



No. 13-167243- -00014-0000

Fecha: 2015-08-20 15:54:45 Dep. 2020 DIR.NUEVASCR
Tra. 11 PATENTEIYII Eve: 378 FASENACIONALI
Act. 412 REPOSPRESRECU Folios: 44

P-3858

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
SUPERINTENDENTE DE INDUSTRIA Y COMERCIO

E. S. D.

Re: Expediente No. 13-167.243

Solicitud de patente a nombre de **REATA PHARMACEUTICALS, INC.**

1. Yo, CAROLINA DAZA MONTALVO identificada como aparece al pie de mi firma, domiciliada en la Carrera 11 No. 86-53, piso 6, obrando como apoderada de conformidad con la sustitución de poder otorgada a mi favor por la apoderada principal y que se acompaña en el presente escrito, de la sociedad **REATA PHARMACEUTICALS, INC.**, domiciliada en Texas, Estados Unidos, solicitante de la patente en referencia, interpongo recurso de reposición en contra del **ARTÍCULO SEGUNDO** de la Resolución No. 33248 del día 30 de junio de 2015, expedida por el Superintendente de Industria y Comercio, para que sea revocada y en consecuencia se conceda el privilegio de patente de invención para las reivindicaciones 1 a 69.

Con el recurso que por este escrito se interpone pretendo:

- 1.1. Que se **revoque** el **ARTÍCULO SEGUNDO** de la Resolución No. 33248 del 30 de junio de 2015;
- 1.2. Que se **otorgue** el privilegio de patente de invención para las reivindicaciones 1 a 69 a favor de mi poderdante la sociedad **REATA PHARMACEUTICALS, INC.**;
- 1.3. Que se **mantenga** en sus demás partes la decisión contenida en la Resolución No. 33248 del 30 de junio de 2015.

2. FUNDAMENTOS DEL RECURSO

Con fundamento en la facultad que confiere el artículo 34 de la Decisión 486 al solicitante para modificar la solicitud de patente en cualquier momento del trámite, presento junto con este recurso de reposición, un nuevo capítulo reivindicatorio que comprende un total de 52 reivindicaciones, en donde se eliminaron las reivindicaciones 14-20, 26, 27, 41, 42, 57, 58 y 61-64 y

adicionalmente, se restringió el alcance de la presente invención al restringir la definición de los radicales R1, R2, R3 y R6 de los compuestos de fórmula III y IV, en un esfuerzo por acelerar el trámite. Solicito a ese Despacho tener en cuenta que la reivindicación 70 previamente otorgada ha sido reenumerada como 52.

En específico, dicha restricción de materia consistió en lo siguiente:

- R1 y R2: sólo pueden ser independientemente, hidrógeno o alquilo($C \leq 12$), eliminando arilos y aralquilos;
- R3 igualmente solo puede ser hidrógeno o alquilo($C \leq 12$), eliminado arilos; y por último
- R6 se restringió a ser halógenos, alquilo($C \leq 12$), alquinilo($C \leq 12$), arilo($C \leq 12$), aralquilo($C \leq 12$), heteroarilo($C \leq 12$) o una versión sustituida de cualquiera de los anteriores, eliminando acilos, alcoxis y amidas.

De esta manera es evidente que el nuevo pliego reivindicatorio de la presente solicitud, no amplía en ningún instante el alcance de la presente solicitud, razón por la cual se informa que este pliego reemplazará por completo al anteriormente presentado, para lo cual se adjunta el recibo de pago por concepto de modificación voluntaria.

2. FUNDAMENTOS DEL RECURSO

2.1 De la indebida determinación de falta de claridad

Su Despacho ha indicado que la reivindicación 69 no cumple con los requisitos exigidos por el artículo 30 de la Decisión 486, por el hecho de que al ser una reivindicación dependiente de la reivindicación 1, ésta reclama compuestos que presentan radicales que no eran contemplados inicialmente en la reivindicación 1.

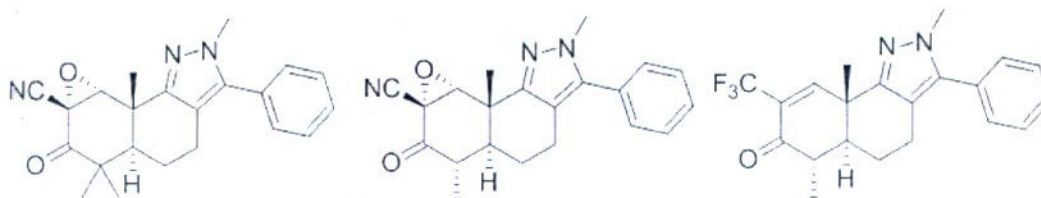
En específico, la reivindicación 1 reclama un compuesto de fórmula (III), en donde se define un átomo de hidrógeno y un grupo ciano en las posiciones 1 y 2 del núcleo tricíclico, pero en la reivindicación 69, se reportan compuestos como el oxabicyclo[4.1.0]heptano entre las posiciones 1 y 2, del núcleo tricíclico en la posición 2.

2.1.2 La presente Invención sí es clara

El artículo 30 de la Dec. 486 de la Comunidad Andina establece:

Artículo 30.- Las reivindicaciones definirán la materia que se desea proteger mediante la patente. Deben ser claras y concisas y estar enteramente sustentadas por la descripción.

En referencia a la objeción hecha por el señor Examinador en dicho respecto, informamos que estamos de acuerdo con su observación, razón por la cual, de dicha reivindicación 69, ahora la nueva reivindicación 51, se eliminaron principalmente los siguientes compuestos (dentro de muchos otros):



Con lo cual se considera se satisface la objeción planteada.

2.2 Del indebido análisis de Nivel Inventivo

De acuerdo con los estamentos de la resolución pendiente, se tiene que los compuestos reclamados en la reivindicación 1, carecen de nivel inventivo en vista de la combinación de las enseñanzas de los documentos citados **D1**: WO03024935 y **D2**: WO03059339.

2.2.2 La invención sí es Inventiva

El artículo 18 de la Decisión 486 señala lo siguiente:

Artículo 18.- Se considerará que una invención tiene nivel inventivo, si para una persona del oficio normalmente versada en la materia técnica correspondiente, esa invención no hubiese resultado obvia ni se hubiese derivado de manera evidente del estado de la técnica.

Una invención se considerará inventiva si la misma no hubiese resultado obvia a partir del arte previo para una persona del oficio normalmente versada en la materia. Esto a su vez implica que el Examinador a cargo de esta evaluación debe colocarse en el momento exacto en el que el inventor buscaba resolver el problema técnico contemplado en la solicitud y para el cual poseía la literatura y el conocimiento disponibles en dicho momento.

Siguiendo entonces los lineamientos de la Jurisprudencia aplicable al análisis del nivel inventivo de una solicitud de patente, se considera que una anterioridad o

una combinación de dos anterioridades puede afectar dicho requerimiento de patentabilidad, si una persona medianamente versada en la materia encuentra enseñanzas o sugerencias claras que, a partir de un conocimiento medio en dicho campo, le permitan llegar de manera obvia a la invención reivindicada. Adicionalmente se considera que una mejora sustancial, un efecto inesperado o una ventaja con respecto al arte previo es un indicio de nivel inventivo, por cuanto aporta un salto tecnológico en la regla técnica.

De acuerdo con el análisis hecho por el señor Examinador, los compuestos divulgados por el documento D2 resultan ser los más cercanos a la presente invención, por tanto, para una persona del oficio versada en la materia, resultaría obvio, reemplazar el anillo-C de los compuestos de D2, por un anillo-C a base de pirazol sustituido con N-arilo, tal y como lo divulga D1, y de este modo se llegaría de forma obvia, a los compuestos reclamados en las reivindicaciones 1 a 69.

En dicho respecto, mi mandante presenta a continuación el siguiente análisis, el cual desvirtúa por completo las conclusiones a las que llegó el señor Examinador. La siguiente tabla, hace una comparación de los compuestos de la invención, con respecto a aquellos divulgados en los documentos citados D1 y D2, haciendo especial énfasis en el anillo-C:

Invención	D2	D1
Compuesto de Formula (III), reivindicación 1: Anillo-C	Fórmula de la reivindicación 1, pag. 82:	Fórmula (I) de la reivindicación 1 on pag. 234:

La tabla anterior, exhibe los compuestos que cobija el nuevo pliego reivindicatorio, los cuales han sido modificados con el fin de diferenciarse aún más de aquellos conocidos en el estado previo de la técnica. De esta manera se tiene que éstos son inventivos, a partir de las siguientes razones, las cuales serán analizadas en detalle posteriormente.

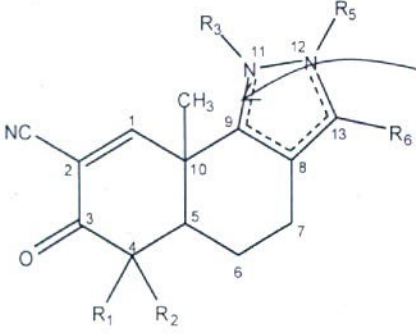
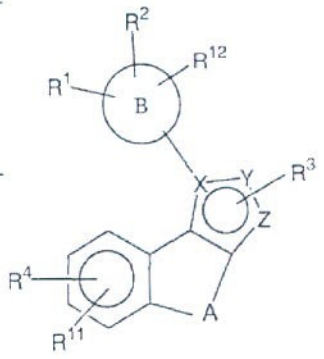
Las razones por las cuales los compuestos de la invención son inventivos sobre los compuestos ya conocidos en D1 y D2, radican en lo siguiente:

- Los compuestos de fórmula (III) reportados en la reivindicación 1, no pueden ser simplemente deducidos al reemplazar el anillo-C de D2 por un anillo-C a base de pirazol sustituido con N-arilo, como se divulga D1, en vista de que requeriría de al menos dos modificaciones adicionales en la estructura química del compuesto; y
- Los compuestos que actualmente se reclaman en esta solicitud, presentan una eficiencia mejorada sobre aquellos divulgados en D2.

(a). Modificaciones adicionales que requieren los radicales R₃ y R₆ de los compuestos de la invención:

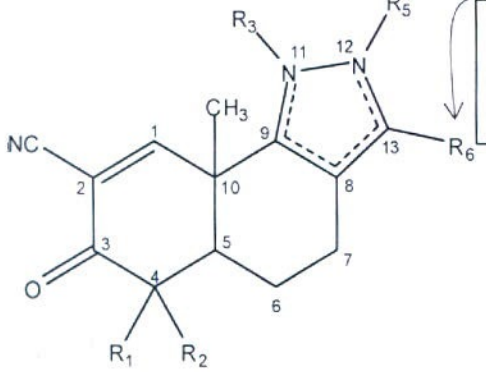
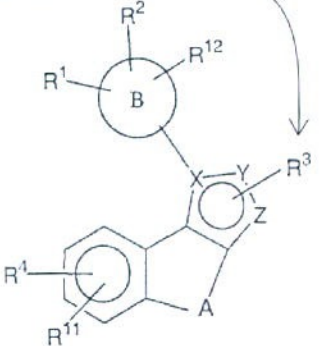
Las modificaciones adicionales que requeriría hacer el experto en la materia, para deducir los compuestos reclamados en esta solicitud, consisten en:

- El experto en la materia, requeriría remover el grupo B (arilo, heteroarilo o heterociclo) del anillo de pirazol en la posición X, divulgado en el documento D1. Cabe resaltar que ninguno de los compuestos que actualmente se reportan en la nueva reivindicación 51, presentan un grupo arilo, heteroarilo o heterocíclico en dicha posición. Como se puede apreciar en la siguiente tabla, R₃ no puede ser sustituido por un arilo (tal y como se reporta en la nueva reivindicación 1 de la presente solicitud):

Invención	D1
Formula (III), reivindicación 1:  R₃ es hydrogeno o alquilo (C_{≤12})	Formula (I), reivindicación 1, pag. 234:  B es un heteroarilo de 5 o 6 miembros, arilo, heterociclo saturado o no-saturado en donde dicho arilo, heteroarilo o heterociclo son opcionalmente sustituidos con R¹ R² y R¹²

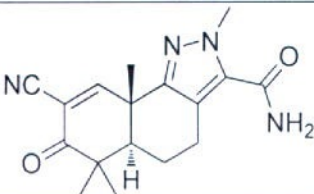
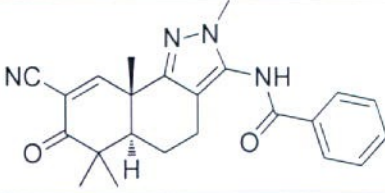
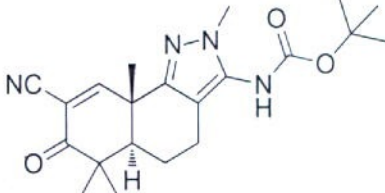
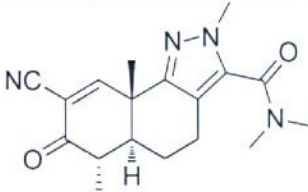
Como se puede apreciar, ni D1 ni D2, mencionan o sugieren remover al grupo B del compuesto. Contrario a dicha suposición, en la reivindicación 1 de D1, se requiere que dicho grupo B sea un grupo arilo, heteroarilo o heterociclo, definición que es completamente diferente a aquella propuesta en la nueva reivindicación 1.

- En otro escenario, se tiene que esa persona experta en el área, a su vez requeriría remover le grupo amida $-\text{CONH}_2$ del átomo marcado como Z, en el compuesto de D1. Es así, como mientras el radical R3 del compuesto de la reivindicación 1 de D1, permite la presencia de diferentes grupos a base de nitrógeno, encontrando así 452 compuestos en dicha referencia que contienen el grupo amida ($-\text{CONH}_2$), en la posición de dicho radical (ver ejemplos 1-452 de las páginas 38-226). Contrario a este escenario, se tiene que el radical R6 del compuesto de fórmula (III) de la invención, el cual es el equivalente para el R3 de D1, no presenta el grupo $-\text{CONH}_2$ o cualquier otro tipo de grupo amida, tal y como se refleja en la siguiente tabla:

Fórmula (III) de la reivindicación 1:	Fórmula (I) de la reivindicación 1, pag. 234:
 R_6 es halo o alquilo_(C≤12), alquinilo_(C≤12), arilo_(C≤12), aralquilo_(C≤12), heteroarilo_(C≤12), o una versión sustituida de cualquiera de estos grupos.	 R_6 y R^3 son diferentes R^3 es seleccionado del grupo que consiste de amidina sustituida o no-sustituida, alquilamina, aminoalquil, CONHR^7, NH_2, NHCOR^6, y $\text{CH}_2\text{NHCOR}^6$ con AL condición de que cuando R^3 es NH_2 o NHCH_3, entonces R^1, R^2, R^4, R^{11} y R^{12} no son híbridos

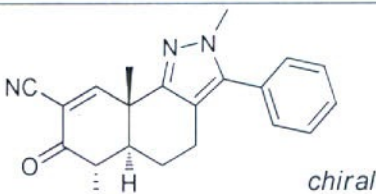
La razón por la cual los grupos amida representan un grupo prohibido para el radical R6 del compuesto de fórmula (III) de la presente solicitud, es que en el caso de llegar a haber presencia de alguno de estos, se disminuye la actividad antiinflamatoria de los mismos. En específico, se tiene que el compuesto TX63619 de la invención, el cual presenta ese grupo amida en el radical R6, muestra poca o casi nula actividad, con un $\text{IC}_{50} > 200 \text{ nM}$, al igual que ocurre con los compuestos TX63531, TX63594 y TX63655 de esta misma solicitud. Lo anterior, se evidencia a continuación en la siguiente tabla, los cuales

corresponden a los valores obtenidos en la tabla 1 del capítulo descriptivo de la presente solicitud:

Compuesto No.	Fórmula Química	NO IC ₅₀ (nM)
TX63619		>200
TX63531		>200
TX63594		>200
TX63655		>200

En este punto se desea resaltar, que ninguno de los compuestos expuestos en la tabla anterior, se encuentran dentro del alcance del actual pliego reivindicatorio.

Contrario a dicho escenario, se tiene que los compuestos de interés en esta solicitud, presentan una actividad antiinflamatoria bastante alta, con un promedio de 29.1 nM, en donde aquel compuesto de mayor actividad es el TX63791, con 2.3 nM:

Compuesto No.	Fórmula Química	NO IC ₅₀ (nM)
TX63791		2.3

Siendo así las cosas, se tiene finalmente que si los compuestos divulgados en D2, se les modificara el anillo-C, con aquel divulgado en D1, los compuestos resultantes presentarían una actividad inflamatoria mucho más baja, que aquella que presentan los compuestos actualmente reclamados en esta solicitud.

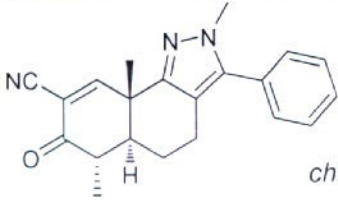
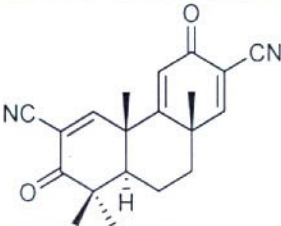
(b). Actividad mejorada sorprendentemente

Como ya se mencionó anteriormente, se tiene que los compuestos definidos en las nuevas reivindicaciones 1 a 51, presentan una actividad antiinflamatoria, mucho más alta que aquella que presentan los compuestos de D2.

Con el ánimo de demostrar experimentalmente dicha afirmación, se debe normalizar los valores, en vista de que los valores de IC₅₀ absoluto, pueden variar de un ensayo a otro.

Dichos valores normalizados fueron obtenidos mediante la comparación con la actividad de un compuesto común en referencia, como por ejemplo lo es el CDDO-Me. Es así, como ambos compuestos de la presente solicitud y aquellos de D2 (TBE) fueron comprados con respecto al CDDO-Me. Los valores normalizados de IC₅₀ de los compuestos de la invención, se muestran en la tabla 1 del capítulo descriptivo de esta solicitud, mientras que los valores normalizados para los compuestos de D2, se calcularon al dividir los valores absolutos que se plasman en la tabla 1 de dicha anterioridad (pag. 52, D2), entre el valor de CDDO-Me, que reporta el artículo de Honda et al., J. Med. Chem., 54(6): 1762-1778, 2011 (ver anexo).

De este modo y después de realizar dicho procedimiento, se tiene que al comparar los compuestos más activos, tanto de la invención como de D2, se concluye que el compuesto **TX63791** es aproximadamente 7.5 veces más activo que (±)-TBE-9 de D2:

Compuesto No.	Estructura Molecular	NO IC ₅₀ (nM)	NO IC ₅₀ Relativo
TX63791		2.3	1.4
(±)-TBE-9		2.1	10.5

Igualmente, cuando los valores de la actividad promedio son comparados, los compuestos que se reportan en la nueva reivindicación 51, resultan ser considerablemente más activos que aquellos divulgados en D2, lo cual se aprecia

tanto en los valores normalizados como absolutos:

	NO IC ₅₀ (nM)	NO IC ₅₀ Relativo
Valores promedio de los compuestos listados en la nueva reivindicación 51 de la presente invención	29.1	17.5
Valores promedio de los compuestos TBE de la tabla 1, D2, excluyendo los tres compuestos menos activos (TBE-1, TBE-2 and TBE-7).	53.8	269

Como se puede apreciar en la tabla anterior, a pesar de excluir del promedio los tres compuestos menos activos de aquellos divulgados en D2, los de la invención, continúan siendo al menos 15 veces más activos, con un valor promedio de IC₅₀ de (17.5) en comparación con el obtenido para los de D2: (269).

Con base en los anteriores argumentos, se puede afirmar que en definitiva no existe la posibilidad de que al combinar las enseñanzas de los documentos D1 y D2, la cual específicamente consiste en reemplazar el anillo-C de D2, con el anillo-C a base de pirazol sustituido con N-arilo, de D1, se obtengan los compuestos acá reclamados, dado que adicionalmente se requeriría remover el grupo arilo del radical R3 y el grupo amida del radical R6, movimientos y/o acciones, que no están mencionadas y/o sugeridas en el estado previo de la técnica. En consecuencia, no se obtendrían compuestos con una actividad antiinflamatoria mejorada, como la que ya se demostró previamente, frente a los compuestos de D2.

De este modo, se le solicita amablemente a este Despacho, tener en consideración los anteriores argumentos y el nuevo capítulo reivindicatorio que se presenta en esta oportunidad, para así confirmar por sí mismo, la superación de todas las objeciones que se planteaban y en consecuencia, revocar la decisión de negativa y proceder con la concesión de patente de esta solicitud.

3. Concesión de solicitudes equivalentes

Si bien entendemos y aceptamos que cada país es autónomo en la concesión o negación de los privilegios de patente, queremos resaltar el hecho que el análisis de los requisitos para el otorgamiento de esos derechos es prácticamente universal. La presente invención fue concedida por la Oficina de Patentes de los Estados Unidos: US8513436 (B2).

Si bien es cierto que cada país es autónomo en conceder o negar el privilegio de patente para una solicitud, **también es cierto que los requisitos de concesión de dichos privilegios son universales**, esto es, novedad, nivel inventivo y aplicación industrial, **como también la forma de evaluarlos**. Así las cosas, el hecho de que la misma solicitud cumpla con los mencionados requisitos en diferentes países, como se mencionó anteriormente, es un claro indicio del merecimiento de la patente en Colombia.

4. PETICIÓN

Teniendo en cuenta lo dicho anteriormente, solicito a usted lo siguiente:

- a) Que de conformidad con lo previsto en los artículo 3, 42 y 80 del Código de Procedimiento Administrativo y de lo Contencioso Administrativo se consideren y se resuelvan TODOS los argumentos y TODAS las cuestiones planteadas así como las que aparezcan con motivo de este recurso.
- b) Que se revoque parcialmente la Resolución No. 33248 del día 30 de junio de 2015 expedida por el Superintendente de Industria y Comercio, y se proceda a conceder en su totalidad el privilegio de patente de invención a la solicitud de mi mandante

Señor Superintendente,


CAROLINA DAZA MONTALVO

C.C. 66.783.790 de Palmira Valle

T.P 141563 del CSJ

notificaciones@clarkemodet.com.co

ANEXOS

- Nuevo capítulo reivindicatorio, compuesto por 52 reivindicaciones.
- Copia del recibo de pago por un valor de \$ 132.000, por concepto de modificaciones voluntarias.
- Artículo: "Tricyclic compounds containing nonenolizable cyano enones. A novel class of highly potent anti-inflammatory and cytoprotective agents", Honda et al., Journal Medicinal Chemistry, 2011, 54, 1762-1778.

**DIRECCIÓN DE NUEVAS CREACIONES
MODIFICACIONES Y/O CORRECCIONES**

1. Identificación del Trámite

☐ Error material

☒ Modificación a una Solicitud en trámite

☒ Patente de Invención

☐ Patente de Modelo de Utilidad

☐ Registro de Diseño Industrial

☐ Esquema de Trazado de Circuitos Integrados

2. Datos del solicitante

Persona Natural ☐

Persona Jurídica ☒

REATA PHARMACEUTICALS INC.

Nombre o denominación / Nombre o razón social completa

Nombre representante legal

Tipo de empresa

Micro ☐

Pequeña ☐

Mediana ☐

Otra ☐

Documento de identificación:

☐ C.C.

☐ C.E.

☐ NIT

☐ Otro Número _____

Nacionalidad/País de constitución ESTADOS UNIDOS	Dirección y domicilio del titular 2801 Gateway Orive, Suite 150,	Ciudad Irving, Texas 75063,
Dirección electrónica	No. Fax	Número telefónico

☐ Autorizo expresamente a la Superintendencia de Industria y Comercio para que todas las comunicaciones sean enviadas a través de la dirección de correo electrónico

3. Datos del apoderado:

CAROLINA DAZA MONTALVO

C.C. 66.783.790

141563 CSJ

Apellidos y Nombre

Documento de identificación

Tarjeta profesional

Dirección y domicilio	Ciudad	
Carrera 11 No. 86-53, Piso 6, Bogotá D.C., Colombia	Bogotá D.C.	
Dirección electrónica	No. Fax	Número telefónico
<u>notificaciones@clarkemodet.com.co</u>	6350824	6181088

En caso de haber presentado poder general para asuntos que se adelanten ante la Delegatura de Propiedad Industrial, sírvase indicar el número de su radicación o protocolo de poder general _____

4. Datos del trámite

Corrección:

Expediente No. 13-167.243

Aparece _____

Debe ser _____

Modificación:

Presentación Solicitud Divisional.

Modificación al capítulo descriptivo.

☐

☒ Modificación al capítulo Reivindicatorio

Presento junto con este memorial nuevo capítulo reivindicatorio, que comprende un total de 52 reivindicaciones.

Modificación al resumen.

☐

Modificación a los dibujos.

☐

Modificación al solicitante.

5. Anexos

☒

Comprobante de pago de la tasa para la presentación de la corrección y/o modificación

☐

Poder, si fuere el caso.

☒

Documento que contiene las modificaciones o correcciones.

☐

Copia de la solicitud y sus anexos en formato magnético.

6. Firma



CAROLINA DAZA MONTALVO

C.C. 66.783.790 de Palmira Valle

TP-141563 CSJ

cdaza@clarkemodet.com.co

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

NIT : 800.176.089-2

- / -



RECIBO DE CAJA

No. 15 - 0078455

Bogotá D.C., Julio 14 de 2015 - 15:45:50

RECIBIDO DE : CLARKE MODET

NI 830.008.643

*** Soporte del Pago ***

TIPO PAGO	BANCO	CUENTA	No. PAGO	FECHA PAGO	VR. PAGO
CONSIGNACION	BANCO DE BOGOTA	062754387	696976140	14/07/2015	10.272.000.00

*** Conceptos Pagados ***

CANT. RENTISTICO	CONCEPTO	Vr.UNDITARIO	Vr.CONCEPTO
1 50005-01-03 CAMBIO DE MODALIDAD Y MODIFICACIONES EN CREACION CORRECCION A SOLICITUD EN TRAMITE / MODIFICA		132.000.00	132.000.00
			\$132.000.00

SON: **CIENTO TREINTA Y DOS MIL PESOS MONEDA CORRIENTE***

Responsable: _____

Recibo de Caja Aplicado al Expediente No. _____

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



No. 13-167243- -00014-0000

Fecha: 2015-08-20 15:54:45 Dep. 2020 DIR.NUEVASCR
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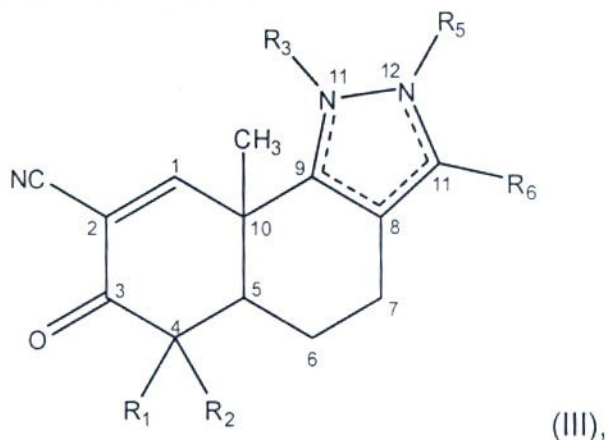
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REIVINDICACIONES

1. El compuesto de la fórmula (III):



En donde:

R₁ y R₂ son independientemente hidrógeno ó alquilo_(C₁₋₁₂); o

R_1 y R_2 se toman juntos y son alcandiilo_(C≤12);

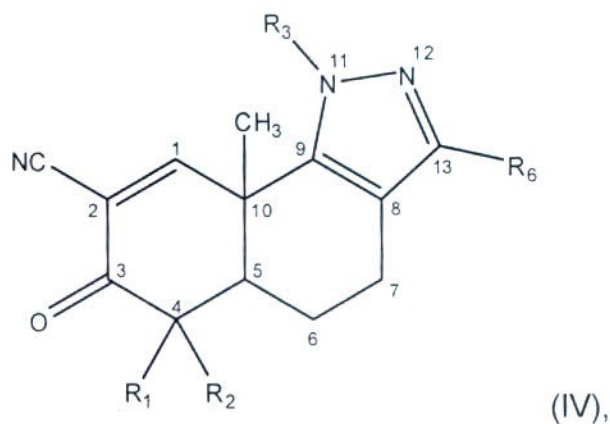
R₃ es nulo, hidrógeno ó alquilo_(C≤12) con la condición de que R₃ se encuentra ausente solamente cuando el átomo al cual está unido forma parte de una unión doble;

R₅ es nulo, hidrógeno; o alquilo_(C≤12), arilo_(C≤12), acilo_(C≤12) o una versión sustituida de cualquiera de estos grupos; con la condición de que R₅ se encuentra ausente solamente cuando el átomo al cual está unido forma parte de una unión doble; y

R₆ es halo o alquilo_(C≤12), alquinilo_(C≤12), arilo_(C≤12), aralquilo_(C≤12), heteroarilo_(C≤12), o una versión sustituida de cualquiera de estos grupos;

o una sal aceptable farmacéuticamente o un tautómero del mismo.

2. El compuesto de la reivindicación 1, CARACTERIZADO PORQUE además se define por medio de la fórmula (IV):



En donde:

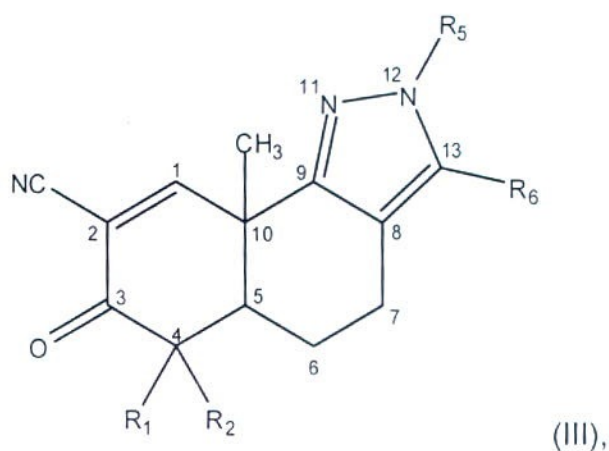
R_1 y R_2 son independientemente hidrógeno ó alquilo_(C≤12);

R_3 es hidrógeno ó alquilo_(C≤12); y

R_6 es halo o alquilo_(C≤12), alquinilo_(C≤12), arilo_(C≤12), aralquilo_(C≤12), heteroarilo_(C≤12) o una versión sustituida de cualquiera de estos grupos;

o una sal farmacéuticamente aceptable o un tautómero del mismo.

3. El compuesto de la reivindicación 1, CARACTERIZADO PORQUE además se define por medio de la fórmula (III):



En donde:

R_1 y R_2 son independientemente hidrógeno ó alquilo_(C≤12); o

R_1 y R_2 se toman juntos y son alcanodiilo_(C≤12);

R_5 es hidrógeno; o alquilo_(C≤12), arilo_(C≤12), acilo_(C≤12) o una versión sustituida de cualquiera de estos grupos; y

R_6 es halo o alquilo_(C≤12), alquinilo_(C≤12), arilo_(C≤12), aralquilo_(C≤12), heteroarilo_(C≤12) o una versión sustituida de cualquiera de estos grupos;

o una sal farmacéuticamente aceptable o un tautómero del mismo.

4. El compuesto de acuerdo con una de las reivindicaciones 1-3, CARACTERIZADO PORQUE R_1 o R_2 son alquilo_(C≤8).
5. El compuesto de la reivindicación 4, CARACTERIZADO PORQUE R_1 es metilo.
6. El compuesto de la reivindicación 5, CARACTERIZADO PORQUE R_1 y R_2 son cada uno metilo.
7. El compuesto de la reivindicación 5, CARACTERIZADO PORQUE R_1 es metilo y R_2 es hidrógeno.
8. El compuesto de la reivindicación 4, CARACTERIZADO PORQUE R_1 es etilo.
9. El compuesto de la reivindicación 8, CARACTERIZADO PORQUE R_1 es etilo y R_2 es hidrógeno.
10. El compuesto de acuerdo con una de las reivindicaciones 1-3, CARACTERIZADO PORQUE R_1 es hidrógeno.
11. El compuesto de la reivindicación 10, CARACTERIZADO PORQUE R_1 y R_2 son cada uno hidrógeno.
12. El compuesto de acuerdo con la reivindicación 1 o 3, CARACTERIZADO PORQUE R_1 y R_2 se toman juntos y son alcanodiilo_(C≤8).
13. El compuesto de la reivindicación 12, CARACTERIZADO PORQUE R_1 y R_2 se toman juntos y son 1,4-butanodiilo.

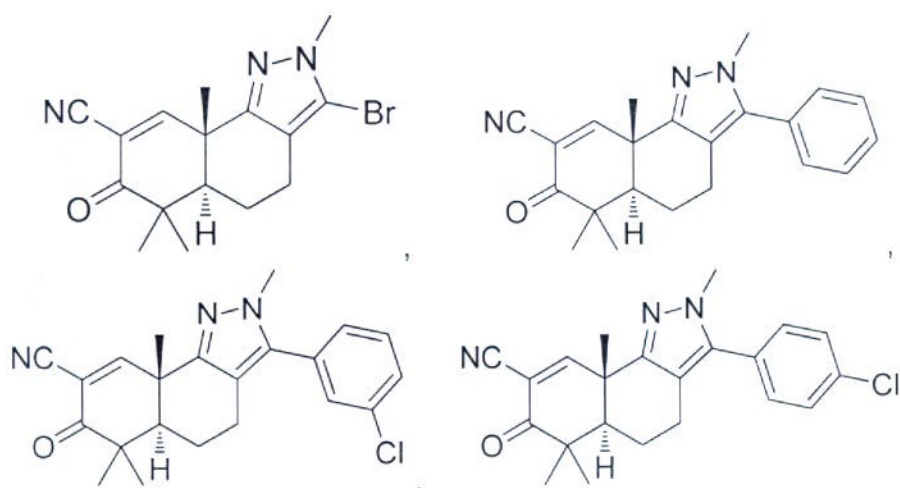
14. El compuesto de acuerdo con una de las reivindicaciones 1 y 13, CARACTERIZADO PORQUE R_3 se encuentra ausente.
15. El compuesto de acuerdo con una de las reivindicaciones 1, 2 y 13, CARACTERIZADO PORQUE R_3 es hidrógeno.
16. El compuesto de acuerdo con una de las reivindicaciones 1, 2 y 13, CARACTERIZADO PORQUE R_3 es alquilo_(C≤8).
17. El compuesto de la reivindicación 16, CARACTERIZADO PORQUE R_3 es alquilo_(C≤4).
18. El compuesto de la reivindicación 17, CARACTERIZADO PORQUE R_3 es metilo.
19. El compuesto de acuerdo con una de las reivindicaciones 1, 4 y 18 CARACTERIZADO PORQUE R_5 se encuentra ausente.
20. El compuesto de acuerdo con una de las reivindicaciones 1, 3 y 18 CARACTERIZADO PORQUE R_5 es hidrógeno.
21. El compuesto de acuerdo con una de las reivindicaciones 1, 3 y 18, CARACTERIZADO PORQUE R_5 es alquilo_(C≤6) o alquilo_(C≤6) sustituido.
22. El compuesto de la reivindicación 21, CARACTERIZADO PORQUE R_5 es alquilo_(C≤6).
23. El compuesto de la reivindicación 21, CARACTERIZADO PORQUE R_5 es metilo, ciclohexilo, o *t*-butilo.
24. El compuesto de la reivindicación 21, CARACTERIZADO PORQUE R_5 es alquilo_(C≤4) sustituido.

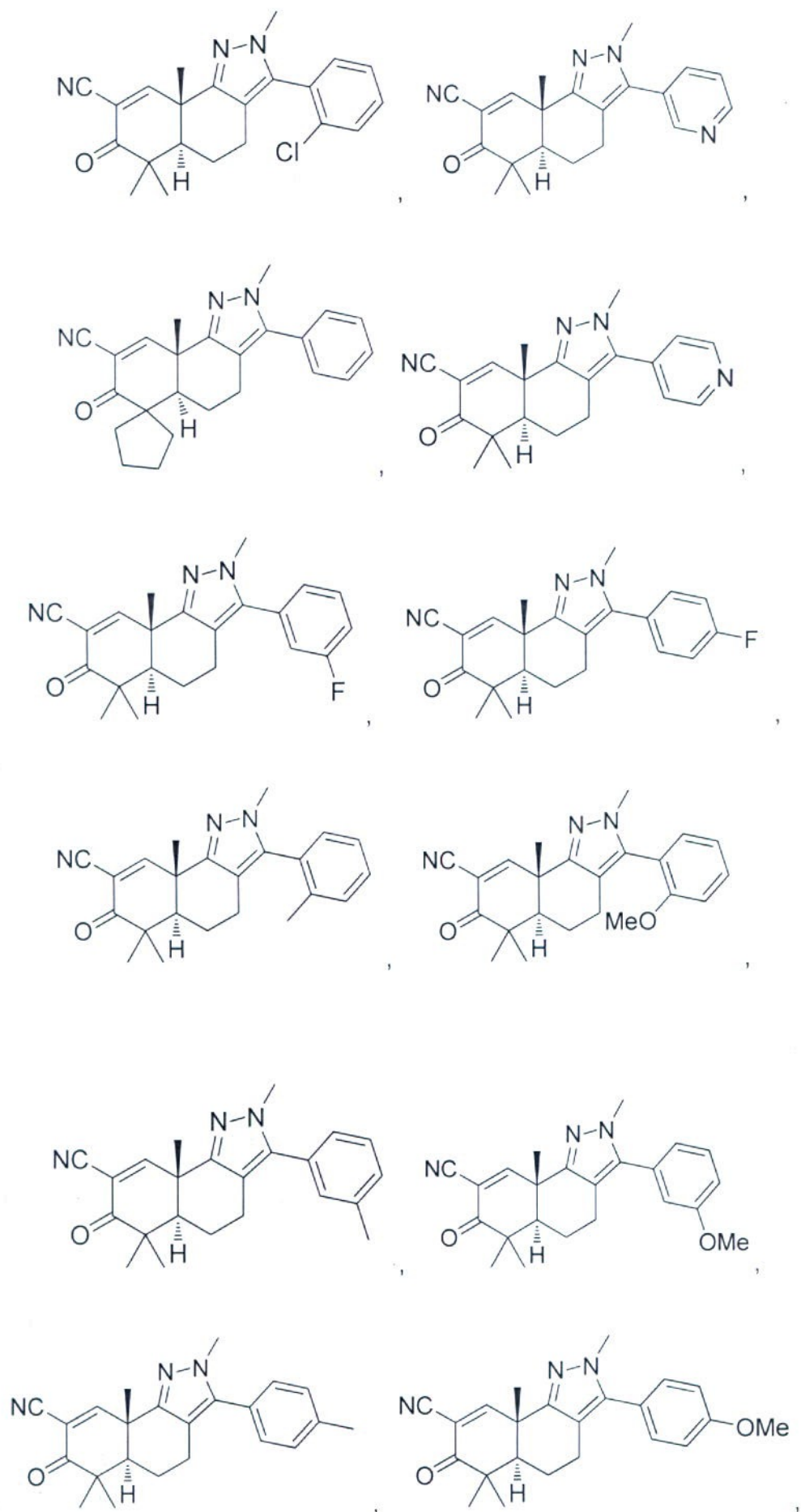
25. El compuesto de la reivindicación 24, CARACTERIZADO PORQUE R_5 es 2-hidroxietilo o 2,2,2-trifluoroetilo.
26. El compuesto de la reivindicación 21, CARACTERIZADO PORQUE R_5 es arilo_(C≤8) o arilo_(C≤8) sustituido.
27. El compuesto de la reivindicación 26, CARACTERIZADO PORQUE R_5 es arilo_(C≤8).
28. El compuesto de la reivindicación 27, CARACTERIZADO PORQUE R_5 es fenilo.
29. El compuesto de la reivindicación 21, CARACTERIZADO PORQUE R_5 es acilo_(C≤8) o acilo_(C≤8) sustituido.
30. El compuesto de la reivindicación 29, CARACTERIZADO PORQUE R_5 es acetilo, metoxicarbonilo, etoxicarbonilo, o fenilcarbonilo.
31. El compuesto de acuerdo con una de las reivindicaciones 1-30, CARACTERIZADO PORQUE R_6 es halo.
32. El compuesto de la reivindicación 31, CARACTERIZADO PORQUE R_6 es cloro o bromo.
33. El compuesto de acuerdo con una de las reivindicaciones 1-30, CARACTERIZADO PORQUE R_6 es alquilo_(C≤6) o alquilo_(C≤6) sustituido.
34. El compuesto de la reivindicación 33, CARACTERIZADO PORQUE R_6 es alquilo_(C≤6).
35. El compuesto de la reivindicación 34, CARACTERIZADO PORQUE R_6 es

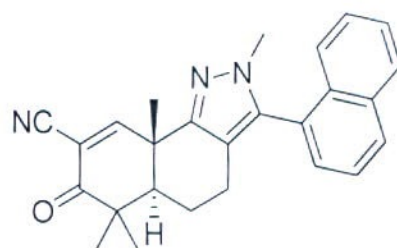
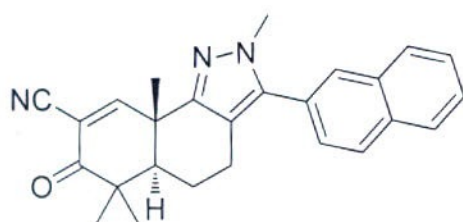
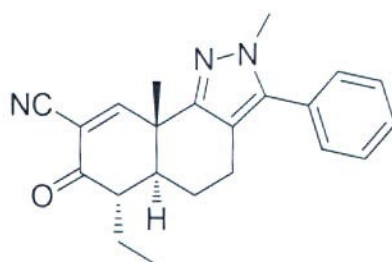
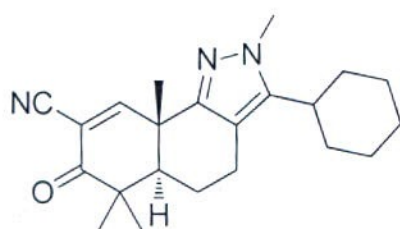
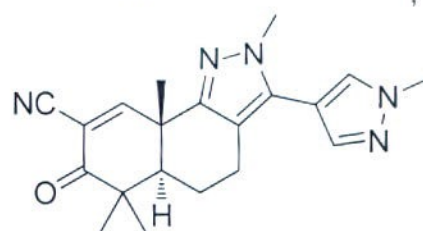
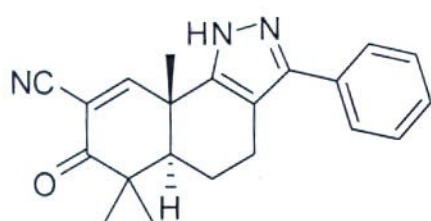
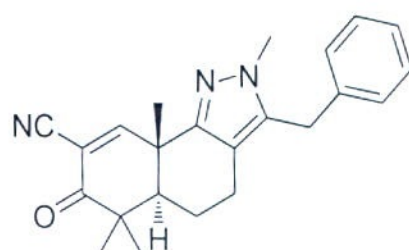
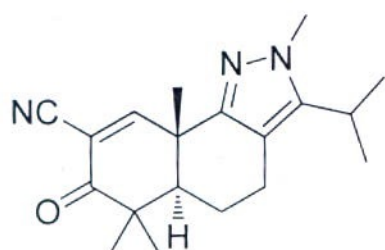
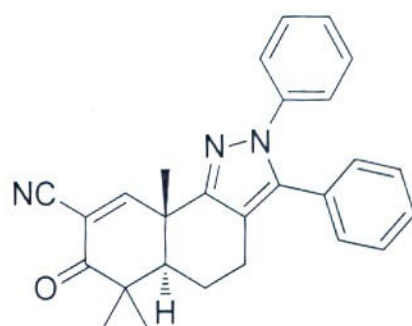
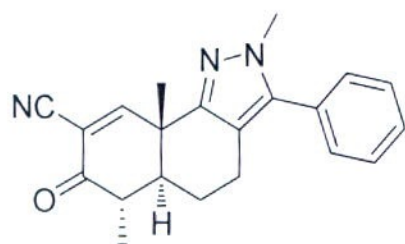
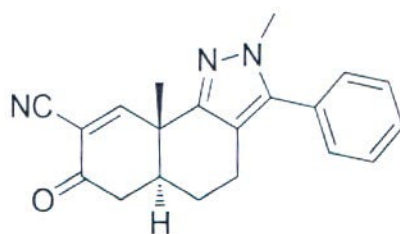
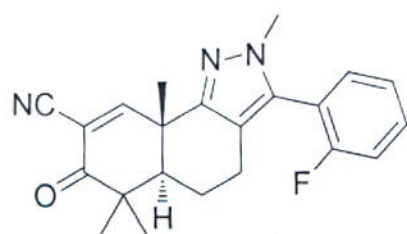
metilo, isopropilo o ciclohexilo.

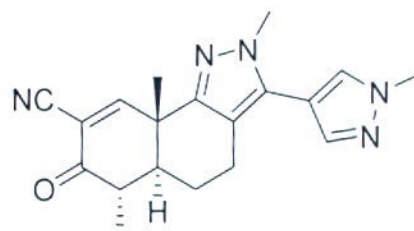
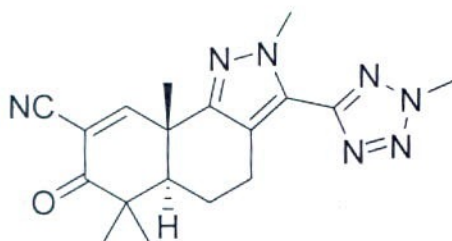
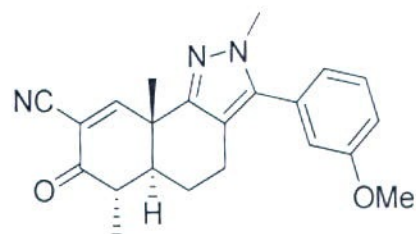
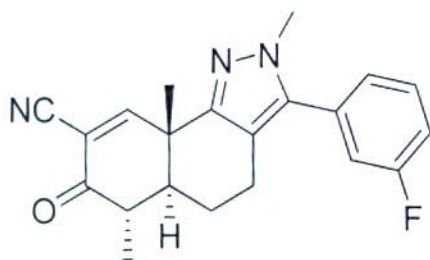
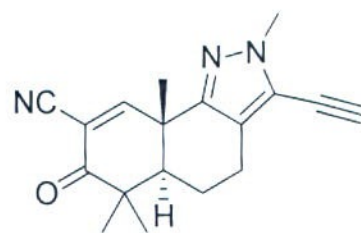
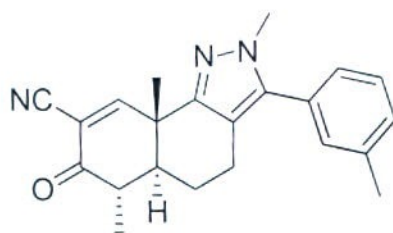
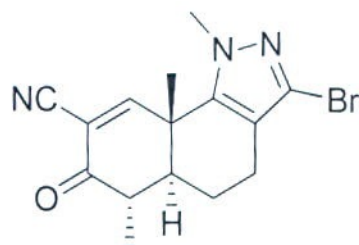
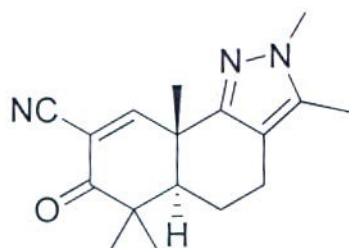
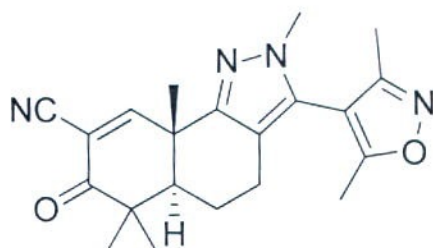
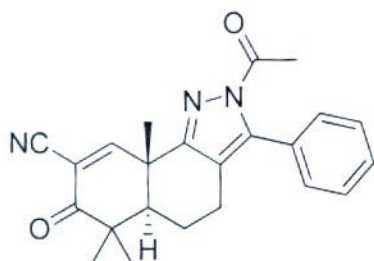
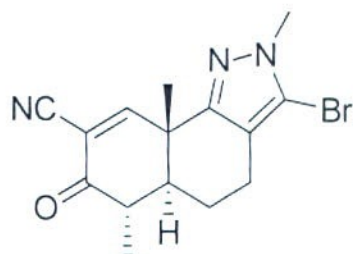
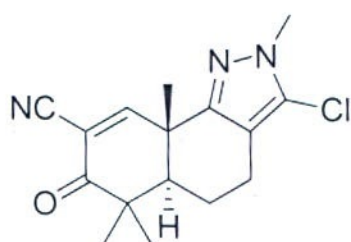
36. El compuesto de acuerdo con una de las reivindicaciones 1-30, CARACTERIZADO PORQUE R_6 es arilo_(C≤8), heteroarilo_(C≤8) o una versión sustituida de cualquiera de estos grupos.
37. El compuesto de una de cualquiera de las reivindicaciones 1-30, CARACTERIZADO PORQUE R_6 es arilo_(C≤10).
38. El compuesto de la reivindicación 37, CARACTERIZADO PORQUE R_6 es fenilo, metilfenilo o naftilo.
39. El compuesto de la reivindicación 36, CARACTERIZADO PORQUE R_6 es arilo_(C≤8) sustituido.
40. El compuesto de la reivindicación 39, CARACTERIZADO PORQUE R_6 es clorofenilo, fluorofenilo o metoxifenilo.
41. El compuesto de la reivindicación 36, CARACTERIZADO PORQUE R_6 es heteroarilo_(C≤8).
42. El compuesto de la reivindicación 41, CARACTERIZADO PORQUE R_6 es piridinilo, pirimidinilo, metilpirazolilo, dimetilisoxazolilo, metiltriazolilo, o metiltetrazolilo.
43. El compuesto de acuerdo con una de las reivindicaciones 1-30, CARACTERIZADO PORQUE R_6 es aralquilo_(C≤12) o aralquilo_(C≤12) sustituido.
44. El compuesto de la reivindicación 43, CARACTERIZADO PORQUE R_6 es bencilo.

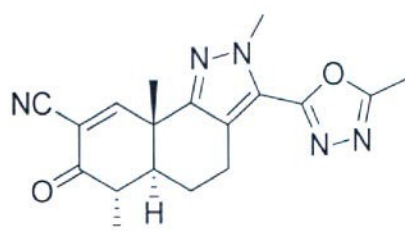
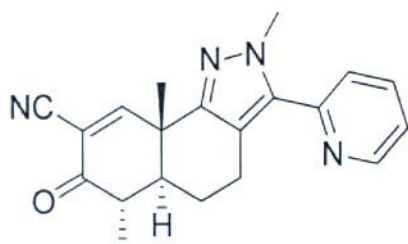
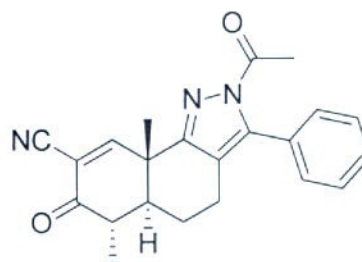
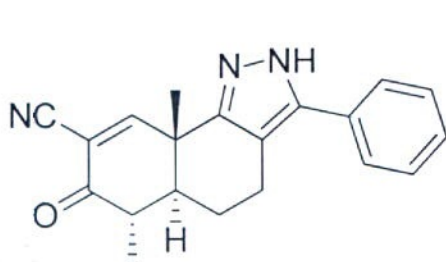
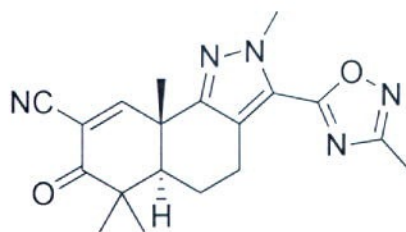
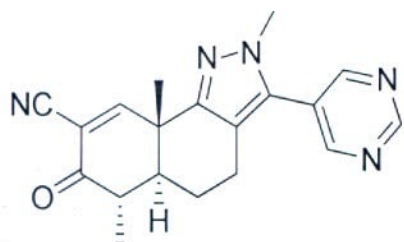
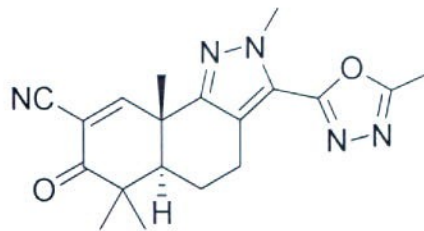
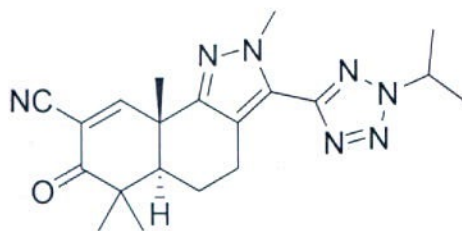
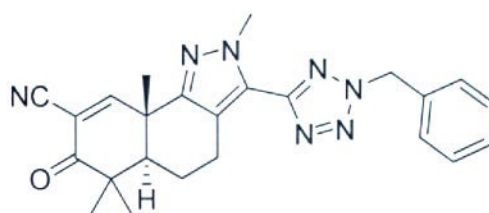
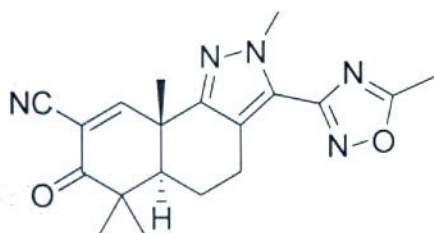
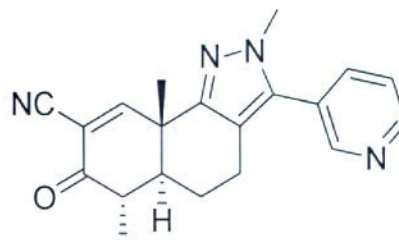
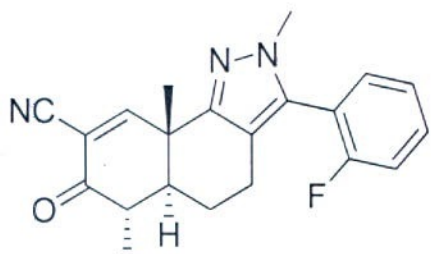
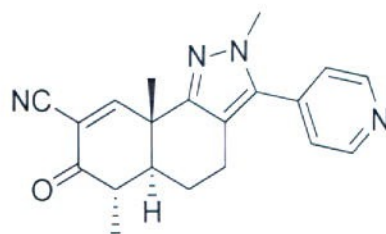
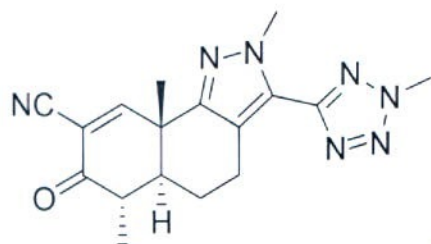
45. El compuesto de acuerdo con una de las reivindicaciones 1-30, CARACTERIZADO PORQUE R_6 es alquínilo_(C_≤8).
46. El compuesto de la reivindicación 45, CARACTERIZADO PORQUE R_6 es etinilo.
47. Los compuestos de acuerdo con cualquiera de las reivindicaciones 1-46, CARACTERIZADO PORQUE el átomo de carbono 10 tiene configuración R.
48. Los compuestos de acuerdo con cualquiera de las reivindicaciones 1-46, CARACTERIZADO PORQUE el átomo de carbono 10 tiene configuración S.
49. Los compuestos de acuerdo con cualquiera de las reivindicaciones 1-48, CARACTERIZADO PORQUE el átomo de carbono 5 tiene configuración R.
50. Los compuestos de acuerdo con cualquiera de las reivindicaciones 1-48, CARACTERIZADO PORQUE el átomo de carbono 5 tiene configuración S.
51. El compuesto de la reivindicación 1, CARACTERIZADO PORQUE además se define de la siguiente manera:

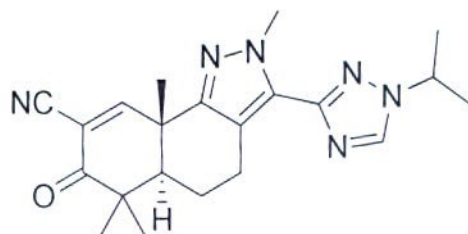
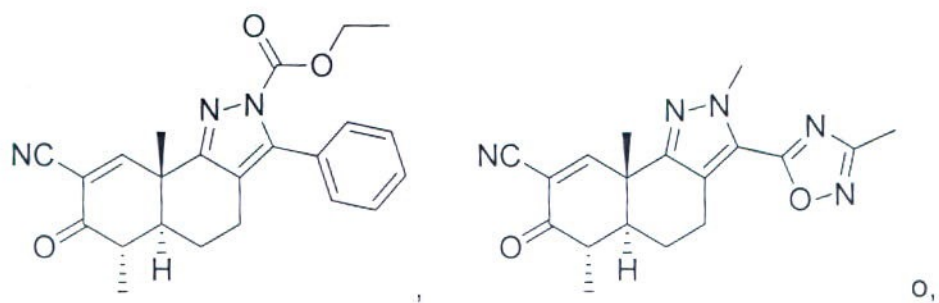




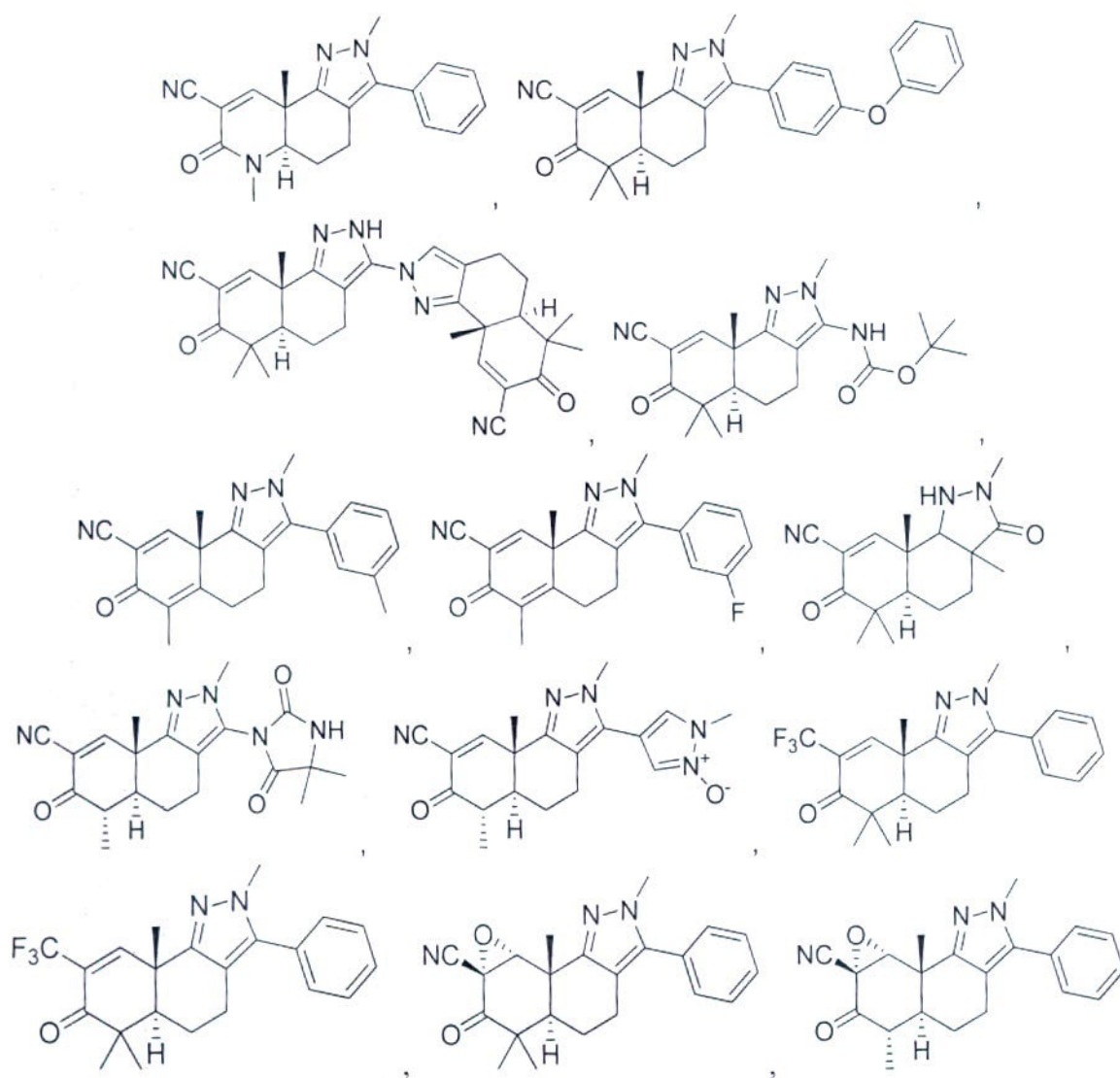


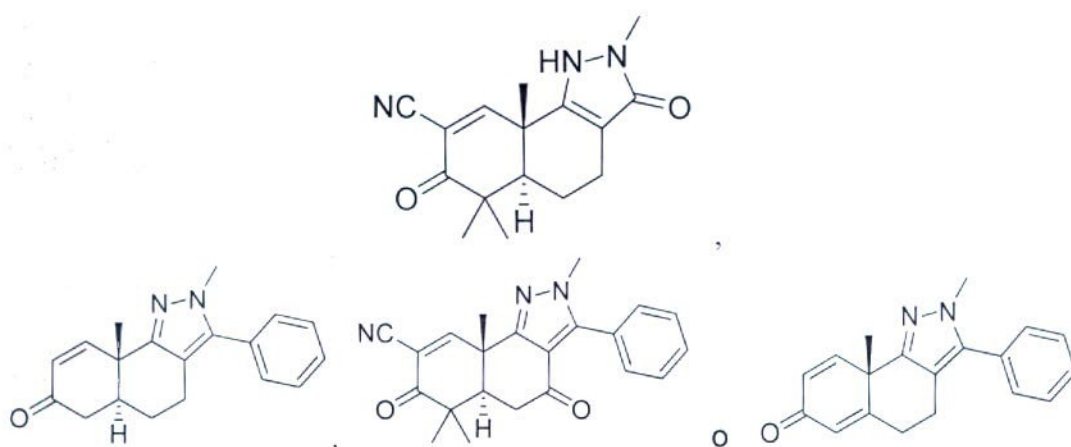






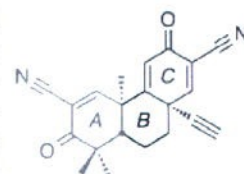
52. Un compuesto CARACTERIZADO PORQUE tiene la fórmula:





**Tricyclic Compounds Containing Nonenolizable Cyano Enones.
A Novel Class of Highly Potent Anti-Inflammatory and
Cytoprotective Agents**⁷⁹Tadashi Honda,^{*,†} Hidenori Yoshizawa,[†] Chitra Sundararajan,[†] Emilie David,[†] Marc J. Lajoie,[†]
Frank G. Favaloro, Jr.,[†] Tomasz Janosik,[†] Xiaobo Su,[†] Yukiko Honda,[†] Bill D. Roebuck,[†] and
Gordon W. Gribble[†][†]Department of Chemistry, Dartmouth College, Hanover, New Hampshire 03755, United States[‡]Department of Pharmacology and Toxicology, Dartmouth Medical School, Hanover, New Hampshire 03755, United States**S** Supporting Information

ABSTRACT: Forty-four novel tricycles containing nonenolizable cyano enones (TCEs) were designed and synthesized on the basis of a semisynthetic pentacyclic triterpenoid, bardoxolone methyl, which is currently being developed in phase II clinical trials for the treatment of severe chronic kidney disease in diabetic patients. Most of the TCEs having two different kinds of nonenolizable cyano enones in rings A and C are highly potent suppressors of induction of inducible nitric oxide synthase stimulated with interferon- γ and are highly potent inducers of the cytoprotective enzymes heme oxygenase-1 and NAD(P)H:quinone oxidoreductase-1. Among these compounds, ($\pm$)-(4bS,8aR,10aS)-10a-ethynyl-4b,8,8-trimethyl-3,7-dioxo-3,4b,7,8,8a,9,10,10a-octahydrophenanthrene-2,6-dicarbonitrile (($\pm$)-31) is the most potent in these bioassays in our pool of drug candidates including semisynthetic triterpenoids and synthetic tricycles. These facts strongly suggest that an essential factor for potency is not a triterpenoid skeleton but the cyano enone functionality. Notably, TCE 31 reduces hepatic tumorigenesis induced with aflatoxin in rats. Further preclinical studies and detailed mechanism studies on 31 are in progress.

**1. INTRODUCTION**

The concept that inflammation and carcinogenesis are related phenomena has been the subject of many studies that have attempted to link these two processes in a mechanistic fashion.^{1,2} The enzyme that mediates the constitutive synthesis of nitric oxide (NO) from arginine has relatively little significance for either inflammation or carcinogenesis. In contrast, inducible nitric oxide synthase (iNOS) has critical roles in the response of tissues to injury or infectious agents and is an essential component of the inflammatory response, the ultimate repair of injury, and carcinogenesis.^{3–5} Although the physiological activity of iNOS may provide a definite benefit to the organism, aberrant or excessive expression of iNOS has been implicated in the pathogenesis of many disease processes, particularly in Alzheimer's disease, Parkinson's disease, multiple sclerosis, rheumatoid arthritis, inflammatory bowel disease, chronic kidney disease, and carcinogenesis.^{6–10} Therefore, we adopted inhibition of NO production in primary mouse macrophages¹¹ and/or RAW 264.7 cells stimulated with interferon- γ (IFN- γ) (the iNOS assay) as initial screening systems for the development and evaluation of our potential anti-inflammatory and cytoprotective agents that we have designed and synthesized.

Heme oxygenase is the rate-limiting phase 2 enzyme in the catabolism of heme, producing biliverdin, iron, and carbon monoxide. Three heme oxygenase isoforms have been described:^{12–14} HO-1, HO-2, and HO-3 [the last two recently described as pseudogenes¹⁵ are constitutively expressed]. HO-1 is induced by a variety of stimuli, including growth factors, cytokines, NO, and

oxidants such as heme, hydrogen peroxide, oxidized lipids, and heavy metals.¹⁶ HO-1 and its breakdown products possess potent anti-inflammatory and cytoprotective properties.^{17–19} Currently, there is major interest in stimulating HO-1 as a protective enzyme in many chronic disease conditions in which inflammation and oxidative stress play an important role. Thus, we have evaluated our compounds, whose potency in the iNOS assay is sufficient, for induction of HO-1 in RAW cells (the HO-1 assay).

NAD(P)H:quinone oxidoreductase (NQO1) is also a representative phase 2 enzyme. It is a widely distributed FAD-dependent flavoprotein that catalyzes the obligatory two-electron reduction of a broad range of substrates, including quinones, quinoneimines, and nitro compounds by using either NADPH or NADH as the hydride donor.²⁰ In addition and independent of its catalytic mechanism, NQO1 also has a "gatekeeping" role in regulating the proteasomal degradation of specific proteins, and this function appears to be important in the stabilization of p53, a broadly functioning tumor suppressor gene. NQO1 is transcriptionally induced in response to various agents, including xenobiotics, oxidants, antioxidants, as well as ultraviolet and ionizing radiation.^{21–24} NQO1, therefore, is important as a cytoprotective enzyme. We have evaluated the potency of our compounds for induction of NQO1 in Hepa1c1c7 murine hepatoma cells (the NQO1 assay).

Over the past decade, we have been engaged in the improvement of the anti-inflammatory and cytoprotective activity of

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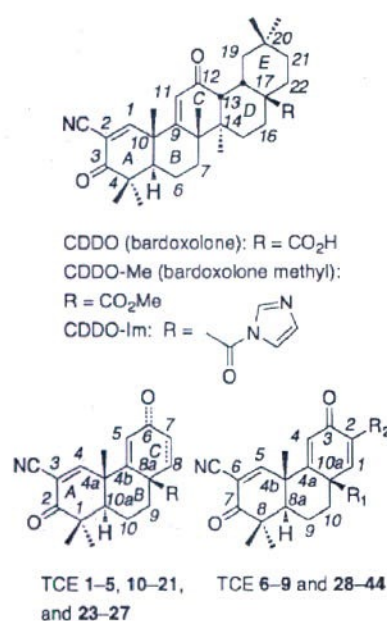
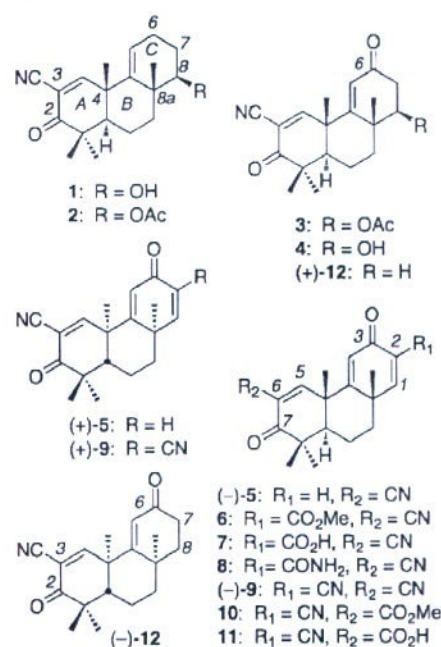


Figure 1. Structures of CDDO, CDDO-Me, and CDDO-Im and general structures of TCEs.

oleanolic acid, a ubiquitous naturally occurring triterpenoid. This led to the discovery of CDDO (Figure 1).^{25–27} CDDO and its related compounds are multifunctional agents. CDDO shows high inhibitory activity in the iNOS assay^{25,26} and induces HO-1 in vitro²⁸ and in vivo and NQO1 in vitro.²⁹ CDDO induces differentiation of human myeloid leukemia cells and mouse 3T3-L1 fibroblasts and enhances nerve-growth-factor-induced neuronal differentiation of rat PC12 cells,^{27,30} inhibits the proliferation of human myeloid leukemia and carcinoma cell lines,²⁷ blocks de novo synthesis of iNOS and inducible cyclooxygenase (COX-2) in mouse macrophages, microglia, and fibroblasts,²⁷ and induces apoptosis of human myeloid leukemia,^{31–33} osteosarcoma,³⁴ lung cancer,³⁵ and CLL cells.³⁶ The acylimidazole of CDDO (CDDO-Im, Figure 1) inhibits aflatoxin-induced tumorigenesis in rats.³⁷ The methyl ester of CDDO (CDDO-Me, bardoxolone methyl, Figure 1) prevents lung cancer induced by vinyl carbamate in A/J mice.³⁸ Presently, bardoxolone methyl is being developed in late phase II clinical trials for the treatment of severe chronic kidney disease in type 2 diabetes mellitus patients. It significantly increases the estimated glomerular filtration rate in more than 90% of diabetic patients.³⁹

We have chemically demonstrated that the nonenolizable cyano enone in ring A of CDDO gives reversible Michael adducts with the SH group of DTT by UV and NMR studies.⁴⁰ We speculate that this characteristic reactivity may imply a molecular mechanism of action. Indeed, our mechanism studies suggest that CDDO and its related compounds regulate proteins affecting inflammation, oxidative stress, differentiation, apoptosis, and proliferation, including Keap1,²⁹ IKK β , and JAK1, to name a few, by reversible Michael addition between their nonenolizable cyano enone functionality in ring A and the SH groups of cysteine moieties on these proteins. Recently, Cys179 in the kinase domain on IKK β was identified as a target of CDDO-Me and CDDO-Im.^{41,42} By binding to this site on IKK β , CDDO-Me inactivates the kinase and ultimately results in blocking of the binding of NF- κ B to DNA and thus inhibits transcriptional activation of NF- κ B-dependent target genes. It has also been

Table 1. Inhibitory Activity of New TCEs 1–12 on NO Production Induced by IFN- γ in Primary Mouse Macrophages^a



compd	IC ₅₀ (nM)	compd	IC ₅₀ (nM)
(±)-1	310	(±)-9	2.1
(±)-2	480	(-)-9	14
(±)-3	53	(+)-9	1.3
(±)-4	75	(±)-10	>60
(±)-5	61	(±)-11	>600
(-)-5	64	(±)-12	19
(+)-5	58	(-)-12	26
(±)-6	91	(+)-12	19
(±)-7	1600	CDDO	0.5
(±)-8	61	CDDO-Me	0.2
		hydrocortisone	10

^a IC₅₀ values of TCEs, CDDO, CDDO-Me, and hydrocortisone were determined in the range 0.1 pM to 10 μ M (10-fold dilutions). Values are an average of two separate experiments. None of the compounds were toxic to primary mouse macrophages at 10 μ M. The experimental protocol is in the Supporting Information. These data have been published and presented in refs 79a–79d.

reported that CDDO-Me inhibits the JAK1 $\rightarrow$ STAT3 pathway by directly binding to JAK1 at Cys1077 and STAT3 at Cys259.⁴³ Small molecule inhibitors of the STAT3 pathway are known to be effective as anticancer agents in vitro and in animal models.

During the development of CDDO, we found the very important structure–activity relationships (SARs) that the nonenolizable cyano enone in ring A and the enone in ring C are essential for the extremely high potency of CDDO [see Figure S1 in the Supporting Information].²⁶ Therefore, we reasoned that the entire oleanane skeleton might not be necessary for potency. Consequently, we have designed tricycles containing nonenolizable cyano enones (TCEs, i.e., compounds 1–44, Figure 1). A literature survey revealed that these compounds were previously unknown.

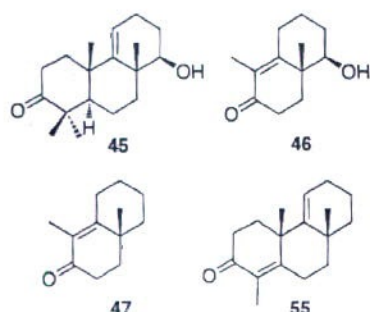


Figure 2. Structures of 45–47 and 55.

Our rationale for pursuing the synthesis of these compounds is as follows. Since they could be synthesized from commercially available small molecules, these compounds with various functionalities at different positions would be easily obtained. Such diversity-oriented synthesis could lead to new potential anti-inflammatory and cytoprotective agents that have high oral potency and high water solubility for ease of administration and formulation, as well as high biological selectivity for avoiding possible side effects. Accordingly, we have synthesized various new TCEs and evaluated them in the iNOS, HO-1, and NQO1 assays. As a result, we have found that TCE 31 (code number in house: TBE-31, (±)-(4bS,8aR,10aS)-10a-ethynyl-4b,8,8-trimethyl-3,7-dioxo-3,4b,7,8,8a,9,10,10a-octahydrophenanthrene-2,6-dicarbonitrile) is the most potent in our collection of our semisynthetic triterpenoids and synthetic tricycles. We herein describe the full account of our synthetic work with these tricycles and their interesting biological results.

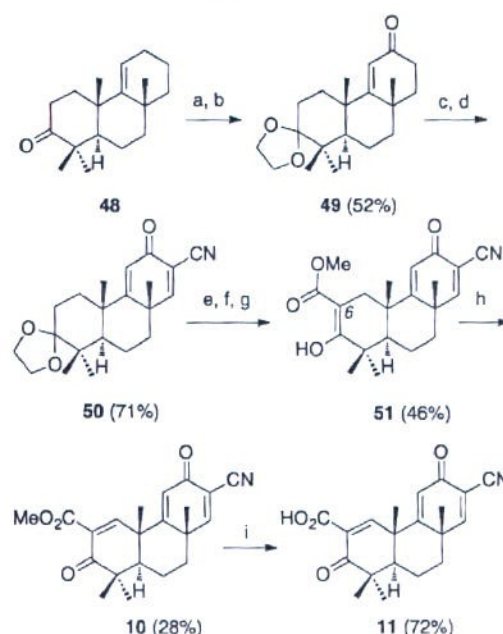
2. CHEMISTRY

2.1. Initial Set of Tricycles. Our initial targets 1–5 (structures, see Table 1) were synthesized in racemic form from known compound 45⁴⁴ (structure, see Figure 2) by synthetic sequence that has been published (syntheses, see Scheme S1 in the Supporting Information).^{79a}

Because 5 in racemic form shows good potency in the iNOS assay among the initial set of the tricycles (Table 1), we have synthesized both (–)-5, with the same configuration as CDDO, and its antipode (+)-5 from the known bicyclic enones (–)-46 and (+)-46⁴⁵ (structure of (–)-46, see Figure 2), respectively, according to the improved synthetic route that is specifically directed toward (–)-5 and (+)-5 (syntheses, see Scheme S2 in the Supporting Information).^{79c}

2.2. Insertion of Electron-Withdrawing Groups at C2 Position of Tricycles. Since 5 shows good potency in the iNOS assay, this compound was thought to be a good scaffold from which to discover new, more potent tricycles. Thus, we targeted 6–9 (structures, see Table 1), analogues of 5 with electron-withdrawing groups at the C2 position, to discern the influence of substituents at the C2 position on biological activity, because we previously found that substitution at the α position of an α,β -unsaturated ketone strongly affects the potency of semisynthetic triterpenoids.⁴⁶

Racemic 6–9 were synthesized from 45 according to the synthetic route that has been published (syntheses, see Scheme S3 in the Supporting Information).^{79a} Of these TCEs, 9 showed high potency (IC_{50} = 1 nM level) and it is approaching the potency of CDDO in the iNOS assay (see Table 1).

Scheme 1. Synthesis of TCEs 10 and 11^a

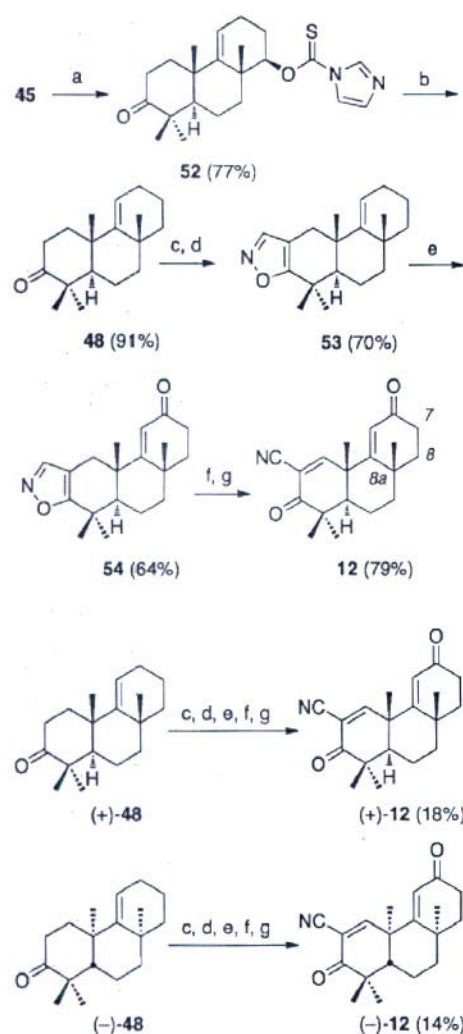
^a Reagents and yields: (a) ethylene glycol, PPTS, PhH, 94%; (b) CrO_3 , *t*-BuOOH, CH_2Cl_2 , 55%; (c) *p*-TsCN, LDA, THF; (d) DDQ, PhH, 71% from 49; (e) aqueous HCl, MeOH, 65%; (f) MMC, DMF, 100%; (g) CH_2N_2 , Et_2O , 71%; (h) PhSeCl, pyridine, CH_2Cl_2 , 30% H_2O_2 , 28%; (i) aqueous KOH, MeOH, 72%.

We efficiently synthesized (–)-9, with the same configuration as the naturally occurring oleanolic acid, and its antipode (+)-9 in seven steps from known bicyclic enones (–)-47 and (+)-47,⁴⁷ respectively (structure of (–)-47, see Figure 2; syntheses, see Scheme S4 in the Supporting Information).^{79c}

2.3. Synthesis of TCEs 10 and 11. We designed 10 and 11 in racemic form as analogues of 9 because 11 and its salts would be soluble in water. TCEs 10 and 11 were synthesized from 48^{79a,c} by the sequence shown in Scheme 1 (also see Scheme S5 in the Supporting Information). Tricyclic 49 was prepared by ketalization of 48, followed by a chromium-mediated allylic oxidation⁴⁸ with CrO_3 and *tert*-butyl hydroperoxide (*t*-BuOOH) in CH_2Cl_2 (52% yield). Cyanation of the enolate of 49, generated using LDA in THF, with *p*-toluenesulfonyl cyanide (*p*-TsCN),⁴⁹ followed by 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) oxidation, gave 50 in 71% yield. Methyl ester 51 was obtained by removal of the ketal of 50, followed by carboxylation at the C6 position with Stiles' reagent⁵⁰ and subsequent methylation with diazomethane (46% yield). TCE 10 was prepared in 28% yield from 51 by the addition of PhSeCl in the presence of pyridine and subsequent oxidation/elimination with H_2O_2 .⁵¹ Cleavage of the methyl ester under basic conditions gave 11 in 72% yield.

2.4. Synthesis of TCE 12. We have designed and synthesized 12, which has the same A, B, and C ring system as that of CDDO (Schemes 2 and S6 in the Supporting Information). Imidazole 1-thiocarboxylate 52 was prepared in 77% yield from 45 with 1,1'-thiocarbonyldiimidazole in THF. Reduction of 52 with tri(*n*-butyl)tin hydride in toluene gave 48 in 91% yield.⁵² This tricyclic 48, which is derived from 45 (whose structure has already been confirmed),⁴⁴ is identical with that obtained by reductive methylation of 55⁵³ (structure, see Figure 2; preparation, see Scheme S4

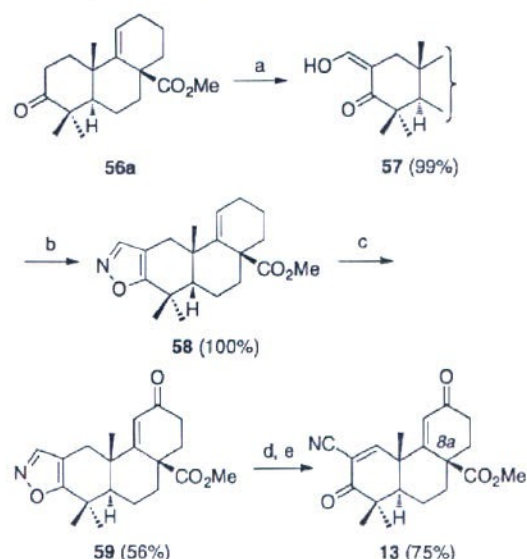
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Scheme 2. Synthesis of Racemic and Optically Active TCE 12^a

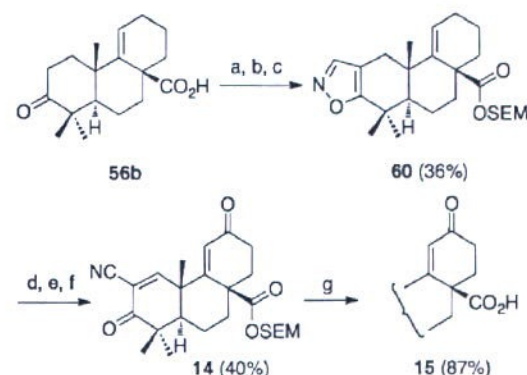
^a Reagents: (a) 1,1'-thiocarbonyldiimidazole, THF; (b) $(n\text{-Bu})_3\text{SnH}$, toluene; (c) HCO_2Et , NaOMe, PhH; (d) $\text{NH}_2\text{OH}\cdot\text{HCl}$, aqueous EtOH; (e) CrO_3 , $t\text{-BuOOH}$, CH_2Cl_2 ; (f) NaOMe, MeOH, Et_2O ; (g) DDQ, PhH.

in the Supporting Information). Consequently, it was proven that the reductive methylation product 48 has the thermodynamically stable *trans*-A/B ring juncture. Isoxazole 53 was obtained in 70% yield by formylation of 48 with ethyl formate in the presence of NaOMe in benzene,⁵⁴ followed by treatment with hydroxylamine hydrochloride in aqueous EtOH.⁵⁵ A chromium-mediated allylic oxidation of 53 with $t\text{-BuOOH}$ produced 54 in 64% yield. The desired TCE 12 was prepared by cleavage of the isoxazole moiety of 54 with NaOMe,⁵⁵ followed by DDQ oxidation (79% yield). We have also synthesized optically active (+)-12 ($[\alpha]_D^{25} +58^\circ$ (c 1.3, CHCl_3), 18% yield in five steps) and (-)-12 ($[\alpha]_D^{25} -66^\circ$ (c 2.0, CHCl_3), 14% yield in five steps), from (+)-48^{79c} and (-)-48,^{79c} respectively, by the same sequence as for ($\pm$)-12.

Since 12 is about 3 times more potent than TCE 5 in the iNOS assay (Table 1), we considered that the 7,8-double bond in ring C is not always necessary for potency. Therefore, we designed and synthesized various C8a functionalized analogues of 12.

Scheme 3. Synthesis of TCE 13^a

^a Reagents: (a) HCO_2Et , NaOMe, PhH; (b) $\text{NH}_2\text{OH}\cdot\text{HCl}$, aqueous EtOH; (c) CrO_3 , $t\text{-BuOOH}$, CH_2Cl_2 ; (d) NaOMe, MeOH, Et_2O ; (e) DDQ, PhH.

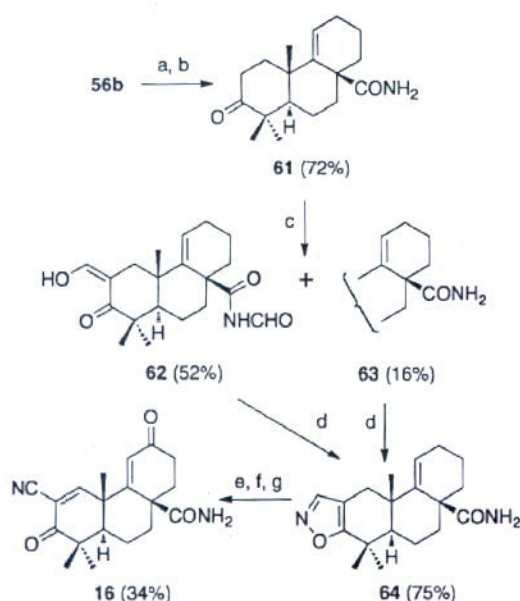
Scheme 4. Synthesis of TCE 15^a

^a Reagents and yields: (a) HCO_2Et , NaOMe, PhH, 45%; (b) $\text{NH}_2\text{OH}\cdot\text{HCl}$, aqueous EtOH, 100%; (c) SEMCl, EDIA, CH_2Cl_2 , 80%; (d) CrO_3 , $t\text{-BuOOH}$, CH_2Cl_2 , 67%; (e) NaOMe, MeOH, Et_2O , 93%; (f) DDQ, PhH, 64%; (g) 48% aqueous $\text{HF}-\text{CH}_3\text{CN}$ (1:9), 87%.

Substitution of functionalities at the C8a position of 12 would be expected to improve the potency and pharmacokinetics because the balance between hydrophilicity and hydrophobicity is shifted.

2.5. Functionality Substitutions at the C8a Position of TCE 12. We synthesized various C8a functionalized TCE analogues using tricycles 56a–c (structures, see Schemes 3, 4, and 6) as starting materials, whose efficient synthesis we have already established for our projected synthesis.⁵⁶ These analogues include typical electron-withdrawing, electron-releasing, hydrophilic, hydrophobic, and bulky groups.

TCE 13 with a methoxycarbonyl group at C8a was synthesized in 42% overall yield via 57, 58, and 59 from 56a by the same sequence as for 12 from 48 (Scheme 3 and Scheme S7 in the Supporting Information). We attempted several methods [KOH , aqueous MeOH (reflux, overnight); LiI , DMF (reflux, 30 min);⁵⁷ KOSiMe_3 , THF (room temp, overnight);⁵⁸ AlBr_3 , Me_2S (room

Scheme 5. Synthesis of TCE 16^a

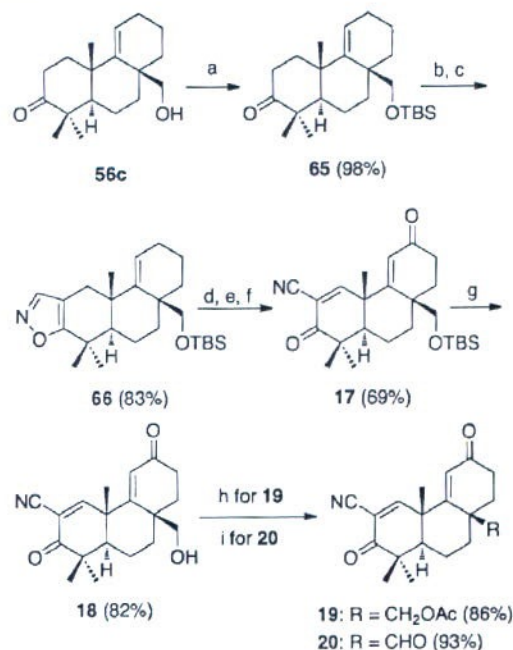
^a Reagents and yields: (a) (COCl)₂, CH₂Cl₂, 90%; (b) NH₃, PhH, 80%; (c) HCO₂Et, NaOMe, PhH; (d) NH₂OH·HCl, aqueous EtOH; (e) CrO₃, *t*-BuOOH, CH₂Cl₂, 50%; (f) NaOMe, MeOH, Et₂O; (g) DDQ, 1,4-dioxane, 68% in two steps (f) and (g).

temp, overnight);⁵⁹ LiS(*n*-Pr), HMPA (room temp, 1 h)⁶⁰] for the cleavage of methyl ester 13 to the corresponding acid 15, but these methods failed to give 15.

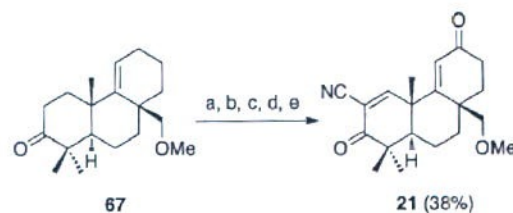
Therefore, we synthesized acid 15 according to the alternative sequence shown in Scheme 4 (Scheme S8 in the Supporting Information). Isoxazole 60 was prepared by formylation of 56b and subsequent isoxazole ring construction, followed by protection of the hydroxyl group with 2-((chloromethoxy)ethyl)-trimethylsilane (SEMCl) in the presence of EDIA in CH₂Cl₂ (36% yield). TCE 14 was obtained in 40% yield from 60 by the same sequence as for 13 from 58. The (trimethylsilyl)-ethoxymethyl (SEM) group was removed from 14 with 48% aqueous HF and CH₃CN (1:9) to give 15 (87% yield). Although we were concerned that 15 might be unstable toward decarboxylation in a medium used for biological testing, in fact, 15 is both soluble and stable in Dulbecco's modified Eagle's medium (10 mM) at room temperature for 4 days at least.

TCE 16 with an aminocarbonyl group was synthesized from 56b (Scheme 5 and Scheme S9 in the Supporting Information). Amide 61 was prepared in 72% yield from 56b by chlorination with oxalyl chloride, followed by the treatment with NH₃ in benzene. Unexpectedly, the formylation of 61 with ethyl formate in the presence of NaOMe gave 62 (52% yield) as a major product and the desired compound 63 (16% yield) as a minor one. However, treatment of 62 with hydroxylamine hydrochloride cleaved the *N*-formyl group to give the desired isoxazole 64 in 75% yield. Under the same conditions, 63 gave 64 in 75% yield. TCE 16 was obtained by allylic oxidation of 64, followed by the cleavage of the isoxazole under basic conditions and subsequent DDQ oxidation in 1,4-dioxane (34% yield). For the DDQ oxidation step, because the precursor of 16 was not soluble in benzene, 1,4-dioxane was used as the solvent.

TCE 18 with a hydroxymethyl group and its derivatives 17 and 19–21 were synthesized according to the sequence shown in

Scheme 6. Synthesis of TCEs 17–20^a

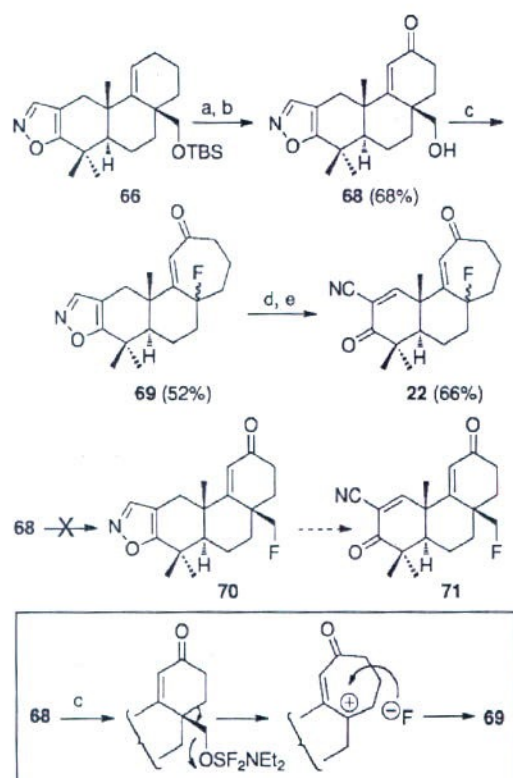
^a Reagents and yields: (a) TBSCl, imidazole, DMF, 98%; (b) HCO₂Et, NaOMe, PhH, 98%; (c) NH₂OH·HCl, NaOAc, aqueous AcOH, 85%; (d) CrO₃, *t*-BuOOH, CH₂Cl₂, 69%; (e) NaOMe, MeOH, Et₂O; (f) DDQ, 1,4-dioxane, 100% in two steps (e) and (f); (g) 48% aqueous HF, CH₃CN, 82%; (h) Ac₂O, pyridine, 86%; (i) CrO₃, pyridine, CH₂Cl₂, 93%.

Scheme 7. Synthesis of TCE 21^a

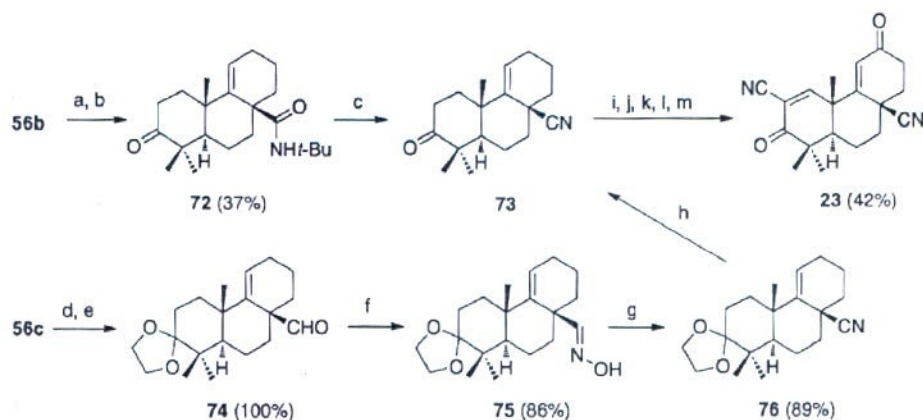
^a Reagents and yields: (a) HCO₂Et, NaOMe, PhH, 100%; (b) NH₂OH·HCl, aqueous EtOH, 100%; (c) CrO₃, *t*-BuOOH, CH₂Cl₂, 68%; (d) NaOMe, MeOH, Et₂O, 97%; (e) DDQ, 1,4-dioxane, 58%.

Schemes 6 and 7 (Schemes S10 and S11 in the Supporting Information). The starting material 56c was protected with *tert*-butylchlorodimethylsilane (TBSCl) to give 65 in 98% yield. Isoxazole 66 was prepared by formylation of 65, followed by treatment with hydroxylamine hydrochloride in the presence of NaOAc in aqueous AcOH (83% yield). At the isoxazole construction step, NaOAc and aqueous AcOH were used instead of aqueous EtOH because the latter conditions gave 66 in low yield. We considered that 1 equiv of HCl, which is produced from the latter conditions, would disrupt this conversion. TCE 17 was obtained in 69% yield from 66 by the same sequence as for 16 from 64. The TBS group of 17 was removed by 48% aqueous HF and CH₃CN (1:9) to give 18 in 82% yield. Acetylation of 18 with acetic anhydride in pyridine afforded 19 in 86% yield. Aldehyde 20 was obtained in 93% yield by Ratcliffe oxidation⁶¹ of 18 with CrO₃ and pyridine in CH₂Cl₂. Methyl ether 21 was synthesized in five steps from a known compound 67⁶² by the same sequence as for 16 from 61 (38% yield, Scheme 7).

We tried to synthesize fluoro derivative **71** from **66** (Scheme 8 and Scheme S12 in the Supporting Information). Tricycle **68** was obtained in 68% yield by allylic oxidation of **66**, followed by removal of the TBS group with 48% aqueous HF and CH₃CN (1:9). However, fluorination of **68** with DAST did not give **70** but a ring expansion product **69** in 52% yield. TCE **22** was prepared in 66% yield by the cleavage of the isoxazole of **69**,

Scheme 8. Synthesis of TCE **22**^a

^a Reagents: (a) CrO₃, *t*-BuOOH, CH₂Cl₂; (b) HF, CH₃CN; (c) DAST, CH₂Cl₂; (d) NaOMe, MeOH, Et₂O; (e) DDQ, 1,4-dioxane.

Scheme 9. Synthesis of TCE **23**^a

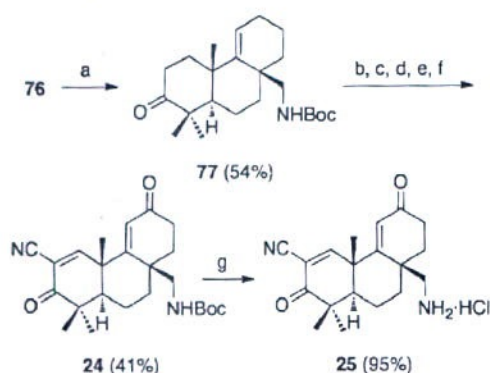
^a Reagents and yields: (a) (COCl)₂, CH₂Cl₂; (b) *t*-BuNH₂, Et₃N, CH₂Cl₂, 37% from **56b**; (c) POCl₃, 100%; (d) ethylene glycol, PPTS, PhH, 100%; (e) Swern oxidation, 100%; (f) NH₂OH·HCl, NaOAc, MeOH, CH₂Cl₂, 86%; (g) 1,1-carbonyldiimidazole, CH₂Cl₂, 89%; (h) aqueous HCl, MeOH, 100%; (i) HCO₂Et, NaOMe, PhH, 72%; (j) NH₂OH·HCl, aqueous EtOH, 100%; (k) CrO₃, *t*-BuOOH, CH₂Cl₂, 83%; (l) NaOMe, MeOH, Et₂O, 88%; (m) DDQ, 1,4-dioxane, 79%.

followed by DDQ oxidation. The structures of **69** and **22** were fully characterized by ¹H and ¹³C NMR, high and low MS, and elemental analysis. We speculate that **69** is produced from **68** according to the mechanisms shown in the box in Scheme 8. The configuration of the fluorine atom has not been determined.

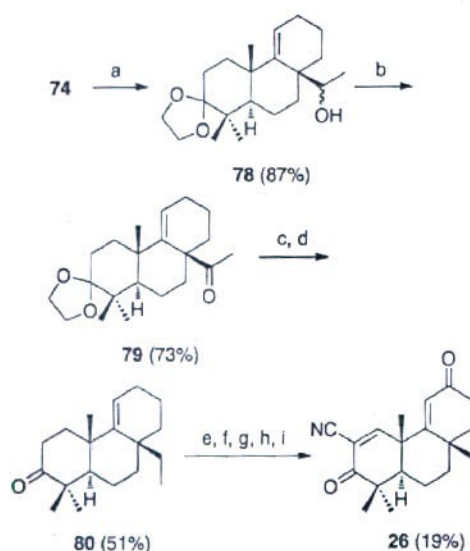
Dicarbonitrile **23** was synthesized from **56b** and **56c** by the different sequence shown in Scheme 9 (Scheme S13 in the Supporting Information). Amide **72** was prepared by chlorination of **56b** with oxalyl chloride, followed by the treatment with *tert*-butylamine (37% yield). Carbonitrile **73** was obtained by the treatment of **72** with POCl₃ (100% yield, 37% overall yield from **56b**).⁶³ However, since this step requires vigorous conditions, the yield varies. Thus, we explored an improved synthesis of **73** from alcohol **56c**. Ketalization of **56c**, followed by Swern oxidation⁶⁴ gave aldehyde **74** (100% yield). Oxime **75** was obtained in 86% yield by the condensation between hydroxylamine and **74** in CH₂Cl₂. Dehydration of **75** with 1,1-carbonyldiimidazole in CH₂Cl₂ provided carbonitrile **76** in 89% yield.⁶⁵ The ketal of **76** was removed under acidic conditions to afford **73** in 100% yield (77% overall yield from **56c**). The overall yield of this sequence is about twice that of **73** from **56b**. Moreover, importantly, the yield of this sequence is reproducible. Dicarbonitrile **23** was synthesized in five steps from **73** by the same sequence as for **16** from **61** (42% yield).

Amine hydrochloride **25** was synthesized from **76** by the sequence shown in Scheme 10 (Scheme S14 in the Supporting Information). Reduction of the cyano group of **76** with LiAlH₄ gave only a complex mixture. In contrast, reduction of **76** with a mixture of NaBH₄ and CoCl₂ (5:1),⁶⁶ followed by workup with 10% aqueous HCl solution and subsequent protection of the amino group with Boc₂O gave **77** in 54% yield. TCE **24** was obtained in five steps from **77** by the same sequence as for **16** from **61** (41% yield). Removal of the Boc group of **24** with 4 M HCl in 1,4-dioxane provided **25** in 95% yield.

TCE **26** with an ethyl group was synthesized from **74** (Scheme 11 and Scheme S15 in the Supporting Information). A Grignard reaction of **74** with MeMgBr in THF gave **78** in 87% yield as a mixture of diastereomers. Ratcliffe oxidation of **78** afforded **79** in 73% yield. A forced Wolff–Kishner reduction⁶⁷ of **79** followed by deketalization under acidic conditions provided

Scheme 10. Synthesis of TCEs 24 and 25^a

^a Reagents and yields: (a) NaBH_4 , CoCl_2 , MeOH, 71%; 10% aqueous HCl, Boc_2O , THF, 76%; (b) HCO_2Et , NaOMe, PhH, 100%; (c) $\text{NH}_2\text{OH}\cdot\text{HCl}$, aqueous EtOH, 76%; (d) CrO_3 , *t*-BuOOH, CH_2Cl_2 , 62%; (e) NaOMe, MeOH, Et_2O , 100%; (f) DDQ, 1,4-dioxane, 86%; (g) 4 M HCl in 1,4-dioxane, 95%.

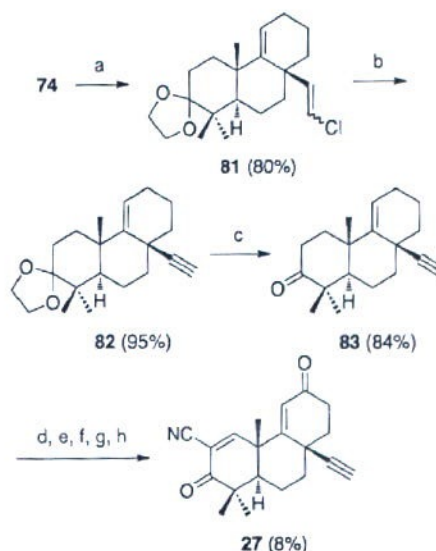
Scheme 11. Synthesis of TCE 26^a

^a Reagents and yields: (a) MeMgBr , THF, 87%; (b) CrO_3 , pyridine, CH_2Cl_2 , 73%; (c) NH_2NH_2 , KOH, diethylene glycol, 51%; (d) aqueous HCl, MeOH, 100%; (e) HCO_2Et , NaOMe, PhH, 75%; (f) $\text{NH}_2\text{OH}\cdot\text{HCl}$, aqueous EtOH, 95%; (g) CrO_3 , *t*-BuOOH, CH_2Cl_2 , 69%; (h) NaOMe, MeOH, Et_2O , 100%; (i) DDQ, 1,4-dioxane, 39%.

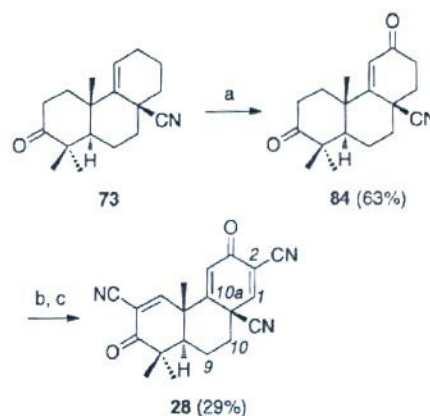
80 in 51% yield. TCE 26 was obtained in five steps from 80 by the same sequence as for 16 from 61 (19% yield).

TCE 27 with an ethynyl group was synthesized from 74 (Scheme 12 and Scheme S16 in the Supporting Information). A Wittig reaction on 74 with (chloromethyl)triphenylphosphonium chloride⁶⁸ gave 81 as a mixture of *E/Z* chlorovinyl isomers (*E/Z* = 4:1) in 80% yield. Dehydrochlorination of 81 with MeLi followed by quenching of the acetylide with aqueous NH_4Cl solution provided 82 in 95% yield.⁶⁸ The ketal of 82 was removed under acidic conditions to afford 83 in 84% yield. TCE 27 was obtained in 8% yield from 83 by the same sequence as for 16 from 61.

2.6. Functionality Substitutions at the C10a position of TCE 9. TCE 28 with a cyano group at C10a, which is a C10a

Scheme 12. Synthesis of TCE 27^a

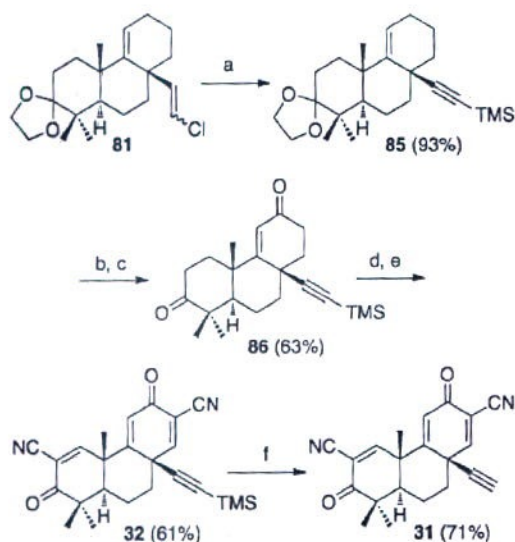
^a Reagents and yields: (a) $\text{Ph}_3\text{PCH}_2\text{Cl}_2$, *n*-BuLi, THF, HMPA, 80%; (b) MeLi, THF; aq NH_4Cl , 95%; (c) 10% aqueous HCl, MeOH, 84%; (d) HCO_2Et , NaOMe, PhH, 76%; (e) $\text{NH}_2\text{OH}\cdot\text{HCl}$, aqueous EtOH, 70%; (f) CrO_3 , *t*-BuOOH, CH_2Cl_2 , 46%; (g) NaOMe, MeOH, Et_2O , 93%; (h) DDQ, 1,4-dioxane, 37%.

Scheme 13. Synthesis of TCE 28^a

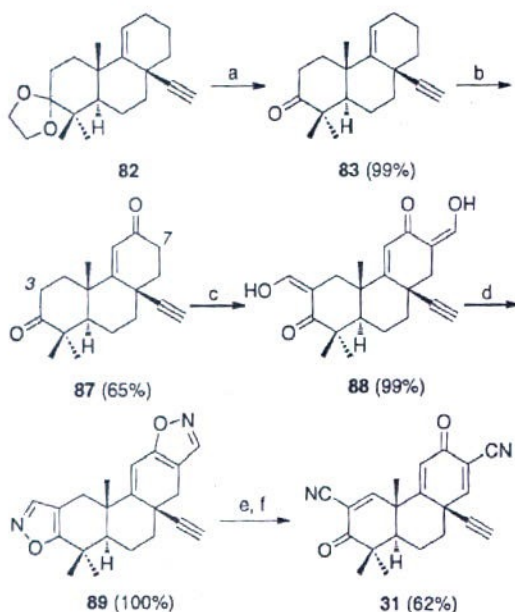
^a Reagents: (a) CrO_3 , *t*-BuOOH, CH_2Cl_2 ; (b) *p*-TsCN, LDA, THF; (c) DDQ, 1,4-dioxane.

functionalized derivative of TCE 9, was synthesized by the sequence shown in Scheme 13 (Scheme S17 in the Supporting Information). A chromium-mediated allylic oxidation of 73 with CrO_3 and *t*-BuOOH in CH_2Cl_2 gave 84 in 63% yield. TCE 28 was obtained by double cyanation of 84 with LDA and *p*-TsCN, followed by DDQ oxidation in 1,4-dioxane (29% yield). TCE 29 with an ethyl group (structure, see Table 3) was synthesized in three steps from 80 by the same sequence as for 28 from 73 (preparation, see Scheme S18 in the Supporting Information).^{79e} TCE 30 with a vinyl group (structure, see Table 3) was obtained in five steps from 74 (preparation, see Scheme S19 in the Supporting Information).^{79e}

TCE 31 with an ethynyl group was synthesized in six steps from 81 (Scheme 14 and Scheme S20 in the Supporting Information). Dehydrochlorination of 81 with MeLi, followed

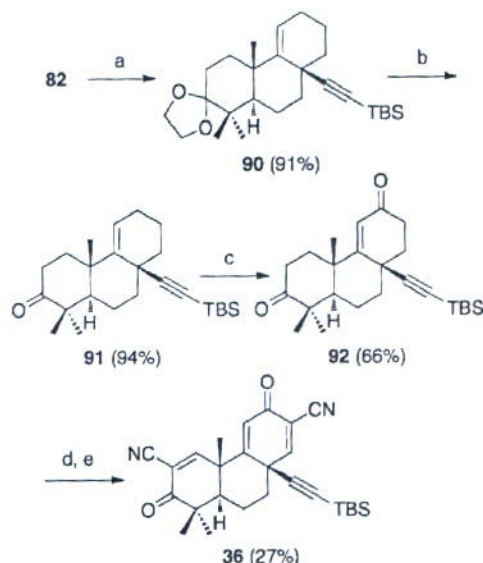
Scheme 14. Synthesis of TCEs 31 and 32^a

^a Reagents: (a) MeLi, THF; TMSCl; (b) aqueous HCl, MeOH; (c) CrO₃, *t*-BuOOH, CH₂Cl₂; (d) *p*-TsCN, LDA, THF; (e) DDQ, PhH; (f) TBAF, THF.

Scheme 15. Improved synthesis of TCE 31^a

^a Reagents: (a) 10% aqueous HCl, MeOH; (b) CrO₃, *t*-BuOOH, CH₂Cl₂; (c) HCO₂Et, NaOMe, PhH; (d) NH₂OH·HCl, aqueous EtOH; (e) NaOMe, MeOH, Et₂O; (f) PhSeCl, pyridine, CH₂Cl₂, 30% H₂O₂, CH₂Cl₂.

by trapping of the acetylide with chlorotrimethylsilane (TMSCl) gave 85 in 93% yield.⁶⁹ Deketalization of 85 under acidic conditions followed by a chromium-mediated allylic oxidation provided 86 in 63% yield. TCE 32 was obtained by double cyanation of 86 with LDA and *p*-TsCN, followed by DDQ oxidation in benzene (61% yield). The trimethylsilyl (TMS) group was removed by tetra(*n*-butyl)ammonium fluoride (TBAF)⁷⁰ to afford 31 in 71% yield (nine steps from 56c, 21% overall yield).

Scheme 16. Synthesis of TCE 36^a

^a Reagents: (a) MeLi, THF, TBSCl; (b) aqueous HCl, MeOH; (c) CrO₃, *t*-BuOOH, CH₂Cl₂; (d) *p*-TsCN, LDA, THF; (e) DDQ, PhH.

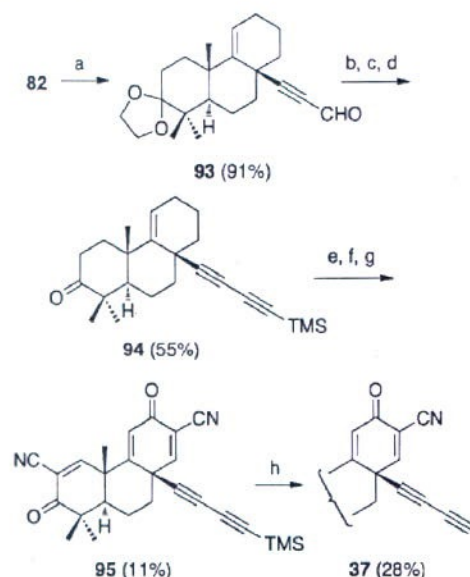
Optically active (–) and (+)-31 and 32 (structures, see Table 3) were synthesized by the sequence that has been published (syntheses, see Scheme S21 in the Supporting Information).^{79e}

2.7. Improved Synthesis of TCE 31. Currently, since TCE 31 is the most potent compound in our pool of semisynthetic triterpenoids and synthetic tricycles that we have evaluated in our bioassays (see Biological Results and Discussion), it is essential for further evaluation to prepare at least 1 g of 31 in a single batch. Although the sequence shown in Scheme 14 is acceptable for a small-scale synthesis (10–200 mg), it is not adequate for a medium-scale synthesis (500 mg to 2 g). Particularly, the double cyanation step using *p*-TsCN is not feasible because *p*-TsCN is a very expensive reagent and the yield of this reaction drastically decreases on a large scale. Thus, we have developed an improved synthesis of 31 (Scheme 15 and Scheme S22 in the Supporting Information) by adopting Johnson's isoxazole method⁵⁵ for the double cyanation, which we used for the synthesis of TCE 12 analogues. With this sequence, 31 is consistently obtained in 30% yield in 10 steps from 56c.

Removal of the ketal of 82 under acidic conditions gave 83 in 99% yield. Enone 87 was prepared in 65% yield from 83 by allylic oxidation. Formyl groups were successfully inserted into the C3 and C7 positions of 87 simultaneously to afford 88 (99% yield) using twice the amount of ethyl formate and NaOMe (11 equiv each) than was used for monoformylation (5.5 equiv each). Treatment of 88 with hydroxylamine hydrochloride provided diisoxazole 89 in quantitative yield. Each isoxazole ring of 89 was converted to a cyano group under basic conditions, which gave 31 upon the addition of PhSeCl and subsequent oxidation/elimination with H₂O₂ (62% yield). This oxidation method was better than the DDQ method for this oxidation with an unprotected acetylene group.

2.8. Synthesis of TCE 31 Analogues. The high potency of 31 and 32 in the iNOS assay (see Table 3) encouraged us to explore the modifications of the ethyne group of TCE 31. TCEs 33–35 (structures, see Table 3) were synthesized in five steps from 82 (syntheses, see Schemes S23–S25 in the Supporting Information).^{79e}

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Scheme 17. Synthesis of TCE 37^a

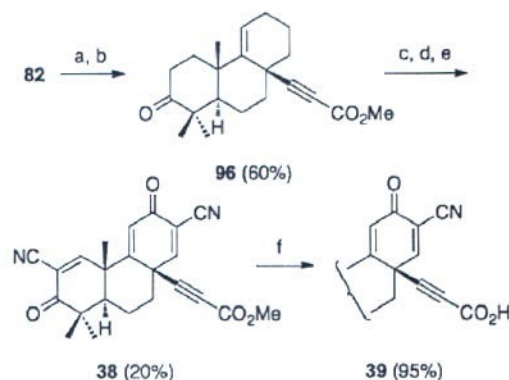
^a Reagents and yields: (a) *n*-BuLi, BF₃·Et₂O, DMF, THF, 91%; (b) Ph₃PCH₂Cl₂, *n*-BuLi, HMPA, THF, 76%; (c) MeLi, THF, TMSCl, 97%; (d) 10% aqueous HCl, MeOH, 74%; (e) CrO₃, *t*-BuOOH, CH₂Cl₂, 50%; (f) *p*-TsCN, LDA, THF; (g) DDQ, PhH, 22% in two steps (f) and (g); (h) TBAF, THF, 28%.

TCE 36 was synthesized in five steps from 82 (Scheme 16 and Scheme S26 in the Supporting Information). Insertion of the TBS group into the acetylene moiety was achieved by treating 82 with MeLi and trapping the resulting anion with TBSCl, to give 90 in 91% yield. The ketal 90 was subjected to acidic conditions to give 91 in 94% yield. Allylic oxidation of 91 afforded 92 in 66% yield. Double cyanation of 92, followed by DDQ oxidation in benzene, gave 36 in 27% yield.

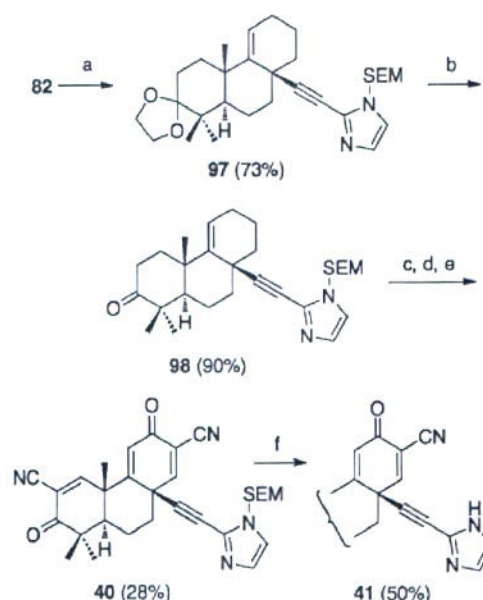
TCE 37 with a buta-1,3-diynyl group was synthesized in eight steps from 82 (Scheme 17 and Scheme S27 in the Supporting Information). The treatment of acetylide 82 with DMF in the presence of BF₃·Et₂O in THF provided aldehyde 93 in 91% yield. Tricycle 94 was prepared by a Wittig reaction of 93 with (chloromethyl)triphenylphosphonium chloride and subsequent treatment with MeLi and TMSCl, followed by deketalization (55% yield). Tricycle 95 was obtained in three steps from 94 by the same sequence as for 36 from 91 (11% yield). The TMS group of 95 was removed with TBAF to give 37 in 28% yield.

TCE 38 with a methoxycarbonyl group and the corresponding acid 39 were synthesized from 82 (Scheme 18 and Scheme S28 in the Supporting Information). Methyl ester 96 was obtained by the treatment of acetylide 82 with methyl chloroformate, followed by deketalization (60% yield). TCE 38 was prepared in three steps from 96 by the same sequence as for 36 from 91 (20% yield). Alkaline hydrolysis of 38 gave 39 in 95% yield.

Acetylenic imidazoles 40 and 41 were synthesized in six steps from 82 (Scheme 19 and Scheme S29 in the Supporting Information). A Sonogashira coupling⁷¹ between 2-iodo-1-((2-(trimethylsilyl)ethoxy)methyl)-1*H*-imidazole⁷² and 82 in the presence of Pd(PPh₃)₂Cl₂ and CuI in Et₃N gave 97 in 73% yield. Deketalization of 97 provided 98 in 90% yield. TCE 40 was obtained by allylic oxidation of 98 and subsequent isoxazole opening under basic conditions, followed by addition of PhSeCl in the presence of pyridine, and subsequent oxidation/

Scheme 18. Synthesis of TCEs 38 and 39^a

^a Reagents and yields: (a) ClCO₂Me, MeLi, THF, 64%; (b) 10% aqueous HCl, MeOH, 93%; (c) CrO₃, *t*-BuOOH, CH₂Cl₂, 60%; (d) *p*-TsCN, LDA, THF; (e) DDQ, PhH, 26% in two steps (d) and (e); (f) aqueous KOH, MeOH, 95%.

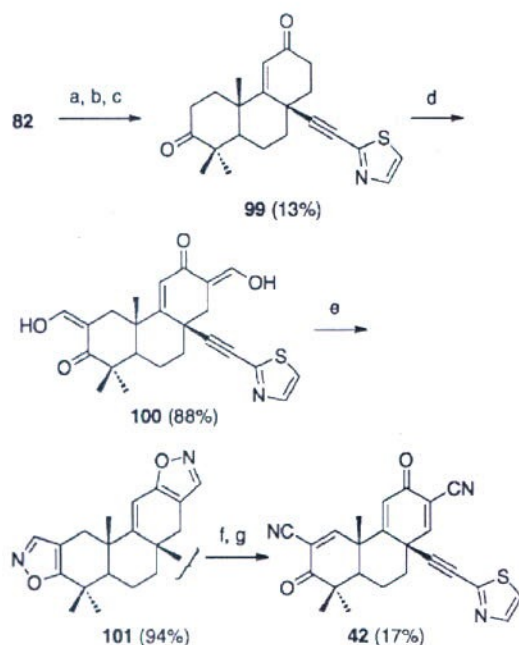
Scheme 19. Synthesis of TCEs 40 and 41^a

^a Reagents and yields: (a) 2-iodo-1-((2-(trimethylsilyl)ethoxy)methyl)-1*H*-imidazole, Pd(PPh₃)₂Cl₂, CuI, Et₃N, 73%; (b) 10% aqueous HCl, MeOH, 90%; (c) CrO₃, *t*-BuOOH, CH₂Cl₂, 43%; (d) *p*-TsCN, LDA, THF, 88%; (e) PhSeCl, pyridine, CH₂Cl₂; 30% H₂O₂, CH₂Cl₂, 75%; (f) CF₃CO₂H–THF (10:1).

elimination with H₂O₂ (28% yield). In this conversion, the PhSeCl and H₂O₂ were used instead of DDQ because the DDQ oxidation gave 40 in a very low yield. Removal of the SEM group of 40 was successfully achieved by CF₃CO₂H–THF (10:1) to afford 41 in 50% yield, after the deprotection using several well-known methods (TBAF, HF–CH₃CN, HF–pyridine, BF₃·Et₂O etc.) was unsuccessful.

Acetylenic thiazole 42 was synthesized in seven steps from 82 (Scheme 20 and Scheme S30 in the Supporting Information). Tricycle 99 was obtained by a Sonogashira coupling between 2-bromothiazole and 82, followed by removal of the ketal and subsequent allylic oxidation (13% yield). Formylation of 99 gave

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Scheme 20. Synthesis of TCE 42^a

^a Reagents and yields: (a) 2-bromothiazole, Pd(PPh₃)₂Cl₂, PPh₃, CuI, Et₃N, PhH, 32%; (b) 10% aqueous HCl, MeOH, 100%; (c) CrO₃, *t*-BuOOH, CH₂Cl₂, 41%; (d) HCO₂Et, NaOMe, PhH, 88%; (e) NH₂OH·HCl, aqueous EtOH, 94%; (f) NaOMe, MeOH, Et₂O; (g) PhSeCl, pyridine, CH₂Cl₂, 30% H₂O₂, CH₂Cl₂, 17% in two steps (f) and (g).

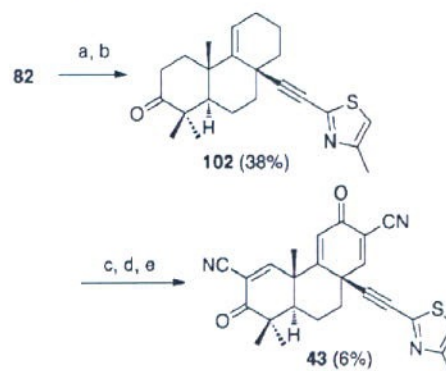
bis(hydroxymethylene) **100** in 88% yield. Treatment of **100** with hydroxylamine provided diisoxazole **101** in 94% yield. TCE **42** was prepared in 17% yield from **101** by the same methods as for **31** from **89** (see Scheme 15). Because double cyanation of **99** with *p*-TsCN followed by DDQ oxidation gave impure **42**, which we could not sufficiently purify, we adopted the Johnson's isoxazole method for this conversion.

Acetylenic methylthiazole **43** was synthesized by a Sonogashira coupling between 2-iodo-4-methylthiazole and **82** and subsequent deketalization, followed by the same sequence from **102** as for **40** from **98** (2% overall yield, Scheme 21 and Scheme S31 in the Supporting Information).

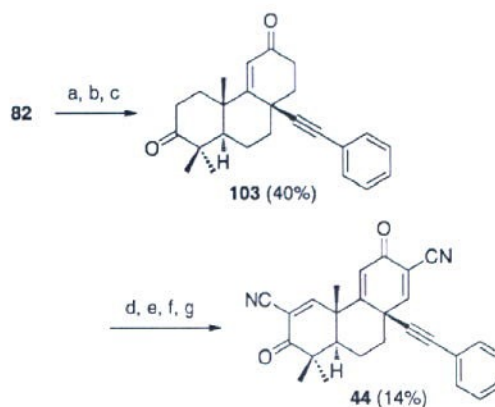
TCE **44** with a phenylethynyl group was synthesized in seven steps from **82** (Scheme 22 and Scheme S32 in the Supporting Information). Enone **103** was obtained by a Sonogashira coupling between iodobenzene and **82** and subsequent deketalization, followed by allylic oxidation (40% yield). TCE **44** was prepared in four steps from **103** by the same sequence as for **42** from **99** (14% yield).

3. BIOLOGICAL RESULTS AND DISCUSSION

3.1. TCEs 1–12 Inhibit NO Production Induced by IFN- γ in Primary Mouse Macrophages. We have evaluated inhibitory activities of the initial set of TCEs on NO production induced by IFN- γ in mouse macrophages. The inhibitory activities [IC₅₀ (nM)] of racemic **1**–**12**, optically active **5**, **9**, and **12**, triterpenoids [CDDO and CDDO-Me], and hydrocortisone (a positive control) are shown in Table 1. Important results, which were obtained from these compounds, are as follows: (1) Among all of the synthetic TCEs, **9** has the highest potency, and next is **12**. TCE **9** approaches the potency of CDDO in this assay; it is only

Scheme 21. Synthesis of TCE 43^a

^a Reagents and yields: (a) 2-iodo-4-methylthiazole, Pd(PPh₃)₂Cl₂, CuI, Et₃N, PhH, 48%; (b) 10% aqueous HCl, MeOH, 80%; (c) CrO₃, *t*-BuOOH, CH₂Cl₂, 49%; (d) *p*-TsCN, LDA, THF; (e) PhSeCl, pyridine, CH₂Cl₂, 30% H₂O₂, CH₂Cl₂, 13% in two steps (d) and (e).

Scheme 22. Synthesis of TCE 44^a

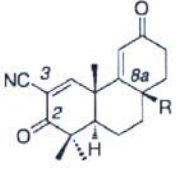
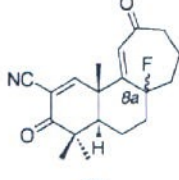
^a Reagents and yields: (a) iodobenzene, Pd(PPh₃)₂Cl₂, CuI, Et₃N, THF, 64%; (b) 10% aqueous HCl, MeOH, 77%; (c) CrO₃, *t*-BuOOH, CH₂Cl₂, 81%; (d) HCO₂Et, NaOMe, PhH, 92%; (e) NH₂OH·HCl, aqueous EtOH, 97%; (f) NaOMe, MeOH, Et₂O, 32%; (g) PhSeCl, pyridine, CH₂Cl₂, 30% H₂O₂, CH₂Cl₂, 50%.

about 4 times less potent than CDDO. A nitrile group at C2 enhances potency among the typical electron-withdrawing groups surveyed at C2. (2) Since **3** and **4** are more potent than **2** and **1**, respectively, the importance of the bis(enone) structure for high potency, even in tricycles, is confirmed. (3) A nitrile group at C6 is essential for high potency (**9** vs **10** and **11**). (4) Both enantiomers of **5** and **12** show similar potency, while (+)-**9** having the same configuration as the CDDO antipode is about 10 times more potent than (–)-**9** having the same configuration as CDDO.

The inhibitory potency of **9** in this assay was not blocked by the glucocorticoid antagonist mifepristone (RU486, at 1 μ M), which reverses the action of hydrocortisone (see Figure S2 in the Supporting Information). This strongly implies that the actions of TCEs in this assay are not mediated by their interaction with the glucocorticoid receptor.

3.2. TCEs 13–44 Inhibit NO Production Induced by IFN- γ in RAW 264.7 Cells. Our results from the initial set of TCEs encouraged us to explore additional TCEs. Thus, we first designed

Table 2. Inhibitory Activity of New TCEs 13–27 on NO Production Induced by IFN- γ in RAW Cells^a

					
compd (racemic)	R	IC ₅₀ (nM)	compd (racemic)	R	IC ₅₀ (nM)
13	CO ₂ Me	290	23	CN	64
14	CO ₂ SEM	150	24	CH ₂ NHBoc	64
15	CO ₂ H	83	25	CH ₂ NH ₂ ·HCl	240
16	CONH ₂	>10000	26	Et	80
17	CH ₂ OTBS	480	27	C≡CH	83
18	CH ₂ OH	350	12	Me	57
19	CH ₂ OAc	85	5	Table 1	87
20	CHO	83	9	Table 1	23
21	CH ₂ OMe	40	CDDO	Table 1	17
22		360	hydrocortisone		61

^a RAW 264.7 cells were treated with various concentrations of compounds and IFN- γ (10 ng/mL) for 24 h. Supernatants were analyzed for NO by the Griess reaction.¹¹ IC₅₀ values are an average of two separate experiments. These data have been published and presented in refs 79f and 79h.

and synthesized the new TCEs 13–27, which have various functionalities at C8a on the basis of 12. These analogues include typical electron-withdrawing, electron-releasing, hydrophilic, hydrophobic, and bulky groups (Table 2). Among them, the lead compound 12 having a methyl group at C8a was still the best. However, we found that hydrocarbon groups are slightly better than functional groups containing heteroatoms.

Second, we designed new TCEs 28–32 on the basis of TCE 9. From the SARs above, we mainly synthesized compounds with hydrocarbon groups at C10a. Also, we prepared optically active 31 and 32 to compare the differences in potency between the enantiomers. Notably and importantly, these compounds have two different nonenolizable cyano enones in rings A and C, which represent two theoretically possible monocyclic nonenolizable cyano enones.

The IC₅₀ (nM) values of these TCEs are shown in Table 3. Remarkably, TCEs 29–32 having hydrocarbon groups are more potent than the lead compound 9, as well as the positive controls CDDO and dexamethasone. In particular, acetylene groups dramatically enhance potency. TCE 31 is much more potent than 9, CDDO, and dexamethasone and is as potent as CDDO-Im, which is the most potent CDDO analogue. Importantly, TCEs having two cyano enones are much more potent than TCEs having only one cyano enone (9 vs 12, 29 vs 26, 31 vs 27). These results suggest that the cyano enone is a very important factor for potency. TCE 28 having a nitrile group at C10a is much less potent than others. The fact that an electron-withdrawing group at C10a decreases potency is identical with the previous results shown in Table 2.

Interestingly, the racemic form and the (+)- and (–)-enantiomers of 32 are slightly less potent than those of 31, although 32 has a much more bulky group than 31.⁷³ Notably, (+)-enantiomers of 31 and 32, having the same configuration as the CDDO antipode, are more potent than (–)-enantiomers of 31 and 32, having the same configuration as CDDO, respectively. These results are identical with those of optically active 9 (Table 1).

We have evaluated 31 and CDDO in the iNOS assay using primary mouse macrophages which are much more sensitive

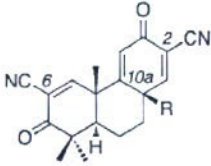
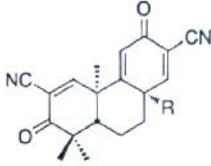
against inhibitors than RAW cells. The IC₅₀ values of 31 and CDDO are 0.056 and 4.3 nM, respectively. Thus, TCE 31 is about 80 times more potent than CDDO in primary mouse macrophages, whereas 31 is about 20 times more potent than CDDO in RAW cells.

Next, we designed and synthesized acetylenic TCEs 33–44 and observed that 31 is still the most potent compound in this series (Table 3). However, TCEs 33, 35, and 37 are nearly equivalent to 31 in potency. Moreover, TCEs 34, 38, 42, and 43 are still more potent than or at least as potent as 9, CDDO, and dexamethasone. TCEs containing a hydrophilic group are much less potent than those with a hydrophobic group (39 vs 38 and 41 vs 40). A TCE with a bulky TBS group is much less potent than with a TMS group (36 vs 32).

3.3. TCEs Induce NQO1 in Hepa1c1c7 Murine Hepatoma Cells. We evaluated some TCEs for induction of the phase 2 cytoprotective enzyme NQO1 in Hepa1c1c7 murine hepatoma cells. TCEs induce NQO1 at 1–100 nM (Table 3). Notably, (+)- and (±)-31 double the specific enzyme activity of NQO1 at 0.9 nM. The potency is higher than the potencies of CDDO and CDDO-Im. Remarkably, even in this assay, (+)-enantiomers of 9, 31, and 32, having the same configuration as the CDDO antipode are more potent than (–)-enantiomers of 9, 31, and 32, having the same configuration as CDDO, respectively. We previously demonstrated a linear correlation between NQO1 inducer potency (CD) and inhibitory activity on NO production (IC₅₀) of oleanolic acid derivatives.²⁹ In this series of TCEs, we also observed a similar correlation (Figure 3), and 31 is the most potent compound in both assays. Most recently, we have demonstrated that incorporation of 31 into the diet of SKH-1 hairless mice dose-dependently induces NQO1 enzyme activity in liver, skin, and stomach.⁷⁴

3.4. TCEs Inhibit the Induction of iNOS in RAW Cells Stimulated with IFN- γ . CDDO blocks de novo synthesis of iNOS protein. We have confirmed that TCEs also inhibit de novo synthesis of iNOS protein (Figure 4). TCEs 31 and 32 significantly inhibit the induction of iNOS at 30 nM. These abilities are

Table 3. Inhibitory Activity of New TCEs 28–44 on NO Production Induced by IFN- γ in RAW Cells and NQO1-Inducing Potency of Some TCEs in Hepa1c1c7 Cells

							
(-)-Enantiomer				(+)Enantiomer			
Compd	R	IC ₅₀ (nM) ^{a,b}	CD (nM) ^c	Compd	R	IC ₅₀ (nM) ^{a,b}	CD (nM) ^c
(±)-5	Table 1	87	160	(±)-33	C≡C-Me	5	7.5
(-)-5	Table 1		170	(±)-34	C≡C-Et	20	
(+)-5	Table 1		140	(±)-35	C≡C-CN	4	
(±)-9	Me	23	18	(±)-36	C≡C-TBS	80	150
(-)-9	Me		150	(±)-37	C≡C-C≡CH	3	
(+)-9	Me		19	(±)-38	C≡C-CO ₂ Me	20	85
(±)-28	CN	115		(±)-39	C≡C-CO ₂ H	100	150
(±)-29	Et	10	18	(±)-40		35	
(±)-30	CH=CH ₂	10	30	(±)-41		400	
(±)-31	C≡CH	1	0.9	(±)-42		10	35
(-)-31	C≡CH	3	2.7	(±)-43		20	
(+)-31	C≡CH	1	0.9	(±)-44		50	
(±)-32	C≡C-TMS	3	3.5	CDDO	Table 1	23	2.3
(-)-32	C≡C-TMS	3	3.0	CDDO-Im	Fig 1	1	3.3
(+)-32	C≡C-TMS	2	2.3	DXM		20	

^a RAW 264.7 cells were treated with various concentrations of compounds and IFN- γ (10 ng/mL) for 24 h. Supernatants were analyzed for NO by the Griess reaction. ^b IC₅₀ values are an average of two separate experiments. ^c These data have been published and presented in refs 79e–79h. ^d Hepa1c1c7 cells were grown for 24 h and then treated with serial dilutions of compounds for 48 h. The concentration required to double (CD) the specific enzyme activity of NQO1 was used to quantify inducer potency.

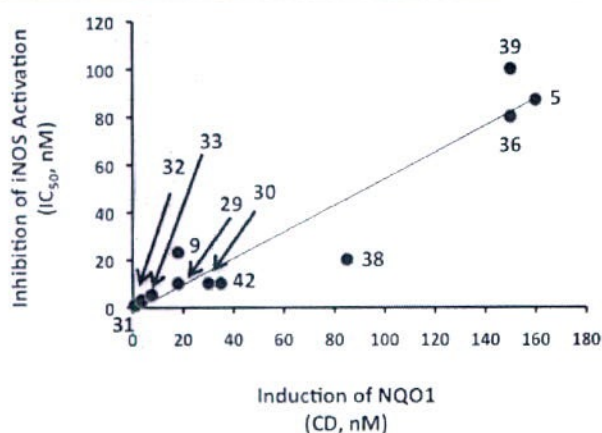


Figure 3. Correlation of potencies of TCEs as inducers of NQO1 in Hepa1c1c7 murine hepatoma cells, expressed as CD values, and for suppression of iNOS induction by IFN- γ in RAW cells, expressed as IC₅₀ values. The linear correlation coefficient is $r^2 = 0.91$.

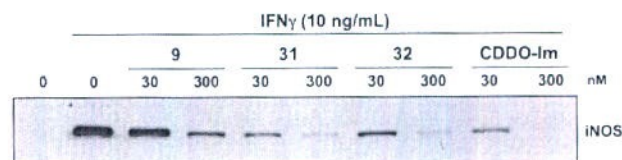


Figure 4. TCEs 9, 31, and 34 inhibit the induction of iNOS in RAW cells stimulated with IFN- γ . Cells were incubated with compounds (30–300 nM) and IFN- γ (10 ng/mL) for 24 h. Total cell lysates were analyzed by Western blot for iNOS. These data have been published in ref 79h.

equivalent to that of CDDO-Im. TCE 9 also inhibits the induction of iNOS at 300 nM.

3.5. TCE 31 Directly and Reversibly Reacts with Cysteine Residues of Keap1. The sensor for inducers of NQO1 and of a network of more than 100 other cytoprotective genes is Keap1, a protein endowed with highly reactive cysteine residues and an essential component of the Keap1/Nrf2/ARE signaling pathway.⁷⁵

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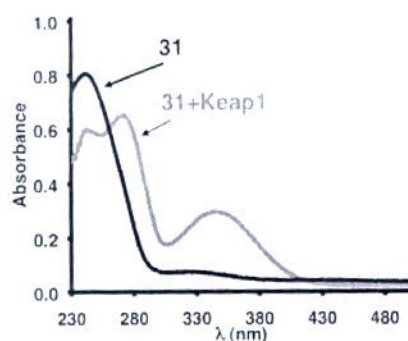


Figure 5. TCE 31 reacts with cysteine residues of Keap1. Absorption spectra of 50 μ M 31 (31, black line) and the reaction mixture of 50 μ M 31 and 10 μ M Keap1 (31 + Keap1, gray line) in 20 mM Tris-HCl/0.005% Tween 20 (pH 8.0) at 25 $^{\circ}$ C against Keap1 blank.

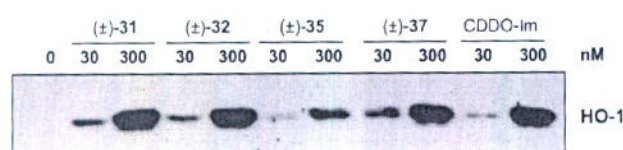


Figure 6. SARs of acetylenic TCEs in the HO-1 assay. Cells were incubated with compounds (30–300 nM) for 6 h. Total cell lysates were analyzed by Western blot for HO-1. These data have been presented and published in refs 79f and 79h.

Recently, we have found that both cyano enones in rings A and C of 31 react with cysteine residues of Keap1 to give Michael adducts.⁷⁴ The absorptions at 265 and 345 nm in the UV spectrum of 31 with Keap1 correspond to a Michael adduct of ring A and ring C with Keap1, respectively (Figure 5).⁷⁴ Also, we have demonstrated by NMR variable temperature studies^{40,76} that the Michael additions of ring A and ring C with DTT are both reversible. The chemical reversibility of these reactions has very significant biological implications: (1) it enhances inducer bioavailability; (2) it allows reversible cysteine modifications of the protein sensor Keap1, which does not need to be permanently inactivated (and possibly subsequently destroyed) but could be easily regenerated without requiring de novo protein synthesis; (3) it leads to a pulse of activation rather than constitutive up-regulation of the pathway; (4) it may explain, at least in part, some of the reasons why TCE 31 is such a potent inducer in vivo, given its ability to react with Keap1 at nanomolar concentrations despite the presence of millimolar concentrations of glutathione.

3.6. TCEs Induce HO-1 in RAW Cells. We have evaluated tricycles with two cyano enones in rings A and C (structures, see Table 3) for induction of the anti-inflammatory and cytoprotective enzyme, HO-1 in RAW cells. These results are shown in Figures 6 and 7. All compounds induce HO-1 at 30 nM. TCEs 31, 32, 37, 38, 42, 43, and 44 are higher inducers at 30 nM. They are superior to CDDO-Im in potency at 30 nM. Although 38, 42, 43, and 44 are about 10–50 times less potent than 31 in the iNOS assay, they are similar to 31 in potency in this HO-1 assay. TCEs 29, 30, 35, and 40 are moderate inducers, and TCEs 36, 39, and 41 are weak inducers. A hydrophilic group and a bulky silyl group decrease the potency. The SARs seen here correlate nicely with the SARs obtained in the iNOS assay.

We have also compared optically active 31 and 32 in this assay (Figure 8), but we do not observe any significant difference

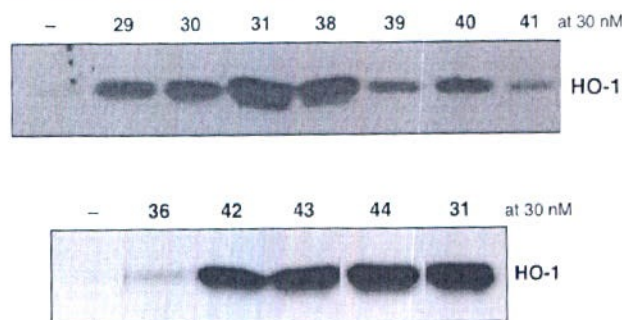


Figure 7. SARs of TCEs 29, 30, acetylenic TCEs 36 and 38–44 in the HO-1 assay. Cells were incubated with compounds (30 nM) for 6 h. Total cell lysates were analyzed by Western blot for HO-1. These data have been presented in ref 79g.

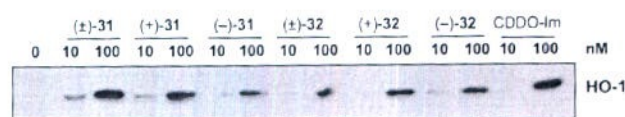


Figure 8. Racemic and optically active 31 and 32 induce HO-1 in RAW cells. Cells were incubated with compounds (10–100 nM) for 6 h. Total cell lysates were analyzed by Western blot for HO-1. These data have been presented in ref 79f.

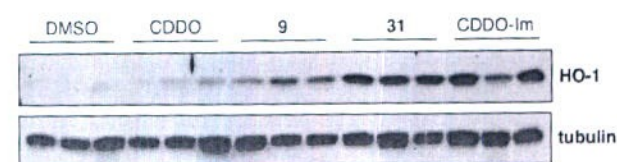


Figure 9. TCE 31 induces HO-1 in liver when given by gavage. Male CD-1 mice (three mice per group) were gavaged with 1 μ mol of the following: TCE 9 or 31, CDDO or CDDO-Im in DMSO. After 6 h, the livers were collected and analyzed by Western blot for HO-1. The tubulin blot is a loading control. These data have been published and presented in refs 79e, 79f, and 79h.



Figure 10. TCE 31 induces HO-1 in stomach when given by gavage. Male CD-1 mice (three mice per group) were gavaged with 1 μ mol of the following: TCE 9 or 31, CDDO or CDDO-Im in DMSO. After 6 h, the stomachs were collected and analyzed by Western blot for HO-1. The tubulin blot is a loading control. These data have been published and presented in refs 79e, 79f, and 79h.

between the two enantiomers. Even in this experiment, racemic and optically active 31 are more potent inducers than CDDO-Im at 10 nM.

3.7. TCE 31 Induces HO-1 in the Liver and Stomach of CD-1 Mouse When Given by Gavage. Subsequent to the HO-1 assay in vitro, we evaluated the potency of 9 and 31 for induction of HO-1 in the liver and stomach using male CD-1 mice by gavage. As shown in Figures 9 and 10, oral dosing of 1 μ mol of 9 and 31 causes significant induction of HO-1 in the liver and stomach while CDDO is markedly less potent at this low dose.

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Notably, TCE 31 is as potent as CDDO-Im in the liver but clearly more potent than CDDO-Im in the stomach, which is the most potent semisynthetic triterpenoid we have developed for induction of HO-1.²⁸ We have also examined optically active 31 in the same in vivo assay, but we did not find a significant difference between both enantiomers in the liver and stomach (see Figures S3 and S4 in the Supporting Information).

3.8. TCE 31 Reduces the Formation of Preneoplastic Foci in the Livers of Rats Challenged with Aflatoxin B₁ (AFB₁). We have evaluated 31 in the short-term model of hepatic tumorigenesis induced with AFB₁ in rats.³⁷ Prior to this experiment, we confirmed that 31 inhibits the formation of hepatic AFB₁-DNA adducts in a dose-dependent manner.⁷⁷ In the model of hepatic tumorigenesis, both 31 and CDDO-Im were well-tolerated, as indicated by the growth rate and final body weight being the same for dosed rats as the controls which were not treated with AFB₁. Both the number ($p = 0.001$) and diameter ($p = 0.024$) of the glutathione *S*-transferase P (GST-P) positive lesions in the liver were significantly decreased with increasing doses of 31. The focal volume percent of foci (analogous to tumor burden) was also significantly ($p < 0.005$) reduced in a dose-dependent manner. There were no foci detected in the AFB₁-treated rats at the highest doses of 31 (i.e., 30 and 60 $\mu\text{mol/kg}$). Even the lowest dose of 31 (0.3 $\mu\text{mol/kg}$) reduced the tumor burden by more than 90% compared to the AFB₁-treated positive control group. TCE 31 and CDDO-Im showed the same potency ($p = 0.6$) at 10 $\mu\text{mol/kg}$. At these doses the focal burden was reduced by greater than 99% (see Figure S5 and S6 in the Supporting Information).

TCE 31 significantly reduced formation of both AFB₁-DNA adducts and preneoplastic foci in the livers of rats challenged with AFB₁. The potency of 31 is similar to or better than that of CDDO-Im for inhibiting hepatic tumorigenesis induced by AFB₁, and this chemoprevention is dependent on activation of Nrf2.³⁷ Notably, both CDDO-Im and 31 are 100 times more potent than what has been reported for oltipraz, which inhibits activation of aflatoxin in humans.⁷⁸

In conclusion, although a series of TCEs was designed based on the ring A and C structures of CDDO initially, the ring C structures of analogues of 9 are clearly different from that of CDDO. It is noteworthy that the analogues of 9 have two different kinds of nonenolizable cyano enones in rings A and C, which work as strong Michael acceptors, while CDDO and its analogues have one nonenolizable cyano enone in ring A. Indeed, most of analogues of 9 are more potent than CDDO in the iNOS assay. Conversely, the triterpenoid oleanolic acid, lacking any cyano enone functional groups, is inactive. These facts strongly suggest that the essential factor for potency is not the triterpenoid or the tricycle skeleton but the functional monocyclic cyano enones that are positioned at a specific orientation relative to each other. TCE 31, an analogue of 9 having an acetylene group at C10a, is the most bioactive compound in both in vitro and in vivo bioassays in our pool of drug candidates, including semisynthetic triterpenoids and synthetic tricycles. Tricyclic compounds, diterpenoids, and triterpenoids with an acetylene group at C10a (C8 in terpenoid nomenclature) have not been reported prior to our synthesis of 31. Therefore, 31 may represent a new class of potential drug candidates having an entirely new structure for prevention and/or treatment of inflammatory diseases and cancers.

Further preclinical studies and detailed mechanism studies including identification of the protein targets of 31 are in progress.

■ ASSOCIATED CONTENT

S Supporting Information. Synthetic procedures and characterization data for new compounds 1–103, experimental procedures for biological evaluation, and Figures S1–S6. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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■ ABBREVIATIONS USED

CDDO, 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid; CDDO-Me, methyl 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oate; CDDO-Im, 1-(2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl)-imidazole; DAST, *N,N*-diethylaminosulfur trifluoride; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; DTT, 1,4-dithiothreitol; DXM, dexamethasone; EDIA, ethyldiisopropylamine; HMPA, hexamethylphosphoric triamide; IKK β , inhibitor of nuclear factor κB kinase β ; JAK1, Janus kinase 1; Keap1, kelchlike ECH-associated protein 1; MTPA, α -methoxy- α -(trifluoromethyl)phenylacetyl; Nf- κB , nuclear factor κB ; Nrf2, nuclear factor (erythroid-derived 2) related factor 2; STAT3, signal transducer and activator of transcription 3

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