of Patents", con el número de código EL267098537US-Washington, DC., 20231.

Nombre del depositante: PAMELA JOHNSTON

Firma: PAMELA JOHNSTON

HOJA DE SOLICITUD PROVISIONAL PARA PATENTE

Esta es una petición para registrar una SOLICITUD PROVISIONAL bajo el código 37 CFR § 1.53 (b)(2):

Número de Caso: 1097P2

INVENTOR(ES) / SOLICITANT(ES)

NOMBRE: Bailon, Pascal Sebastian

RESIDENCIA: Florham Park, Morris County, new Jersey

OFICINA CORREO POSTAL: 21 Woodbine Road

Florham Park, New Jersey 07932

*Eduardo Celado*  
FOR THE  
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☐ Los inventores adicionales se encuentran mencionados en una hoja adjunta a la presente.

TITULO DE LA INVENCION

**CONJUGADOS DE ERYTHROPOIETIN**

---

DIRECCION PARA CORRESPONDENCIA

George W. Jonhston

Hoffmann-La Roche Inc.

340 Kingsland Street

Nutley, NJ 07110

PARTES DE LA SOLICITUD INCLUIDAS

(Verificar que todas apliquen)

|                                                          |                                                            |
|----------------------------------------------------------|------------------------------------------------------------|
| <input checked="" type="checkbox"/> ESPECIFICACIONES     | <input checked="" type="checkbox"/> [20] Número de Páginas |
| <input checked="" type="checkbox"/> REIVINDICACIONES     | No. de Reivindicaciones 29 (4 páginas)                     |
| <input checked="" type="checkbox"/> LISTADO DE SECUENCIA | <input checked="" type="checkbox"/> [2] Número de Páginas  |
| <input checked="" type="checkbox"/> Declaración          | <input checked="" type="checkbox"/> [4] Número de Páginas  |
| <input type="checkbox"/> GRAFICA (S)                     | <input type="checkbox"/> [ ] Hojas de Gráficas             |
| <input checked="" type="checkbox"/> Otros                | <input type="checkbox"/> [ ] <u>Resumen (1 página)</u>     |

FORMA DE DE PAGO DE HONORARIOS PARA LA SOLICITUD PROVISIONAL DE  
ESTA PATENTE (Señalar uno)

*Edmundo Calado*  
Edmundo Calado  
TRADUCCION  
INGLES AL ESPAÑOL  
Rev 541

114  
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☒ El comisionado por la presente se encuentra autorizado a cobrar honorarios de registro y crédito al número de Cuenta Bancaria **08-2525**

Honorarios provisionales para registro = \$150.000

La invencion fué realizada por una agencia del Gobierno de los Estados Unidos o bajo contrato con una agencia del Gobierno de los Estados Unidos.

☒ No

☐ Sí, el nombre de la Agencia del Gobierno de los Estados Unidos y número de contrato son los siguientes:

Presentado atentamente,

FIRMA: Lewis J. Kreisler

No. de Registro: 38522

Telefono: (973) 235-4387

Telefax: (973) 235-2363

Fecha: Agosto 33, 1999

*Edwardo Calzado*

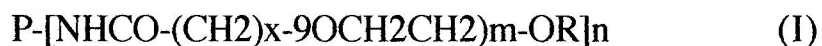


## REIVINDICACIONES :

1. Un conjugado, dicho conjugado consta de glicoproteína erithropoietin el cual tiene por lo menos un grupo de amino ácidos, el cual tiene una actividad biológica *in vivo* la cual produce en las células en la médula del hueso un incremento en la producción de reticulocitos y célula y glóbulos rojos, seleccionados de un grupo que consiste de erithropoietinas y análogos del mismo los cuales tienen una secuencia de erithropoietinas humanas modificadas mediante la adición desde 1 hasta 6 sitios de glicosilaciones o un reordenamiento de por lo menos un sitio de glicocilación; dicha glicoproteína siendo enlazada de covalentemente a grupos de "n" poli(etileno glicol) de la formula  $-CO-(CH_2)_x-9OCH_2CH_2)_m-$  o OR con el  $-CO$  de cada grupo de poli(etileno glicol) formando un enlace de amida con uno de los grupos mencionados grupos de amino; en el cual R es bajo alquilo; x es 2 o 3; m es aproximadamente desde 450 hasta alrededor de 450 hasta aproximadamente 900; n es desde 1 hasta 3; y n y m son escogidos para que el peso molecular del de negativo conjugado de la glicoproteína erithropoietina sea de 20 kilodaltones hasta 100 kilodaltones.

Eduardo Calcedo

2. El conjugado de acuerdo a la reivindicación 1, de la formula:



en el cual m, n y R son como anteriormente se definieron, y P es un residuo de la glicoproteína sin el grupo(s) n de amino, cuya forma de amidos enlaza con el grupo de poli(etileno glicol).

3. El conjugado de acuerdo a la reivindicación 2, en el cual la glicoproteína es un eritropoietina humana.

4. El conjugado de acuerdo a la reivindicación 3, en la cual la glicoproteína eritropoietina humana se expresa mediante activación de genes endógenos.

5. El conjugado de acuerdo a la reivindicación 3, en el cual la glicoproteína tiene una secuencia SEQ ID NO:1.

6. El conjugado de acuerdo a la reivindicación 5, en el cual la R es metilo.

*Eduardo Palacios*

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7. El conjugado de acuerdo a la reivindicación 5, en el cual m es desde aproximadamente 650 hasta alrededor de 750.
8. El conjugado de acuerdo a la reivindicación 5, en la cual la n es 1.
9. El conjugado de acuerdo a la reivindicación 5, en la cual R es metilo; m es aproximadamente desde 650 hasta 750; y n es 1.
10. El conjugado de acuerdo a la reivindicación 2, en la cual la glicoproteína tiene una secuencia de eritropoietina humana modificada por la adición de aproximadamente desde 1 hasta 6 sitios de glicosilación.
11. El conjugado de acuerdo a la reivindicación 10, en la cual la glicoproteína tiene una secuencia de eritropoietina humana modificada mediante una modificación seleccionada de un grupo que consiste de:

Asn30 Thr32;

Eduardo Caludek

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Asn51 Thr53;

Asn57 Thr59;

Asn69;

Asn69 Thr71

Ser68Asn69 Thr71

Val87 Asn88 Thr90;

Ser87Asn88 Thr90

Ser87 Asn88 Gly89 Thr90;

Ser87 Asn88 Thr90 Thr92;

Ser87 Asn88 Thr90 Ala162;

Asn69 Thr71 Ser87 Asn88 Thr90;

Asn30 Thr32 Val87 Asn88 Thr90;

Asn89 Ile90 Thr91;

Ser87 Asn89 Ile90 Thr91;

Asn136 Thr138;

Asn138 Thr138;

Asn138 Thr140;

Thr125; y

Pro124Thr125.

12. El conjugado de acuerdo a la reivindicación 11, en el cual R  
es metilo.

*Eduardo Calado*  
Firma: Eduardo Calado  
C.I. 12.123.456  
P.O. 311 - Caracas - Venezuela

13. El conjugado de acuerdo a la reivindicación 11, en el cual m es aproximadamente desde 650 hasta alrededor de 750.

14. El conjugado de acuerdo a la reivindicación 11, en el cual n es 1.

15. El conjugado de acuerdo a la reivindicación 11, en el cual R es metilo; m es desde aproximadamente 650 hasta alrededor de 750; y n es 1.

16. El conjugado de acuerdo a la reivindicación 10, en el cual la glicoproteína tiene una secuencia que consiste de una eritropoietina humana y una segunda secuencia en el terminus de carboxi de una secuencia de una eritropoietina humana, en la cual la segunda secuencia contiene por lo menos un sitio de glicosilización.

17. El conjugado de acuerdo a la reivindicación 16, en el cual la segunda secuencia consiste de una secuencia que se deriva de la secuencia terminal de carboxi de una gonadotropina corionica humana.

Edwardo Caludek  
"EDUARDO CALUDEK"  
"EDUARDO CALUDEK"  
"EDUARDO CALUDEK"  
"EDUARDO CALUDEK"

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18. El conjugado de acuerdo a la reivindicación 17, en el cual la glicoproteína tiene una secuencia seleccionada desde un grupo que consiste de:

(a) la secuencia de eritropoietina humana y la secuencia SEQ ID NO:3 en el terminus de carboxi de la secuencia eritropoietina humana;

(b) la secuencia en (a) modificada por Ser87 Asn88 Thr90; y

(c) la secuencia en (a) modificada por Asn30 Thr32 Val87 Asn88 Thr90.

19. El conjugado de acuerdo a la reivindicación 18, en el cual R es metilo.

20. El conjugado de acuerdo a la reivindicación 18, en el cual la m es aproximadamente desde 650 hasta alrededor de 750.

21. El conjugado de acuerdo a la reivindicación 18, en el cual n es 1.

*Edwards Calado*  
Edwards Calado  
Patent Attorney  
1000 10th St, Suite 100  
San Francisco, CA 94103  
Tel: 415.774.1111  
Fax: 415.774.1112  
www.edwards-calado.com

22. El conjugado de acuerdo a la reivindicación 18, en el cual R es metilo; m es desde aproximadamente 650 hasta alrededor de 750; y n es 1.

23. El conjugado de acuerdo a la reivindicación 2, en el cual la glicoproteína tiene la secuencia humana de la arthropoietina modificada para reordenamiento de por lo menos un sitio de glicosilación.

24. El conjugado de acuerdo a la reivindicación 23, en el cual la reordenación consiste en la anulación de cualquier sitio de enlace N de glicosilación en arthropoietina humana de un sitio de glicosilación en la posición 88 en la secuencia de erithropoietina humana.

25. El conjugado de acuerdo a la reivindicación 24, en el cual la glicoproteína tiene una secuencia de erithropoietina humana modificada por una modificación seleccionada de un grupo que consiste de:

Gln24 Ser87 Asn88 Thr90;

*Edwardo Calado*  
INVENTOR  
1996

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Gln38 Ser87 Asn88 Thr90; y

Gln83 Ser87 Asn88 Thr90.

26. El conjugado de acuerdo a la reivindicación 25, en el cual R es metilo.

27. El conjugado de acuerdo a la reivindicación 25, en el cual m es desde aproximadamente 650 hasta alrededor de 750.

28. El conjugado de acuerdo a la reivindicación 25, en el cual n es 1.

29. El conjugado de acuerdo a la reivindicación 25, en el cual la R es metilo; m es aproximadamente desde 650 hasta alrededor de 750; y n es 1.

*Edwardo Calado*



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Caso:No1097P2

DECLARACION Y PODER  
PARA LA SOLICITUD DE PATENTES DE LOS ESTADOS UNIDOS

Yo en mi calidad de abajo mencionado inventor, por la presente  
declaro:

Mi residencia, dirección de Oficina Postal y ciudadanía son mencionados a  
continuación como también mi nombre.

Yo creo ser el único y primer inventor (si solo se encuentra un nombre en  
la lista) o original, primero y co-inventor del tópico en cuestión, el cual se  
reivindica pidiendo la solicitud de patente de invención con el título:

TITULO DE LA INVENCION (280 Caracteres máximo)

CONJUGADOS DE ERITHROPOIETINA

cuya especificaciones:

(Indique uno)

[x] se encuentra adjunto.

[ ] Fue registrado en \_\_\_\_\_ como

No Serial de la Solicitud \_\_\_\_\_

y fue revisado en \_\_\_\_\_

Edoardo Calado  
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(si aplica)

Yo por medio del presente documento declaro que he revisado y entiendo el contenido de las especificaciones anteriormente mencionadas, incluyendo las reivindicaciones.

Yo tengo conocimiento sobre mi obligación de revelar toda información que sea parte del material patentable tal como lo define el Artículo 37 C.F.R. §1.56.

Yo por la presente reivindico beneficios de prioridad bajo el Título 35, del Código de los Estados Unidos, Sección §119 (a) -(d) de cualquiera de la solicitud(es) extranjera para patente (s) o certificado como inventor o § 365 (a) de cualquier Solicitud para Patente Internacional (PCT), para la cual se ha nombrado por lo menos a un país además de los Estados Unidos, como es anteriormente mencionado e identificado, Indicando con una indicación en las cajillas , de cualquier solicitud extranjera para Patente certificado como inventor, o solicitud Internacional PCT con una fecha de registro anterior de aquella solicitud que aparece en la cual se reivindica prioridad.

Solicitudes extranjeras con una fecha de registro anterior (es)

Prioridades Reivindicadas

\_\_\_\_\_  
(Nombre)                      (País)                      Día/Mes/año de registro

[ ]    [ ]  
Si      No

*Edvardo Palacios* 14

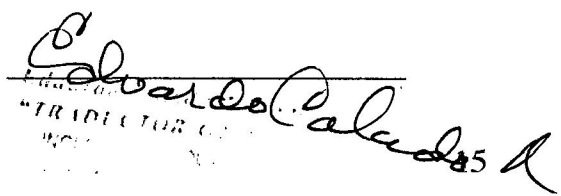
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|           |        |                         |     |     |
|-----------|--------|-------------------------|-----|-----|
| _____     | _____  | _____                   | [ ] | [ ] |
| (Nombre)  | (País) | Día/Mes/año de registro | Si  | No  |
| _____     | _____  | _____                   | [ ] | [ ] |
| (Nombre ) | (País) | Día/Mes/año de registro | Si  | No  |

Yo por medio de la presente reivindico los beneficios bajo 35 U.S.C. § 119 (e) sobre solicitudes anteriores de los Estados Unidos mencionadas a continuación:

|                 |                     |                |                     |
|-----------------|---------------------|----------------|---------------------|
| _____           | _____               | _____          | _____               |
| (No Solicitud ) | (Fecha de Registro) | (No Solicitud) | (Fecha de registro) |

Yo por medio de la presente reivindico beneficios bajo el Código 35 USC § 120 de solicitud de Patente registrada en los Estados Unidos, o §365(c) de cualquier solicitud Internacional de patentes PCT en la manera requeridas tal como se menciona anteriormente y respecto al tópico en cuestión de cada una de las reivindicaciones de esta solicitud la cual no se haya revelada con anterioridad en solicitudes anteriores en los Estados Unidos de acuerdo a lo establecido por el primer párrafo de la norma del Código 35CFR. § 112, tengo conocimiento de mi obligación de revelar toda información que se considere parte del material patentable a la Oficina de Marcas y Patentes tal como lo define la norma del Código 37 C.F.R. §1.56 el cual se hizo efectiva entre las fechas de registro de la solicitud anterior y la fecha de registro Internacional PCT de esta solicitud.

\_\_\_\_\_  
\_\_\_\_\_  
  
"TRADUCTOR"  
"NCL"

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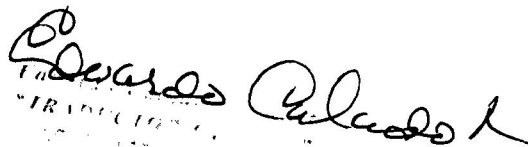
(No Serial Solicitud ) (Fecha de Registro) (estatus)(patentado,pendiente,  
abandonado)

\_\_\_\_\_  
(No Serial Solicitud ) (Fecha de Registro) (Estatus)(patentado,pendiente,  
abandonado)

Yo, por la presente afirmo que toda declaración presentada es de mi  
entero conocimiento, fiel y verdadera y tengo presente que toda  
falsa declaración es castigada mediante una multa o el  
encarcelamiento, o ambos, bajo la Sección 1001 del Título 18 del  
Código de los Estados Unidos, y que cualquier declaración puede  
comprometer la validez de esta solicitud o de cualquier patente  
expedida inmediatamente después.

**PODER :**

Como Inventor, yo por la presente nombro al (los) siguiente (s)  
abogados y /o representante (s) para procesar esta solicitud y llevar a  
cabo toda transacción de negocios en la Oficina de Marcas y Patentes  
relacionadas con la presente. (*listado de nombres y números de  
registro*)

  
Eduardo Palacios  
16

127  
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No. de Registro

|                             |         |       |
|-----------------------------|---------|-------|
| GEORGE W. JOHNSTON          | Reg No. | 28090 |
| WILLIAM H. EPSTEIN          | Reg No. | 20008 |
| LEWIS J. KREISLER           | Reg No. | 38522 |
| DENNIS P. TRAMALONI         | Reg No. | 28542 |
| PATRICIA S. ROCHA-TRAMALONI | Reg No. | 31054 |
| JOHN P. PARISE              | Reg No. | 34403 |

ENVIAR LA CORRESPONDENCIA A:

George W, Johnston, Esq.

HOFFMANN LA ROCHE INC.,

350 KINGSLAND STREET

NUTLEY, NEW JERSEY 07110-1199

llamadas telefónicas:Lewis J. Kreisler (973) 253-4387

Nombre:PASACAL SEBASTIAN BAILON

Residencia: Florham Park, Morris County, new Jersey

Ciudadanía:Estados Unidos de America

Oficina Postal:21 Woodbine Road, Florham Park, New Jersey 07932

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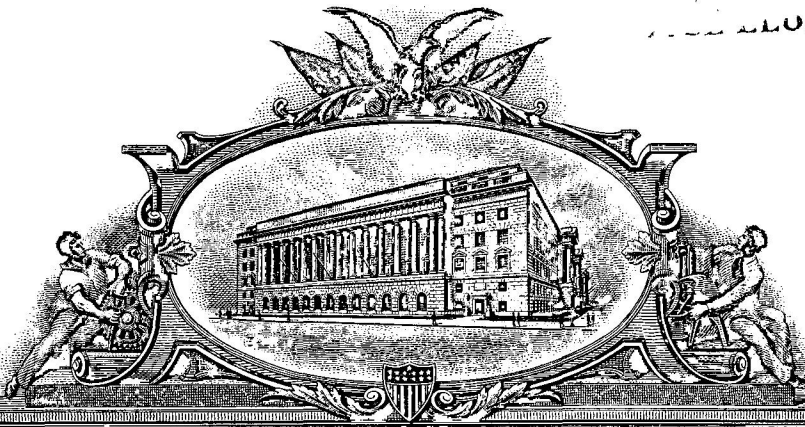
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**APPLICATION NUMBER: 60/150,225**

**FILING DATE: August 23, 1999**



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I hereby certify that this New Application Transmittal and the documents referred to as enclosed therein are being deposited with the United States Postal Service on this date August 23, 1999 in an envelope as "Express Mail Post Office to Addressee" Mailing Label Number EL267098537US addressed to the: Assistant Commissioner for Patents, Washington, D.C. 20231.

Pamela Johnston  
(Print Name)

(Signature)

*Pamela Johnston*

PROVISIONAL APPLICATION COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION under 37 CFR 1.53(b)(2).

Docket No. 1097P2

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☐ Additional inventors are being named on sheets attached hereto

TITLE OF THE INVENTION

ERYTHROPOIETIN CONJUGATES

CORRESPONDENCE ADDRESS

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ENCLOSED APPLICATION PARTS (check all that apply)

|                                                      |                                   |                                                 |                                |
|------------------------------------------------------|-----------------------------------|-------------------------------------------------|--------------------------------|
| <input checked="" type="checkbox"/> Specification    | No. of Pages <u>20</u>            | <input checked="" type="checkbox"/> Declaration | No. of Pages <u>4</u>          |
| <input checked="" type="checkbox"/> Claims           | No. of Claims <u>29 (4 pages)</u> | <input type="checkbox"/> Drawings               | Sheets of Drawings <u>    </u> |
| <input checked="" type="checkbox"/> Sequence Listing | No. of Pages <u>2</u>             | <input checked="" type="checkbox"/> Other       | <u>Abstract (1 page)</u>       |

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NO. 08-2525

OUR ORDER NO. 2695

08/23/99  
JCS64 U.S. PTO

JCS64 U.S. PTO  
08/23/99

08/23/99 10:22:25



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METHOD OF PAYMENT

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☒ No

☐ Yes, the name of the U.S. Government agency and the Government contract number are: \_\_\_\_\_  
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Respectfully submitted,



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**ERYTHROPOIETIN CONJUGATES**

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**BACKGROUND OF THE INVENTION**

Erythropoiesis is the production of red blood cells, which occurs to offset cell destruction. Erythropoiesis is a controlled physiological mechanism that enables sufficient red blood cells to be available for proper tissue oxygenation. Naturally occurring human erythropoietin (hEPO) is produced in the kidney and is the humoral plasma factor which stimulates red blood cell production (Carnot, P and Deflandre, C (1906) C.R. Acad. Sci. 143: 432; Erslev, AJ (1953 Blood 8: 349; Reissmann, KR (1950) Blood 5: 372; Jacobson, LO, Goldwasser, E, Freid, W and Plzak, LF (1957) Nature 179: 6331-4). Naturally occurring EPO stimulates the division and differentiation of committed erythroid progenitors in the bone marrow and exerts its biological activity by binding to receptors on erythroid precursors (Krantz, BS (1991) Blood 77: 419).

Erythropoietin has been manufactured biosynthetically using recombinant DNA technology (Egrie, JC, Strickland, TW, Lane, J et al. (1986) Immunobiol. 72: 213-224) and is the product of a cloned human EPO gene inserted into and expressed in the ovarian tissue cells of the chinese hamster (CHO cells). The primary structure of the predominant, fully processed form of hEPO is illustrated in SEQ ID NO:1. There are two disulfide bridges between Cys<sup>7</sup>-Cys<sup>161</sup> and Cys<sup>29</sup>-Cys<sup>33</sup>. The molecular weight of the polypeptide chain of EPO without the sugar moieties is 18,236 Da. In the intact EPO molecule, approximately 40% of the molecular weight are accounted for by the carbohydrate groups that glycosylate the protein at glycosylation sites on the protein (Sasaki, H, Bothner, B, Dell, A and Fukuda, M (1987) J. Biol. Chem. 262: 12059).

Because human erythropoietin is essential in red blood cell formation, the hormone is useful in the treatment of blood disorders characterized by low or defective red blood cell production. Clinically, EPO is used in the treatment of anemia in chronic

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renal failure patients (CRF) (Eschbach, JW, Egri, JC, Downing, MR et al. (1987) NEJM 316: 73-78; Eschbach, JW, Abdulhadi, MH, Browne, JK et al. (1989) Ann. Intern. Med. 111: 992; Egrie, JC, Eschbach, JW, McGuire, T, Adamson, JW (1988) Kidney Intl. 33: 262; Lim, VS, Degowin, RL, Zavala, D et al. (1989) Ann. Intern. Med. 110: 108-114) and in AIDS and cancer patients undergoing chemotherapy (Danna, RP, Rudnick, SA, Abels, RI In: MB, Garnick, ed. Erythropoietin in Clinical Applications-An International Perspective. New York, NY: Marcel Dekker; 1990: p. 301-324). However, the bioavailability of commercially available protein therapeutics such as EPO is limited by their short plasma half-life and susceptibility to protease degradation. These shortcomings prevent them from attaining maximum clinical potency.

### SUMMARY OF THE INVENTION

This invention provides an erythropoietin conjugate, said conjugate comprising an erythropoietin glycoprotein having at least one free amino group and having the *in vivo* biological activity of causing bone marrow cells to increase production of reticulocytes and red blood cells and selected from the group consisting of human erythropoietin and analogs thereof which have sequence of human erythropoietin modified by the addition of from 1 to 6 glycosylation sites or a rearrangement of at least one glycosylation site; said glycoprotein being covalently linked to "n" poly(ethylene glycol) groups of the formula  $-CO-(CH_2)_x-(OCH_2CH_2)_m-OR$  with the  $-CO$  (i.e. carbonyl) of each poly(ethylene glycol) group forming an amide bond with one of said amino groups; wherein R is lower alkyl; x is 2 or 3; m is from about 450 to about 900; n is from 1 to 3; and n and m are chosen so that the molecular weight of the conjugate minus the erythropoietin glycoprotein is from 20 kilodaltons to 100 kilodaltons.

Compared to unmodified EPO (i.e., EPO without a PEG attached) and conventional PEG-EPO conjugates, the present conjugates have an increased circulating half-life and plasma residence time, decreased clearance, and increased clinical activity *in vivo*. The conjugates of this invention have the same uses as EPO. In particular, the conjugates of this invention are useful to treat patients by stimulating the division and

differentiation of committed erythroid progenitors in the bone marrow in the same way EPO is used to treat patients.

## 5 DETAILED DESCRIPTION OF THE INVENTION

This invention provides conjugates, said conjugates comprising an erythropoietin glycoprotein having at least one free amino group and having the *in vivo* biological activity of causing bone marrow cells to increase production of reticulocytes and red blood cells and selected from the group consisting of human erythropoietin and analogs thereof which have sequence of human erythropoietin modified by the addition of from 1 to 6 glycosylation sites or a rearrangement of at least one glycosylation site; said glycoprotein being covalently linked to "n" poly(ethylene glycol) groups of the formula  $-\text{CO}-(\text{CH}_2)_x-(\text{OCH}_2\text{CH}_2)_m-\text{OR}$  with the  $-\text{CO}$  (i.e. carbonyl) of each poly(ethylene glycol) group forming an amide bond with one of said amino groups; wherein R is lower alkyl; x is 2 or 3; m is from about 450 to about 900; n is from 1 to 3; and n and m are chosen so that the molecular weight of the conjugate minus the erythropoietin glycoprotein is from 20 kilodaltons to 100 kilodaltons.

It has been found that the conjugates of this invention can be used in the same manner as unmodified EPO. However, the conjugates of this invention have an increased circulating half-life and plasma residence time, decreased clearance, and increased clinical activity *in vivo*. Because of these improved properties, the conjugates of this invention can be administered once weekly instead of the three times weekly for unmodified EPO. Decreased frequency of administration is expected to result in improved patient compliance leading to improved treatment outcomes, as well as improved patient quality of life. Compared to conventional conjugates of EPO linked to poly(ethylene glycol) it has been found that conjugates having the molecular weight and linker structure of the conjugates of this invention have an improved potency, stability, AUC, circulating half-life, and cost of goods profile.

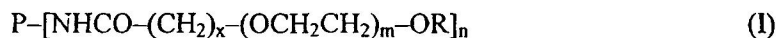
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The conjugates in accordance of this invention can be administered in a therapeutically effective amount to patients in the same way EPO is administered. The therapeutically effective amount is that amount of conjugate necessary for the *in vivo* biological activity of causing bone marrow cells to increase production of reticulocytes and red blood cells. The exact amount of conjugate is a matter of preference subject to such factors as the exact type of condition being treated, the condition of the patient being treated, as well as the other ingredients in the composition. The pharmaceutical compositions containing the conjugate may be formulated at a strength effective for administration by various means to a human patient experiencing blood disorders characterized by low or defective red blood cell production. Average therapeutically effective amounts of the conjugate may vary and in particular should be based upon the recommendations and prescription of a qualified physician.

The erythropoietin glycoprotein products prepared in accordance with this invention may be prepared in pharmaceutical compositions suitable for injection with a pharmaceutically acceptable carrier or vehicle by methods known in the art. Among the preferred pharmaceutically acceptable carriers for formulating the products of the invention are human serum albumin, human plasma proteins, etc.

The term "erythropoietin" or "EPO" refers to a glycoprotein, having the amino acid sequence set out in (SEQ ID NO: 1) or (SEQ ID NO: 2) or an amino acid sequence substantially homologous thereto, whose biological properties relate to the stimulation of red blood cell production and the stimulation of the division and differentiation of committed erythroid progenitors in the bone marrow. As used herein, these terms include such proteins modified deliberately, as for example, by site directed mutagenesis or accidentally through mutations. These terms also include analogs having from 1 to 6 additional sites for glycosylation, analogs having at least one additional amino acid at the carboxy terminal end of the glycoprotein, wherein the additional amino acid includes at least one glycosylation site, and analogs having an amino acid sequence which includes a rearrangement of at least one site for glycosylation. These terms include both natural and recombinantly produced human erythropoietin.

The erythropoietin conjugates of this invention can be represented by Formula 1:



5 wherein x, m, n and R are as above. In Formula I, P is the residue of an erythropoietin glycoprotein described herein, (i.e. without the amino group or amino groups which form an amide linkage with the carbonyl shown in Formula I), having the *in vivo* biological activity of causing bone marrow cells to increase production of reticulocytes and red blood cells. P may be selected from the group consisting of residues of human  
10 erythropoietin and analogs thereof having from 1 to 6 additional sites for glycosylation. As set out in detail below, the preparation and purification of EPO are well known in the art. By EPO is meant the natural or recombinant protein, preferably human, as obtained from any conventional source such as tissues, protein synthesis, cell culture with natural or recombinant cells. Any protein having the activity of EPO, such as muteins or  
15 otherwise modified proteins, is encompassed. Recombinant EPO may be prepared via expression in CHO-, BHK- or HeLa cell lines, by recombinant DNA technology or by endogenous gene activation. Expression of proteins, including EPO, by endogenous gene activation is well known in the art and is disclosed, for example in U.S. Patents Nos. 5,733,761, 5,641,670, and 5,733,746, the contents of each of which are incorporated  
20 herein by reference. The preferred EPO species for the preparation of erythropoietin glycoprotein products are human EPO species. More preferably, the EPO species is the human EPO having the amino acid sequence set out in SEQ ID NO:1 or SEQ ID NO:2, more preferably the amino acid sequence SEQ ID NO:1.

25 In an embodiment, P may be the residue of a glycoprotein analog having from 1 to 6 additional sites for glycosylation. Glycosylation of a protein, with one or more oligosaccharide groups, occurs at specific locations along a polypeptide backbone and greatly affects the physical properties of the protein such as protein stability, secretion, subcellular localization, and biological activity. Glycosylation is usually of two types.  
30 O-linked oligosaccharides are attached to serine or threonine residues and N-linked oligosaccharides are attached to asparagine residues. One type of oligosaccharide found

on both N-linked and O-linked oligosaccharides is N-acetylneuraminic acid (sialic acid), which is a family of amino sugars containing 9 or more carbon atoms. Sialic acid is usually the terminal residue on both N-linked and O-linked oligosaccharides and, because it bears a negative charge, confers acidic properties to the glycoprotein. Human erythropoietin, having 165 amino acids, contains three N-linked and one O-linked oligosaccharide chains which comprise about 40% of the total molecular weight of the glycoprotein. N-linked glycosylation occurs at asparagine residues located at positions 24, 38, and 83 and O-linked glycosylation occurs at a serine residue located at position 126. The oligosaccharide chains are modified with terminal sialic acid residues. Enzymatic removal of all sialic acid residues from the glycosylated erythropoietin results in loss of *in vivo* activity but not *in vitro* activity because sialylation of erythropoietin prevents its binding, and subsequent clearance, by hepatic binding protein.

The glycoproteins of the present invention include analogs of human erythropoietin with one or more changes in the amino acid sequence of human erythropoietin which result in an increase in the number of sites for sialic acid attachment. These glycoprotein analogs may be generated by site-directed mutagenesis having additions, deletions, or substitutions of amino acid residues that increase or alter sites that are available for glycosylation. Glycoprotein analogs having levels of sialic acid greater than those found in human erythropoietin are generated by adding glycosylation sites which do not perturb the secondary or tertiary conformation required for biological activity. The glycoproteins of the present invention also include analogs having increased levels of carbohydrate attachment at a glycosylation site which usually involve the substitution of one or more amino acids in close proximity to an N-linked or O-linked site. The glycoproteins of the present invention also include analogs having one or more amino acids extending from the carboxy terminal end of erythropoietin and providing at least one additional carbohydrate site. The glycoproteins of the present invention also include analogs having an amino acid sequence which includes a rearrangement of at least one site for glycosylation. Such a rearrangement of glycosylation site involves the deletion of one or more glycosylation sites in human erythropoietin and the addition of one or more non-naturally occurring glycosylation sites. Increasing the number of carbohydrate chains on

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erythropoietin, and therefore the number of sialic acids per erythropoietin molecules may confer advantageous properties such as increased solubility, greater resistance to proteolysis, reduced immunogenicity, increased serum half-life, and increased biological activity. Erythropoietin analogs with additional glycosylation sites are disclosed in more  
5 detail in European Patent Application 640 619, to Elliot published March 1, 1995.

In a preferred embodiment, the glycoproteins of the present invention comprise an amino acid sequence which includes at least one additional site for glycosylation such as, but not limited to, erythropoietins comprising the sequence of human erythropoietin  
10 modified by a modification selected from the following:

- Asn<sup>30</sup>Thr<sup>32</sup>;  
Asn<sup>51</sup>Thr<sup>53</sup>;  
Asn<sup>57</sup>Thr<sup>59</sup>;  
Asn<sup>69</sup>;  
15 Asn<sup>69</sup>Thr<sup>71</sup>;  
Ser<sup>68</sup>Asn<sup>69</sup>Thr<sup>71</sup>;  
Val<sup>87</sup>Asn<sup>88</sup>Thr<sup>90</sup>;  
Ser<sup>87</sup>Asn<sup>88</sup>Thr<sup>90</sup>;  
Ser<sup>87</sup>Asn<sup>88</sup>Gly<sup>89</sup>Thr<sup>90</sup>;  
20 Ser<sup>87</sup>Asn<sup>88</sup>Thr<sup>90</sup>Thr<sup>92</sup>;  
Ser<sup>87</sup>Asn<sup>88</sup>Thr<sup>90</sup>Ala<sup>162</sup>;  
Asn<sup>69</sup>Thr<sup>71</sup>Ser<sup>87</sup>Asn<sup>88</sup>Thr<sup>90</sup>;  
Asn<sup>30</sup>Thr<sup>32</sup>Val<sup>87</sup>Asn<sup>88</sup>Thr<sup>90</sup>;  
Asn<sup>89</sup>Ile<sup>90</sup>Thr<sup>91</sup>;  
25 Ser<sup>87</sup>Asn<sup>89</sup>Ile<sup>90</sup>Thr<sup>91</sup>;  
Asn<sup>136</sup>Thr<sup>138</sup>;  
Asn<sup>138</sup>Thr<sup>140</sup>;  
Thr<sup>125</sup>; and  
Pro<sup>124</sup>Thr<sup>125</sup>.

30 The notation used herein for modification of amino acid sequence means that the position(s) of the corresponding unmodified protein (e.g. hEPO of SEQ ID NO:1 or SEQ



ID NO:2) indicated by the superscripted number(s) is changed to the amino acid(s) that immediately precede the respective superscripted number(s).

The glycoprotein may also be an analog having at least one additional amino acid  
5 at the carboxy terminal end of the glycoprotein, wherein the additional amino acid includes at least one glycosylation site. The additional amino acid may comprise a peptide fragment derived from the carboxy terminal end of human chorionic gonadotropin. Preferably, the glycoprotein is an analog selected from the group consisting of (a) human erythropoietin having the amino acid sequence, Ser Ser Ser Ser  
10 Lys Ala Pro Pro Pro Ser Leu Pro Ser Pro Ser Arg Leu Pro Gly Pro Ser Asp Thr Pro Ile Leu Pro Gln (SEQ ID NO:3), extending from the carboxy terminus; (b) the analog in (a) further comprising Ser<sup>87</sup> Asn<sup>88</sup> Thr<sup>90</sup> EPO; and (c) the analog in (a) further comprising Asn<sup>30</sup> Thr<sup>32</sup> Val<sup>87</sup> Asn<sup>88</sup> Thr<sup>90</sup> EPO.

15 The glycoprotein may also be an analog having an amino acid sequence which includes a rearrangement of at least one site for glycosylation. The rearrangement may comprise a deletion of any of the N-linked carbohydrate sites in human erythropoietin and an addition of an N-linked carbohydrate site at position 88 of the amino acid sequence of human erythropoietin. Preferably, the glycoprotein is an analog selected  
20 from the group consisting of Gln<sup>24</sup> Ser<sup>87</sup> Asn<sup>88</sup> Thr<sup>90</sup> EPO; Gln<sup>38</sup> Ser<sup>87</sup> Asn<sup>88</sup> Thr<sup>90</sup> EPO; and Gln<sup>83</sup> Ser<sup>87</sup> Asn<sup>88</sup> Thr<sup>90</sup> EPO.

As used herein, "lower alkyl" means a linear or branched alkyl group having from one to six carbon atoms. Examples of lower alkyl groups include methyl, ethyl and  
25 isopropyl. In accordance with this invention, R is any lower alkyl. Conjugates in which R is methyl are preferred.

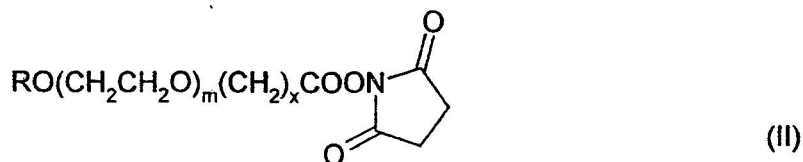
The symbol "m" represents the number of ethylene oxide residues (OCH<sub>2</sub>CH<sub>2</sub>) in the poly(ethylene oxide) group. A single PEG subunit of ethylene oxide has a molecular  
30 weight of about 44 daltons. Thus, the molecular weight of the conjugate (excluding the molecular weight of the EPO) depends on the number "m". In the conjugates of this

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invention "m" is from about 450 to about 900 (corresponding to a molecular weight of about 20 kDa to about 40 kDa), preferably from about 650 to about 750 (corresponding to a molecular weight of about 30 kDa). The number m is selected such that the resulting conjugate of this invention has a physiological activity comparable to unmodified EPO, which activity may represent the same as, more than, or a fraction of the corresponding activity of unmodified EPO. A molecular weight of "about" a certain number means that it is within a reasonable range of that number as determined by conventional analytical techniques. The number "m" is selected so that the molecular weight of each poly(ethylene glycol) group covalently linked to the erythropoietin glycoprotein is from about 20kDa to about 40kDa, and is preferably about 30kDa.

In the conjugates of this invention, the number "n" is the number of polyethylene glycol groups covalently bound to free amino groups (including  $\epsilon$ -amino groups of a lysine amino acid and/or the amino-terminal amino group) of an erythropoietin protein *via* amide linkage(s). A conjugate of this invention may have one, two, or three PEG groups per molecule of EPO. "n" is an integer ranging from 1 to 3, preferably "n" is 1 or 2, and more preferably "n" is 1.

The compound of Formula I can be prepared from the known polymeric material:



in which R and m are as described above, by condensing the compound of Formula II with the erythropoietin glycoprotein. Compounds of Formula II in which x is 3 are alpha-lower alkoxy, butyric acid succinimidyl esters of poly(ethylene glycol) (lower alkoxy-PEG-SBA). Compounds of Formula II in which x is 2 are alpha-lower alkoxy, propionic acid succinimidyl esters of poly(ethylene glycol) (lower alkoxy-PEG-SPA).

Any conventional method of reacting an activated ester with an amine to form an amide can be utilized. In the reaction described above, the exemplified succinimidyl ester is a leaving group causing the amide formation. The use of succinimidyl esters such as the compounds of formula II to produce conjugates with proteins are disclosed in U.S. Patent  
5 No. 5,672,662, issued September 30, 1997 (Harris, et al.).

Human EPO contains nine free amino groups, the amino-terminal amino group plus the  $\epsilon$ -amino groups of 8 lysine residues. When the pegylation reagent was combined with a SBA compound of Formula II, it has been found that at pH 7.5, a protein:PEG  
10 ratio of 1:3, and a reaction temperature of from 20-25°C, a mixture of mono-, di-, and trace amounts of the tri-pegylated species were produced. When the pegylation reagent was a SPA compound of Formula II, at similar conditions except that the protein:PEG ratio was 1:2, primarily the mono-pegylated species is produced. The pegylated EPO can be administered as a mixture, or as the cation exchange chromatography separated  
15 different pegylated species. By manipulating the reaction conditions (e.g., ratio of reagents, pH, temperature, protein concentration, time of reaction etc.), the relative amounts of the different pegylated species can be varied.

Human erythropoietin (EPO) is a human glycoprotein which stimulates the  
20 formation of erythrocytes. Its preparation and therapeutic application are described in detail for example in U.S. Patent Nos. 5,547,933 and 5,621,080, EP-B 0 148 605, Huang, S.L., Proc. Natl. Acad. Sci. USA (1984) 2708-2712, EP-B 0 205 564, EP-B 0 209 539 and EP-B 0 411 678 as well as Lai, P.H. et al., J. Biol. Chem. 261 (1986) 3116-3121, an Sasaki, H. et al., J. Biol. Chem. 262 (1987) 12059-12076. Erythropoietin for therapeutic  
25 uses may be produced by recombinant means (EP-B 0 148 605, EP-B 0 209 539 and Egrie, J.C., Strickland, T.W., Lane, J. et al. (1986) Immunobiol. 72: 213-224).

Methods for the expression and preparation of erythropoietin in serum free medium are described for example in WO 96/35718, to Burg published 14 November 1996, and in  
30 European Patent Publication No. 513 738, to Koch published 12 June 1992. In addition to the publications mentioned above, it is known that a serum-free fermentation of

recombinant CHO cells which contain an EPO gene can be carried out. Such methods are described for example in EP-A 0 513 738, EP-A 0 267 678 and in a general form by Kawamoto, T. et al., Analytical Biochem. 130 (1983) 445-453, EP-A 0 248 656, Kowar, J. and Franek, F., Methods in Enzymology 421 (1986) 277-292, Bavister, B., Expology  
5 271 (1981) 45-51, EP-A 0 481 791, EP-A 0 307 247, EP-A 0 343 635, WO 88/00967.

In EP-A 0 267 678 an ion exchange chromatography on S-Sepharose, a preparative reverse phase HPLC on a Cg column and a gel filtration chromatography are described for the purification of EPO produced in serum-free culture after dialysis. In this connection the gel filtration chromatography step can be replaced by ion exchange  
10 chromatography on S-Sepharose fast flow. It is also proposed that a dye chromatography on a Blue Trisacryl column be carried out before the ion exchange chromatography.

A process for the purification of recombinant EPO is described by Nobuo, I. et al., J. Biochem. 107 (1990) 352-359. In this process EPO is treated however with a solution of  
15 Tween<sup>®</sup> 20, phenylmethylsulfonyl fluoride, ethylmaleimide, pepstatin A, copper sulfate and oxamic acid prior to the purification steps. Publications, including WO 96/35718, to Burg published 14 November 1996, discloses a process for preparing erythropoietin in a serum free fermentation process (EPOsf).

The specific activity of EPO or EPO conjugates in accordance with this invention  
20 can be determined by various assays known in the art. The biological activity of the purified EPO proteins of this invention are such that administration of the EPO protein by injection to human patients results in bone marrow cells increasing production of reticulocytes and red blood cells compared to non-injected or control groups of subjects.  
25 The biological activity of the EPO proteins, or fragments thereof, obtained and purified in accordance with this invention can be tested by methods according to Annable, et al., Bull. Wld. Hlth. Org. (1972) 47: 99-112 and Pharm. Europa Spec. Issue Erythropoietin BRP Bio 1997(2). Another biological assay for determining the activity of EPO protein, the normocythaemic mouse assay, is described in Example 4.

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The invention will be better understood by reference to the following examples which illustrate but do not limit the invention described herein.

### EXAMPLE 1

#### 5 Fermentation And Purification Of Human EPO

##### a) Inoculum Preparation and Fermentation

One vial of the Working Cell Bank, originating from an EPO-producing CHO cell  
10 line (ATCC CRL8695, disclosed in EP 411 678 (Genetics Institute) can be used) is taken from the gas phase of the liquid nitrogen storage tank. The cells are transferred into glass spinner flasks and cultivated in a hydrogen carbonate-buffered medium in a humidified CO<sub>2</sub> incubator. Typical serum free media used for the inoculum preparation and fermentation are disclosed in European Patent Application 513 738, to Koch published 12  
15 June 1992, or WO 96/35718, to Burg published 14 November 1996, for example contain as medium DMEM/F12 (e.g. JRH Biosciences/Hazleton Biologics, Denver, US, order No. 57-736) and additionally sodium hydrogencarbonate, L+glutamine, D+glucose, recombinant insulin, sodium selenite, diaminobutane, hydrocortisone, iron(II) sulfate, asparagine, aspartic acid, serine and a stabilizer for mammalian cells such as e.g.  
20 polyvinyl alcohol, methyl cellulose, polydextran, polyethylene glycol, Pluronic F68, plasma expander polygelin (HEMACCEL<sup>®</sup>) or polyvinyl pyrrolidone (WO 96/35718).

The cultures are microscopically checked for the absence of contaminating microorganisms, and the cell densities are determined. These tests are performed at each splitting step.

25

After the initial growth period, the cell culture is diluted with fresh medium to the starting cell density and undergoes another growth cycle. This procedure is repeated until a culture volume of approximately 2 l per glass spinner flask has been obtained. After approx. 12 doublings 1 to 5 liter of this culture is available which then is used as  
30 inoculum for the 10 l inoculum fermenter.

After 3 - 5 days, the culture in the 10 l fermenter can be used as inoculum for the 100 l inoculum fermenter.

After additional 3 - 5 days of cultivation, the culture in the 100 l fermenter can be used as inoculum for the 1000 l production fermenter.

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#### b) Harvesting and Cell Separation

A batch refeed process is used, i.e. when the desired cell density is reached, approx. 80 % of the culture is harvested. The remaining culture is replenished with fresh culture medium and cultivated until the next harvest. One production run consists of a maximum of 10 subsequent harvests: 9 partial harvests and 1 overall harvest at the end of fermentation. Harvesting takes place every 3 - 4 days.

The determined harvest volume is transferred into a cooled vessel. The cells are removed by centrifugation or filtration and discarded. The EPO containing supernatant of the centrifugation step is in-line filtered and collected in a second cooled vessel. Each harvest is processed separately during purification.

A typical process for the purification of EPO-protein is disclosed in WO 96/35718, to Burg published 14 November 1996. The purification process is explained in the following.

#### 25 a) Blue Sepharose Chromatography

Blue Sepharose (Pharmacia) consists of Sepharose beads to the surface of which the Cibacron blue dye is covalently bound. Since EPO binds more strongly to Blue Sepharose than most non-proteinaceous contaminants, some proteinaceous impurities and PVA, EPO can be enriched in this step. The elution of the Blue Sepharose column is performed by increasing the salt concentration as well as the pH.

The column is filled with 80 - 100 l of Blue Sepharose, regenerated with NaOH and equilibrated with equilibration buffer (sodium/ calcium chloride and sodium acetate). The acidified and filtered fermenter supernatant is loaded. After completion of the loading, the column is washed first with a buffer similar to the equilibration buffer containing a higher sodium chloride concentration and consecutively with a Tris-base buffer. The product is eluted with a Tris-base buffer and collected in a single fraction in accordance with the master elution profile.

10 b) Butyl Toyopearl Chromatography

The Butyl Toyopearl 650 C (Toso Haas) is a polystyrene based matrix to which aliphatic butyl-residues are covalently coupled. Since EPO binds more strongly to this gel than most of the impurities and PVA, it has to be eluted with a buffer containing isopropanol.

The column is packed with 30 - 40 l of Butyl Toyopearl 650 C, regenerated with NaOH, washed with a Tris-base buffer and equilibrated with a Tris-base buffer containing isopropanol.

The Blue Sepharose eluate is adjusted to the concentration of isopropanol in the column equilibration buffer and loaded onto the column. Then the column is washed with equilibration buffer with increased isopropanol concentration. The product is eluted with elution buffer (Tris-base buffer with high isopropanol content) and collected in a single fraction in accordance with the master elution profile.

c) Hydroxyapatite Ultrogel Chromatography

The Hydroxyapatite Ultrogel (Biosepra) consists of hydroxyapatite which is incorporated in an agarose matrix to improve the mechanical properties. EPO has a low

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affinity to hydroxyapatite and can therefore be eluted at lower phosphate concentrations than protein impurities.

5 The column is filled with 30 - 40 l of Hydroxyapatite Ultrogel and regenerated with a potassium phosphate/ calcium chloride buffer and NaOH followed by a Tris-base buffer. Then it is equilibrated with a Tris-base buffer containing a low amount of isopropanol and sodium chloride.

10 The EPO containing eluate of the Butyl Toyopearl chromatography is loaded onto the column. Subsequently the column is washed with equilibration buffer and a Tris-base buffer without isopropanol and sodium chloride. The product is eluted with a Tris-base buffer containing a low concentration of potassium phosphate and collected in a single fraction in accordance with the master elution profile.

15 d) Reversed Phase HPLC on Vydac C4

20 The RP-HPLC material Vydac C4 (Vydac) consists of silica gel particles, the surfaces of which carry C4-alkyl chains. The separation of EPO from the proteinaceous impurities is based on differences in the strength of hydrophobic interactions. Elution is performed with an acetonitrile gradient in diluted trifluoroacetic acid.

25 Preparative HPLC is performed using a stainless steel column (filled with 2.8 to 3.2 liter of Vydac C4 silicagel). The Hydroxyapatite Ultrogel eluate is acidified by adding trifluoro-acetic acid and loaded onto the Vydac C4 column. For washing and elution an acetonitrile gradient in diluted trifluoroacetic acid is used. Fractions are collected and immediately neutralized with phosphate buffer. The EPO fractions which are within the IPC limits are pooled.

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#### e) DEAE Sepharose Chromatography

The DEAE Sepharose (Pharmacia) material consists of diethylaminoethyl (DEAE) - groups which are covalently bound to the surface of Sepharose beads. The binding of EPO to the DEAE groups is mediated by ionic interactions. Acetonitrile and trifluoroacetic acid pass through the column without being retained. After these substances have been washed off, trace impurities are removed by washing the column with acetate buffer at a low pH. Then the column is washed with neutral phosphate buffer and EPO is eluted with a buffer with increased ionic strength.

The column is packed with DEAE Sepharose fast flow. The column volume is adjusted to assure an EPO load in the range of 3 - 10 mg EPO/ml gel. The column is washed with water and equilibration buffer (sodium/ potassium phosphate). The pooled fractions of the HPLC eluate are loaded and the column is washed with equilibration buffer. Then the column is washed with washing buffer (sodium acetate buffer) followed by washing with equilibration buffer. Subsequently, EPO is eluted from the column with elution buffer (sodium chloride, sodium/ potassium phosphate) and collected in a single fraction in accordance with the master elution profile.

The eluate of the DEAE Sepharose column is adjusted to the specified conductivity. The resulting drug substance is sterile filtered into Teflon bottles and stored at -70 °C.

#### EXAMPLE 2

##### Pegylation of EPO with mPEG-SBA

EPO purified in accordance with the serum free procedure of Example 1 (EPOsf) was homogeneous as determined by analytical methods and showed the typical isoform pattern consisting of 8 isoforms. It had a specific biological activity of 190,000 IU/mg as determined by the normocythaemic mouse assay. The pegylation reagent used was a methoxy-PEG-SBA, which is a compound of Formula II in which R is methyl; x is 3; and

m is from 650 to 750 (avg. about 680, corresponding to an average molecular weight of about 30 kDa).

### Pegylation Reaction

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To one hundred milligrams of EPOsf (9.71 ml of a 10.3 mg/ml EPOsf stock , 5.48  $\mu$ mol) 10 ml of 0.1 M potassium phosphate buffer, pH, 7.5 containing 506 mg of 30kDa methoxy-PEG-SBA (16.5  $\mu$  mol) (obtained from Shearwater Polymers, Inc., Huntsville, Alabama) was added and mixed for 2h at room temperature (20-23 °C). The final protein  
10 concentration was 5 mg/ml and the protein:PEG reagent ratio was 1:3. After two hours, the reaction was stopped by adjusting the pH to 4.5 with glacial acetic acid and stored at -20°C, until ready for purification.

### Purification

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1. **Conjugate Mixture:** Approximately 28 ml of SP-SEPHAROSE FF (sulfo-propyl cation exchange resin) was packed into an AMICON glass column (2.2 x 7.5 cm) and equilibrated with 20 mM acetate buffer pH, 4.5 at a flowrate of 150 ml/h. Six milliliters of the reaction mixture containing 30 mg protein was diluted 5-fold with  
20 the equilibration buffer and applied onto the column. Unadsorbed materials were washed away with the buffer and the adsorbed PEG conjugate mixture was eluted from the column with 0.175 M NaCl in the equilibration buffer. Unmodified EPOsf still remaining on the column was eluted with 750 mM NaCl. Column was reequilibrated in the starting buffer. Samples were analyzed by SDS-PAGE and their  
25 degree of pegylation were determined. It was found that the 0.175M NaCl eluate contained, mono- as well as di- and trace amounts of the tri-pegylated species, whereas the 750 mM NaCl eluate contained unmodified EPOsf.

25

2. **Di-PEG and Mono-PEG-EPOsf:** The purified conjugate mixture eluted from the  
30 column in the previous step was diluted 4-fold with the buffer and reapplied onto the column and washed as described. Di-PEG-EPOsf and mono-PEG-EPOsf were

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separately eluted from the column with 0.1M NaCl and 0.175 M NaCl, respectively. Elution was also performed with 750mM NaCl to elute any remaining unmodified EPOsf.

- 5      Alternatively, the reaction mixture was diluted 5-fold with the acetate buffer and applied onto the SP-Sepharose column (~0.5 mg protein/ml gel). Column was washed and adsorbed mono-PEG-EPOsf, di-PEG-EPOsf and unmodified EPOsf were eluted as described in the previous section.

## 10      **Results**

- PEG-EPOsf was synthesized by chemically conjugating a linear PEG molecule with a number average molecular weight of 30 kDa. PEG-EPOsf was derived from the reaction between the primary amino groups of EPOsf and the succinimidyl ester derivative of a 30  
15      kDa PEG-butyric acid, resulting in an amide bond.

- Results are summarized in Table1. Purified conjugate mixture comprised of mono- and di-PEG-EPOsf and was free of unmodified EPOsf as determined by SDS-PAGE analysis. Conjugate mixture accounted for 23.4 mg or 78% of the starting material. Cation  
20      exchange chromatographic separation of mono- and di-PEG-EPOsf indicated that mono- to di-PEG ratio in the conjugate mixture was almost 1:1. After completion of the reaction, ratio of the individual components of Mono: Di: Unmodified were 40: 38: 20 (%). Overall yield was almost quantitative.

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Table 1. Summary of results of EPOsf pegylation

|    | Sample      | Protein (mg) | Yield (%) |
|----|-------------|--------------|-----------|
| 5  | Rxn. Mix.   | 30           | 100       |
|    | Mono-       | 12.0         | 40        |
|    | Di-         | 11.4         | 38        |
| 10 | Unmod.      | 6.0          | 20        |
|    | Conju. Mix. | 23.4         | 78        |
| 15 |             |              |           |

EXAMPLE 3

Pegylation of EPO with mPEG-SPA

20 A different aliquot of the EPOsf used in Example 2 was reacted with 30 kDa methoxy-PEG-SPA (Shearwater Polymers, Inc., Huntsville, Alabama). Reaction was performed at a protein:reagent ratio of 1:2 and purification techniques were in accordance with Example 2. Primarily the mono-pegylated species was produced.

25

EXAMPLE 4

In-vivo activity of pegylated EPO determined by the normocythaemic mouse assay

30 The normocythaemic mouse bioassay is known in the art (Pharm. Europa Spec. Issue Erythropoietin BRP Bio 1997(2)) and a method in the monography of erythropoietin of Ph. Eur. BRP. The samples were diluted with BSA-PBS. Normal healthy mice, 7-15 weeks old, were administered s.c. 0.2 ml of the EPO-fraction containing un-pegylated EPO or tri-, di- or mono-pegylated EPO from Example 2 or 3. Over a period of 6 days,  
35 blood was drawn by puncture of the tail vein and diluted such that 1 µl of blood was

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present in 1 ml of an 0.15  $\mu$ mol acridine orange staining solution. The staining time was 3 to 10 minutes. The reticulocyte counts were carried out microfluorometrically in a flow cytometer by analysis of the red fluorescence histogram. The reticulocyte counts were given in terms of absolute figures (per 30,000 blood cells analyzed). For the data presented, each group consisted of 5 mice per day, and the mice were bled only once.

In separate experiments, a single dose of unmodified EPO (25 ng of EPO), the PEG(SBA)-EPO mixture from Example 2 (10 ng of conjugate), mono- and di- pegylated EPOs from Example 2 (10 ng of conjugate), the PEG(SPA)-EPO from Example 3 (10 ng of conjugate), and buffer solution were administered to mice. The results are shown in Table 2. The results show the superior activity and the prolonged half life of the pegylated EPO species indicated by the significantly increased amounts of reticulocytes and the shift of the reticulocytes count maximum using the same dose per mouse (10 ng), compared to a dose of 25 ng for unmodified EPO.

TABLE 2

|      | EPO<br>(Unmodified) | 30 kDa SPA<br>PEG | Mono 30K<br>SBA | Di 30K<br>SBA | PEG-EPO<br>SBA Conjugate<br>Mixture | Control<br>Buffer |
|------|---------------------|-------------------|-----------------|---------------|-------------------------------------|-------------------|
| 72h  | 1000                | 1393              | 1411            | 994           | 1328                                | 857               |
| 96h  | 500                 | 1406              | 1501            | 926           | 1338                                | 697               |
| 120h | ~200                | 1100              | 1182            | 791           | 944                                 | 701               |
| 144h | ~0                  | 535               | 607             | 665           | 660                                 | 708               |
|      |                     |                   |                 |               |                                     |                   |

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SEQUENCE LISTING

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15

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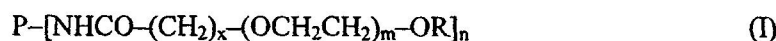
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20 25

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What is claimed is:

1. A conjugate, said conjugate comprising an erythropoietin glycoprotein having at least one free amino group and having the *in vivo* biological activity of causing bone marrow cells to increase production of reticulocytes and red blood cells and selected from the group consisting of human erythropoietin and analogs thereof which have sequence of human erythropoietin modified by the addition of from 1 to 6 glycosylation sites or a rearrangement of at least one glycosylation site; said glycoprotein being covalently linked to "n" poly(ethylene glycol) groups of the formula  
10  $-\text{CO}-(\text{CH}_2)_x-(\text{OCH}_2\text{CH}_2)_m-\text{OR}$  with the  $-\text{CO}$  of each poly(ethylene glycol) group forming an amide bond with one of said amino groups; wherein R is lower alkyl; x is 2 or 3; m is from about 450 to about 900; n is from 1 to 3; and n and m are chosen so that the molecular weight of the conjugate minus the erythropoietin glycoprotein is from 20 kilodaltons to 100 kilodaltons.

15 2. The conjugate of claim 1, of the formula:



20 wherein m, n and R are as above, and P is the residue of the glycoprotein without the n amino group(s) which form amide linkage(s) with the poly(ethylene glycol) group(s).

3. The conjugate of claim 2, wherein the glycoprotein is a human erythropoietin.

25 4. The conjugate of claim 3, wherein the human erythropoietin glycoprotein is expressed by endogenous gene activation.

5. The conjugate of claim 3, wherein the glycoprotein has the sequence SEQ ID NO:1.

30 6. The conjugate of claim 5, wherein R is methyl.



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7. The conjugate of claim 5, wherein m is from about 650 to about 750.

8. The conjugate of claim 5, wherein n is 1.

5

9. The conjugate of claim 5, wherein R is methyl; m is from about 650 to about 750; and n is 1.

10. The conjugate of claim 2, wherein the glycoprotein has the sequence of human erythropoietin modified by the addition of from 1 to 6 glycosylation sites.

11. The conjugate of claim 10, wherein the glycoprotein has the sequence of human erythropoietin modified by a modification selected from the group consisting of:

Asn<sup>30</sup>Thr<sup>32</sup>;

15 Asn<sup>51</sup>Thr<sup>53</sup>,

Asn<sup>57</sup>Thr<sup>59</sup>;

Asn<sup>69</sup>;

Asn<sup>69</sup>Thr<sup>71</sup>;

Ser<sup>68</sup>Asn<sup>69</sup>Thr<sup>71</sup>;

20 Val<sup>87</sup>Asn<sup>88</sup>Thr<sup>90</sup>;

Ser<sup>87</sup>Asn<sup>88</sup>Thr<sup>90</sup>;

Ser<sup>87</sup>Asn<sup>88</sup>Gly<sup>89</sup>Thr<sup>90</sup>;

Ser<sup>87</sup>Asn<sup>88</sup>Thr<sup>90</sup>Thr<sup>92</sup>;

Ser<sup>87</sup>Asn<sup>88</sup>Thr<sup>90</sup>Ala<sup>162</sup>;

25 Asn<sup>69</sup>Thr<sup>71</sup>Ser<sup>87</sup>Asn<sup>88</sup>Thr<sup>90</sup>;

Asn<sup>30</sup>Thr<sup>32</sup>Val<sup>87</sup>Asn<sup>88</sup>Thr<sup>90</sup>;

Asn<sup>89</sup>Ile<sup>90</sup>Thr<sup>91</sup>;

Ser<sup>87</sup>Asn<sup>89</sup>Ile<sup>90</sup>Thr<sup>91</sup>;

Asn<sup>136</sup>Thr<sup>138</sup>;

30 Asn<sup>138</sup>Thr<sup>140</sup>;

Thr<sup>125</sup>; and

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Pro<sup>124</sup>Thr<sup>125</sup>.

12. The conjugate of claim 11, wherein R is methyl.

5 13. The conjugate of claim 11, wherein m is from about 650 to about 750.

14. The conjugate of claim 11, wherein n is 1.

15. The conjugate of claim 11, wherein R is methyl; m is from about 650 to about  
10 750; and n is 1.

16. The conjugate of claim 10, wherein the glycoprotein has a sequence comprising  
the sequence of human erythropoietin and a second sequence at the carboxy terminus of  
the human erythropoietin sequence, wherein the second sequence contains at least one  
15 glycosylation site.

17. The conjugate of claim 16, wherein the second sequences comprises a sequence  
derived from the carboxy terminal sequence of human chorionic gonadotropin.

20 18. The conjugate of claim 17, wherein the glycoprotein has a sequence selected from  
the group consisting of:

- (a) the sequence of human erythropoietin and the sequence SEQ ID NO:3 at the  
carboxy terminus of the human erythropoietin sequence;  
(b) the sequence in (a) modified by Ser<sup>87</sup> Asn<sup>88</sup> Thr<sup>90</sup>; and  
25 (c) the sequence in (a) modified by Asn<sup>30</sup> Thr<sup>32</sup> Val<sup>87</sup> Asn<sup>88</sup> Thr<sup>90</sup>.

19. The conjugate of claim 18, wherein R is methyl.

20. The conjugate of claim 18, wherein m is from about 650 to about 750.

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21. The conjugate of claim 18, wherein n is 1.

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23. The conjugate of claim 2, wherein the glycoprotein has the sequence of human erythropoietin modified by a rearrangement of at least one glycosylation site.

24. The conjugate of claim 23, wherein the rearrangement comprises deletion of any of the N-linked glycosylation sites in human erythropoietin and addition of an N-linked glycosylation site at position 88 of the sequence of human erythropoietin.

25. The conjugate of claim 24, wherein the glycoprotein has the sequence of human erythropoietin modified by a modification selected from the group consisting of:

Gln<sup>24</sup> Ser<sup>87</sup> Asn<sup>88</sup> Thr<sup>90</sup>;

Gln<sup>38</sup> Ser<sup>87</sup> Asn<sup>88</sup> Thr<sup>90</sup>; and

Gln<sup>83</sup> Ser<sup>87</sup> Asn<sup>88</sup> Thr<sup>90</sup>.

26. The conjugate of claim 25, wherein R is methyl.

27. The conjugate of claim 25, wherein m is from about 650 to about 750.

28. The conjugate of claim 25, wherein  $n$  is 1.

29. The conjugate of claim 25, wherein R is methyl; m is from about 650 to about 750; and n is 1.

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## ABSTRACT OF THE INVENTION

Conjugates of erythropoietin with poly(ethylene glycol) comprise an erythropoietin glycoprotein having at least one free amino group and having the *in vivo* biological activity of causing bone marrow cells to increase production of reticulocytes and red blood cells and selected from the group consisting of human erythropoietin and analogs thereof which have sequence of human erythropoietin modified by the addition of from 1 to 6 glycosylation sites or a rearrangement of at least one glycosylation site; said glycoprotein being covalently linked to "n" poly(ethylene glycol) groups of the formula  $-\text{CO}-(\text{CH}_2)_x(\text{OCH}_2\text{CH}_2)_m-\text{OR}$  with the carbonyl of each poly(ethylene glycol) group forming an amide bond with one of said amino groups; wherein R is lower alkyl; x is 2 or 3; m is about 450 to about 900; n is from 1 to 3; and n and m are chosen so that the molecular weight of the conjugate minus the erythropoietin glycoprotein is from 20 kilodaltons to 100 kilodaltons.

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I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s), or § 365(c) of any P International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR § 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

|                                   |                        |                                                  |
|-----------------------------------|------------------------|--------------------------------------------------|
| _____<br>(Application Serial No.) | _____<br>(Filing Date) | _____<br>(Status) (patented, pending, abandoned) |
|-----------------------------------|------------------------|--------------------------------------------------|

|                                   |                        |                                                  |
|-----------------------------------|------------------------|--------------------------------------------------|
| _____<br>(Application Serial No.) | _____<br>(Filing Date) | _____<br>(Status) (patented, pending, abandoned) |
|-----------------------------------|------------------------|--------------------------------------------------|

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. *(list name and registration number)*

|                    |                  |                             |                  |
|--------------------|------------------|-----------------------------|------------------|
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| William H. Epstein | (Reg. No. 20008) | Patricia S. Rocha-Tramaloni | (Reg. No. 31054) |
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Second Inventor's signature Date

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Full name of third joint inventor, if any

Third Inventor's signature Date

Residence

Citizenship

Post Office Address

Full name of fourth joint inventor, if any

Fourth Inventor's signature Date

Residence

Citizenship

Post Office Address

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159

Full name of fifth joint inventor, if any

Fifth Inventor's signature

Date

Residence

Citizenship

Post Office Address

Title 37, Code of Federal Regulations, §1.56, duty to disclose information material to patentability provides, in part, that each individual associated with the filing and prosecution of a patent application has a duty of candor and good faith in dealing with the Office, which includes a duty to disclose to the Office all information known to that individual to be material to patentability as defined in this section. The duty to disclose information exists with respect to each pending claim until the claim is cancelled or withdrawn from consideration, or the application becomes abandoned.

Under this section, information is material to patentability when it is not cumulative to information already of record or being made of record in the application, and

- (1) It establishes, by itself or in combination with other information, a prima facie case of unpatentability of a claim; or
- (2) It refutes, or is inconsistent with, a position the applicant takes in:
  - (i) Opposing an argument of unpatentability relied on by the Office, or
  - (ii) Asserting an argument of patentability.

Junio 14, 2000  
Traducción Oficial No.0273  
REF:F. HOFFMANN-LA ROCHE AG  
Patente No.60/151,548

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F. Hoffmann  
Caso-20431

DEPARTAMENTO DE COMERCIO DE LOS ESTADOS UNIDOS

OFICINA DE MARCAS Y PATENTES

Abril 14, 2000

Por medio del presente documento se certifica que adjunta se encuentra una fiel copia de los registros de la Oficina de Marcas y Patentes de los Estados Unidos, correspondientes a los documentos para la solicitud tal como se presentaron, con el fin de asignarle una fecha de registro bajo la norma del código 35 USC 111.

Número de Solicitud: 60/151,548

Fecha de Registro: Agosto 31, 1999

Autorizado por:

COMISIONADO DE MARCAS Y PATENTES

Firma:L. Eleden

Funcionario Encargado de Certificaciones

Hay sello realzado de la Oficina de Marcas y Patentes de color dorado.

*Eduardo Valadez*  
Eduardo Valadez  
Comisionado de Marcas y Patentes  
Departamento de Comercio  
Estados Unidos



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Jersey 07932

*Edwards Caludeh*

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JAN 10 1964

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☐ Los inventores adicionales se mencionaran en una hoja adjunta a la presente.

TITULO DE LA INVENCION  
**CONJUGADOS DE ERYTHROPOIETIN**

---

DIRECCION PARA CORRESPONDENCIA

George W. Jonhston

Hoffmann-La Roche Inc.

340 Kingsland Street

Nutley, NJ 07110

PARTES DE LA SOLICITUD INCLUIDAS

(Verificar que todas apliquen)

|                                                          |                                                        |
|----------------------------------------------------------|--------------------------------------------------------|
| <input checked="" type="checkbox"/> ESPECIFICACIONES     | <input type="checkbox"/> [20] Número de Páginas        |
| <input checked="" type="checkbox"/> REIVINDICACIONES     | No. de Reivindicaciones 29 (4 páginas)                 |
| <input checked="" type="checkbox"/> LISTADO DE SECUENCIA | <input type="checkbox"/> [2] Número de Páginas         |
| <input checked="" type="checkbox"/> Declaración          | <input type="checkbox"/> [4] Número de Páginas         |
| <input checked="" type="checkbox"/> GRAFICA (S)          | <input type="checkbox"/> [ ] Hojas de Gráficas         |
| <input checked="" type="checkbox"/> Otros                | <input type="checkbox"/> [ ] <u>Resumen (1 página)</u> |

FORMA DE DE PAGO DE HONORARIOS PARA LA SOLICITUD PROVISIONAL DE  
ESTA PATENTE (Señalar uno)

☒ El comisionado por la presente se encuentra autorizado a cobrar honorarios de registro y crédito al número de Cuenta Bancaria **08-2525**

*Edw. de Calasch*  
EDWARD DE CALASCH  
ATTORNEY AT LAW  
NEW YORK, N.Y.  
Rm.

Honorarios provisionales para registro = \$150.000

La invencion fué realizada por una agencia del Gobierno de los Estados Unidos o bajo contrato con una agencia del Gobierno de los Estados Unidos.

☒ No

☐ Sí, el nombre de la Agencia del Gobierno de los Estados Unidos y número de contrato son los siguientes:

Presentado atentamente,

FIRMA: Lewis J. Kreisler

No. de Registro: 38522

Telefono: (973) 235-4387

Telefax: (973) 235-2363

Fecha: Agosto 31, 1999

*Edwards Paladino*  
100-38522-1  
100-38522-2  
100-38522-3  
100-38522-4  
100-38522-5  
100-38522-6  
100-38522-7  
100-38522-8  
100-38522-9  
100-38522-10

## REIVINDICACIONES :

1. Un conjugado, dicho conjugado consta de glicoproteína erithropoietin el cual tiene por lo menos un grupo de amino ácidos, el cual tiene una actividad biológica *in vivo* la cual produce en las células en la médula del hueso un incremento en la producción de reticulocitos y célula y glóbulos rojos, seleccionados de un grupo que consiste de erithropoietinas y análogos del mismo los cuales tienen una secuencia de erithropoietinas humanas modificadas mediante la adición desde 1 hasta 6 sitios de glicosilaciones o un reordenamiento de por lo menos un sitio de glicocilación; dicha glicoproteína siendo enlazada de covalentemente a grupos de "n" poli(etileno glicol) de la formula  $-CO-(CH_2)_x-9OCH_2CH_2)_m-$  o OR con el  $-CO$  de cada grupo de poli(etileno glicol) formando un enlace de amida con uno de los grupos mencionados grupos de amino; en el cual R es bajo alquilo; x es 2 o 3; m es aproximadamente desde 450 hasta alrededor de 450 hasta aproximadamente 900; n es desde 1 hasta 3; y n y m son escogidos para que el peso molecular del de negativo conjugado de la glicoproteína erithropoietina sea de 20 kilodaltones hasta 100 kilodaltones.

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2. El conjugado de acuerdo a la reivindicación 1, de la formula:



en el cual m, n y R son como anteriormente se definieron, y P es un residuo de la glicoproteína sin el grupo(s) n de amino, cuya forma de amidos enlaza con el grupo de poli(etileno glicol).

3. El conjugado de acuerdo a la reivindicación 2, en el cual la glicoproteína es un erithropoietina humana.

4. El conjugado de acuerdo a la reivindicación 3, en la cual la glicoproteina erithropoietina humana se expresa mediante activación de genes endógenos.

5. El conjugado de acuerdo a la reivindicación 3, en el cual la glicoproteina tiene una secuencia SEQ ID NO:1.

6. El conjugado de acuerdo a la reivindicación 6, en el cual la R es metilo.

*Adriano C. C. C.*  
E. 1. 1. 1.

7. El conjugado de acuerdo a la reivindicación 5, en el cual m es desde aproximadamente 650 hasta alrededor de 750.

8. El conjugado de acuerdo a la reivindicación 5, en la cual la n es 1.

9. El conjugado de acuerdo a la reivindicación 5, en la cual R es metilo; m es aproximadamente desde 650 hasta 750; y n es 1.

10. El conjugado de acuerdo a la reivindicación 2, en la cual la glicoproteína tiene una secuencia de eritropoietina humana modificada por la adición de aproximadamente desde 1 hasta 6 sitios de glicolisación.

11. El conjugado de acuerdo a la reivindicación 10, en la cual la glicoproteína tiene una secuencia de eritropoietina humana modificada mediante una modificación seleccionada de un grupo que consiste de:

Asn30 Thr32;

Asn51 Thr53;

*Edwardo Calado*

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Asn57 Thr59;

Asn69;

Asn69 Thr71

Ser68Asn69 Thr71

Val87 Asn88 Thr90;

Ser87Asn88 Thr90

Ser87 Asn88 Gly89 Thr90;

Ser87 Asn88 Thr90 Thr92;

Ser87 Asn88 Thr90 Ala162;

Asn69 Thr71 Ser87 Asn88 Thr90;

Asn30 Thr32 Val87 Asn88 Thr90;

Asn89 Ile90 Thr91;

Ser87 Asn89 Ile90 Thr91;

Asn136 Thr138;

Asn138 Thr138;

Asn138 Thr140;

Thr125; y

Pro124Thr125.

12. El conjugado de acuerdo a la reivindicación 11, en el cual R es metilo.

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"EDWARDS"

13. El conjugado de acuerdo a la reivindicación 11, en el cual m es aproximadamente desde 650 hasta alrededor de 750.

14. El conjugado de acuerdo a la reivindicación 11, en el cual n es 1.

15. El conjugado de acuerdo a la reivindicación 11, en el cual R es metilo; m es desde aproximadamente 650 hasta alrededor de 750; y n es 1.

16. El conjugado de acuerdo a la reivindicación 10, en el cual la glicoproteína tiene una secuencia que consiste de una eritropoietina humana y una segunda secuencia en el terminus de carboxi de una secuencia de una eritropoietina humana, en la cual la segunda secuencia contiene por lo menos un sitio de glicosilización.

17. El conjugado de acuerdo a la reivindicación 16, en el cual la segunda secuencia consiste de una secuencia que se deriva de la secuencia terminal de carboxi de una gonadotropina corionica humana.

*Eduardo Calado*  
Eduardo Calado  
RADIOLAB  
BOCA RATON, FLORIDA  
U.S.A.



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18. El conjugado de acuerdo a la reivindicación 17, en el cual la glicoproteína tiene una secuencia seleccionada desde un grupo que consiste de:

(a) la secuencia de eritropoietina humana y la secuencia SEQ ID NO:3 en el terminus de carboxi de la secuencia eritropoietina humana;

(b) la secuencia en (a) modificada por Ser87 Asn88 Thr90; y

(c) la secuencia en (a) modificada por Asn30 Thr32 Val87 Asn88 Thr90.

19. El conjugado de acuerdo a la reivindicación 18, en el cual R es metilo.

20. El conjugado de acuerdo a la reivindicación 18, en el cual la m es aproximadamente desde 650 hasta alrededor de 750.

21. El conjugado de acuerdo a la reivindicación 18, en el cual n es 1.

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1. 2. 3. 4. 5. 6.

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22. El conjugado de acuerdo a la reivindicación 18, en el cual R es metilo; m es desde aproximadamente 650 hasta alrededor de 750; y n es 1.

23. El conjugado de acuerdo a la reivindicación 2, en el cual la glicoproteína tiene la secuencia humana de la aritropoietina modificada para reordenamiento de por lo menos un sitio de glicosilación.

24. El conjugado de acuerdo a la reivindicación 23, en el cual la reordenación consiste en la anulación de cualquier sitio de enlace N de glicosilación en aritropoietina humana de un sitio de glicosilación en la posición 88 en la secuencia de eritropoietina humana.

25. El conjugado de acuerdo a la reivindicación 24, en el cual la glicoproteína tiene una secuencia de eritropoietina humana modificada por una modificación seleccionada de un grupo que consiste de:

Gln24 Ser87 Asn88 Thr90;

*Arturo Paladino*

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Gln38 Ser87 Asn88 Thr90; y

Gln83 Ser87 Asn88 Thr90.

26. El conjugado de acuerdo a la reivindicación 25, en el cual R es metilo.

27. El conjugado de acuerdo a la reivindicación 25, en el cual m es desde aproximadamente 650 hasta alrededor de 750.

28. El conjugado de acuerdo a la reivindicación 25, en el cual n es 1.

29. El conjugado de acuerdo a la reivindicación 25, en el cual la R es metilo; m es aproximadamente desde 650 hasta alrededor de 750; y n es 1.

*Eduardo Calado*

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Caso:No1097P4

DECLARACION Y PODER  
PARA LA SOLICITUD DE PATENTES DE LOS ESTADOS UNIDOS

Yo en mi calidad de abajo mencionado inventor, por la presente declaro:

Mi residencia, dirección de Oficina Postal y ciudadanía son mencionados a continuación como también mi nombre.

Yo creo ser el único y primer inventor (si solo se encuentra un nombre en la lista) o original, primero y co-inventor del tópico en cuestión, el cual se reivindica pidiendo la solicitud de patente de invención con el título:

TITULO DE LA INVENCION (280 Caracteres máximo)  
CONJUGADOS DE ERITHROPOIETINA

cuya especificaciones:

(Indique uno)

☒ se encuentra adjunto.

☐ Fue registrado en \_\_\_\_\_ como

No Serial de la Solicitud \_\_\_\_\_

*Edwardo Calado*  
Eduardo Calado  
"OFICIAL"  
CLUB DE  
1996

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y fue revisado en \_\_\_\_\_  
(si aplica)

Yo por medio del presente documento declaro que he revisado y  
entiendo el contenido de las especificaciones anteriormente  
mencionadas, incluyendo las reivindicaciones.

Yo tengo conocimiento sobre mi obligación de revelar toda  
información que sea parte del material patentable tal como lo define  
el Artículo 37 C.F.R. §1.56.

Yo por la presente reivindico beneficios de prioridad bajo el Título  
35, del Código de los Estados Unidos, Sección 119 (a) -(d) de  
cualquiera de la solicitud(es) extranjera para patente (s) o  
certificado como inventor o S 365 (a) de cualquier Solicitud para  
Patente Internacional (PCT), para la cual se ha nombrado por lo  
menos a un país además de los Estados Unidos, como es  
anteriormente mencionado e identificado, Indicando con una  
indicación en las cajillas , de cualquier solicitud extranjera para  
Patente certificado como inventor, o solicitud Internacional PCT con  
una fecha de registro anterior de aquella solicitud que aparece en la  
cual se reivindica prioridad.

Solicitudes extranjeras con una fecha de registro anterior (es)  
\_\_\_\_\_

Prioridades Reivindicadas  
[ ] [ ]

*Eduardo Celado* 15  
[Stamp: RECEIVED, MAR 14 1996, U.S. PATENT & TRADEMARK OFFICE]

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|           |        |                         |     |     |
|-----------|--------|-------------------------|-----|-----|
| (Nombre)  | (País) | Día/Mes/año de registro | Si  | No  |
| _____     | _____  | _____                   | [ ] | [ ] |
| (Nombre)  | (País) | Día/Mes/año de registro | Si  | No  |
| _____     | _____  | _____                   | [ ] | [ ] |
| (Nombre ) | (País) | Día/Mes/año de registro | Si  | No  |

Yo por medio de la presente reivindico los beneficios bajo 35 U.S.C. § 119 (e) sobre solicitudes anteriores de los Estados Unidos mencionadas a continuación:

|                 |                     |                |                     |
|-----------------|---------------------|----------------|---------------------|
| _____           | _____               | _____          | _____               |
| (No Solicitud ) | (Fecha de Registro) | (No Solicitud) | (Fecha de registro) |

Yo por medio de la presente reivindico beneficios bajo el Código 35 USC § 120 de solicitud de Patente registrada en los Estados Unidos, o §365(c) de cualquier solicitud Internacional de patentes PCT en la manera requeridas tal como se menciona anteriormente y respecto al tópico en cuestión de cada una de las reivindicaciones de esta solicitud la cual no se haya revelada con anterioridad en solicitudes anteriores en los Estados Unidos de acuerdo a lo establecido por el primer párrafo de la norma del Código 35CFR. § 112, tengo conocimiento de mi obligación de revelar toda información que se considere parte del material patentable a la Oficina de Marcas y Patentes tal como lo define la norma del Código 37 C.F.R. §1.56 el cual se hizo efectiva entre las fechas de registro de la solicitud anterior y la fecha de registro Internacional PCT de esta solicitud.

*Eduardo Salazar*  
Eduardo Salazar, S.A. de C.V.  
"TRADUCTOR OFICIAL"  
INGLES-ESPAÑOL-INGLES  
EX-521 Febrero de 1976

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\_\_\_\_\_  
(No Serial Solicitud) (Fecha de Registro) (estatus)(patentado,pendiente,  
abandonado)

\_\_\_\_\_  
(No Serial Solicitud) (Fecha de Registro) (Estatus)(patentado,pendiente,  
abandonado)

Yo, por la presente afirmo que toda declaración presentada es de mi entero conocimiento, fiel y verdadera y tengo presente que toda falsa declaración es castigada mediante una multa o el encarcelamiento, o ambos, bajo la Sección 1001 del Título 18 del Código de los Estados Unidos, y que cualquier declaración puede comprometer la validez de esta solicitud o de cualquier patente expedida inmediatamente después.

**PODER :**

Como Inventor, yo por la presente nombro al (los) siguiente (s) abogados y /o representante (s) para procesar esta solicitud y llevar a cabo toda transacción de negocios en la Oficina de Marcas y Patentes

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"Edwards Calados"

relacionadas con la presente. (*listado de nombres y números de registro*)

No. de Registro

|                             |         |       |
|-----------------------------|---------|-------|
| GEORGE W. JOHNSTON          | Reg No. | 28090 |
| WILLIAM H. EPSTEIN          | Reg No. | 20008 |
| LEWIS J. KREISLER           | Reg No. | 38522 |
| DENNIS P. TRAMALONI         | Reg No. | 28542 |
| PATRICIA S. ROCHA-TRAMALONI | Reg No. | 31054 |
| JOHN P. PARISE              | Reg No. | 34403 |

ENVIAR LA CORRESPONDENCIA A:

George W, Johnston, Esq.  
HOFFMANN LA ROCHE INC.,  
350 KINGSLAND STREET  
NUTLEY, NEW JERSEY 07110-1199

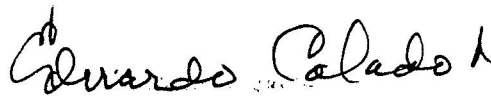
llamadas telefónicas:Lewis J. Kreisler (973) 253-4387

Nombre:PASACAL SEBASTIAN BAILON

Residencia: Florham Park, Morris County, new Jersey

Ciudadanía:Estados Unidos de America

Oficina Postal:21 Woodbine Road, Florham Park, New Jersey 07932

  
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1981-1982



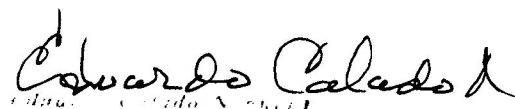
Fecha: 8/31/99

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De acuerdo al Título 37, del Código de Regulaciones Federales, § 1.56, tengo conocimiento sobre mi obligación de proteger y revelar información de material patentable el cual especifica que cada persona asociada con el registro y la continuación de la solicitud de patente tiene un deber de cumplimiento y buena fe en llevar a cabo todas las negociaciones con la Oficina de Patentes, la cual se debe proteger y revelar toda información de material patentable tal como lo define ésta sección. El deber de revelar toda información respecto a cada reivindicación pendiente hasta que la reivindicación sea cancelada o retirada o la solicitud sea abandonada.

Bajo esta sección la información es material patentable hasta cuando no es cumulativo sobre información que aparezca en los registros o sean parte de los registros en la solicitud, y

- (1) Reconoce que por si sola o conjuntamente con otra información o pruebas suficientes de la patentabilidad de una reivindicación; o
- (2) Si refuta o es inconsistente con la posición de un solicitante:

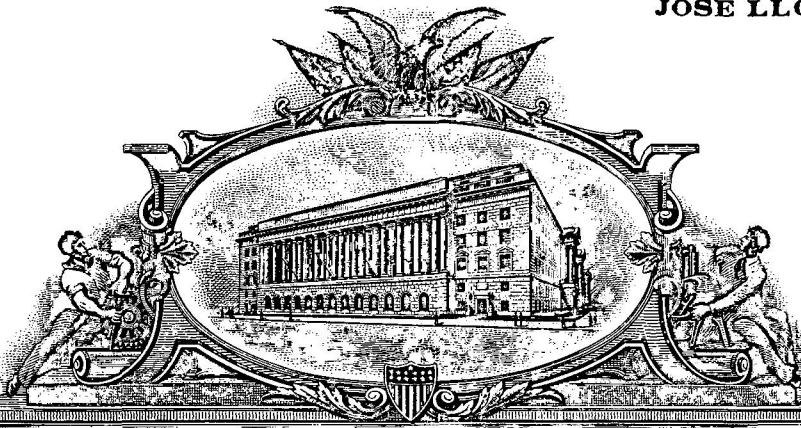
  
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"Eduardo Calado"

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- (i) Oponerse a un argumento de no patentabilidad propuesto por la Oficina, o
  - (ii) Afirmación de un argumento sobre patentabilidad.

*Eduardo Calado*  
Eduardo Calado  
ABogado  
11 de Febrero de 1980

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PA 233356



**THE UNITED STATES OF AMERICA**

**TO ALL TO WHOM THESE PRESENTS SHALL COME:**

**UNITED STATES DEPARTMENT OF COMMERCE**

**United States Patent and Trademark Office**

**April 14, 2000**

**THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM  
THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK  
OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT  
APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A  
FILING DATE UNDER 35 USC 111.**

**APPLICATION NUMBER: 60/151,548**

**FILING DATE: August 31, 1999**



**By Authority of the  
COMMISSIONER OF PATENTS AND TRADEMARKS**

*L. Edelen*

**L. EDELEN  
Certifying Officer**

CERTIFICATION UNDER 37 CFR 1.10

A18ROV

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JCS83 U.S. PTO  
60/151548  
08/31/99

I hereby certify that this New Application Transmittal and the documents referred to as enclosed therein are being deposited with the United States Postal Service on this date August 31, 1999 in an envelope as "Express Mail Post Office to Addressee" Mailing Label Number EL261866783US addressed to the: Assistant Commissioner for Patents, Washington, D.C. 20231.

Pamela Johnston  
(Print Name)

*Pamela Johnston*  
(Signature)

PROVISIONAL APPLICATION COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION under 37 CFR 1.53(b)(2).

Docket No. 1097P3

INVENTOR(s)/APPLICANT(s)

Name: Bailon, Pascal Sebastian  
Residence: Florham Park, Morris County, New Jersey  
Post Office Address: 21 Woodbine Road  
Florham Park, New Jersey 07932

☐ Additional inventors are being named on sheets attached hereto

TITLE OF THE INVENTION

ERYTHROPOIETIN CONJUGATES

CORRESPONDENCE ADDRESS

George W. Johnston  
Hoffmann-La Roche Inc.  
340 Kingsland Street  
Nutley, NJ 07110

ENCLOSED APPLICATION PARTS (check all that apply)

|                                                      |                                   |                                                 |                                |
|------------------------------------------------------|-----------------------------------|-------------------------------------------------|--------------------------------|
| <input checked="" type="checkbox"/> Specification    | No. of Pages <u>20</u>            | <input checked="" type="checkbox"/> Declaration | No. of Pages <u>4</u>          |
| <input checked="" type="checkbox"/> Claims           | No. of Claims <u>29 (4 pages)</u> | <input type="checkbox"/> Drawings               | Sheets of Drawings <u>    </u> |
| <input checked="" type="checkbox"/> Sequence Listing | No. of Pages <u>2</u>             | <input checked="" type="checkbox"/> Other       | <u>Abstract (1 page)</u>       |

DEPOSIT ACCOUNT  
NO. 08-2525

OUR ORDER NO. 2716

*[The page contains faint, illegible markings or bleed-through from the reverse side.]*

[ ] Yes, the name of the U.S. Government agency and the Government contract number are: \_\_\_\_\_

  
Attorney of Record  
Lewis J. Kreisler  
(Reg. No. 38522)  
Telephone: (973) 235-4387  
Telefax: (973) 235-2363  
Date: August 31, 1999

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## ERYTHROPOIETIN CONJUGATES

### 5 BACKGROUND OF THE INVENTION

Erythropoiesis is the production of red blood cells, which occurs to offset cell destruction. Erythropoiesis is a controlled physiological mechanism that enables sufficient red blood cells to be available for proper tissue oxygenation. Naturally occurring human erythropoietin (hEPO) is produced in the kidney and is the humoral plasma factor which stimulates red blood cell production (Carnot, P and Deflandre, C (1906) C.R. Acad. Sci. 143: 432; Erslev, AJ (1953 Blood 8: 349; Reissmann, KR (1950) Blood 5: 372; Jacobson, LO, Goldwasser, E, Freid, W and Plzak, LF (1957) Nature 179: 6331-4). Naturally occurring EPO stimulates the division and differentiation of committed erythroid progenitors in the bone marrow and exerts its biological activity by binding to receptors on erythroid precursors (Krantz, BS (1991) Blood 77: 419).

Erythropoietin has been manufactured biosynthetically using recombinant DNA technology (Egrie, JC, Strickland, TW, Lane, J et al. (1986) Immunobiol. 72: 213-224) and is the product of a cloned human EPO gene inserted into and expressed in the ovarian tissue cells of the chinese hamster (CHO cells). The primary structure of the predominant, fully processed form of hEPO is illustrated in SEQ ID NO:1. There are two disulfide bridges between Cys<sup>7</sup>-Cys<sup>161</sup> and Cys<sup>29</sup>-Cys<sup>33</sup>. The molecular weight of the polypeptide chain of EPO without the sugar moieties is 18,236 Da. In the intact EPO molecule, approximately 40% of the molecular weight are accounted for by the carbohydrate groups that glycosylate the protein at glycosylation sites on the protein (Sasaki, H, Bothner, B, Dell, A and Fukuda, M (1987) J. Biol. Chem. 262: 12059).

Because human erythropoietin is essential in red blood cell formation, the hormone is useful in the treatment of blood disorders characterized by low or defective red blood cell production. Clinically, EPO is used in the treatment of anemia in chronic

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renal failure patients (CRF) (Eschbach, JW, Egri, JC, Downing, MR et al. (1987) NEJM 316: 73-78; Eschbach, JW, Abdulhadi, MH, Browne, JK et al. (1989) Ann. Intern. Med. 111: 992; Egrie, JC, Eschbach, JW, McGuire, T, Adamson, JW (1988) Kidney Intl. 33: 262; Lim, VS, Degowin, RL, Zavala, D et al. (1989) Ann. Intern. Med. 110: 108-114) and in AIDS and cancer patients undergoing chemotherapy (Danna, RP, Rudnick, SA, Abels, RI In: MB, Garnick, ed. Erythropoietin in Clinical Applications-An International Perspective. New York, NY: Marcel Dekker; 1990: p. 301-324). However, the bioavailability of commercially available protein therapeutics such as EPO is limited by their short plasma half-life and susceptibility to protease degradation. These shortcomings prevent them from attaining maximum clinical potency.

### SUMMARY OF THE INVENTION

This invention provides an erythropoietin conjugate, said conjugate comprising an erythropoietin glycoprotein having at least one free amino group and having the *in vivo* biological activity of causing bone marrow cells to increase production of reticulocytes and red blood cells and selected from the group consisting of human erythropoietin and analogs thereof which have sequence of human erythropoietin modified by the addition of from 1 to 6 glycosylation sites or a rearrangement of at least one glycosylation site; said glycoprotein being covalently linked to "n" poly(ethylene glycol) groups of the formula  $-\text{CO}-(\text{CH}_2)_x-(\text{OCH}_2\text{CH}_2)_m-\text{OR}$  with the  $-\text{CO}$  (i.e. carbonyl) of each poly(ethylene glycol) group forming an amide bond with one of said amino groups; wherein R is lower alkyl; x is 2 or 3; m is from about 450 to about 900; n is from 1 to 3; and n and m are chosen so that the molecular weight of the conjugate minus the erythropoietin glycoprotein is from 20 kilodaltons to 100 kilodaltons.

Compared to unmodified EPO (i.e., EPO without a PEG attached) and conventional PEG-EPO conjugates, the present conjugates have an increased circulating half-life and plasma residence time, decreased clearance, and increased clinical activity *in vivo*. The conjugates of this invention have the same uses as EPO. In particular, the conjugates of this invention are useful to treat patients by stimulating the division and

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differentiation of committed erythroid progenitors in the bone marrow in the same way EPO is used to treat patients.

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## DETAILED DESCRIPTION OF THE INVENTION

10 This invention provides conjugates, said conjugates comprising an erythropoietin glycoprotein having at least one free amino group and having the *in vivo* biological activity of causing bone marrow cells to increase production of reticulocytes and red blood cells and selected from the group consisting of human erythropoietin and analogs thereof which have sequence of human erythropoietin modified by the addition of from 1 to 6 glycosylation sites or a rearrangement of at least one glycosylation site; said glycoprotein being covalently linked to "n" poly(ethylene glycol) groups of the formula  $-\text{CO}-(\text{CH}_2)_x-(\text{OCH}_2\text{CH}_2)_m-\text{OR}$  with the  $-\text{CO}$  (i.e. carbonyl) of each  
15 poly(ethylene glycol) group forming an amide bond with one of said amino groups; wherein R is lower alkyl; x is 2 or 3; m is from about 450 to about 900; n is from 1 to 3; and n and m are chosen so that the molecular weight of the conjugate minus the erythropoietin glycoprotein is from 20 kilodaltons to 100 kilodaltons.

20 It has been found that the conjugates of this invention can be used in the same manner as unmodified EPO. However, the conjugates of this invention have an increased circulating half-life and plasma residence time, decreased clearance, and increased clinical activity *in vivo*. Because of these improved properties, the conjugates of this invention can be administered once weekly instead of the three times weekly for  
25 unmodified EPO. Decreased frequency of administration is expected to result in improved patient compliance leading to improved treatment outcomes, as well as improved patient quality of life. Compared to conventional conjugates of EPO linked to poly(ethylene glycol) it has been found that conjugates having the molecular weight and linker structure of the conjugates of this invention have an improved potency, stability,  
30 AUC, circulating half-life, and cost of goods profile.

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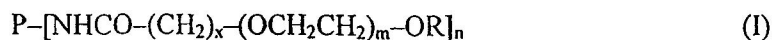
5 The conjugates in accordance of this invention can be administered in a therapeutically effective amount to patients in the same way EPO is administered. The therapeutically effective amount is that amount of conjugate necessary for the *in vivo* biological activity of causing bone marrow cells to increase production of reticulocytes and red blood cells. The exact amount of conjugate is a matter of preference subject to such factors as the exact type of condition being treated, the condition of the patient being treated, as well as the other ingredients in the composition. The pharmaceutical compositions containing the conjugate may be formulated at a strength effective for administration by various means to a human patient experiencing blood disorders characterized by low or defective red blood cell production. Average therapeutically effective amounts of the conjugate may vary and in particular should be based upon the recommendations and prescription of a qualified physician.

15 The erythropoietin glycoprotein products prepared in accordance with this invention may be prepared in pharmaceutical compositions suitable for injection with a pharmaceutically acceptable carrier or vehicle by methods known in the art. Among the preferred pharmaceutically acceptable carriers for formulating the products of the invention are human serum albumin, human plasma proteins, etc.

20 The term "erythropoietin" or "EPO" refers to a glycoprotein, having the amino acid sequence set out in (SEQ ID NO: 1) or (SEQ ID NO: 2) or an amino acid sequence substantially homologous thereto, whose biological properties relate to the stimulation of red blood cell production and the stimulation of the division and differentiation of committed erythroid progenitors in the bone marrow. As used herein, these terms include such proteins modified deliberately, as for example, by site directed mutagenesis or accidentally through mutations. These terms also include analogs having from 1 to 6 additional sites for glycosylation, analogs having at least one additional amino acid at the carboxy terminal end of the glycoprotein, wherein the additional amino acid includes at least one glycosylation site, and analogs having an amino acid sequence which includes a rearrangement of at least one site for glycosylation. These terms include both natural and recombinantly produced human erythropoietin.

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The erythropoietin conjugates of this invention can be represented by Formula 1:



5 wherein x, m, n and R are as above. In Formula I, P is the residue of an erythropoietin glycoprotein described herein, (i.e. without the amino group or amino groups which form an amide linkage with the carbonyl shown in Formula I), having the *in vivo* biological activity of causing bone marrow cells to increase production of reticulocytes and red blood cells. P may be selected from the group consisting of residues of human  
10 erythropoietin and analogs thereof having from 1 to 6 additional sites for glycosylation. As set out in detail below, the preparation and purification of EPO are well known in the art. By EPO is meant the natural or recombinant protein, preferably human, as obtained from any conventional source such as tissues, protein synthesis, cell culture with natural or recombinant cells. Any protein having the activity of EPO, such as muteins or  
15 otherwise modified proteins, is encompassed. Recombinant EPO may be prepared via expression in CHO-, BHK- or HeLa cell lines, by recombinant DNA technology or by endogenous gene activation. Expression of proteins, including EPO, by endogenous gene activation is well known in the art and is disclosed, for example in U.S. Patents Nos. 5,733,761, 5,641,670, and 5,733,746, and international patent publication Nos. WO  
20 93/09222, WO 94/12650, WO 95/31560, WO 90/11354, WO 91/06667 and WO 91/09955, the contents of each of which are incorporated herein by reference. The preferred EPO species for the preparation of erythropoietin glycoprotein products are human EPO species. More preferably, the EPO species is the human EPO having the amino acid sequence set out in SEQ ID NO:1 or SEQ ID NO:2, more preferably the  
25 amino acid sequence SEQ ID NO:1.

In an embodiment, P may be the residue of a glycoprotein analog having from 1 to 6 additional sites for glycosylation. Glycosylation of a protein, with one or more oligosaccharide groups, occurs at specific locations along a polypeptide backbone and  
30 greatly affects the physical properties of the protein such as protein stability, secretion, subcellular localization, and biological activity. Glycosylation is usually of two types.

O-linked oligosaccharides are attached to serine or threonine residues and N-linked oligosaccharides are attached to asparagine residues. One type of oligosaccharide found on both N-linked and O-linked oligosaccharides is N-acetylneuraminic acid (sialic acid), which is a family of amino sugars containing 9 or more carbon atoms. Sialic acid is usually the terminal residue on both N-linked and O-linked oligosaccharides and, because it bears a negative charge, confers acidic properties to the glycoprotein. Human erythropoietin, having 165 amino acids, contains three N-linked and one O-linked oligosaccharide chains which comprise about 40% of the total molecular weight of the glycoprotein. N-linked glycosylation occurs at asparagine residues located at positions 24, 38, and 83 and O-linked glycosylation occurs at a serine residue located at position 126. The oligosaccharide chains are modified with terminal sialic acid residues. Enzymatic removal of all sialic acid residues from the glycosylated erythropoietin results in loss of *in vivo* activity but not *in vitro* activity because sialylation of erythropoietin prevents its binding, and subsequent clearance, by hepatic binding protein.

The glycoproteins of the present invention include analogs of human erythropoietin with one or more changes in the amino acid sequence of human erythropoietin which result in an increase in the number of sites for sialic acid attachment. These glycoprotein analogs may be generated by site-directed mutagenesis having additions, deletions, or substitutions of amino acid residues that increase or alter sites that are available for glycosylation. Glycoprotein analogs having levels of sialic acid greater than those found in human erythropoietin are generated by adding glycosylation sites which do not perturb the secondary or tertiary conformation required for biological activity. The glycoproteins of the present invention also include analogs having increased levels of carbohydrate attachment at a glycosylation site which usually involve the substitution of one or more amino acids in close proximity to an N-linked or O-linked site. The glycoproteins of the present invention also include analogs having one or more amino acids extending from the carboxy terminal end of erythropoietin and providing at least one additional carbohydrate site. The glycoproteins of the present invention also include analogs having an amino acid sequence which includes a rearrangement of at least one site for glycosylation. Such a rearrangement of glycosylation site involves the deletion of one or

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more glycosylation sites in human erythropoietin and the addition of one or more non-naturally occurring glycosylation sites. Increasing the number of carbohydrate chains on erythropoietin, and therefore the number of sialic acids per erythropoietin molecules may confer advantageous properties such as increased solubility, greater resistance to  
5 proteolysis, reduced immunogenicity, increased serum half-life, and increased biological activity. Erythropoietin analogs with additional glycosylation sites are disclosed in more detail in European Patent Application 640 619, to Elliot published March 1, 1995.

In a preferred embodiment, the glycoproteins of the present invention comprise an  
10 amino acid sequence which includes at least one additional site for glycosylation such as, but not limited to, erythropoietins comprising the sequence of human erythropoietin modified by a modification selected from the following:

- Asn<sup>30</sup>Thr<sup>32</sup>;  
Asn<sup>51</sup>Thr<sup>53</sup>;  
15 Asn<sup>57</sup>Thr<sup>59</sup>;  
Asn<sup>69</sup>;  
Asn<sup>69</sup>Thr<sup>71</sup>;  
Ser<sup>68</sup>Asn<sup>69</sup>Thr<sup>71</sup>;  
Val<sup>87</sup>Asn<sup>88</sup>Thr<sup>90</sup>;  
20 Ser<sup>87</sup>Asn<sup>88</sup>Thr<sup>90</sup>;  
Ser<sup>87</sup>Asn<sup>88</sup>Gly<sup>89</sup>Thr<sup>90</sup>;  
Ser<sup>87</sup>Asn<sup>88</sup>Thr<sup>90</sup>Thr<sup>92</sup>;  
Ser<sup>87</sup>Asn<sup>88</sup>Thr<sup>90</sup>Ala<sup>162</sup>;  
Asn<sup>69</sup>Thr<sup>71</sup>Ser<sup>87</sup>Asn<sup>88</sup>Thr<sup>90</sup>;  
25 Asn<sup>30</sup>Thr<sup>32</sup>Val<sup>87</sup>Asn<sup>88</sup>Thr<sup>90</sup>;  
Asn<sup>89</sup>Ile<sup>90</sup>Thr<sup>91</sup>;  
Ser<sup>87</sup>Asn<sup>89</sup>Ile<sup>90</sup>Thr<sup>91</sup>;  
Asn<sup>136</sup>Thr<sup>138</sup>;  
Asn<sup>138</sup>Thr<sup>140</sup>;  
30 Thr<sup>125</sup>; and  
Pro<sup>124</sup>Thr<sup>125</sup>.

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The notation used herein for modification of amino acid sequence means that the position(s) of the corresponding unmodified protein (e.g. hEPO of SEQ ID NO:1 or SEQ ID NO:2) indicated by the superscripted number(s) is changed to the amino acid(s) that immediately precede the respective superscripted number(s).

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The glycoprotein may also be an analog having at least one additional amino acid at the carboxy terminal end of the glycoprotein, wherein the additional amino acid includes at least one glycosylation site. The additional amino acid may comprise a peptide fragment derived from the carboxy terminal end of human chorionic gonadotropin. Preferably, the glycoprotein is an analog selected from the group consisting of (a) human erythropoietin having the amino acid sequence, Ser Ser Ser Ser Lys Ala Pro Pro Pro Ser Leu Pro Ser Pro Ser Arg Leu Pro Gly Pro Ser Asp Thr Pro Ile Leu Pro Gln (SEQ ID NO:3), extending from the carboxy terminus; (b) the analog in (a) further comprising Ser<sup>87</sup> Asn<sup>88</sup> Thr<sup>90</sup> EPO; and (c) the analog in (a) further comprising Asn<sup>30</sup> Thr<sup>32</sup> Val<sup>87</sup> Asn<sup>88</sup> Thr<sup>90</sup> EPO.

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The glycoprotein may also be an analog having an amino acid sequence which includes a rearrangement of at least one site for glycosylation. The rearrangement may comprise a deletion of any of the N-linked carbohydrate sites in human erythropoietin and an addition of an N-linked carbohydrate site at position 88 of the amino acid sequence of human erythropoietin. Preferably, the glycoprotein is an analog selected from the group consisting of Gln<sup>24</sup> Ser<sup>87</sup> Asn<sup>88</sup> Thr<sup>90</sup> EPO; Gln<sup>38</sup> Ser<sup>87</sup> Asn<sup>88</sup> Thr<sup>90</sup> EPO; and Gln<sup>83</sup> Ser<sup>87</sup> Asn<sup>88</sup> Thr<sup>90</sup> EPO.

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As used herein, "lower alkyl" means a linear or branched alkyl group having from one to six carbon atoms. Examples of lower alkyl groups include methyl, ethyl and isopropyl. In accordance with this invention, R is any lower alkyl. Conjugates in which R is methyl are preferred.

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The symbol "m" represents the number of ethylene oxide residues (OCH<sub>2</sub>CH<sub>2</sub>) in the poly(ethylene oxide) group. A single PEG subunit of ethylene oxide has a molecular

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alkoxy-PEG-SBA). Compounds of Formula II in which x is 2 are alpha-lower alkoxy, propionic acid succinimidyl esters of poly(ethylene glycol) (lower alkoxy-PEG-SPA). Any conventional method of reacting an activated ester with an amine to form an amide can be utilized. In the reaction described above, the exemplified succinimidyl ester is a leaving group causing the amide formation. The use of succinimidyl esters such as the compounds of formula II to produce conjugates with proteins are disclosed in U.S. Patent No. 5,672,662, issued September 30, 1997 (Harris, et al.).

Human EPO contains nine free amino groups, the amino-terminal amino group plus the  $\epsilon$ -amino groups of 8 lysine residues. When the pegylation reagent was combined with a SBA compound of Formula II, it has been found that at pH 7.5, a protein:PEG ratio of 1:3, and a reaction temperature of from 20-25°C, a mixture of mono-, di-, and trace amounts of the tri-pegylated species were produced. When the pegylation reagent was a SPA compound of Formula II, at similar conditions except that the protein:PEG ratio was 1:2, primarily the mono-pegylated species is produced. The pegylated EPO can be administered as a mixture, or as the cation exchange chromatography separated different pegylated species. By manipulating the reaction conditions (e.g., ratio of reagents, pH, temperature, protein concentration, time of reaction etc.), the relative amounts of the different pegylated species can be varied.

Human erythropoietin (EPO) is a human glycoprotein which stimulates the formation of erythrocytes. Its preparation and therapeutic application are described in detail for example in U.S. Patent Nos. 5,547,933 and 5,621,080, EP-B 0 148 605, Huang, S.L., Proc. Natl. Acad. Sci. USA (1984) 2708-2712, EP-B 0 205 564, EP-B 0 209 539 and EP-B 0 411 678 as well as Lai, P.H. et al., J. Biol. Chem. 261 (1986) 3116-3121, an Sasaki, H. et al., J. Biol. Chem. 262 (1987) 12059-12076. Erythropoietin for therapeutic uses may be produced by recombinant means (EP-B 0 148 605, EP-B 0 209 539 and Egrie, J.C., Strickland, T.W., Lane, J. et al. (1986) Immunobiol. 72: 213-224).

Methods for the expression and preparation of erythropoietin in serum free medium are described for example in WO 96/35718, to Burg published 14 November 1996, and in

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European Patent Publication No. 513 738, to Koch published 12 June 1992. In addition to the publications mentioned above, it is known that a serum-free fermentation of recombinant CHO cells which contain an EPO gene can be carried out. Such methods are described for example in EP-A 0 513 738, EP-A 0 267 678 and in a general form by  
5 Kawamoto, T. et al., Analytical Biochem. 130 (1983) 445-453, EP-A 0 248 656, Kowar, J. and Franek, F., Methods in Enzymology 421 (1986) 277-292, Bavister, B., Expcology 271 (1981) 45-51, EP-A 0 481 791, EP-A 0 307 247, EP-A 0 343 635, WO 88/00967.

In EP-A 0 267 678 an ion exchange chromatography on S-Sepharose, a preparative reverse phase HPLC on a Cg column and a gel filtration chromatography are described  
10 for the purification of EPO produced in serum-free culture after dialysis. In this connection the gel filtration chromatography step can be replaced by ion exchange chromatography on S-Sepharose fast flow. It is also proposed that a dye chromatography on a Blue Trisacryl column be carried out before the ion exchange chromatography.

A process for the purification of recombinant EPO is described by Nobuo, I. et al., J.  
15 Biochem. 107 (1990) 352-359. In this process EPO is treated however with a solution of Tween<sup>®</sup> 20, phenylmethylsulfonyl fluoride, ethylmaleimide, pepstatin A, copper sulfate and oxamic acid prior to the purification steps. Publications, including WO 96/35718, to  
Burg published 14 November 1996, discloses a process for preparing erythropoietin in a  
20 serum free fermentation process (EPOsf).

The specific activity of EPO or EPO conjugates in accordance with this invention can be determined by various assays known in the art. The biological activity of the purified EPO proteins of this invention are such that administration of the EPO protein by  
25 injection to human patients results in bone marrow cells increasing production of reticulocytes and red blood cells compared to non-injected or control groups of subjects. The biological activity of the EPO proteins, or fragments thereof, obtained and purified in accordance with this invention can be tested by methods according to Annable, et al., Bull. Wld. Hlth. Org. (1972) 47: 99-112 and Pharm. Europa Spec. Issue Erythropoietin  
30 BRP Bio 1997(2). Another biological assay for determining the activity of EPO protein, the normocythaemic mouse assay, is described in Example 4.



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The invention will be better understood by reference to the following examples which illustrate but do not limit the invention described herein.

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### EXAMPLE 1

#### Fermentation And Purification Of Human EPO

##### 10 a) Inoculum Preparation and Fermentation

One vial of the Working Cell Bank, originating from an EPO-producing CHO cell line (ATCC CRL8695, disclosed in EP 411 678 (Genetics Institute) can be used) is taken from the gas phase of the liquid nitrogen storage tank. The cells are transferred into glass spinner flasks and cultivated in a hydrogen carbonate-buffered medium in a humidified  
15 CO<sub>2</sub> incubator. Typical serum free media used for the inoculum preparation and fermentation are disclosed in European Patent Application 513 738, to Koch published 12 June 1992, or WO 96/35718, to Burg published 14 November 1996, for example contain as medium DMEM/F12 (e.g. JRH Biosciences/Hazleton Biologics, Denver, US, order No. 57-736) and additionally sodium hydrogencarbonate, L+glutamine, D+glucose,  
20 recombinant insulin, sodium selenite, diaminobutane, hydrocortisone, iron(II) sulfate, asparagine, aspartic acid, serine and a stabilizer for mammalian cells such as e.g. polyvinyl alcohol, methyl cellulose, polydextran, polyethylene glycol, Pluronic F68, plasma expander polygelin (HEMACCEL<sup>®</sup>) or polyvinyl pyrrolidone (WO 96/35718).

The cultures are microscopically checked for the absence of contaminating  
25 microorganisms, and the cell densities are determined. These tests are performed at each splitting step.

After the initial growth period, the cell culture is diluted with fresh medium to the starting cell density and undergoes another growth cycle. This procedure is repeated until  
30 a culture volume of approximately 2 l per glass spinner flask has been obtained. After approx. 12 doublings 1 to 5 liter of this culture is available which then is used as inoculum for the 10 l inoculum fermenter.

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After 3 - 5 days, the culture in the 10 l fermenter can be used as inoculum for the 100 l inoculum fermenter.

After additional 3 - 5 days of cultivation, the culture in the 100 l fermenter can be used as inoculum for the 1000 l production fermenter.

#### b) Harvesting and Cell Separation

A batch refeed process is used, i.e. when the desired cell density is reached, approx. 80 % of the culture is harvested. The remaining culture is replenished with fresh culture medium and cultivated until the next harvest. One production run consists of a maximum of 10 subsequent harvests: 9 partial harvests and 1 overall harvest at the end of fermentation. Harvesting takes place every 3 - 4 days.

The determined harvest volume is transferred into a cooled vessel. The cells are removed by centrifugation or filtration and discarded. The EPO containing supernatant of the centrifugation step is in-line filtered and collected in a second cooled vessel. Each harvest is processed separately during purification.

A typical process for the purification of EPO-protein is disclosed in WO 96/35718, to Burg published 14 November 1996. The purification process is explained in the following.

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#### a) Blue Sepharose Chromatography

Blue Sepharose (Pharmacia) consists of Sepharose beads to the surface of which the Cibacron blue dye is covalently bound. Since EPO binds more strongly to Blue Sepharose than most non-proteinaceous contaminants, some proteinaceous impurities and

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PVA, EPO can be enriched in this step. The elution of the Blue Sepharose column is performed by increasing the salt concentration as well as the pH.

5 The column is filled with 80 - 100 l of Blue Sepharose, regenerated with NaOH and equilibrated with equilibration buffer (sodium/ calcium chloride and sodium acetate). The acidified and filtered fermenter supernatant is loaded. After completion of the loading, the column is washed first with a buffer similar to the equilibration buffer containing a higher sodium chloride concentration and consecutively with a Tris-base buffer. The product is eluted with a Tris-base buffer and collected in a single fraction in accordance  
10 with the master elution profile.

#### b) Butyl Toyopearl Chromatography

15 The Butyl Toyopearl 650 C (Toso Haas) is a polystyrene based matrix to which aliphatic butyl-residues are covalently coupled. Since EPO binds more strongly to this gel than most of the impurities and PVA, it has to be eluted with a buffer containing isopropanol.

20 The column is packed with 30 - 40 l of Butyl Toyopearl 650 C, regenerated with NaOH, washed with a Tris-base buffer and equilibrated with a Tris-base buffer containing isopropanol.

25 The Blue Sepharose eluate is adjusted to the concentration of isopropanol in the column equilibration buffer and loaded onto the column. Then the column is washed with equilibration buffer with increased isopropanol concentration. The product is eluted with elution buffer (Tris-base buffer with high isopropanol content) and collected in a single fraction in accordance with the master elution profile.

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#### c) Hydroxyapatite Ultrogel Chromatography

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The Hydroxyapatite Ultrogel (Biosepra) consists of hydroxyapatite which is incorporated in an agarose matrix to improve the mechanical properties. EPO has a low affinity to hydroxyapatite and can therefore be eluted at lower phosphate concentrations than protein impurities.

The column is filled with 30 - 40 l of Hydroxyapatite Ultrogel and regenerated with a potassium phosphate/ calcium chloride buffer and NaOH followed by a Tris-base buffer. Then it is equilibrated with a Tris-base buffer containing a low amount of isopropanol and sodium chloride.

The EPO containing eluate of the Butyl Toyopearl chromatography is loaded onto the column. Subsequently the column is washed with equilibration buffer and a Tris-base buffer without isopropanol and sodium chloride. The product is eluted with a Tris-base buffer containing a low concentration of potassium phosphate and collected in a single fraction in accordance with the master elution profile.

#### d) Reversed Phase HPLC on Vydac C4

The RP-HPLC material Vydac C4 (Vydac) consists of silica gel particles, the surfaces of which carry C4-alkyl chains. The separation of EPO from the proteinaceous impurities is based on differences in the strength of hydrophobic interactions. Elution is performed with an acetonitrile gradient in diluted trifluoroacetic acid.

Preparative HPLC is performed using a stainless steel column (filled with 2.8 to 3.2 liter of Vydac C4 silicagel). The Hydroxyapatite Ultrogel eluate is acidified by adding trifluoro-acetic acid and loaded onto the Vydac C4 column. For washing and elution an acetonitrile gradient in diluted trifluoroacetic acid is used. Fractions are collected and immediately neutralized with phosphate buffer. The EPO fractions which are within the IPC limits are pooled.

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#### e) DEAE Sepharose Chromatography

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The DEAE Sepharose (Pharmacia) material consists of diethylaminoethyl (DEAE) - groups which are covalently bound to the surface of Sepharose beads. The binding of EPO to the DEAE groups is mediated by ionic interactions. Acetonitrile and trifluoroacetic acid pass through the column without being retained. After these  
10 substances have been washed off, trace impurities are removed by washing the column with acetate buffer at a low pH. Then the column is washed with neutral phosphate buffer and EPO is eluted with a buffer with increased ionic strength.

The column is packed with DEAE Sepharose fast flow. The column volume is  
15 adjusted to assure an EPO load in the range of 3 - 10 mg EPO/ml gel. The column is washed with water and equilibration buffer (sodium/ potassium phosphate). The pooled fractions of the HPLC eluate are loaded and the column is washed with equilibration buffer. Then the column is washed with washing buffer (sodium acetate buffer) followed by washing with equilibration buffer. Subsequently, EPO is eluted from the column with  
20 elution buffer (sodium chloride, sodium/ potassium phosphate) and collected in a single fraction in accordance with the master elution profile.

The eluate of the DEAE Sepharose column is adjusted to the specified conductivity. The resulting drug substance is sterile filtered into Teflon bottles and stored at -70 °C.

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#### EXAMPLE 2

##### Pegylation of EPO with mPEG-SBA

EPO purified in accordance with the serum free procedure of Example 1 (EPOsf) was  
30 homogeneous as determined by analytical methods and showed the typical isoform pattern consisting of 8 isoforms. It had a specific biological activity of 190,000 IU/mg as

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determined by the normocythaemic mouse assay. The pegylation reagent used was a methoxy-PEG-SBA, which is a compound of Formula II in which R is methyl; x is 3; and m is from 650 to 750 (avg. about 680, corresponding to an average molecular weight of about 30 kDa).

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#### Pegylation Reaction

To one hundred milligrams of EPOsf (9.71 ml of a 10.3 mg/ml EPOsf stock , 5.48  $\mu$ mol) 10 ml of 0.1 M potassium phosphate buffer, pH, 7.5 containing 506 mg of 30kDa methoxy-PEG-SBA (16.5  $\mu$  mol) (obtained from Shearwater Polymers, Inc., Huntsville, Alabama) was added and mixed for 2h at room temperature (20-23 °C). The final protein concentration was 5 mg/ml and the protein:PEG reagent ratio was 1:3. After two hours, the reaction was stopped by adjusting the pH to 4.5 with glacial acetic acid and stored at -20 °C, until ready for purification.

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#### Purification

1. **Conjugate Mixture:** Approximately 28 ml of SP-SEPHAROSE FF (sulfo-propyl cation exchange resin) was packed into an AMICON glass column (2.2 x 7.5 cm) and equilibrated with 20 mM acetate buffer pH, 4.5 at a flowrate of 150 ml/h. Six milliliters of the reaction mixture containing 30 mg protein was diluted 5-fold with the equilibration buffer and applied onto the column. Unadsorbed materials were washed away with the buffer and the adsorbed PEG conjugate mixture was eluted from the column with 0.175 M NaCl in the equilibration buffer. Unmodified EPOsf still remaining on the column was eluted with 750 mM NaCl. Column was reequilibrated in the starting buffer. Samples were analyzed by SDS-PAGE and their degree of pegylation were determined. It was found that the 0.175M NaCl eluate contained, mono- as well as di- and trace amounts of the tri-pegylated species, whereas the 750 mM NaCl eluate contained unmodified EPOsf.

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2. **Di-PEG and Mono-PEG-EPOsf:** The purified conjugate mixture eluted from the column in the previous step was diluted 4-fold with the buffer and reapplied onto the column and washed as described. Di-PEG-EPOsf and mono-PEG-EPOsf were separately eluted from the column with 0.1M NaCl and 0.175 M NaCl, respectively.  
5 Elution was also performed with 750mM NaCl to elute any remaining unmodified EPOsf.

Alternatively, the reaction mixture was diluted 5-fold with the acetate buffer and applied onto the SP-Sepharose column (~0.5 mg protein/ml gel). Column was washed  
10 and adsorbed mono-PEG-EPOsf, di-PEG-EPOsf and unmodified EPOsf were eluted as described in the previous section.

### Results

15 PEG-EPOsf was synthesized by chemically conjugating a linear PEG molecule with a number average molecular weight of 30 kDa. PEG-EPOsf was derived from the reaction between the primary amino groups of EPOsf and the succinimidyl ester derivative of a 30 kDa PEG-butyric acid, resulting in an amide bond.

20 Results are summarized in Table 1. Purified conjugate mixture comprised of mono- and di-PEG-EPOsf and was free of unmodified EPOsf as determined by SDS-PAGE analysis. Conjugate mixture accounted for 23.4 mg or 78% of the starting material. Cation exchange chromatographic separation of mono- and di-PEG-EPOsf indicated that mono- to di-PEG ratio in the conjugate mixture was almost 1:1. After completion of the reaction,  
25 ratio of the individual components of Mono: Di: Unmodified were 40: 38: 20 (%). Overall yield was almost quantitative.

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**Table 1. Summary of results of EPOsf pegylation**

|    | Sample      | Protein (mg) | Yield (%) |
|----|-------------|--------------|-----------|
| 5  | Rxn. Mix.   | 30           | 100       |
|    | Mono-       | 12.0         | 40        |
|    | Di-         | 11.4         | 38        |
| 10 | Unmod.      | 6.0          | 20        |
|    | Conju. Mix. | 23.4         | 78        |

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### EXAMPLE 3

#### Pegylation of EPO with mPEG-SPA

20 A different aliquot of the EPOsf used in Example 2 was reacted with 30 kDa methoxy-PEG-SPA (Shearwater Polymers, Inc., Huntsville, Alabama). Reaction was performed at a protein:reagent ratio of 1:2 and purification techniques were in accordance with Example 2. Primarily the mono-pegylated species was produced.

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### EXAMPLE 4

#### In-vivo activity of pegylated EPO determined by the normocythaemic mouse assay

30 The normocythaemic mouse bioassay is known in the art (Pharm. Europa Spec. Issue Erythropoietin BRP Bio 1997(2)) and a method in the monography of erythropoietin of Ph. Eur. BRP. The samples were diluted with BSA-PBS. Normal healthy mice, 7-15 weeks old, were administered s.c. 0.2 ml of the EPO-fraction containing un-pegylated EPO or tri-, di- or mono-pegylated EPO from Example 2 or 3. Over a period of 6 days,  
35 blood was drawn by puncture of the tail vein and diluted such that 1 µl of blood was



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present in 1 ml of an 0.15  $\mu$ mol acridine orange staining solution. The staining time was 3 to 10 minutes. The reticulocyte counts were carried out microfluorometrically in a flow cytometer by analysis of the red fluorescence histogram. The reticulocyte counts were given in terms of absolute figures (per 30,000 blood cells analyzed). For the data presented, each group consisted of 5 mice per day, and the mice were bled only once.

In separate experiments, a single dose of unmodified EPO (25 ng of EPO), the PEG(SBA)-EPO mixture from Example 2 (10 ng of conjugate), mono- and di- pegylated EPOs from Example 2 (10 ng of conjugate), the PEG(SPA)-EPO from Example 3 (10 ng of conjugate), and buffer solution were administered to mice. The results are shown in Table 2. The results show the superior activity and the prolonged half life of the pegylated EPO species indicated by the significantly increased amounts of reticulocytes and the shift of the reticulocytes count maximum using the same dose per mouse (10 ng), compared to a dose of 25 ng for unmodified EPO.

TABLE 2

|      | EPO<br>(Unmodified) | 30 kDa SPA<br>PEG | Mono 30K<br>SBA | Di 30K<br>SBA | PEG-EPO<br>SBA Conjugate<br>Mixture | Control<br>Buffer |
|------|---------------------|-------------------|-----------------|---------------|-------------------------------------|-------------------|
| 72h  | 1000                | 1393              | 1411            | 994           | 1328                                | 857               |
| 96h  | 500                 | 1406              | 1501            | 926           | 1338                                | 697               |
| 120h | ~200                | 1100              | 1182            | 791           | 944                                 | 701               |
| 144h | ~0                  | 535               | 607             | 665           | 660                                 | 708               |

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SEQUENCE LISTING

5 <110> Bailon, Pascal S  
<120> Erythropoietin Conjugates  
<130> 1097P3  
10 <140>  
<141>  
<160> 3  
15 <170> PatentIn Ver. 2.0  
<210> 1  
<211> 165  
<212> PRT  
20 <213> Homo sapiens  
<400> 1  
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1 5 10 15  
25 Leu Glu Ala Lys Glu Ala Glu Asn Ile Thr Thr Gly Cys Ala Glu His  
20 25 30  
30 Cys Ser Leu Asn Glu Asn Ile Thr Val Pro Asp Thr Lys Val Asn Phe  
35 40 45  
Tyr Ala Trp Lys Arg Met Glu Val Gly Gln Gln Ala Val Glu Val Trp  
50 55 60  
35 Gln Gly Leu Ala Leu Leu Ser Glu Ala Val Leu Arg Gly Gln Ala Leu  
65 70 75 80  
Leu Val Asn Ser Ser Gln Pro Trp Glu Pro Leu Gln Leu His Val Asp  
85 90 95  
40 Lys Ala Val Ser Gly Leu Arg Ser Leu Thr Thr Leu Leu Arg Ala Leu  
100 105 110  
Gly Ala Gln Lys Glu Ala Ile Ser Pro Pro Asp Ala Ala Ser Ala Ala  
115 120 125  
Pro Leu Arg Thr Ile Thr Ala Asp Thr Phe Arg Lys Leu Phe Arg Val  
130 135 140  
50 Tyr Ser Asn Phe Leu Arg Gly Lys Leu Lys Leu Tyr Thr Gly Glu Ala  
145 150 155 160  
Cys Arg Thr Gly Asp  
165  
55

SEQUENCE LISTING

206  
203

<210> 2  
<211> 166  
<212> PRT  
<213> Homo sapiens

5

<400> 2  
Ala Pro Pro Arg Leu Ile Cys Asp Ser Arg Val Leu Glu Arg Tyr Leu  
1 5 10 15

10 Leu Glu Ala Lys Glu Ala Glu Asn Ile Thr Thr Gly Cys Ala Glu His  
20 25 30

Cys Ser Leu Asn Glu Asn Ile Thr Val Pro Asp Thr Lys Val Asn Phe  
35 40 45

15

Tyr Ala Trp Lys Arg Met Glu Val Gly Gln Gln Ala Val Glu Val Trp  
50 55 60

20 Gln Gly Leu Ala Leu Leu Ser Glu Ala Val Leu Arg Gly Gln Ala Leu  
65 70 75 80

Leu Val Asn Ser Ser Gln Pro Trp Glu Pro Leu Gln Leu His Val Asp  
85 90 95

25 Lys Ala Val Ser Gly Leu Arg Ser Leu Thr Thr Leu Leu Arg Ala Leu  
100 105 110

Gly Ala Gln Lys Glu Ala Ile Ser Pro Pro Asp Ala Ala Ser Ala Ala  
115 120 125

30

Pro Leu Arg Thr Ile Thr Ala Asp Thr Phe Arg Lys Leu Phe Arg Val  
130 135 140

35 Tyr Ser Asn Phe Leu Arg Gly Lys Leu Lys Leu Tyr Thr Gly Glu Ala  
145 150 155 160

Cys Arg Thr Gly Asp Arg  
165

40

<210> 3  
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<212> PRT  
<213> Homo sapiens

45

<400> 3  
Ser Ser Ser Ser Lys Ala Pro Pro Pro Ser Leu Pro Ser Pro Ser Arg  
1 5 10 15

50 Leu Pro Gly Pro Ser Asp Thr Pro Ile Leu Pro Gln  
20 25

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What is claimed is:

1. A conjugate, said conjugate comprising an erythropoietin glycoprotein having at least one free amino group and having the *in vivo* biological activity of causing bone marrow cells to increase production of reticulocytes and red blood cells and selected from the group consisting of human erythropoietin and analogs thereof which have sequence of human erythropoietin modified by the addition of from 1 to 6 glycosylation sites or a rearrangement of at least one glycosylation site; said glycoprotein being covalently linked to "n" poly(ethylene glycol) groups of the formula  
10  $-\text{CO}-(\text{CH}_2)_x-(\text{OCH}_2\text{CH}_2)_m-\text{OR}$  with the  $-\text{CO}$  of each poly(ethylene glycol) group forming an amide bond with one of said amino groups; wherein R is lower alkyl; x is 2 or 3; m is from about 450 to about 900; n is from 1 to 3; and n and m are chosen so that the molecular weight of the conjugate minus the erythropoietin glycoprotein is from 20 kilodaltons to 100 kilodaltons.  
15
2. The conjugate of claim 1, of the formula:  
$$\text{P}-[\text{NHCO}-(\text{CH}_2)_x-(\text{OCH}_2\text{CH}_2)_m-\text{OR}]_n \quad (\text{I})$$
  
20 wherein m, n and R are as above, and P is the residue of the glycoprotein without the n amino group(s) which form amide linkage(s) with the poly(ethylene glycol) group(s).
3. The conjugate of claim 2, wherein the glycoprotein is a human erythropoietin.
- 25 4. The conjugate of claim 3, wherein the human erythropoietin glycoprotein is expressed by endogenous gene activation.
5. The conjugate of claim 3, wherein the glycoprotein has the sequence SEQ ID NO:1.  
30
6. The conjugate of claim 5, wherein R is methyl.

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7. The conjugate of claim 5, wherein m is from about 650 to about 750.
8. The conjugate of claim 5, wherein n is 1.
- 5
9. The conjugate of claim 5, wherein R is methyl; m is from about 650 to about 750; and n is 1.
10. The conjugate of claim 2, wherein the glycoprotein has the sequence of human erythropoietin modified by the addition of from 1 to 6 glycosylation sites.
- 10
11. The conjugate of claim 10, wherein the glycoprotein has the sequence of human erythropoietin modified by a modification selected from the group consisting of:
- Asn<sup>30</sup>Thr<sup>32</sup>;
- 15 Asn<sup>51</sup>Thr<sup>53</sup>;
- Asn<sup>57</sup>Thr<sup>59</sup>;
- Asn<sup>69</sup>;
- Asn<sup>69</sup>Thr<sup>71</sup>;
- Ser<sup>68</sup>Asn<sup>69</sup>Thr<sup>71</sup>;
- 20 \* Val<sup>87</sup>Asn<sup>88</sup>Thr<sup>90</sup>;
- Ser<sup>87</sup>Asn<sup>88</sup>Thr<sup>90</sup>;
- Ser<sup>87</sup>Asn<sup>88</sup>Gly<sup>89</sup>Thr<sup>90</sup>;
- Ser<sup>87</sup>Asn<sup>88</sup>Thr<sup>90</sup>Thr<sup>92</sup>;
- Ser<sup>87</sup>Asn<sup>88</sup>Thr<sup>90</sup>Ala<sup>162</sup>;
- 25 Asn<sup>69</sup>Thr<sup>71</sup>Ser<sup>87</sup>Asn<sup>88</sup>Thr<sup>90</sup>;
- Asn<sup>30</sup>Thr<sup>32</sup>Val<sup>87</sup>Asn<sup>88</sup>Thr<sup>90</sup>;
- Asn<sup>89</sup>Ile<sup>90</sup>Thr<sup>91</sup>;
- Ser<sup>87</sup>Asn<sup>89</sup>Ile<sup>90</sup>Thr<sup>91</sup>;
- Asn<sup>136</sup>Thr<sup>138</sup>;
- 30 Asn<sup>138</sup>Thr<sup>140</sup>;
- Thr<sup>125</sup>; and

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Pro<sup>124</sup>Thr<sup>125</sup>.

12. The conjugate of claim 11, wherein R is methyl.
- 5 13. The conjugate of claim 11, wherein m is from about 650 to about 750.
14. The conjugate of claim 11, wherein n is 1.
15. The conjugate of claim 11, wherein R is methyl; m is from about 650 to about  
10 750; and n is 1.
16. The conjugate of claim 10, wherein the glycoprotein has a sequence comprising  
the sequence of human erythropoietin and a second sequence at the carboxy terminus of  
the human erythropoietin sequence, wherein the second sequence contains at least one  
15 glycosylation site.
17. The conjugate of claim 16, wherein the second sequences comprises a sequence  
derived from the carboxy terminal sequence of human chorionic gonadotropin.
- 20 18. The conjugate of claim 17, wherein the glycoprotein has a sequence selected from  
the group consisting of:
- (a) the sequence of human erythropoietin and the sequence SEQ ID NO:3 at the  
carboxy terminus of the human erythropoietin sequence;
- (b) the sequence in (a) modified by Ser<sup>87</sup> Asn<sup>88</sup> Thr<sup>90</sup>; and
- 25 (c) the sequence in (a) modified by Asn<sup>30</sup> Thr<sup>32</sup> Val<sup>87</sup> Asn<sup>88</sup> Thr<sup>90</sup>.
19. The conjugate of claim 18, wherein R is methyl.
20. The conjugate of claim 18, wherein m is from about 650 to about 750.
- 30 21. The conjugate of claim 18, wherein n is 1.

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22. The conjugate of claim 18, wherein R is methyl; m is from about 650 to about 750; and n is 1.
- 5 23. The conjugate of claim 2, wherein the glycoprotein has the sequence of human erythropoietin modified by a rearrangement of at least one glycosylation site.
24. The conjugate of claim 23, wherein the rearrangement comprises deletion of any of the N-linked glycosylation sites in human erythropoietin and addition of an N-linked  
10 glycosylation site at position 88 of the sequence of human erythropoietin.
25. The conjugate of claim 24, wherein the glycoprotein has the sequence of human erythropoietin modified by a modification selected from the group consisting of:  
Gln<sup>24</sup> Ser<sup>87</sup> Asn<sup>88</sup> Thr<sup>90</sup>;  
15 Gln<sup>38</sup> Ser<sup>87</sup> Asn<sup>88</sup> Thr<sup>90</sup>; and  
Gln<sup>83</sup> Ser<sup>87</sup> Asn<sup>88</sup> Thr<sup>90</sup>.
26. The conjugate of claim 25, wherein R is methyl.
- 20 27. The conjugate of claim 25, wherein m is from about 650 to about 750.
28. The conjugate of claim 25, wherein n is 1.
29. The conjugate of claim 25, wherein R is methyl; m is from about 650 to about  
25 750; and n is 1.

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### Declaration and Power of Attorney for Patent Application

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

#### ERYTHROPOIETIN CONJUGATES

the specification of which

(check one)

☒ is attached hereto.

☐ was filed on \_\_\_\_\_ as

Application Serial No. \_\_\_\_\_

and was amended on \_\_\_\_\_  
(if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR § 1.56.

I hereby claim foreign priority benefits under 35 U.S.C. § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or § 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or PCT International application having a filing date before that of the application on which priority is claimed.

#### Prior Foreign Application(s)

#### Priority Claimed

|                   |                    |                                 |                                 |                                |
|-------------------|--------------------|---------------------------------|---------------------------------|--------------------------------|
| _____<br>(Number) | _____<br>(Country) | _____<br>(Day/Month/Year Filed) | <input type="checkbox"/><br>Yes | <input type="checkbox"/><br>No |
| _____<br>(Number) | _____<br>(Country) | _____<br>(Day/Month/Year Filed) | <input type="checkbox"/><br>Yes | <input type="checkbox"/><br>No |
| _____<br>(Number) | _____<br>(Country) | _____<br>(Day/Month/Year Filed) | <input type="checkbox"/><br>Yes | <input type="checkbox"/><br>No |

I hereby claim the benefit under 35 U.S.C. § 119(e) of any United States provisional application(s) listed below.

|                            |                        |                            |                        |
|----------------------------|------------------------|----------------------------|------------------------|
| _____<br>(Application No.) | _____<br>(Filing Date) | _____<br>(Application No.) | _____<br>(Filing Date) |
|----------------------------|------------------------|----------------------------|------------------------|

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I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s), or § 365(c) of any P International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR § 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

(Application Serial No.)

(Filing Date)

(Status) (patented, pending, abandoned)

(Application Serial No.)

(Filing Date)

(Status) (patented, pending, abandoned)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. (list name and registration number)

|                    |                  |                             |                  |
|--------------------|------------------|-----------------------------|------------------|
| George W. Johnston | (Reg. No. 28090) | Dennis P. Tramaloni         | (Reg. No. 28542) |
| William H. Epstein | (Reg. No. 20008) | Patricia S. Rocha-Tramaloni | (Reg. No. 31054) |
| Lewis J. Kreisler  | (Reg. No. 38522) | John P. Parise              | (Reg. No. 34403) |

Send Correspondence to:

George W. Johnston, Esq., Hoffmann-La Roche Inc., 340 Kingsland Street,  
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Direct Telephone Calls to: (name and telephone number)

Lewis J. Kreisler (973) 235-4387

Pascal Sebastian Bailon

Full name of sole or first inventor

Pascal Sebastian Bailon

Inventor's signature

8/31/99

Date

Florham Park, Morris County, New Jersey

Residence

U.S.A.

Citizenship

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Post Office Address

214  
211

Full name of second joint inventor, if any

Second Inventor's signature

Date

Residence

Citizenship

Post Office Address

Full name of third joint inventor, if any

Third Inventor's signature

Date

Residence

Citizenship

Post Office Address

Full name of fourth joint inventor, if any

Fourth Inventor's signature

Date

Residence

Citizenship

Post Office Address

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212

Full name of fifth joint inventor, if any

Fifth Inventor's signature

Date

Residence

Citizenship

Post Office Address

Title 37, Code of Federal Regulations, §1.56, duty to disclose information material to patentability provides, in part, that each individual associated with the filing and prosecution of a patent application has a duty of candor and good faith in dealing with the Office, which includes a duty to disclose to the Office all information known to that individual to be material to patentability as defined in this section. The duty to disclose information exists with respect to each pending claim until the claim is cancelled or withdrawn from consideration, or the application becomes abandoned.

Under this section, information is material to patentability when it is not cumulative to information already of record or being made of record in the application, and

- (1) It establishes, by itself or in combination with other information, a prima facie case of unpatentability of a claim; or
- (2) It refutes, or is inconsistent with, a position the applicant takes in:
  - (i) Opposing an argument of unpatentability relied on by the Office, or
  - (ii) Asserting an argument of patentability.

Junio 14, 2000  
Traducción Oficial No.0269  
REF:F. HOFFMANN-LA ROCHE AG  
Patente No. 60/166,151

F. Hoffmann  
Caso-20431

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DEPARTAMENTO DE COMERCIO DE LOS ESTADOS UNIDOS

OFICINA DE MARCAS Y PATENTES

Abril 12, 2000

Por medio del presente documento se certifica que adjunta se encuentra una fiel copia de los registros de la Oficina de Marcas y Patentes de los Estados Unidos, correspondientes a los documentos para la solicitud tal como se presentaron, con el fin de asignarle una fecha de registro bajo la norma del código 35 USC 111.

Número de Solicitud: 60/166,151

Fecha de Registro: Noviembre 17, 1999

Autorizado por:

COMISIONADO DE MARCAS Y PATENTES

Firma: W. Montgomery

Funcionario Encargado de Certificaciones

Hay sello realizado de la Oficina de Marcas y Patentes de color dorado.

*Eduardo Calenda*  
Eduardo Calenda  
Director  
Oficina de Marcas y Patentes  
E. S. S. 1-800-768-6868

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CERTIFICACION BAJO EL CODIGO 37 Codigo de Regulaciones  
federales 1.10

Yo por medio del presente documento certifico que éste ha sido enviado por Correo Postal - "CORREO POSTAL AL DESTINATARIO" de los Estados Unidos el 17 de Noviembre de 1999, en un sobre rotulado al "Assistant Commissioner of Patents", Washington, DC., 20231.

Nombre del depositante: PAMELA JOHNSTON

Firma: PAMELA JOHNSTON

HOJA DE SOLICITUD PROVISIONAL PARA PATENTE

Esta es una petición para registrar una SOLICITUD PROVISIONAL bajo el código 37 CFR § 1.53 (b)(2):

Número de Caso: 1097P4

INVENTORES / SOLICITANTES

NOMBRE: Bailon, Pascal Sebastian

RESIDENCIA: Florham Park, Morris County, new Jersey

OFICINA CORREO POSTAL: 21 Woodbine Road

Florham Park, New Jersey 07932

*Eduardo Calandak*

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[ ] Los inventores adicionales se mencionaran en una hoja adjunta a la presente.

TITULO DE LA INVENCIÓN  
**CONJUGADOS DE ERYTHROPOIETIN**

---

DIRECCIÓN PARA CORRESPONDENCIA

George W. Jonhston  
Hoffmann-La Roche Inc.  
340 Kingsland Street  
Nutley, NJ 07110

PARTES DE LA SOLICITUD INCLUIDAS

(Verificar que todas apliquen)

- |     |                                 |      |                                        |
|-----|---------------------------------|------|----------------------------------------|
| [X] | ESPECIFICACIONES                | [22] | Número de Páginas                      |
| [x] | REIVINDICACIONES                |      | No. de Reivindicaciones 60 (9 páginas) |
| [x] | LISTADO DE SECUENCIA            | [2]  | Número de Páginas                      |
| [x] | Declaración                     | [4]  | Número de Páginas                      |
| [x] | GRAFICA (S)                     | [ ]  | Hojas de Gráficas                      |
| [x] | Otros <u>resumen (1 página)</u> |      |                                        |

*Edward Calandrot*  
SECRET  
RECEIVED  
JAN 19 1966

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FORMA DE DE PAGO DE HONORARIOS PARA LA SOLICITUD  
PROVISIONAL DE ESTA PATENTE (Señalar uno)

☒ El comisionado por la presente se encuentra autorizado a cobrar honorarios de  
registro y crédito al número de Cuenta Bancaria **08-2525**

Honorarios provisionales para registro = \$150.000

La invencion fué realizada por una agencia del Gobierno de los Estados Unidos o bajo  
contrato con una agencia del Gobierno de los Estados Unidos.

☒ No

☐ Sí, el nombre de la Agencia del Gobierno de los Estados Unidos y número de  
contrato son los siguientes:

Presentado atentamente,

FIRMA: Lewis J. Kreisler

No. de Registro: 38522

Telefono: (973) 235-4387

Telefax: (973) 235-2363

Fecha: Noviembre 17, 1999

*Eduardo Calado*  
Eduardo Calado  
Director General  
Instituto de Patentes  
y Marcas Registradas



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## REIVINDICACIONES :

1. Un conjugado, dicho conjugado consta de glicoproteína erithropoietin el cual tiene por lo menos un grupo de amino ácidos, el cual tiene una actividad biológica *in vivo* la cual produce en las células en la médula del hueso un incremento en la producción de reticulocitos y célula y glóbulos rojos, seleccionados de un grupo que consiste de erithropoietinas y análogos del mismo los cuales tienen una secuencia de erithropoietinas humanas modificadas mediante la adición desde 1 hasta 6 sitios de glicosilaciones o un reordenamiento de por lo menos un sitio de glicocilación; dicha glicoproteína siendo enlazada de covalentemente a grupos de "n" poli(etileno glicol) de la formula  $-CO-(CH_2)_x-9OCH_2CH_2)_m-$  o OR con el  $-CO$  de cada grupo de poli(etileno glicol) formando un enlace de amida con uno de los grupos mencionados grupos de amino; en el cual R es bajo alquilo; x es 2 o 3; m es aproximadamente desde 450 hasta alrededor de 450 hasta aproximadamente 900; n es desde 1 hasta 3; y n y m son escogidos para que el peso molecular del de negativo conjugado de la glicoproteína erithropoietina sea de 20 kilodaltones hasta 100 kilodaltones.

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2. El conjugado de acuerdo a la reivindicación 1, de la formula:



en el cual m, n y R son como anteriormente se definieron, y P es un residuo de la glicoproteína sin el grupo(s) n de amino, cuya forma de amidos enlaza con el grupo de poli(etileno glicol).

3. El conjugado de acuerdo a la reivindicación 2, en el cual la glicoproteína es un erithropoietina humana.

4. El conjugado de acuerdo a la reivindicación 3, en la cual la glicoproteina erithropoietina humana se expresa mediante activación de genes endógenos.

5. El conjugado de acuerdo a la reivindicación 3, en el cual la glicoproteina tiene una secuencia SEQ ID NO:1.

6. El conjugado de acuerdo a la reivindicación 6, en el cual la R es metilo.

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Eduardo Caludo  
PANEL-6  
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7. El conjugado de acuerdo a la reivindicación 5, en el cual m es desde aproximadamente 650 hasta alrededor de 750.
8. El conjugado de acuerdo a la reivindicación 5, en la cual la n es 1.
9. El conjugado de acuerdo a la reivindicación 5, en la cual R es metilo; m es aproximadamente desde 650 hasta 750; y n es 1.
10. El conjugado de acuerdo a la reivindicación 2, en la cual la glicoproteína tiene una secuencia de eritropoietina humana modificada por la adición de aproximadamente desde 1 hasta 6 sitios de glicosilación.
11. El conjugado de acuerdo a la reivindicación 10, en la cual la glicoproteína tiene una secuencia de eritropoietina humana modificada mediante una modificación seleccionada de un grupo que consiste de:

Asn30 Thr32;

Asn51 Thr53;

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Asn57 Thr59;

Asn69;

Asn69 Thr71

Ser68Asn69 Thr71

Val87 Asn88 Thr90;

Ser87Asn88 Thr90

Ser87 Asn88 Gly89 Thr90;

Ser87 Asn88 Thr90 Thr92;

Ser87 Asn88 Thr90 Ala162;

Asn69 Thr71 Ser87 Asn88 Thr90;

Asn30 Thr32 Val87 Asn88 Thr90;

Asn89 Ile90 Thr91;

Ser87 Asn89 Ile90 Thr91;

Asn136 Thr138;

Asn138 Thr138;

Asn138 Thr140;

Thr125; y

Pro124Thr125.

12. El conjugado de acuerdo a la reivindicación 11, en el cual R es metilo.

*Eduardo Caludo*

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13. El conjugado de acuerdo a la reivindicación 11, en el cual m es aproximadamente desde 650 hasta alrededor de 750.

14. El conjugado de acuerdo a la reivindicación 11, en el cual n es 1.

15. El conjugado de acuerdo a la reivindicación 11, en el cual R es metilo; m es desde aproximadamente 650 hasta alrededor de 750; y n es 1.

16. El conjugado de acuerdo a la reivindicación 10, en el cual la glicoproteína tiene una secuencia que consiste de una eritropoietina humana y una segunda secuencia en el terminus de carboxi de una secuencia de una eritropoietina humana, en la cual la segunda secuencia contiene por lo menos un sitio de glicosilación.

17. El conjugado de acuerdo a la reivindicación 16, en el cual la segunda secuencia consiste de una secuencia que se deriva de la secuencia terminal de carboxi de una gonadotropina corionica humana.

Edwards Calado

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18. El conjugado de acuerdo a la reivindicación 17, en el cual la glicoproteína tiene una secuencia seleccionada desde un grupo que consiste de:

- (a) la secuencia de eritropoietina humana y la secuencia SEQ ID NO:3 en el terminus de carboxi de la secuencia aritropoietina humana;
- (b) la secuencia en (a) modificada por Ser87 Asn88 Thr90; y
- (c) la secuencia en (a) modificada por Asn30 Thr32 Val87 Asn88 Thr90.

19. El conjugado de acuerdo a la reivindicación 18, en el cual R es metilo.

20. El conjugado de acuerdo a la reivindicación 18, en el cual la m es aproximadamente desde 650 hasta alrededor de 750.

21. El conjugado de acuerdo a la reivindicación 18, en el cual n es 1.

*Eduardo Calado*

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22. El conjugado de acuerdo a la reivindicación 18, en el cual R es metilo; m es desde aproximadamente 650 hasta alrededor de 750; y n es 1.

23. El conjugado de acuerdo a la reivindicación 2, en el cual la glicoproteína tiene la secuencia humana de la arithropoietina modificada para reordenamiento de por lo menos un sitio de glicosilación.

24. El conjugado de acuerdo a la reivindicación 23, en el cual la reordenación consiste en la anulación de cualquier sitio de enlace N de glicosilación en arithropoietina humana de un sitio de glicosilación en la posición 88 en la secuencia de erithropoietina humana.

25. El conjugado de acuerdo a la reivindicación 24, en el cual la glicoproteína tiene una secuencia de erithropoietina humana modificada por una modificación seleccionada de un grupo que consiste de:

Gln24 Ser87 Asn88 Thr90;

*Eduardo Calado*

Gln38 Ser87 Asn88 Thr90; y

Gln83 Ser87 Asn88 Thr90.

26. El conjugado de acuerdo a la reivindicación 25, en el cual R es metilo.

27. El conjugado de acuerdo a la reivindicación 25, en el cual m es desde aproximadamente 650 hasta alrededor de 750.

28. El conjugado de acuerdo a la reivindicación 25, en el cual n es 1.

29. El conjugado de acuerdo a la reivindicación 25, en el cual la R es metilo; m es aproximadamente desde 650 hasta alrededor de 750; y n es 1.

30. Un conjugado, dicho conjugado consta de glicoproteína eritropoietin el cual tiene por lo menos un grupo de amino ácidos,

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Eduardo Calado  
"TRADUCCION"  
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1991



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una secuencia de eritropoietinas humanas modificadas mediante la adición desde 1 hasta 6 sitios de glicosilaciones o un reordenamiento de por lo menos un sitio de glicosilación; dicha glicoproteína siendo enlazada de covalentemente a grupos de "n" poli(etileno glicol) de la formula  $-\text{CO}-(\text{CH}_2)_x-\text{OCH}_2\text{CH}_2)_m-$  o OR con el  $-\text{CO}$  de cada grupo de poli(etileno glicol) formando un enlace de amida con uno de los grupos mencionados grupos de amino; en el cual R es bajo alquilo; x es 2 o 3; m es aproximadamente desde 450 hasta alrededor de 450 hasta aproximadamente 900; n es desde 1 hasta 3; y n y m son escogidos para que el peso molecular del de negativo conjugado de la glicoproteína eritropoietina sea de 20 kilodaltones hasta 100 kilodaltones; el porcentaje se conjuga donde n es 1 y por o menos un 90 porciento.

31. El conjugado de acuerdo a la reivindicación 30, en el cual el porcentaje de conjugados donde n es 1 es de por lo menos un noventa y dos porciento.

32. El conjugado de acuerdo a la reivindicación 31, en el cual el porcentaje de conjugados donde n es 1 es de por lo menos un noventa y dos porciento.

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Eduardo Calado  
"TRADUCCION"  
R.S.S.

33. El conjugado de acuerdo a la reivindicación 30, en el cual el porcentaje de conjugados donde n es 1 es de por lo menos un noventa y dos por ciento.

34. El conjugado de acuerdo a la reivindicación 30, de la formula:



en el cual m, n y R son como anteriormente se definieron, y P es un residuo de la glicoproteína sin el grupo(s) n de amino, cuya forma de amidos enlaza con el grupo de poli(etileno glicol).

35. El conjugado de acuerdo a la reivindicación 34, en el cual el porcentaje de conjugados donde n es 1 es de por lo menos un noventa y dos por ciento.

36. El conjugado de acuerdo a la reivindicación 35, en el cual el porcentaje de conjugados donde n es 1 es de por lo menos un noventa y dos por ciento.

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37. El conjugado de acuerdo a la reivindicación 34, en el cual el porcentaje de los conjugados donde n es 1 es de por lo menos un noventa y dos por ciento.

38. El conjugado de acuerdo a la reivindicación 34, en el cual cada uno de los conjugados de glicoproteína es un eritropoietina humana.

39. El conjugado de acuerdo a la reivindicación 38, en el cual cada uno de los conjugados de glicoproteína eritropoietina humana se expresa mediante una activación de genes endógena.

40. La composición de acuerdo a la reivindicación 38, en el cual cada conjugado de glicoproteína tiene una secuencia SEQ ID NO:1.

41. La composición de acuerdo a la reivindicación 40, en el cual cada conjugado R es metilo.

42. La composición de acuerdo a la reivindicación 40, en el cual cada conjugado m es aproximadamente desde 650 hasta alrededor de 750.

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43. La composición de acuerdo a la reivindicación 40, en el cual cada conjugado R es metilo y m es desde aproximadamente 650 hasta 750.

44. La composición de acuerdo a la reivindicación 34, en el cual en cada conjugado de glicoproteína tiene una secuencia de arithropoietina modificada mediante la adición desde 1 hasta 6 sitios de glicosilación.

45. El conjugado de acuerdo a la reivindicación 44, en la cual la glicoproteína tiene una secuencia de erithropoietina humana modificada mediante una modificación seleccionada de un grupo que consiste de:

Asn30 Thr32;

Asn51 Thr53;

Asn57 Thr59;

Asn69;

Asn69 Thr71

Ser68Asn69 Thr71

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Val87 Asn88 Thr90;

Ser87Asn88 Thr90

Ser87 Asn88 Gly89 Thr90;

Ser87 Asn88 Thr90 Thr92;

Ser87 Asn88 Thr90 Ala162;

Asn69 Thr71 Ser87 Asn88 Thr90;

Asn30 Thr32 Val87 Asn88 Thr90;

Asn89 Ile90 Thr91;

Ser87 Asn89 Ile90 Thr91;

Asn136 Thr138;

Asn138 Thr138;

Asn138 Thr140;

Thr125; y

Pro124Thr125.

46. El conjugado de acuerdo a la reivindicación 45, en el cual R es metilo.

46. El conjugado de acuerdo a la reivindicación 45, en el cual m es aproximadamente desde 650 hasta alrededor de 750.

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RECIBO

47. El conjugado de acuerdo a la reivindicación 45, en el cual R es metilo; m es desde aproximadamente 650 hasta alrededor de 750;

48. El conjugado de acuerdo a la reivindicación 45, en el cual cada conjugado R es metilo y m es desde aproximadamente 650 hasta 750.

49. La composición de acuerdo a la reivindicación 44, en la cual cada conjugado de glicoproteína tiene una secuencia que consiste de una eritropoietina humana y una segunda secuencia en el terminus de carboxi de una secuencia de una eritropoietina humana, en la cual la segunda secuencia contiene por lo menos un sitio de glicosilización.

50. El conjugado de acuerdo a la reivindicación 49, en el cual la segunda secuencia consiste de una secuencia que se deriva de la secuencia terminal de carboxi de una gonadotropina corionica humana.

51. El conjugado de acuerdo a la reivindicación 50, en el cual cada conjugado de glicoproteína tiene una secuencia seleccionada desde un grupo que consiste de:

ne una secuencia seleccionada

*Adolfo Calado*

Adolfo Calado

DIRECTOR OF

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(a) la secuencia de erithrpoietina humana y la secuencia SEQ ID NO:3 en el terminus de carboxi de la secuencia arithropoietina humana;

(b) la secuencia en (a) modificada por Ser87 Asn88 Thr90; y

(c) la secuencia en (a) modificada por Asn30 Thr32 Val87 Asn88 Thr90.

52. El conjugado de acuerdo a la reivindicación 51, en el cual R es metilo.

53. El conjugado de acuerdo a la reivindicación 51, en el cual la m es aproximadamente desde 650 hasta alrededor de 750.

54. El conjugado de acuerdo a la reivindicación 51, en el cual R es metilo; m es desde aproximadamente 650 hasta alrededor de 750.

55. El conjugado de acuerdo a la reivindicación 34, en el cual la glicoproteina tiene la secuencia humana de la arithropoietina modificada para reordenamiento de por lo menos un sitio de glicosilación.

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56. El conjugado de acuerdo a la reivindicación 55, en el cual la reordenación de cada conjugado consiste en la anulación de cualquier sitio de enlace N de glicosilación en arithropoietina humana de un sitio de glicosilación en la posición 88 en la secuencia de erithropoietina humana.

57. El conjugado de acuerdo a la reivindicación 56, en el cual la glicoproteína tiene una secuencia de erithropoietina humana modificada por una modificación seleccionada de un grupo que consiste de:

Gln24 Ser87 Asn88 Thr90;

Gln38 Ser87 Asn88 Thr90; y

Gln83 Ser87 Asn88 Thr90.

58. La composición de acuerdo a la reivindicación 57, en el cual cada conjugado es R es metilo.

59. La composición de acuerdo a la reivindicación 57, en el cual cada conjugado m es desde 650 hasta alrededor de 750.

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60. La composición de acuerdo a la reivindicación 57, donde  
cada conjugado R es metilo y m es aproximadamente desde 650 hasta  
750.

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aviso de 196

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Caso: No 1097P4

DECLARACION Y PODER  
PARA LA SOLICITUD DE PATENTES DE LOS ESTADOS UNIDOS

Yo en mi calidad de abajo mencionado inventor, por la presente declaro:

Mi residencia, dirección de Oficina Postal y ciudadanía son mencionados a continuación como también mi nombre.

Yo creo ser el único y primer inventor (si solo se encuentra un nombre en la lista) o original, primero y co-inventor del tópico en cuestión, el cual se reivindica pidiendo la solicitud de patente de invención con el título:

TITULO DE LA INVENCION (280 Caracteres máximo)

CONJUGADOS DE ERITHROPOIETINA

cuya especificaciones:

(Indique uno)

☒ se encuentra adjunto.

☐ Fue registrado en \_\_\_\_\_ como

No Serial de la Solicitud \_\_\_\_\_

Edvarado Calado N.  
SECRETARIO DE ESTADO  
DE ECONOMIA Y FINANZAS  
22  
24 Febrero 1984

240,  
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y fue revisado en \_\_\_\_\_  
(si aplica)

Yo por medio del presente documento declaro que he revisado y entiendo el contenido de las especificaciones anteriormente mencionadas, incluyendo las reivindicaciones.

Yo tengo conocimiento sobre mi obligación de revelar toda información que sea parte del material patentable tal como lo define el Artículo 37 C.F.R. B1.56.

Yo por la presente reivindico beneficios de prioridad bajo el Título 35, del Código de los Estados Unidos, Sección 119 (a) -(d) de cualquiera de la solicitud(es) extranjera para patente (s) o certificado como inventor o S 365 (a) de cualquier Solicitud para Patente Internacional (PCT), para la cual se ha nombrado por lo menos a un país además de los Estados Unidos, como es anteriormente mencionado e identificado, Indicando con una indicación en las cajillas , de cualquier solicitud extranjera para Patente certificado como inventor, o solicitud Internacional PCT con una fecha de registro anterior de aquella solicitud que aparece en la cual se reivindica prioridad.

Solicitudes extranjeras con una fecha de registro anterior (es)

Prioridades Reivindicadas

\_\_\_\_\_

[ ] [ ]

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|           |        |                         |     |     |
|-----------|--------|-------------------------|-----|-----|
| (Nombre)  | (País) | Día/Mes/año de registro | Si  | No  |
| _____     | _____  | _____                   | [ ] | [ ] |
| (Nombre)  | (País) | Día/Mes/año de registro | Si  | No  |
| _____     | _____  | _____                   | [ ] | [ ] |
| (Nombre ) | (País) | Día/Mes/año de registro | Si  | No  |

Yo por medio de la presente reivindico los beneficios bajo 35 U.S.C. § 119 (e)  
sobre solicitudes anteriores de los Estados Unidos mencionadas a continuación:

|                 |                     |                |                     |
|-----------------|---------------------|----------------|---------------------|
| _____           | _____               | _____          | _____               |
| (No Solicitud ) | (Fecha de Registro) | (No Solicitud) | (Fecha de registro) |

Yo por medio de la presente reivindico beneficios bajo el Código 35 USC § 120 de solicitud de Patente registrada en los Estados Unidos, o §365(c) de cualquier solicitud Internacional de patentes PCT en la manera requeridas tal como se menciona anteriormente y respecto al tópico en cuestión de cada una de las reivindicaciones de esta solicitud la cual no se haya revelada con anterioridad en solicitudes anteriores en los Estados Unidos de acuerdo a lo establecido por el primer párrafo de la norma del Código 35CFR. § 112, tengo conocimiento de mi obligación de revelar toda información que se considere parte del material patentable a la Oficina de Marcas y Patentes tal como lo define la norma del Código 37 C.F.R. §1.56 el cual se hizo efectiva entre las fechas de registro de la solicitud anterior y la fecha de registro Internacional PCT de esta solicitud.

*Edvardo Calado K*

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\_\_\_\_\_  
(No Serial Solicitud) (Fecha de Registro) (estatus)(patentado,pendiente,  
abandonado)

\_\_\_\_\_  
(No Serial Solicitud) (Fecha de Registro) (Estatus)(patentado,pendiente,  
abandonado)

Yo, por la presente afirmo que toda declaración presentada es de mi  
entero conocimiento, fiel y verdadera y tengo presente que toda  
falsa declaración es castigada mediante una multa o el  
encarcelamiento, o ambos, bajo la Sección 1001 del Título 18 del  
Código de los Estados Unidos, y que cualquier declaración puede  
comprometer la validez de esta solicitud o de cualquier patente  
expedida inmediatamente después.

**PODER :**

Como Inventor, yo por la presente nombro al (los) siguiente (s)  
abogados y /o representante (s) para procesar esta solicitud y llevar a  
cabo toda transacción de negocios en la Oficina de Marcas y Patentes

*Eduardo Calado*  
Eduardo Calado  
"IRACOR" O. S. A.  
"IRACOR" O. S. A.  
"IRACOR" O. S. A.

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relacionadas con la presente. (*listado de nombres y números de registro*)

|                             | No. de Registro |       |
|-----------------------------|-----------------|-------|
| GEORGE W. JOHNSTON          | Reg No.         | 28090 |
| WILLIAM H. EPSTEIN          | Reg No.         | 20008 |
| LEWIS J. KREISLER           | Reg No.         | 38522 |
| DENNIS P. TRAMALONI         | Reg No.         | 28542 |
| PATRICIA S. ROCHA-TRAMALONI | Reg No.         | 31054 |
| JOHN P. PARISE              | Reg No.         | 34403 |

ENVIAR LA CORRESPONDENCIA A:

George W, Johnston, Esq.  
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350 KINGSLAND STREET  
NUTLEY, NEW JERSEY 07110-1199

llamadas telefónicas: Lewis J. Kreisler (973) 253-4387

Nombre: PASACAL SEBASTIAN BAILON

Residencia: Florham Park, Morris County, new Jersey

Ciudadanía: Estados Unidos de America

Oficina Postal: 21 Woodbine Road, Florham Park, New Jersey 07932

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Fecha: 11/17/99

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De acuerdo al Título 37, del Código de Regulaciones Federales, § 1.56, tengo conocimiento sobre mi obligación de proteger y revelar información de material patentable el cual especifica que cada persona asociada con el registro y la continuación de la solicitud de patente tiene un deber de cumplimiento y buena fe en llevar a cabo todas las negociaciones con la Oficina de Patentes, la cual se debe proteger y revelar toda información de material patentable tal como lo define ésta sección. El deber de revelar toda información respecto a cada reivindicación pendiente hasta que la reivindicación sea cancelada o retirada o la solicitud sea abandonada.

Bajo esta sección la información es material patentable hasta cuando no es cumulativo sobre información que aparezca en los registros o sean parte de los registros en la solicitud, y

- (1) Reconoce que por si sola o conjuntamente con otra información o pruebas suficientes de la patentabilidad de una reivindicación; o
- (2) Si refuta o es inconsistente con la posición de un solicitante:

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- (i) Oponerse a un argumento de no patentabilidad propuesto por la Oficina, o
- (ii) Afirmación de un argumento sobre patentabilidad.

*Eduardo Calado*  
SECRETARÍA DE ECONOMÍA  
DIRECCIÓN GENERAL DE PATENTES  
MEXICO, D.F.



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PA 232783



# THE UNITED STATES OF AMERICA

**TO ALL TO WHOM THESE PRESENTS SHALL COME:**

**UNITED STATES DEPARTMENT OF COMMERCE**

**United States Patent and Trademark Office**

**April 12, 2000**

**THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A FILING DATE UNDER 35 USC 111.**

**APPLICATION NUMBER: 60/166,151**

**FILING DATE: November 17, 1999**



**By Authority of the**

**COMMISSIONER OF PATENTS AND TRADEMARKS**

*W. Montgomery*  
**W. MONTGOMERY**

**Certifying Officer**

CERTIFICATION UNDER 37 CFR 1.10

A/PROV 247  
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I hereby certify that this New Application Transmittal and the documents referred to as enclosed therein are being deposited with the United States Postal Service on this date November 17, 1999 in an envelope as "Express Mail Post Office to Addressee" Mailing Label Number EL261866664US addressed to the: Assistant Commissioner for Patents, Washington, D.C. 20231.

11/17/99  
1600 U.S. PTO

Pamela Johnston  
(Print Name)  
*Pamela Johnston*  
(Signature)

1600 U.S. PTO  
11/17/99

PROVISIONAL APPLICATION COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION under 37 CFR 1.53(b)(2).

Docket No. 1097P4

INVENTOR(S)/APPLICANT(S)

Name: Bailon, Pascal Sebastian  
Residence: Florham Park, Morris County, New Jersey  
Post Office Address: 21 Woodbine Road  
Florham Park, New Jersey 07932

☐ Additional inventors are being named on sheets attached hereto

TITLE OF THE INVENTION

ERYTHROPOIETIN CONJUGATES

CORRESPONDENCE ADDRESS

George W. Johnston  
Hoffmann-La Roche Inc.  
340 Kingsland Street  
Nutley, NJ 07110

ENCLOSED APPLICATION PARTS (check all that apply)

|                                                      |                                   |                                                 |                                |
|------------------------------------------------------|-----------------------------------|-------------------------------------------------|--------------------------------|
| <input checked="" type="checkbox"/> Specification    | No. of Pages <u>22</u>            | <input checked="" type="checkbox"/> Declaration | No. of Pages <u>4</u>          |
| <input checked="" type="checkbox"/> Claims           | No. of Claims <u>60 (9 pages)</u> | <input type="checkbox"/> Drawings               | Sheets of Drawings <u>    </u> |
| <input checked="" type="checkbox"/> Sequence Listing | No. of Pages <u>2</u>             | <input checked="" type="checkbox"/> Other       | <u>Abstract (1 page)</u>       |

DEPOSIT ACCOUNT  
NO. 03-2525  
OUR ORDER NO. 2822

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METHOD OF PAYMENT

[X] The Commissioner is hereby authorized to  
charge any fees or credit any overpayment  
to Deposit Account Number: 08-2525

Provisional  
Filing Fee  
Amount \$150.00

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

[X] No

[ ] Yes, the name of the U.S. Government agency and the Government contract number are: \_\_\_\_\_

\_\_\_\_\_  
\_\_\_\_\_  
\_\_\_\_\_

Respectfully submitted,



Attorney of Record

Lewis J. Kreisler

(Reg. No. 38522)

Telephone: (973) 235-4387

Telefax: (973) 235-2363

Date: November 17, 1999

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## ERYTHROPOIETIN CONJUGATES

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### BACKGROUND OF THE INVENTION

Erythropoiesis is the production of red blood cells, which occurs to offset cell destruction. Erythropoiesis is a controlled physiological mechanism that enables sufficient red blood cells to be available for proper tissue oxygenation. Naturally occurring human erythropoietin (hEPO) is produced in the kidney and is the humoral plasma factor which stimulates red blood cell production (Carnot, P and Deflandre, C (1906) C.R. Acad. Sci. 143: 432; Erslev, AJ (1953 Blood 8: 349; Reissmann, KR (1950) Blood 5: 372; Jacobson, LO, Goldwasser, E, Freid, W and Plzak, LF (1957) Nature 179: 6331-4). Naturally occurring EPO stimulates the division and differentiation of committed erythroid progenitors in the bone marrow and exerts its biological activity by binding to receptors on erythroid precursors (Krantz, BS (1991) Blood 77: 419).

Erythropoietin has been manufactured biosynthetically using recombinant DNA technology (Egrie, JC, Strickland, TW, Lane, J et al. (1986) Immunobiol. 72: 213-224) and is the product of a cloned human EPO gene inserted into and expressed in the ovarian tissue cells of the chinese hamster (CHO cells). The primary structure of the predominant, fully processed form of hEPO is illustrated in SEQ ID NO:1. There are two disulfide bridges between Cys<sup>7</sup>-Cys<sup>161</sup> and Cys<sup>29</sup>-Cys<sup>33</sup>. The molecular weight of the polypeptide chain of EPO without the sugar moieties is 18,236 Da. In the intact EPO molecule, approximately 40% of the molecular weight are accounted for by the carbohydrate groups that glycosylate the protein at glycosylation sites on the protein (Sasaki, H, Bothner, B, Dell, A and Fukuda, M (1987) J. Biol. Chem. 262: 12059).

Because human erythropoietin is essential in red blood cell formation, the hormone is useful in the treatment of blood disorders characterized by low or defective red blood cell production. Clinically, EPO is used in the treatment of anemia in chronic

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renal failure patients (CRF) (Eschbach, JW, Egri, JC, Downing, MR et al. (1987) NEJM 316: 73-78; Eschbach, JW, Abdulhadi, MH, Browne, JK et al. (1989) Ann. Intern. Med. 111: 992; Egrie, JC, Eschbach, JW, McGuire, T, Adamson, JW (1988) Kidney Intl. 33: 262; Lim, VS, Degowin, RL, Zavala, D et al. (1989) Ann. Intern. Med. 110: 108-114) and in AIDS and cancer patients undergoing chemotherapy (Danna, RP, Rudnick, SA, Abels, RI In: MB, Garnick, ed. Erythropoietin in Clinical Applications-An International Perspective. New York, NY: Marcel Dekker; 1990: p. 301-324). However, the bioavailability of commercially available protein therapeutics such as EPO is limited by their short plasma half-life and susceptibility to protease degradation. These shortcomings prevent them from attaining maximum clinical potency.

### SUMMARY OF THE INVENTION

This invention provides an erythropoietin conjugate, said conjugate comprising an erythropoietin glycoprotein having at least one free amino group and having the *in vivo* biological activity of causing bone marrow cells to increase production of reticulocytes and red blood cells and selected from the group consisting of human erythropoietin and analogs thereof which have sequence of human erythropoietin modified by the addition of from 1 to 6 glycosylation sites or a rearrangement of at least one glycosylation site; said glycoprotein being covalently linked to "n" poly(ethylene glycol) groups of the formula  $-\text{CO}-(\text{CH}_2)_x-(\text{OCH}_2\text{CH}_2)_m-\text{OR}$  with the  $-\text{CO}$  (i.e. carbonyl) of each poly(ethylene glycol) group forming an amide bond with one of said amino groups; wherein R is lower alkyl; x is 2 or 3; m is from about 450 to about 900; n is from 1 to 3; and n and m are chosen so that the molecular weight of the conjugate minus the erythropoietin glycoprotein is from 20 kilodaltons to 100 kilodaltons. This invention further provides compositions containing conjugates described herein in which the percentage of conjugates in the composition in which n is 1 is at least ninety percent.

Compared to unmodified EPO (i.e., EPO without a PEG attached) and conventional PEG-EPO conjugates, the present conjugates have an increased circulating

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half-life and plasma residence time, decreased clearance, and increased clinical activity *in vivo*. The conjugates of this invention have the same uses as EPO. In particular, the conjugates of this invention are useful to treat patients by stimulating the division and differentiation of committed erythroid progenitors in the bone marrow in the same way

5 EPO is used to treat patients.

### DETAILED DESCRIPTION OF THE INVENTION

10 This invention provides conjugates, said conjugates comprising an erythropoietin glycoprotein having at least one free amino group and having the *in vivo* biological activity of causing bone marrow cells to increase production of reticulocytes and red blood cells and selected from the group consisting of human erythropoietin and analogs thereof which have sequence of human erythropoietin modified by the addition  
15 of from 1 to 6 glycosylation sites or a rearrangement of at least one glycosylation site; said glycoprotein being covalently linked to "n" poly(ethylene glycol) groups of the formula  $-\text{CO}-(\text{CH}_2)_x-(\text{OCH}_2\text{CH}_2)_m-\text{OR}$  with the  $-\text{CO}$  (i.e. carbonyl) of each poly(ethylene glycol) group forming an amide bond with one of said amino groups; wherein R is lower alkyl; x is 2 or 3; m is from about 450 to about 900; n is from 1 to 3;  
20 and n and m are chosen so that the molecular weight of the conjugate minus the erythropoietin glycoprotein is from 20 kilodaltons to 100 kilodaltons.

It has been found that the conjugates of this invention can be used in the same manner as unmodified EPO. However, the conjugates of this invention have an increased  
25 circulating half-life and plasma residence time, decreased clearance, and increased clinical activity *in vivo*. Because of these improved properties, the conjugates of this invention can be administered once weekly instead of the three times weekly for unmodified EPO. Decreased frequency of administration is expected to result in improved patient compliance leading to improved treatment outcomes, as well as  
30 improved patient quality of life. Compared to conventional conjugates of EPO linked to poly(ethylene glycol) it has been found that conjugates having the molecular weight and

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linker structure of the conjugates of this invention have an improved potency, stability, AUC, circulating half-life, and cost of goods profile.

5 The conjugates in accordance of this invention can be administered in a therapeutically effective amount to patients in the same way EPO is administered. The therapeutically effective amount is that amount of conjugate necessary for the *in vivo* biological activity of causing bone marrow cells to increase production of reticulocytes and red blood cells. The exact amount of conjugate is a matter of preference subject to such factors as the exact type of condition being treated, the condition of the patient being  
10 treated, as well as the other ingredients in the composition. The pharmaceutical compositions containing the conjugate may be formulated at a strength effective for administration by various means to a human patient experiencing blood disorders characterized by low or defective red blood cell production. Average therapeutically effective amounts of the conjugate may vary and in particular should be based upon the  
15 recommendations and prescription of a qualified physician.

The erythropoietin glycoprotein products prepared in accordance with this invention may be prepared in pharmaceutical compositions suitable for injection with a pharmaceutically acceptable carrier or vehicle by methods known in the art. Among the  
20 preferred pharmaceutically acceptable carriers for formulating the products of the invention are human serum albumin, human plasma proteins, etc.

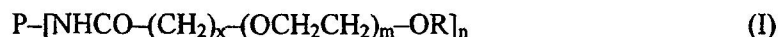
The term "erythropoietin" or "EPO" refers to a glycoprotein, having the amino acid sequence set out in (SEQ ID NO: 1) or (SEQ ID NO: 2) or an amino acid sequence  
25 substantially homologous thereto, whose biological properties relate to the stimulation of red blood cell production and the stimulation of the division and differentiation of committed erythroid progenitors in the bone marrow. As used herein, these terms include such proteins modified deliberately, as for example, by site directed mutagenesis or accidentally through mutations. These terms also include analogs having from 1 to 6  
30 additional sites for glycosylation, analogs having at least one additional amino acid at the carboxy terminal end of the glycoprotein, wherein the additional amino acid includes at

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least one glycosylation site, and analogs having an amino acid sequence which includes a rearrangement of at least one site for glycosylation. These terms include both natural and recombinantly produced human erythropoietin.

The erythropoietin conjugates of this invention can be represented by Formula 1:

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wherein x, m, n and R are as above. In Formula I, P is the residue of an erythropoietin glycoprotein described herein, (i.e. without the amino group or amino groups which form an amide linkage with the carbonyl shown in Formula I), having the *in vivo* biological activity of causing bone marrow cells to increase production of reticulocytes and red blood cells. P may be selected from the group consisting of residues of human erythropoietin and analogs thereof having from 1 to 6 additional sites for glycosylation. As set out in detail below, the preparation and purification of EPO are well known in the art. By EPO is meant the natural or recombinant protein, preferably human, as obtained from any conventional source such as tissues, protein synthesis, cell culture with natural or recombinant cells. Any protein having the activity of EPO, such as muteins or otherwise modified proteins, is encompassed. Recombinant EPO may be prepared via expression in CHO-, BHK- or HeLa cell lines, by recombinant DNA technology or by endogenous gene activation. Expression of proteins, including EPO, by endogenous gene activation is well known in the art and is disclosed, for example in U.S. Patents Nos. 5,733,761, 5,641,670, and 5,733,746, and international patent publication Nos. WO 93/09222, WO 94/12650, WO 95/31560, WO 90/11354, WO 91/06667 and WO 91/09955, the contents of each of which are incorporated herein by reference. The preferred EPO species for the preparation of erythropoietin glycoprotein products are human EPO species. More preferably, the EPO species is the human EPO having the amino acid sequence set out in SEQ ID NO:1 or SEQ ID NO:2, more preferably the amino acid sequence SEQ ID NO:1.

In an embodiment, P may be the residue of a glycoprotein analog having from 1 to 6 additional sites for glycosylation. Glycosylation of a protein, with one or more



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oligosaccharide groups, occurs at specific locations along a polypeptide backbone and greatly affects the physical properties of the protein such as protein stability, secretion, subcellular localization, and biological activity. Glycosylation is usually of two types. O-linked oligosaccharides are attached to serine or threonine residues and N-linked oligosaccharides are attached to asparagine residues. One type of oligosaccharide found on both N-linked and O-linked oligosaccharides is N-acetylneuraminic acid (sialic acid), which is a family of amino sugars containing 9 or more carbon atoms. Sialic acid is usually the terminal residue on both N-linked and O-linked oligosaccharides and, because it bears a negative charge, confers acidic properties to the glycoprotein. Human erythropoietin, having 165 amino acids, contains three N-linked and one O-linked oligosaccharide chains which comprise about 40% of the total molecular weight of the glycoprotein. N-linked glycosylation occurs at asparagine residues located at positions 24, 38, and 83 and O-linked glycosylation occurs at a serine residue located at position 126. The oligosaccharide chains are modified with terminal sialic acid residues. Enzymatic removal of all sialic acid residues from the glycosylated erythropoietin results in loss of *in vivo* activity but not *in vitro* activity because sialylation of erythropoietin prevents its binding, and subsequent clearance, by hepatic binding protein.

The glycoproteins of the present invention include analogs of human erythropoietin with one or more changes in the amino acid sequence of human erythropoietin which result in an increase in the number of sites for sialic acid attachment. These glycoprotein analogs may be generated by site-directed mutagenesis having additions, deletions, or substitutions of amino acid residues that increase or alter sites that are available for glycosylation. Glycoprotein analogs having levels of sialic acid greater than those found in human erythropoietin are generated by adding glycosylation sites which do not perturb the secondary or tertiary conformation required for biological activity. The glycoproteins of the present invention also include analogs having increased levels of carbohydrate attachment at a glycosylation site which usually involve the substitution of one or more amino acids in close proximity to an N-linked or O-linked site. The glycoproteins of the present invention also include analogs having one or more amino acids extending from the carboxy terminal end of erythropoietin and providing at least one additional

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carbohydrate site. The glycoproteins of the present invention also include analogs having an amino acid sequence which includes a rearrangement of at least one site for glycosylation. Such a rearrangement of glycosylation site involves the deletion of one or more glycosylation sites in human erythropoietin and the addition of one or more non-naturally occurring glycosylation sites. Increasing the number of carbohydrate chains on erythropoietin, and therefore the number of sialic acids per erythropoietin molecules may confer advantageous properties such as increased solubility, greater resistance to proteolysis, reduced immunogenicity, increased serum half-life, and increased biological activity. Erythropoietin analogs with additional glycosylation sites are disclosed in more detail in European Patent Application 640 619, to Elliot published March 1, 1995.

In a preferred embodiment, the glycoproteins of the present invention comprise an amino acid sequence which includes at least one additional site for glycosylation such as, but not limited to, erythropoietins comprising the sequence of human erythropoietin modified by a modification selected from the following:

- Asn<sup>30</sup>Thr<sup>32</sup>;
- Asn<sup>51</sup>Thr<sup>53</sup>;
- Asn<sup>57</sup>Thr<sup>59</sup>;
- Asn<sup>69</sup>;
- Asn<sup>69</sup>Thr<sup>71</sup>;
- Ser<sup>68</sup>Asn<sup>69</sup>Thr<sup>71</sup>;
- Val<sup>87</sup>Asn<sup>88</sup>Thr<sup>90</sup>;
- Ser<sup>87</sup>Asn<sup>88</sup>Thr<sup>90</sup>;
- Ser<sup>87</sup>Asn<sup>88</sup>Gly<sup>89</sup>Thr<sup>90</sup>;
- Ser<sup>87</sup>Asn<sup>88</sup>Thr<sup>90</sup>Thr<sup>92</sup>;
- Ser<sup>87</sup>Asn<sup>88</sup>Thr<sup>90</sup>Ala<sup>162</sup>;
- Asn<sup>69</sup>Thr<sup>71</sup>Ser<sup>87</sup>Asn<sup>88</sup>Thr<sup>90</sup>;
- Asn<sup>30</sup>Thr<sup>32</sup>Val<sup>87</sup>Asn<sup>88</sup>Thr<sup>90</sup>;
- Asn<sup>89</sup>Ile<sup>90</sup>Thr<sup>91</sup>;
- Ser<sup>87</sup>Asn<sup>89</sup>Ile<sup>90</sup>Thr<sup>91</sup>;
- Asn<sup>136</sup>Thr<sup>138</sup>;

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Asn<sup>138</sup>Thr<sup>140</sup>;  
Thr<sup>125</sup>; and  
Pro<sup>124</sup>Thr<sup>125</sup>.

The notation used herein for modification of amino acid sequence means that the  
5 position(s) of the corresponding unmodified protein (e.g. hEPO of SEQ ID NO:1 or SEQ  
ID NO:2) indicated by the superscripted number(s) is changed to the amino acid(s) that  
immediately precede the respective superscripted number(s).

The glycoprotein may also be an analog having at least one additional amino acid  
10 at the carboxy terminal end of the glycoprotein, wherein the additional amino acid  
includes at least one glycosylation site. The additional amino acid may comprise a  
peptide fragment derived from the carboxy terminal end of human chorionic  
gonadotropin. Preferably, the glycoprotein is an analog selected from the group  
consisting of (a) human erythropoietin having the amino acid sequence, Ser Ser Ser Ser  
15 Lys Ala Pro Pro Pro Ser Leu Pro Ser Pro Ser Arg Leu Pro Gly Pro Ser Asp Thr Pro Ile  
Leu Pro Gln (SEQ ID NO:3), extending from the carboxy terminus; (b) the analog in (a)  
further comprising Ser<sup>87</sup> Asn<sup>88</sup> Thr<sup>90</sup> EPO; and (c) the analog in (a) further comprising  
Asn<sup>30</sup> Thr<sup>32</sup> Val<sup>87</sup> Asn<sup>88</sup> Thr<sup>90</sup> EPO.

The glycoprotein may also be an analog having an amino acid sequence which  
20 includes a rearrangement of at least one site for glycosylation. The rearrangement may  
comprise a deletion of any of the N-linked carbohydrate sites in human erythropoietin  
and an addition of an N-linked carbohydrate site at position 88 of the amino acid  
sequence of human erythropoietin. Preferably, the glycoprotein is an analog selected  
25 from the group consisting of Gln<sup>24</sup> Ser<sup>87</sup> Asn<sup>88</sup> Thr<sup>90</sup> EPO; Gln<sup>38</sup> Ser<sup>87</sup> Asn<sup>88</sup> Thr<sup>90</sup> EPO;  
and Gln<sup>83</sup> Ser<sup>87</sup> Asn<sup>88</sup> Thr<sup>90</sup> EPO.

As used herein, "lower alkyl" means a linear or branched alkyl group having from  
one to six carbon atoms. Examples of lower alkyl groups include methyl, ethyl and  
30 isopropyl. In accordance with this invention, R is any lower alkyl. Conjugates in which  
R is methyl are preferred.

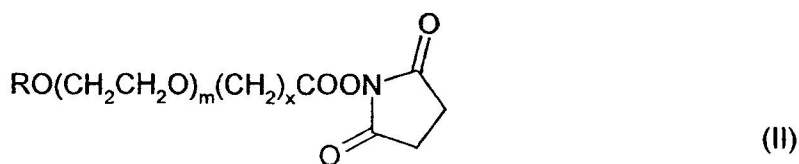
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The symbol "m" represents the number of ethylene oxide residues (OCH<sub>2</sub>CH<sub>2</sub>) in the poly(ethylene oxide) group. A single PEG subunit of ethylene oxide has a molecular weight of about 44 daltons. Thus, the molecular weight of the conjugate (excluding the molecular weight of the EPO) depends on the number "m". In the conjugates of this invention "m" is from about 450 to about 900 (corresponding to a molecular weight of about 20 kDa to about 40 kDa), preferably from about 650 to about 750 (corresponding to a molecular weight of about 30 kDa). The number m is selected such that the resulting conjugate of this invention has a physiological activity comparable to unmodified EPO, which activity may represent the same as, more than, or a fraction of the corresponding activity of unmodified EPO. A molecular weight of "about" a certain number means that it is within a reasonable range of that number as determined by conventional analytical techniques. The number "m" is selected so that the molecular weight of each poly(ethylene glycol) group covalently linked to the erythropoietin glycoprotein is from about 20kDa to about 40kDa, and is preferably about 30kDa.

In the conjugates of this invention, the number "n" is the number of polyethylene glycol groups covalently bound to free amino groups (including ε-amino groups of a lysine amino acid and/or the amino-terminal amino group) of an erythropoietin protein via amide linkage(s). A conjugate of this invention may have one, two, or three PEG groups per molecule of EPO. "n" is an integer ranging from 1 to 3, preferably "n" is 1 or 2, and more preferably "n" is 1.

The compound of Formula I can be prepared from the known polymeric material:



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in which R and m are as described above, by condensing the compound of Formula II with the erythropoietin glycoprotein. Compounds of Formula II in which x is 3 are alpha-lower alkoxy, butyric acid succinimidyl esters of poly(ethylene glycol) (lower alkoxy-PEG-SBA). Compounds of Formula II in which x is 2 are alpha-lower alkoxy, propionic acid succinimidyl esters of poly(ethylene glycol) (lower alkoxy-PEG-SPA). Any conventional method of reacting an activated ester with an amine to form an amide can be utilized. In the reaction described above, the exemplified succinimidyl ester is a leaving group causing the amide formation. The use of succinimidyl esters such as the compounds of formula II to produce conjugates with proteins are disclosed in U.S. Patent No. 5,672,662, issued September 30, 1997 (Harris, et al.).

Human EPO contains nine free amino groups, the amino-terminal amino group plus the  $\epsilon$ -amino groups of 8 lysine residues. When the pegylation reagent was combined with a SBA compound of Formula II, it has been found that at pH 7.5, a protein:PEG ratio of 1:3, and a reaction temperature of from 20-25°C, a mixture of mono-, di-, and trace amounts of the tri-pegylated species were produced. When the pegylation reagent was a SPA compound of Formula II, at similar conditions except that the protein:PEG ratio was 1:2, primarily the mono-pegylated species is produced. The pegylated EPO can be administered as a mixture, or as the cation exchange chromatography separated different pegylated species. By manipulating the reaction conditions (e.g., ratio of reagents, pH, temperature, protein concentration, time of reaction etc.), the relative amounts of the different pegylated species can be varied.

Human erythropoietin (EPO) is a human glycoprotein which stimulates the formation of erythrocytes. Its preparation and therapeutic application are described in detail for example in U.S. Patent Nos. 5,547,933 and 5,621,080, EP-B 0 148 605, Huang, S.L., Proc. Natl. Acad. Sci. USA (1984) 2708-2712, EP-B 0 205 564, EP-B 0 209 539 and EP-B 0 411 678 as well as Lai, P.H. et al., J. Biol. Chem. 261 (1986) 3116-3121, an Sasaki, H. et al., J. Biol. Chem. 262 (1987) 12059-12076. Erythropoietin for therapeutic uses may be produced by recombinant means (EP-B 0 148 605, EP-B 0 209 539 and Egrie, J.C., Strickland, T.W., Lane, J. et al. (1986) Immunobiol. 72: 213-224).

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Methods for the expression and preparation of erythropoietin in serum free medium are described for example in WO 96/35718, to Burg published 14 November 1996, and in European Patent Publication No. 513 738, to Koch published 12 June 1992. In addition  
5 to the publications mentioned above, it is known that a serum-free fermentation of recombinant CHO cells which contain an EPO gene can be carried out. Such methods are described for example in EP-A 0 513 738, EP-A 0 267 678 and in a general form by Kawamoto, T. et al., Analytical Biochem. 130 (1983) 445-453, EP-A 0 248 656, Kowar, J. and Franek, F., Methods in Enzymology 421 (1986) 277-292, Bavister, B., Expcology  
10 271 (1981) 45-51, EP-A 0 481 791, EP-A 0 307 247, EP-A 0 343 635, WO 88/00967.

In EP-A 0 267 678 an ion exchange chromatography on S-Sepharose, a preparative reverse phase HPLC on a C<sub>8</sub> column and a gel filtration chromatography are described for the purification of EPO produced in serum-free culture after dialysis. In this connection the gel filtration chromatography step can be replaced by ion exchange  
15 chromatography on S-Sepharose fast flow. It is also proposed that a dye chromatography on a Blue Trisacryl column be carried out before the ion exchange chromatography.

A process for the purification of recombinant EPO is described by Nobuo, I. et al., J. Biochem. 107 (1990) 352-359. In this process EPO is treated however with a solution of  
20 Tween<sup>®</sup> 20, phenylmethylsulfonyl fluoride, ethylmaleimide, pepstatin A, copper sulfate and oxamic acid prior to the purification steps. Publications, including WO 96/35718, to Burg published 14 November 1996, discloses a process for preparing erythropoietin in a serum free fermentation process (EPOsf).

25 The specific activity of EPO or EPO conjugates in accordance with this invention can be determined by various assays known in the art. The biological activity of the purified EPO proteins of this invention are such that administration of the EPO protein by injection to human patients results in bone marrow cells increasing production of reticulocytes and red blood cells compared to non-injected or control groups of subjects.  
30 The biological activity of the EPO proteins, or fragments thereof, obtained and purified in accordance with this invention can be tested by methods according to Annable, et al.,

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Bull. Wld. Hlth. Org. (1972) 47: 99-112 and Pharm. Europa Spec. Issue Erythropoietin BRP Bio 1997(2). Another biological assay for determining the activity of EPO protein, the normocythaemic mouse assay, is described in Example 4.

- 5 This invention provides a composition comprised of conjugates as described above. A composition containing at least ninety percent mono-PEG conjugates, i.e. in which n is 1, can be prepared as shown in Example 5. Usually mono-PEG conjugates of erythropoietin glycoproteins are desirable because they tend to have higher activity than di-PEG conjugates.. The percentage of mono-PEG conjugates as well as the ratio of mono- and
- 10 di-PEG species can be controlled by pooling broader fractions around the elution peak to decrease the percentage of mono-PEG or narrower fractions to increase the percentage of mono-PEG in the composition. About ninety percent mono-PEG conjugates is a good balance of yield and activity. Sometimes compositions in which, for example, at least ninety-two percent or at least ninety-six percent of the conjugates are mono-PEG species
- 15 (n equals 1) may be desired. In an embodiment of this invention the percentage of conjugates where n is 1 is from ninety percent to ninety-six percent.

The invention will be better understood by reference to the following examples which illustrate but do not limit the invention described herein.

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#### EXAMPLE 1

##### Fermentation And Purification Of Human EPO

- 25 a) Inoculum Preparation and Fermentation

- One vial of the Working Cell Bank, originating from an EPO-producing CHO cell line (ATCC CRL8695, disclosed in EP 411 678 (Genetics Institute) can be used) is taken from the gas phase of the liquid nitrogen storage tank. The cells are transferred into glass spinner flasks and cultivated in a hydrogen carbonate-buffered medium in a humidified
- 30 CO<sub>2</sub> incubator. Typical serum free media used for the inoculum preparation and fermentation are disclosed in European Patent Application 513 738, to Koch published 12 June 1992, or WO 96/35718, to Burg published 14 November 1996, for example contain as medium DMEM/F12 (e.g. JRH Biosciences/Hazleton Biologics, Denver, US, order

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No. 57-736) and additionally sodium hydrogencarbonate, L+glutamine, D+glucose, recombinant insulin, sodium selenite, diaminobutane, hydrocortisone, iron(II) sulfate, asparagine, aspartic acid, serine and a stabilizer for mammalian cells such as e.g. polyvinyl alcohol, methyl cellulose, polydextran, polyethylene glycol, Pluronic F68, plasma expander polygelin (HEMACCEL<sup>®</sup>) or polyvinyl pyrrolidone (WO 96/35718).

The cultures are microscopically checked for the absence of contaminating microorganisms, and the cell densities are determined. These tests are performed at each splitting step.

After the initial growth period, the cell culture is diluted with fresh medium to the starting cell density and undergoes another growth cycle. This procedure is repeated until a culture volume of approximately 2 l per glass spinner flask has been obtained. After approx. 12 doublings 1 to 5 liter of this culture is available which then is used as inoculum for the 10 l inoculum fermenter.

After 3 - 5 days, the culture in the 10 l fermenter can be used as inoculum for the 100 l inoculum fermenter.

After additional 3 - 5 days of cultivation, the culture in the 100 l fermenter can be used as inoculum for the 1000 l production fermenter.

#### b) Harvesting and Cell Separation

A batch refeed process is used, i.e. when the desired cell density is reached, approx. 80 % of the culture is harvested. The remaining culture is replenished with fresh culture medium and cultivated until the next harvest. One production run consists of a maximum of 10 subsequent harvests: 9 partial harvests and 1 overall harvest at the end of fermentation. Harvesting takes place every 3 - 4 days.

The determined harvest volume is transferred into a cooled vessel. The cells are removed by centrifugation or filtration and discarded. The EPO containing supernatant



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of the centrifugation step is in-line filtered and collected in a second cooled vessel. Each harvest is processed separately during purification.

- 5 A typical process for the purification of EPO-protein is disclosed in WO 96/35718, to Burg published 14 November 1996. The purification process is explained in the following.

10 a) Blue Sepharose Chromatography

- Blue Sepharose (Pharmacia) consists of Sepharose beads to the surface of which the Cibacron blue dye is covalently bound. Since EPO binds more strongly to Blue Sepharose than most non-proteinaceous contaminants, some proteinaceous impurities and  
15 PVA, EPO can be enriched in this step. The elution of the Blue Sepharose column is performed by increasing the salt concentration as well as the pH.

- The column is filled with 80 - 100 l of Blue Sepharose, regenerated with NaOH and equilibrated with equilibration buffer (sodium/ calcium chloride and sodium acetate). The  
20 acidified and filtered fermenter supernatant is loaded. After completion of the loading, the column is washed first with a buffer similar to the equilibration buffer containing a higher sodium chloride concentration and consecutively with a Tris-base buffer. The product is eluted with a Tris-base buffer and collected in a single fraction in accordance with the master elution profile.

25

b) Butyl Toyopearl Chromatography

- The Butyl Toyopearl 650 C (Toso Haas) is a polystyrene based matrix to which  
30 aliphatic butyl-residues are covalently coupled. Since EPO binds more strongly to this gel

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than most of the impurities and PVA, it has to be eluted with a buffer containing isopropanol.

The column is packed with 30 - 40 l of Butyl Toyopearl 650 C, regenerated with NaOH, washed with a Tris-base buffer and equilibrated with a Tris-base buffer containing isopropanol.

The Blue Sepharose eluate is adjusted to the concentration of isopropanol in the column equilibration buffer and loaded onto the column. Then the column is washed with equilibration buffer with increased isopropanol concentration. The product is eluted with elution buffer (Tris-base buffer with high isopropanol content) and collected in a single fraction in accordance with the master elution profile.

#### c) Hydroxyapatite Ultrogel Chromatography

The Hydroxyapatite Ultrogel (Biosepra) consists of hydroxyapatite which is incorporated in an agarose matrix to improve the mechanical properties. EPO has a low affinity to hydroxyapatite and can therefore be eluted at lower phosphate concentrations than protein impurities.

The column is filled with 30 - 40 l of Hydroxyapatite Ultrogel and regenerated with a potassium phosphate/ calcium chloride buffer and NaOH followed by a Tris-base buffer. Then it is equilibrated with a Tris-base buffer containing a low amount of isopropanol and sodium chloride.

The EPO containing eluate of the Butyl Toyopearl chromatography is loaded onto the column. Subsequently the column is washed with equilibration buffer and a Tris-base buffer without isopropanol and sodium chloride. The product is eluted with a Tris-base buffer containing a low concentration of potassium phosphate and collected in a single fraction in accordance with the master elution profile.

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#### d) Reversed Phase HPLC on Vydac C4

5        The RP-HPLC material Vydac C4 (Vydac) consists of silica gel particles, the surfaces of which carry C4-alkyl chains. The separation of EPO from the proteinaceous impurities is based on differences in the strength of hydrophobic interactions. Elution is performed with an acetonitrile gradient in diluted trifluoroacetic acid.

10       Preparative HPLC is performed using a stainless steel column (filled with 2.8 to 3.2 liter of Vydac C4 silicagel). The Hydroxyapatite Ultrogel eluate is acidified by adding trifluoro-acetic acid and loaded onto the Vydac C4 column. For washing and elution an acetonitrile gradient in diluted trifluoroacetic acid is used. Fractions are collected and immediately neutralized with phosphate buffer. The EPO fractions which are within the  
15    IPC limits are pooled.

#### e) DEAE Sepharose Chromatography

20       The DEAE Sepharose (Pharmacia) material consists of diethylaminoethyl (DEAE) - groups which are covalently bound to the surface of Sepharose beads. The binding of EPO to the DEAE groups is mediated by ionic interactions. Acetonitrile and trifluoroacetic acid pass through the column without being retained. After these  
25    substances have been washed off, trace impurities are removed by washing the column with acetate buffer at a low pH. Then the column is washed with neutral phosphate buffer and EPO is eluted with a buffer with increased ionic strength.

30       The column is packed with DEAE Sepharose fast flow. The column volume is adjusted to assure an EPO load in the range of 3 - 10 mg EPO/ml gel. The column is washed with water and equilibration buffer (sodium/ potassium phosphate). The pooled

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fractions of the HPLC eluate are loaded and the column is washed with equilibration buffer. Then the column is washed with washing buffer (sodium acetate buffer) followed by washing with equilibration buffer. Subsequently, EPO is eluted from the column with elution buffer (sodium chloride, sodium/ potassium phosphate) and collected in a single  
5 fraction in accordance with the master elution profile.

The eluate of the DEAE Sepharose column is adjusted to the specified conductivity. The resulting drug substance is sterile filtered into Teflon bottles and stored at  $-70^{\circ}\text{C}$ .

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## EXAMPLE 2

### Pegylation of EPO with mPEG-SBA

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EPO purified in accordance with the serum free procedure of Example 1 (EPOsf) was homogeneous as determined by analytical methods and showed the typical isoform pattern consisting of 8 isoforms. It had a specific biological activity of 190,000 IU/mg as  
determined by the normocythaemic mouse assay. The pegylation reagent used was a methoxy-PEG-SBA, which is a compound of Formula II in which R is methyl; x is 3; and m is from 650 to 750 (avg. about 680, corresponding to an average molecular weight of about 30 kDa).

20

### Pegylation Reaction

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To one hundred milligrams of EPOsf (9.71 ml of a 10.3 mg/ml EPOsf stock, 5.48  $\mu\text{mol}$ ) 10 ml of 0.1 M potassium phosphate buffer, pH, 7.5 containing 506 mg of 30kDa methoxy-PEG-SBA (16.5  $\mu\text{mol}$ ) (obtained from Shearwater Polymers, Inc., Huntsville, Alabama) was added and mixed for 2h at room temperature ( $20-23^{\circ}\text{C}$ ). The final protein concentration was 5 mg/ml and the protein:PEG reagent ratio was 1:3. After two hours, the reaction was stopped by adjusting the pH to 4.5 with glacial acetic acid and stored at  $-20^{\circ}\text{C}$ , until ready for purification.

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## Purification

1. **Conjugate Mixture:** Approximately 28 ml of SP-SEPHAROSE FF (sulfo-propyl cation exchange resin) was packed into an AMICON glass column (2.2 x 7.5 cm) and equilibrated with 20 mM acetate buffer pH, 4.5 at a flowrate of 150 ml/h. Six milliliters of the reaction mixture containing 30 mg protein was diluted 5-fold with the equilibration buffer and applied onto the column. Unadsorbed materials were washed away with the buffer and the adsorbed PEG conjugate mixture was eluted from the column with 0.175 M NaCl in the equilibration buffer. Unmodified EPOsf still remaining on the column was eluted with 750 mM NaCl. Column was reequilibrated in the starting buffer. Samples were analyzed by SDS-PAGE and their degree of pegylation were determined. It was found that the 0.175M NaCl eluate contained, mono- as well as di- and trace amounts of the tri-pegylated species, whereas the 750 mM NaCl eluate contained unmodified EPOsf.

2. **Di-PEG and Mono-PEG-EPOsf:** The purified conjugate mixture eluted from the column in the previous step was diluted 4-fold with the buffer and reapplied onto the column and washed as described. Di-PEG-EPOsf and mono-PEG-EPOsf were separately eluted from the column with 0.1M NaCl and 0.175 M NaCl, respectively. Elution was also performed with 750mM NaCl to elute any remaining unmodified EPOsf.

Alternatively, the reaction mixture was diluted 5-fold with the acetate buffer and applied onto the SP-Sepharose column (~0.5 mg protein/ml gel). Column was washed and adsorbed mono-PEG-EPOsf, di-PEG-EPOsf and unmodified EPOsf were eluted as described in the previous section.

## Results

PEG-EPOsf was synthesized by chemically conjugating a linear PEG molecule with a number average molecular weight of 30 kDa. PEG-EPOsf was derived from the reaction

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between the primary amino groups of EPOsf and the succinimidyl ester derivative of a 30 kDa PEG-butyric acid, resulting in an amide bond.

Results are summarized in Table1. Purified conjugate mixture comprised of mono- and di-PEG-EPOsf and was free of unmodified EPOsf as determined by SDS-PAGE analysis. Conjugate mixture accounted for 23.4 mg or 78% of the starting material. Cation exchange chromatographic separation of mono- and di-PEG-EPOsf indicated that mono- to di-PEG ratio in the conjugate mixture was almost 1:1. After completion of the reaction, ratio of the individual components of Mono: Di: Unmodified were 40: 38: 20 (%). Overall yield was almost quantitative.

Table 1. Summary of results of EPOsf pegylation

|    | Sample      | Protein (mg) | Yield (%) |
|----|-------------|--------------|-----------|
| 15 | Rxn. Mix.   | 30           | 100       |
|    | Mono-       | 12.0         | 40        |
| 20 | Di-         | 11.4         | 38        |
|    | Unmod.      | 6.0          | 20        |
| 25 | Conju. Mix. | 23.4         | 78        |

EXAMPLE 3

Pegylation of EPO with mPEG-SPA

A different aliquot of the EPOsf used in Example 2 was reacted with 30 kDa methoxy-PEG-SPA (Shearwater Polymers, Inc., Huntsville, Alabama). Reaction was performed at a protein:reagent ratio of 1:2 and purification techniques were in accordance with Example 2. Primarily the mono-pegylated species was produced.

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### e normocythaemic mouse assay

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The normocythaemic mouse bioassay is known as Erythropoietin BRP Bio 1997(2)) and a method Ph. Eur. BRP. The samples were diluted with 10 weeks old, were administered s.c. 0.2 ml of 100 U EPO or tri-, di- or mono-pegylated EPO from 10 weeks old. Blood was drawn by puncture of the tail vein and present in 1 ml of an 0.15  $\mu$ mol acridine orange solution for 3 to 10 minutes. The reticulocyte counts were determined by analysis of the red fluorescence with a flow cytometer by analysis of the red fluorescence given in terms of absolute figures (per 30,000 cells presented, each group consisted of 5 mice per

In separate experiments, a single dose PEG(SBA)-EPO mixture from Example 2 (10 ng of conjugate), EPOs from Example 2 (10 ng of conjugate), and buffer solution were administered to mice. The results show the superior activity of the pegylated EPO species indicated by the significant increase in the reticulocytes count and the shift of the reticulocytes count maximum compared to a dose of 25 ng for unmodified EPO.

the art (Pharm. Europa Spec. Issue  
the monography of erythropoietin of  
PBS. Normal healthy mice, 7-15  
O-fraction containing un-pegylated  
ple 2 or 3. Over a period of 6 days,  
diluted such that 1  $\mu$ l of blood was  
ing solution. The staining time was  
d out microfluorometrically in a flow  
gram. The reticulocyte counts were  
lood cells analyzed). For the data  
and the mice were bled only once.

modified EPO (25 ng of EPO), the conjugate), mono- and di- pegylated G(SPA)-EPO from Example 3 (10 ng) was administered to mice. The results are shown in Table 1 and the prolonged half life of the conjugate and the increased amounts of reticulocytes resulting from the same dose per mouse (10 ng),

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**TABLI**

|      | EPO<br>(Unmodified) | 30 kDa SPA<br>PEG | Mono 30K<br>SBA |
|------|---------------------|-------------------|-----------------|
| 72h  | 1000                | 1393              | 1411            |
| 96h  | 500                 | 1406              | 1501            |
| 120h | ~200                | 1100              | 1182            |
| 144h | ~0                  | 535               | 607             |

| 30K<br>3A | PEG-EPO<br>SBA Conjugate<br>Mixture | Control<br>Buffer |
|-----------|-------------------------------------|-------------------|
| 94        | 1328                                | 857               |
| 26        | 1338                                | 697               |
| 91        | 944                                 | 701               |
| 65        | 660                                 | 708               |

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**EXAMI**

**Preparation of Predominantly mono-PEG-**

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**Pegylation Reaction**

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Starting with 100 mg (5.48  $\mu$ mol) of EPO in 100 mM potassium phosphate buffer pH 7.5 prepared in accordance with Table 1, there was added 329 mg (10.96  $\mu$ mol) of 30 kDa PEG-SBA reagent dissolved in 3 ml 1 mM HCL. Enough 100 mM potassium phosphate buffer pH 7.5 was added to make the reaction mixture volume to 20 ml. The final protein concentration was 5 mg/ml and the protein : PEG reagent ratio was 1:2. The reaction mixture was mixed for 2 hours at room temperature (20 - 22°C). After 2h, the reaction was stopped by adjusting the pH to 4.5 with glacial acetic acid and stored frozen at -20°C until ready for purification.

**Purification**

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The reaction mixture from the previous step was diluted 1:5 with 10 mM sodium acetate, pH 4.5 and applied to 300 ml SP4B (sulfopropyl cation exchange resin) packed into a 4.2 x 19 cm column. The column was previously equilibrated with the same buffer. Column effluents were monitored at 280 nm with a Gilson UV monitor.

dsf in 100 mM potassium phosphate buffer pH 7.5 prepared in accordance with Table 1, there was added 329 mg (10.96  $\mu$ mol) of 30 kDa PEG-SBA reagent dissolved in 3 ml 1 mM HCL. Enough 100 mM potassium phosphate buffer pH 7.5 was added to make the reaction mixture volume to 20 ml. The final protein concentration was 5 mg/ml and the protein : PEG reagent ratio was 1:2. The reaction mixture was mixed for 2 hours at room temperature (20 - 22°C). After 2h, the reaction was stopped by adjusting the pH to 4.5 with glacial acetic acid and stored frozen at -20°C until ready for purification.

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and recorded with a Kipp and Zonen recorder. The column was washed with 300 ml or 1 bed volume of equilibration buffer to remove excess reagents, reaction byproducts and oligomeric PEG-EPO. It was followed by wash with 2 bed volumes of 100 mM NaCl to remove di-PEG-EPO. Mono-PEG-EPO was then eluted with 200 mM NaCl. During elution of the mono-PEG-EPO, the first 50 ml of the protein peak was discarded and the mono-PEG-EPO was collected as a 150 ml fraction. Unmodified EPOsf remaining on the column was eluted with 750 mM NaCl. All elution buffers were made in the equilibration buffer. All eluted samples were analyzed by SDS-PAGE and by high performance Size Exclusion Chromatography (SEC). The mono-PEG-EPO pool obtained from the 150 ml fraction, which had no detectable unmodified EPOsf, was then concentrated to ~ 4.5 – 7.5 mg/ml and diafiltered into the storage buffer (10 mM potassium phosphate, 100 mM NaCl, pH 7.5). Concentration/Diafiltration was performed with Millipore Labscale™ TFF System fitted with 50 kDa cut off Millipore Pellicon XL Biomax 50 membrane at ambient temperature. Concentrated mono-PEG-EPO was sterile filtered and stored frozen at -20°C.

Approximately 75% of EPOsf was purified. After purification, total yield was ~30% mono-PEG-EPO with no detectable unmodified EPOsf and around 25% di-PEG-EPO. Oligomers, and unpegylated EPOsf accounted for the remaining protein. The mono-PEG-EPO pool obtained from the 150 ml fraction contained approximately 90% mono-PEG-EPO and approximately 10% di-PEG-EPO.

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SEQUENCE LISTING

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20 25 30  
30 Cys Ser Leu Asn Glu Asn Ile Thr Val Pro Asp Thr Lys Val Asn Phe  
35 40 45  
Tyr Ala Trp Lys Arg Met Glu Val Gly Gln Gln Ala Val Glu Val Trp  
50 55 60  
35 Gln Gly Leu Ala Leu Leu Ser Glu Ala Val Leu Arg Gly Gln Ala Leu  
65 70 75 80  
Leu Val Asn Ser Ser Gln Pro Trp Glu Pro Leu Gln Leu His Val Asp  
85 90 95  
40 Lys Ala Val Ser Gly Leu Arg Ser Leu Thr Thr Leu Leu Arg Ala Leu  
100 105 110  
45 Gly Ala Gln Lys Glu Ala Ile Ser Pro Pro Asp Ala Ala Ser Ala Ala  
115 120 125  
Pro Leu Arg Thr Ile Thr Ala Asp Thr Phe Arg Lys Leu Phe Arg Val  
130 135 140  
50 Tyr Ser Asn Phe Leu Arg Gly Lys Leu Lys Leu Tyr Thr Gly Glu Ala  
145 150 155 160  
Cys Arg Thr Gly Asp  
165  
55

SEQUENCE LISTING

273  
69

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5

<400> 2  
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1 5 10 15

10 Leu Glu Ala Lys Glu Ala Glu Asn Ile Thr Thr Gly Cys Ala Glu His  
20 25 30

Cys Ser Leu Asn Glu Asn Ile Thr Val Pro Asp Thr Lys Val Asn Phe  
35 40 45

15

Tyr Ala Trp Lys Arg Met Glu Val Gly Gln Gln Ala Val Glu Val Trp  
50 55 60

20 Gln Gly Leu Ala Leu Leu Ser Glu Ala Val Leu Arg Gly Gln Ala Leu  
65 70 75 80

Leu Val Asn Ser Ser Gln Pro Trp Glu Pro Leu Gln Leu His Val Asp  
85 90 95

25 Lys Ala Val Ser Gly Leu Arg Ser Leu Thr Thr Leu Leu Arg Ala Leu  
100 105 110

Gly Ala Gln Lys Glu Ala Ile Ser Pro Pro Asp Ala Ala Ser Ala Ala  
115 120 125

30

Pro Leu Arg Thr Ile Thr Ala Asp Thr Phe Arg Lys Leu Phe Arg Val  
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35 Tyr Ser Asn Phe Leu Arg Gly Lys Leu Lys Leu Tyr Thr Gly Glu Ala  
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Cys Arg Thr Gly Asp Arg  
165

40

<210> 3  
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45

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1 5 10 15

50 Leu Pro Gly Pro Ser Asp Thr Pro Ile Leu Pro Gln  
20 25

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What is claimed is:

1. A conjugate, said conjugate comprising an erythropoietin glycoprotein having at least one free amino group and having the *in vivo* biological activity of causing bone marrow cells to increase production of reticulocytes and red blood cells and selected from the group consisting of human erythropoietin and analogs thereof which have sequence of human erythropoietin modified by the addition of from 1 to 6 glycosylation sites or a rearrangement of at least one glycosylation site; said glycoprotein being covalently linked to "n" poly(ethylene glycol) groups of the formula
   

$$-\text{CO}-(\text{CH}_2)_x-(\text{OCH}_2\text{CH}_2)_m-\text{OR}$$
 with the -CO of each poly(ethylene glycol) group forming an amide bond with one of said amino groups; wherein R is lower alkyl; x is 2 or 3; m is from about 450 to about 900; n is from 1 to 3; and n and m are chosen so that the molecular weight of the conjugate minus the erythropoietin glycoprotein is from 20 kilodaltons to 100 kilodaltons.
2. The conjugate of claim 1, of the formula:
 
$$\text{P}-[\text{NHCO}-(\text{CH}_2)_x-(\text{OCH}_2\text{CH}_2)_m-\text{OR}]_n \quad (\text{I})$$
 wherein m, n and R are as above, and P is the residue of the glycoprotein without the n amino group(s) which form amide linkage(s) with the poly(ethylene glycol) group(s).
3. The conjugate of claim 2, wherein the glycoprotein is a human erythropoietin.
4. The conjugate of claim 3, wherein the human erythropoietin glycoprotein is expressed by endogenous gene activation.
5. The conjugate of claim 3, wherein the glycoprotein has the sequence SEQ ID NO:1.
6. The conjugate of claim 5, wherein R is methyl.

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7. The conjugate of claim 5, wherein m is from about 650 to about 750.

8. The conjugate of claim 5, wherein n is 1.

5

9. The conjugate of claim 5, wherein R is methyl; m is from about 650 to about 750; and n is 1.

10. The conjugate of claim 2, wherein the glycoprotein has the sequence of human erythropoietin modified by the addition of from 1 to 6 glycosylation sites.

11. The conjugate of claim 10, wherein the glycoprotein has the sequence of human erythropoietin modified by a modification selected from the group consisting of:

- Asn<sup>30</sup>Thr<sup>32</sup>;
- 15 Asn<sup>51</sup>Thr<sup>53</sup>;
- Asn<sup>57</sup>Thr<sup>59</sup>;
- Asn<sup>69</sup>;
- Asn<sup>69</sup>Thr<sup>71</sup>;
- Ser<sup>68</sup>Asn<sup>69</sup>Thr<sup>71</sup>;
- 20 Val<sup>87</sup>Asn<sup>88</sup>Thr<sup>90</sup>;
- Ser<sup>87</sup>Asn<sup>88</sup>Thr<sup>90</sup>;
- Ser<sup>87</sup>Asn<sup>88</sup>Gly<sup>89</sup>Thr<sup>90</sup>;
- Ser<sup>87</sup>Asn<sup>88</sup>Thr<sup>90</sup>Thr<sup>92</sup>;
- Ser<sup>87</sup>Asn<sup>88</sup>Thr<sup>90</sup>Ala<sup>162</sup>;
- 25 Asn<sup>69</sup>Thr<sup>71</sup>Ser<sup>87</sup>Asn<sup>88</sup>Thr<sup>90</sup>;
- Asn<sup>30</sup>Thr<sup>32</sup>Val<sup>87</sup>Asn<sup>88</sup>Thr<sup>90</sup>;
- Asn<sup>89</sup>Ile<sup>90</sup>Thr<sup>91</sup>;
- Ser<sup>87</sup>Asn<sup>89</sup>Ile<sup>90</sup>Thr<sup>91</sup>;
- Asn<sup>136</sup>Thr<sup>138</sup>;
- 30 Asn<sup>138</sup>Thr<sup>140</sup>;
- Thr<sup>125</sup>; and

Pro<sup>124</sup>Thr<sup>125</sup>.

12. The conjugate of claim 11, wherein R is methyl.

5 13. The conjugate of claim 11, wherein m is from about 650 to about 750.

14. The conjugate of claim 11, wherein n is 1.

15. The conjugate of claim 11, wherein R is methyl; m is from about 650 to about  
10 750; and n is 1.

16. The conjugate of claim 10, wherein the glycoprotein has a sequence comprising  
the sequence of human erythropoietin and a second sequence at the carboxy terminus of  
the human erythropoietin sequence, wherein the second sequence contains at least one  
15 glycosylation site.

17. The conjugate of claim 16, wherein the second sequences comprises a sequence  
derived from the carboxy terminal sequence of human chorionic gonadotropin.

20 18. The conjugate of claim 17, wherein the glycoprotein has a sequence selected from  
the group consisting of:

- (a) the sequence of human erythropoietin and the sequence SEQ ID NO:3 at the  
carboxy terminus of the human erythropoietin sequence;  
(b) the sequence in (a) modified by Ser<sup>87</sup> Asn<sup>88</sup> Thr<sup>90</sup>; and  
25 (c) the sequence in (a) modified by Asn<sup>30</sup> Thr<sup>32</sup> Val<sup>87</sup> Asn<sup>88</sup> Thr<sup>90</sup>.

19. The conjugate of claim 18, wherein R is methyl.

20. The conjugate of claim 18, wherein m is from about 650 to about 750.

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21. The conjugate of claim 18, wherein n is 1.

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22. The conjugate of claim 18, wherein R is methyl; m is from about 650 to about 750; and n is 1.
- 5 23. The conjugate of claim 2, wherein the glycoprotein has the sequence of human erythropoietin modified by a rearrangement of at least one glycosylation site.
24. The conjugate of claim 23, wherein the rearrangement comprises deletion of any of the N-linked glycosylation sites in human erythropoietin and addition of an N-linked  
10 glycosylation site at position 88 of the sequence of human erythropoietin.
25. The conjugate of claim 24, wherein the glycoprotein has the sequence of human erythropoietin modified by a modification selected from the group consisting of:  
Gln<sup>24</sup> Ser<sup>87</sup> Asn<sup>88</sup> Thr<sup>90</sup>;  
15 Gln<sup>38</sup> Ser<sup>87</sup> Asn<sup>88</sup> Thr<sup>90</sup>; and  
Gln<sup>83</sup> Ser<sup>87</sup> Asn<sup>88</sup> Thr<sup>90</sup>.
26. The conjugate of claim 25, wherein R is methyl.
- 20 27. The conjugate of claim 25, wherein m is from about 650 to about 750.
28. The conjugate of claim 25, wherein n is 1.
29. The conjugate of claim 25, wherein R is methyl; m is from about 650 to about  
25 750; and n is 1.
30. A composition comprising conjugates, each of said conjugates comprising an erythropoietin glycoprotein having at least one free amino group and having the *in vivo* biological activity of causing bone marrow cells to increase production of reticulocytes and red blood cells and selected from the group consisting of human erythropoietin and  
30 analogs thereof which have sequence of human erythropoietin modified by the addition

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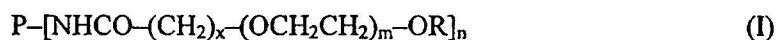
of from 1 to 6 glycosylation sites or a rearrangement of at least one glycosylation site; the glycoprotein in each said conjugate being covalently linked to "n" poly(ethylene glycol) groups of the formula  $-\text{CO}-(\text{CH}_2)_x-(\text{OCH}_2\text{CH}_2)_m-\text{OR}$  with the  $-\text{CO}$  of each poly(ethylene glycol) group forming an amide bond with one of said amino groups; where in each of said conjugates R is lower alkyl; x is 2 or 3; m is from about 450 to about 900; n is from 1 to 3; n and m are chosen so that the molecular weight of each conjugate minus the erythropoietin glycoprotein is from 20 kilodaltons to 100 kilodaltons; the percentage of conjugates where n is 1 is at least ninety percent.

31. The composition of claim 30 wherein the percentage of conjugates where n is 1 is at least ninety-two percent.

32. The composition of claim 31 wherein the percentage of conjugates where n is 1 is at least ninety-six percent.

33. The composition of claim 30 wherein the percentage of conjugates where n is 1 is from ninety percent to ninety-six percent.

34. The composition of claim 30, wherein each of said conjugates has the formula:



wherein m, n and R are as above, and P in each conjugate is the residue of the glycoprotein without the n amino group(s) which form amide linkage(s) with the poly(ethylene glycol) group(s).

35. The composition of claim 34 wherein the percentage of conjugates where n is 1 is at least ninety-two percent.

36. The composition of claim 35 wherein the percentage of conjugates where n is 1 is at least ninety-six percent.



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37. The composition of claim 34 wherein the percentage of conjugates where n is 1 is from ninety percent to ninety-six percent.

5 38. The composition of claim 34, where in each conjugate the glycoprotein is a human erythropoietin.

39. The composition of claim 38, where in each conjugate the human erythropoietin glycoprotein is expressed by endogenous gene activation.

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40. The composition of claim 38, where in each conjugate the glycoprotein has the sequence SEQ ID NO:1.

41. The composition of claim 40, where in each conjugate R is methyl.

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42. The composition of claim 40, where in each conjugate m is from about 650 to about 750.

43. The composition of claim 40, where in each conjugate R is methyl and m is from about 650 to about 750.

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44. The composition of claim 34, where in each conjugate the glycoprotein has the sequence of human erythropoietin modified by the addition of from 1 to 6 glycosylation sites.

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45. The composition of claim 44, where in each conjugate the glycoprotein has the sequence of human erythropoietin modified by a modification selected from the group consisting of:

Asn<sup>30</sup>Thr<sup>32</sup>;

30 Asn<sup>51</sup>Thr<sup>53</sup>;

Asn<sup>57</sup>Thr<sup>59</sup>;

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- Asn<sup>69</sup>;  
Asn<sup>69</sup>Thr<sup>71</sup>;  
Ser<sup>68</sup>Asn<sup>69</sup>Thr<sup>71</sup>;  
Val<sup>87</sup>Asn<sup>88</sup>Thr<sup>90</sup>;  
5 Ser<sup>87</sup>Asn<sup>88</sup>Thr<sup>90</sup>;  
Ser<sup>87</sup>Asn<sup>88</sup>Gly<sup>89</sup>Thr<sup>90</sup>;  
Ser<sup>87</sup>Asn<sup>88</sup>Thr<sup>90</sup>Thr<sup>92</sup>;  
Ser<sup>87</sup>Asn<sup>88</sup>Thr<sup>90</sup>Ala<sup>162</sup>;  
Asn<sup>69</sup>Thr<sup>71</sup>Ser<sup>87</sup>Asn<sup>88</sup>Thr<sup>90</sup>;  
10 Asn<sup>30</sup>Thr<sup>32</sup>Val<sup>87</sup>Asn<sup>88</sup>Thr<sup>90</sup>;  
Asn<sup>89</sup>Ile<sup>90</sup>Thr<sup>91</sup>;  
Ser<sup>87</sup>Asn<sup>89</sup>Ile<sup>90</sup>Thr<sup>91</sup>;  
Asn<sup>136</sup>Thr<sup>138</sup>;  
Asn<sup>138</sup>Thr<sup>140</sup>;  
15 Thr<sup>125</sup>; and  
Pro<sup>124</sup>Thr<sup>125</sup>.

46. The composition of claim 45, where in each conjugate R is methyl.
- 20 47. The composition of claim 45, where in each conjugate m is from about 650 to about 750.
48. The composition of claim 45, where in each conjugate R is methyl and m is from about 650 to about 750.
- 25 49. The composition of claim 44, where in each conjugate the glycoprotein has a sequence comprising the sequence of human erythropoietin and a second sequence at the carboxy terminus of the human erythropoietin sequence, wherein the second sequence contains at least one glycosylation site.

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50. The composition of claim 49, where in each conjugate the second sequences comprises a sequence derived from the carboxy terminal sequence of human chorionic gonadotropin.

5 51. The composition of claim 50, where in each conjugate the glycoprotein has a sequence selected from the group consisting of:

(a) the sequence of human erythropoietin and the sequence SEQ ID NO:3 at the carboxy terminus of the human erythropoietin sequence;

(b) the sequence in (a) modified by Ser<sup>87</sup> Asn<sup>88</sup> Thr<sup>90</sup>; and

10 (c) the sequence in (a) modified by Asn<sup>30</sup> Thr<sup>32</sup> Val<sup>87</sup> Asn<sup>88</sup> Thr<sup>90</sup>.

52. The composition of claim 51, where in each conjugate R is methyl.

53. The composition of claim 51, where in each conjugate m is from about 650 to  
15 about 750.

54. The composition of claim 51, where in each conjugate R is methyl and m is from about 650 to about 750.

20 55. The composition of claim 34, where in each conjugate the glycoprotein has the sequence of human erythropoietin modified by a rearrangement of at least one glycosylation site.

56. The composition of claim 55, where in each conjugate the rearrangement  
25 comprises deletion of any of the N-linked glycosylation sites in human erythropoietin and addition of an N-linked glycosylation site at position 88 of the sequence of human erythropoietin.

57. The composition of claim 56, where in each conjugate the glycoprotein has the  
30 sequence of human erythropoietin modified by a modification selected from the group consisting of:



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**Declaration and Power of Attorney for Patent Application**

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

**ERYTHROPOIETIN CONJUGATES**

the specification of which

(check one)

☒ is attached hereto.

☐ was filed on \_\_\_\_\_ as

Application Serial No. \_\_\_\_\_

and was amended on \_\_\_\_\_  
(if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR § 1.56.

I hereby claim foreign priority benefits under 35 U.S.C. § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or § 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or PCT International application having a filing date before that of the application on which priority is claimed.

**Prior Foreign Application(s)**

**Priority Claimed**

|                   |                    |                                 |                                 |                                |
|-------------------|--------------------|---------------------------------|---------------------------------|--------------------------------|
| _____<br>(Number) | _____<br>(Country) | _____<br>(Day/Month/Year Filed) | <input type="checkbox"/><br>Yes | <input type="checkbox"/><br>No |
| _____<br>(Number) | _____<br>(Country) | _____<br>(Day/Month/Year Filed) | <input type="checkbox"/><br>Yes | <input type="checkbox"/><br>No |
| _____<br>(Number) | _____<br>(Country) | _____<br>(Day/Month/Year Filed) | <input type="checkbox"/><br>Yes | <input type="checkbox"/><br>No |

I hereby claim the benefit under 35 U.S.C. § 119(e) of any United States provisional application(s) listed below.

|                            |                        |                            |                        |
|----------------------------|------------------------|----------------------------|------------------------|
| _____<br>(Application No.) | _____<br>(Filing Date) | _____<br>(Application No.) | _____<br>(Filing Date) |
|----------------------------|------------------------|----------------------------|------------------------|

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I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s), or § 365(c) of any P International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR § 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

|                                 |                      |                                                |
|---------------------------------|----------------------|------------------------------------------------|
| <u>(Application Serial No.)</u> | <u>(Filing Date)</u> | <u>(Status) (patented, pending, abandoned)</u> |
|---------------------------------|----------------------|------------------------------------------------|

|                                 |                      |                                                |
|---------------------------------|----------------------|------------------------------------------------|
| <u>(Application Serial No.)</u> | <u>(Filing Date)</u> | <u>(Status) (patented, pending, abandoned)</u> |
|---------------------------------|----------------------|------------------------------------------------|

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. *(list name and registration number)*

|                                     |                                              |
|-------------------------------------|----------------------------------------------|
| George W. Johnston (Reg. No. 28090) | Dennis P. Tramaloni (Reg. No. 28542)         |
| William H. Epstein (Reg. No. 20008) | Patricia S. Rocha-Tramaloni (Reg. No. 31054) |
| Lewis J. Kreisler (Reg. No. 38522)  | John P. Parise (Reg. No. 34403)              |

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Lewis J. Kreisler (973) 235-4387

Pascal Sebastian Bailon

Full name of sole or first inventor

Pascal Sebastian Bailon

11/17/99

Inventor's signature

Date

Florham Park, Morris County, New Jersey

Residence

U.S.A.

Citizenship

21 Woodbine Road, Florham Park, New Jersey 07932

Post Office Address

286  
230

Full name of second joint inventor, if any

Second Inventor's signature Date

Residence

Citizenship

Post Office Address

Full name of third joint inventor, if any

Third Inventor's signature Date

Residence

Citizenship

Post Office Address

Full name of fourth joint inventor, if any

Fourth Inventor's signature Date

Residence

Citizenship

Post Office Address



282  
131

Full name of fifth joint inventor, if any

Fifth Inventor's signature

Date

Residence

Citizenship

Post Office Address

Title 37, Code of Federal Regulations, §1.56, duty to disclose information material to patentability provides, in part, that each individual associated with the filing and prosecution of a patent application has a duty of candor and good faith in dealing with the Office, which includes a duty to disclose to the Office all information known to that individual to be material to patentability as defined in this section. The duty to disclose information exists with respect to each pending claim until the claim is cancelled or withdrawn from consideration, or the application becomes abandoned.

Under this section, information is material to patentability when it is not cumulative to information already of record or being made of record in the application, and

- (1) It establishes, by itself or in combination with other information, a prima facie case of unpatentability of a claim; or
- (2) It refutes, or is inconsistent with, a position the applicant takes in:
  - (i) Opposing an argument of unpatentability relied on by the Office, or
  - (ii) Asserting an argument of patentability.



REQUISITOS MINIMOS PARA ADMISION A TRAMITE

PATENTE DE INVENCION [X]

MODELO DE UTILIDAD [ ]

- Nombre e identificación del Solicitante ( 1 )
- Nombre e identificación del Inventor ( 1 )
- Título o nombre de la Invención ( 1 )
- Descripción ( 6 )
- Reivindicaciones ( 46 )
- Resumen ( 5 )
- Comprobante de Pago ( 2 )

[X]

[X]

[X]

[X]

[X]

[X]

[X]

COMPLETA [X]

INCOMPLETA [ ]

DISEÑO INDUSTRIAL [ ]

- Nombre e identificación del peticionario [ ]
- Nombre e identificación del inventor o diseñador [ ]
- Indicación del género de productos para los cuales debe utilizarse [ ]
- Representación gráfica [ ]
- Comprobante de pago [ ]

COMPLETA [ ]

INCOMPLETA [ ]

Firma Responsable

Fecha

233

## INFORME DE PROTOCOLO

**Expediente** 00-48963

**Fecha:** 30 de Junio del 2000.

Se certifica que:

Desde fecha 22 de junio de 1994,  
del folio 76.618 al folio 76.623 del  
libro de Protocolo N° 259, obra el poder  
N° 15.256 conferido por F. HOFFMANN LA  
ROCHE A.G.

domiciliado(a) en Basilea - Suiza

al doctor JOSE LLOREDA CAMACHO y/o ALFONSO  
LLOREDA CAMACHO y/o JOSE ANTONIO LLOREDA

Representante en Santafé de Bogotá D.C. Los  
mismos

Facultad para desistir SI.

Revisado \_\_\_\_\_.

234  
430  
2000-06-30  
DIA 235  
292

**DIVISION DE NUEVAS CREACIONES**  
**PRIMER EXAMEN DE FORMA**

**EXPEDIENTE 0-48963**

Practicado el examen jurídico de acuerdo con lo establecido en el art. 21 de la Decisión 344 se determinó que esta solicitud de Patente de Invención:

7-13

- |                                        |                             |                                                                                                                                                                                                                    |
|----------------------------------------|-----------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| SI <input checked="" type="checkbox"/> | NO <input type="checkbox"/> | NO APLICABLE <input type="checkbox"/> Presentó los poderes necesarios en forma correcta (Decisión 344, art. 14-a).                                                                                                 |
| SI <input checked="" type="checkbox"/> | NO <input type="checkbox"/> | NO APLICABLE <input type="checkbox"/> Acreditó, en forma correcta, la existencia y representación de la persona jurídica solicitante (art. 14-c de la Decisión 344; literal g) del art. 4 del Decreto 117/94).     |
| SI <input checked="" type="checkbox"/> | NO <input type="checkbox"/> | NO APLICABLE <input type="checkbox"/> Presentó, en forma correcta, el comprobante de pago por la tasa de presentación establecida (art. 13-f de la Decisión 344 y la Resolución 02 del 4 de Enero del 2000)        |
| SI <input checked="" type="checkbox"/> | NO <input type="checkbox"/> | EXTEMPORANEAMENTE <input type="checkbox"/> Reivindicó prioridad, dentro del plazo y de acuerdo con lo previsto en el Artículo 4 del Convenio de Paris.                                                             |
| SI <input checked="" type="checkbox"/> | NO <input type="checkbox"/> | NO APLICABLE <input type="checkbox"/> Presentó copia, certificada su conformidad por la Administración que recibió la solicitud de la cual reivindica prioridad, al momento de radicar la solicitud nacional.      |
| SI <input checked="" type="checkbox"/> | NO <input type="checkbox"/> | NO APLICABLE <input type="checkbox"/> Presentó la traducción del capítulo reivindicatorio de la solicitud de la cual se reivindica prioridad (Art. 4, D-3 Convenio de París; Concepto: 5 de enero de 1999 S.I.C.). |
| SI <input checked="" type="checkbox"/> | NO <input type="checkbox"/> | CUMPLE LOS REQUISITOS JURIDICOS                                                                                                                                                                                    |

5/12

esta

**OBSERVACIONES**

En consecuencia, se conceptúa que esta solicitud:

SI ☒ NO ☐ Se ajusta a los aspectos FORMALES indicados en la Decisión 344 y por tanto,

SI ☒ NO ☐ Se recomienda PUBLICAR el extracto correspondiente.

Santafé de Bogotá, D.C., 30 de Junio del 2000

**EXAMINADOR JURIDICO.**  
202022

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Conmutador 334 1221-2-3-4  
Fax 281 3125  
Santafé de Bogotá, D.C. - Colombia

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