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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



No. 06-100763-A-00006-0000

Fecha: 2013-05-17 16:37:50 Dep. 2020 DIR.NUEVASCR
Tra. 11 PATENTEIYII Eve: 379 FASENACIONALII
Act. 412 REPOSPRESRECU Folios: 45 46

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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

DIVISIÓN DE NUEVAS CREACIONES

E.

S.

D.

Re: Expediente No. 06-100.763A

Solicitud de patente de invención titulada: "EMULSIONES DE ACEITE EN AGUA
MICROFLUIDIZADAS Y COMPOSICIONES DE LAS VACUNAS".

Solicitante: PFIZER PRODUCTS INC.

Nuestra Ref.: P2006/000215A

RECURSO DE REPOSICION

CARLOS R. OLARTE, mayor de edad y vecino de Bogotá, D.C., identificado como aparece al pie de mi firma, en mi condición de apoderado de la sociedad solicitante, y dentro del término legal previsto por el artículo 51 del Código Contencioso Administrativo, me permito interponer RECURSO DE REPOSICIÓN en contra de la Resolución No. 14596, notificada por edicto desfijado el 09 de mayo de 2013.

1. ANTECEDENTES

La solicitud de patente ventilada en este expediente contiene un capítulo reivindicatorio con 8 reivindicaciones (radicado el 27 de junio de 2012, en el momento de presentación de esta Solicitud Divisional). Según la Resolución No. 14596, el Despacho niega la totalidad de las reivindicaciones por supuesta falta de novedad.

2. OBJETO DEL RECURSO

Que se revoque la resolución recurrida, y en su lugar se conceda el privilegio de patente de invención para el Capítulo Reivindicatorio Modificado adjunto al presente recurso de reposición.

3. FUNDAMENTO DEL RECURSO

Con referencia a los argumentos presentados por la SIC para desvirtuar la novedad de la presente solicitud en la resolución aquí recurrida, respetuosamente difiero del análisis expuesto, y procedo a exponer las motivaciones técnicas que demuestran que las reivindicaciones adjuntas cumplen con los requisitos legales de patentabilidad.

3.1. En primer lugar, con el ánimo de brindar mayor claridad a la materia reivindicada, me permito presentar un Capítulo Reivindicatorio Modificado, que reemplaza en su totalidad el Capítulo Reivindicatorio radicado el 27 de junio de 2012.

Así entonces, el Capítulo Reivindicatorio Modificado adjunto está conformado por 8 Reivindicaciones, en donde en la Reivindicación 1 se limitó la proporción entre el glicósido de saponina y el esteroles, correspondiendo al rango entre 1:100 a 5:1, lo cual encuentra soporte en el Capítulo Descriptivo, página 39, líneas 1 y 11.

Valga anotar que las modificaciones efectuadas al Capítulo Reivindicatorio cumplen a cabalidad con lo estipulado en el Artículo 34 de la Decisión 486, ya que éstas de ninguna manera implican una ampliación de la protección correspondiente a la divulgación contenida en la solicitud inicial.

3.2 a- La SIC insiste en que las patentes WO9633739 (D1), US20030095974 (D2) y WO2004017990 (D3), afectan la novedad de la presente invención.

La SIC presenta la siguiente tabla comparando las características esenciales de las composiciones divulgadas en D1 y D2 y las composiciones de la presente invención:

Características esenciales	Esterol (colesterol) D1	Esterol (colesterol) D2
Composición de vacuna	Composición de vacuna	Composición de vacuna
Glucósido de saponina	Fracción de saponina inmunológicamente activa (QS21)	Fracción inmunológicamente activa de saponina (Quil-A)
Un esteroles		
Un antígeno	Antígeno	Antígeno

La SIC argumenta que D1 divulga composiciones de vacuna que comprenden un antígeno, una fracción inmunológicamente activa de saponina que corresponde a QS21 y un estero, de preferencia, colesterol, en un vehículo farmacéuticamente aceptable, que comprende una emulsión aceite en agua que se caracteriza por ser una micro-emulsión en forma de liposoma con un tamaño de partícula menor a 1 nm.

De la misma forma, el Despacho señala que D1 divulga un procedimiento de preparación de la composición de vacuna en donde se tienen en cuenta la combinación entre el estero y el glicósido de saponina, y establece que la presencia conjunta e inicial entre el colesterol y el QS21 forman liposomas o micelas estables que pueden permanecer en suspensión por más tiempo, y dicha mezcla o asociación produce la estabilización de los glicósidos de saponina, lo que conlleva una menor reactogenicidad. Además, D1 divulga que la mezcla del antígeno se hace posteriormente a la mezcla colesterol-QS21, donde dicho antígeno está mezclado pero no integrado en micelas o liposomas generados por microemulsión, lo cual anticipa la materia reclamada en la Reivindicación 1.

De otra parte, la SIC argumenta que D2 divulga una composición de vacuna que comprende una fracción inmunológicamente activa de saponina que corresponde a Quil-A o un derivado como QS21, en un vehículo que comprende una emulsión submicronizada de aceite en agua con un tamaño de gota de 1 micrómetro. Dicha composición además contiene un antígeno de naturaleza bacteriana o viral y un estero, de preferencia, el colesterol, lo cual anticipa la materia reclamada en la Reivindicación 1.

b- La SIC además presenta la siguiente tabla comparando las características esenciales de las vacunas divulgadas en D3 y las vacunas reclamadas en la Reivindicación 7 de la presente invención:

Características esenciales (Rv 7)	D3
Vacuna	Vacuna
Glucósido de saponina	Glucósido de saponina (Quil-A/GPI-0100)
Un estero	Esterol (Colesterol)
Un antígeno (agente vírico, un agente bacteriano o una combinación de los mismos)	Un antígeno que comprende: BVDV tipo I ó II, Virus de herpes

La SIC además señala que D3 divulga composiciones inmunogénicas y vacunas que comprenden la combinación de a) un agente viral como el virus de herpes bovina (BHV-1); b) un antígeno de BVDV tipo I y tipo II; c) un adyuvante seleccionado entre glucósidos de saponina o sus derivados (Quil-A/GPI-0100), colesterol, citoquinas, etc, un vehículo veterinariamente aceptable

que involucra emulsiones aceite en agua microfluidizadas, lo cual anticipa la materia reclamada en la Reivindicación 7.

c- Adicionalmente, me permito resaltar que la tabla comparativa presentada por el Despacho no es clara puesto que presenta unas superposiciones en el texto y un desorden generalizado en cuanto a los títulos de las columnas, lo cual resulta completamente ilegible y por tanto no permite realizar un análisis certero de la información allí contenida, lo que supone una desventaja al momento de contestar el presente recurso de reposición, puesto que no se cuenta con la información veraz necesaria.

Características esenciales	Esterol (colesterol) D1	Esterol (colesterol) B2
Composición de vacuna	Composición de vacuna	Composición de vacuna
Glucósido de saponina	Fracción de saponina inmunológicamente activa (QS21)	Fracción inmunológicamente activa de saponina (Quil-A)
Un esteroi		
Un antígeno	Antígeno	Antígeno

3.3. Adicionalmente, me permito dar respuesta a cada una de las observaciones que presenta la SIC en el examen de fondo en respuesta a mis argumentos, como se muestra a continuación:

a- i) La SIC afirma que “la formación de complejos en forma de micelas helicoidales, no puede considerarse como una característica técnica esencial de la composición, puesto que esta conformación helicoidal está dada no solo por las características físicoquímicas de los componentes de la composición, sino por el proceso de elaboración de dicha composición.”

Además, la SIC argumenta que “este despacho reitera que esta característica corresponde a un resultado a alcanzar puesto que dicha conformación u organización en forma de micela helicoidal se obtiene luego de hacer la respectiva transformación de los componentes mediante un proceso de mezclado, microfluidización y filtración (Ejemplo 20 y 21) y no sólo se debe a las características lipofílicas o hidrofílicas de los componentes, como pretende establecerlo el solicitante.”

ii) Contrario a lo afirmado por la SIC, las micelas helicoidales no son simplemente un resultado a alcanzar, sino una característica estructural esencial. La forma de los complejos de glucósido de saponina-esterol, de ninguna manera pueden ser considerados como

un resultado a alcanzar, por el contrario, esta característica define una particularidad estructural de la organización dentro de la composición.

En este sentido, el Manual Andino de Patentes (MAP) establece que las características técnicas de la invención en las reivindicaciones pueden ser estructurales (como la forma) o funcionales.¹ Adicionalmente, debe tenerse en cuenta que la práctica andina permite que las reivindicaciones incluyan definiciones de producto en términos de su proceso de fabricación.² Así entonces, puede entenderse que la Reivindicación 1 además comprende una cierta condición de proceso para la obtención de micelas helicoidales.

b- i) La SIC señala que el hecho que no se establezcan las proporciones específicas en que están cada uno de los componentes dentro de la composición adyuvante, no diferencia las composiciones reclamadas de las divulgadas en el estado de la técnica, lo cual lleva a la conclusión que cualquier proporción, cantidad y/o relación peso/peso o volumen/volumen de los componentes resuelve el problema técnico planteado como es que las composiciones presenten buena capacidad inmunogénica, sean seguras y estables.

ii) En respuesta, me permito subrayar que la presente invención se refiere única e inequívocamente a una composición de vacuna que comprende un glicósido de saponina, un esterol (en un rango entre 1:100 y 5:1 peso a peso) y un antígeno, en donde (al formarse dicha composición) dicho esterol es adicionado a dicho glicósido de saponina, los cuales se asocian entre sí para formar complejos micelares de forma helicoidal y en donde dicho antígeno está mezclado pero no integrado en las micelas helicoidales.

c- i) La SIC argumenta que de acuerdo con el Capítulo Descriptivo, sólo influyen en la formación de las micelas helicoidales los procesos de mezclado (orden de adición de los componentes) y microfluidización (Ejemplos 20, 21). Por tal razón solamente mediante la caracterización del proceso de elaboración de dichas composiciones, se puede establecer cómo el glicósido de saponina y el esterol (colesterol) se asocian entre sí para formar complejos estables en forma de micela helicoidales y de esta manera lograr composiciones estables, seguras, etc.

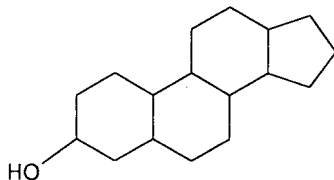
ii) Para dar respuesta a lo anterior, me permito señalar que las saponinas actúan formando espuma en el agua, y podrían precipitar el colesterol, si éste se encuentra en el

¹ Manual Andino de Patentes. (2004). Sección 4.2 (disponible en <http://www.comunidadandina.org/public/patentes.pdf>).

² Manual Andino de Patentes. (2004). Sección 4.6.8 (disponible en <http://www.comunidadandina.org/public/patentes.pdf>).

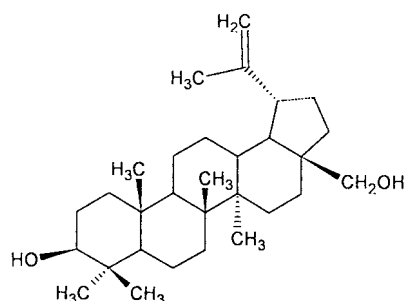
agua debido a que es una molécula hidrofóbica. De acuerdo con la invención (Ejemplo 21), una solución de colesterol preparada en etanol, al ser diluida en agua, volvería el agua turbia; sin embargo, tras la adición de saponinas, el colesterol precipita. Por el contrario, si una pequeña cantidad de la solución de colesterol en el alcohol se añade lentamente al agua que ya contiene saponinas, el colesterol y saponinas interactúan entre sí para formar las micelas helicoidales de la Reivindicación 1. El colesterol, debido a sus características estructurales, tiene una afinidad muy fuerte por los lípidos. Así, los métodos en los que se combine el colesterol con un lípido, darían lugar a un complejo de colesterol/lípidos (por ejemplo, liposomas), por lo que el colesterol no estaría disponible para la formación de micelas helicoidales como sucede en la presente invención. Además, el Capítulo Descriptivo de la presente solicitud proporciona una descripción detallada de las micelas helicoidales de la invención (ver Ejemplo 21), describiendo en detalle la composición de las micelas helicoidal y el método de fabricación de éstas.

Ahora, en relación con Quil-A y el colesterol, es importante resaltar que una persona medianamente versada en la materia podría extrapolar con facilidad que las micelas helicoidales formadas a partir de Quil-A y colesterol, como lo reclama la presente invención, se pueden formar a partir de variados esteroides y saponinas. Es decir, una persona medianamente versada en la materia estaría en capacidad de predecir los resultados descritos en el Capítulo Descriptivo, los cuales podrían ser extrapolados a otros esteroides y saponinas, si se tiene en cuenta dos factores: primero, todas las saponinas comparten el mismo núcleo, es decir, el núcleo de aglicona, y segundo, todos los esteroides tienen el mismo núcleo, es decir, el núcleo de esteroide.



Núcleo de esteroide

El hecho de que los esteroides sean plenamente conocidos es suficiente para que una persona normalmente versada en la materia reconozca que dicho término abarca toda la familia de compuestos que comparten el mismo núcleo, porque evidentemente compartirían la misma actividad. Del mismo modo, el hecho de que Quil-A también sea una molécula conocida, representa para la persona normalmente versada en la materia que el término abarca toda la familia de compuestos que comparten núcleo de aglicona.



Estructura de Betulina

(Triterpeno que está presente en la naturaleza)

Particularmente, la razón para que una persona medianamente versada en la materia sea capaz de llegar a tal conclusión se encuentra en la divulgación de los compuestos y en la implicación de que los compuestos estructuralmente similares probablemente presentan propiedades similares.

Me permito adjuntar la declaración del Dr. Paul Dominowski, presentada durante el trámite de contraparte europea de la presente solicitud. En dicha declaración, el Dr. Dominowski explica y deja claro que los esteroides y las saponinas presentan núcleos comunes, los cuales permiten extrapolar la actividad a cualquier miembro de las respectivas familias de compuestos. Así las cosas, una persona normalmente versada en la materia realizaría el mismo análisis que el Dr. Dominowski sin dificultades excesivas. Por lo tanto, es claro que la información divulgada en el Capítulo Descriptivo y la información disponible de fuentes fácilmente disponibles (por ejemplo, las fórmulas de los esteroides) es suficiente para establecer dicho razonamiento. De la misma forma, me permito adjuntar 5 referencias que soportan lo mencionado, así como la hoja de vida del Dr. Dominowski.

3.4 a- Con respecto al arte previo, el Despacho encuentra que el proceso de preparación del adyuvante es equivalente entre la solicitud y D1, puesto que si bien en D1 el colesterol está en una solución madre con solvente orgánico en lugar de alcohol, nada en D1 indica que el colesterol se precipita al preparar el adyuvante. Por tanto, la forma de preparación de la composición, en la cual no es relevante el solvente empleado con el colesterol, no es diferente entre los dos documentos.

Además, la SIC sostiene que, al igual como sucede con D1, la forma de preparación del adyuvante es equivalente entre la solicitud y D2. El uso de un aceite metabolizable tal como el escualeno, frente al uso de un aceite no metabolizable de la invención, no resultan relevantes puesto que la composición reclamada no menciona nada sobre las características del aceite, evidenciando así que esta no es una característica esencial de la composición.

Finalmente, la SIC argumenta que la formación de micelas helicoidales, o de cualquier otra conformación, forma agregados que no son estáticos, sino que se disocian, reagrupan y reagregan rápidamente. Hay un equilibrio dinámico (un intercambio incesante) entre las moléculas aisladas del tensoactivo en solución, las micelas y las moléculas adsorbidas en monocapa en las interfaces. De esta manera, resulta evidente que la organización de las micelas no puede considerarse una característica esencial para la composición.

b- i) En respuesta a lo anterior, en cuanto al arte previo citado, en primer lugar, es importante entender que los vehículos en composiciones adyuvantes enseñados por las referencias citadas, se selecciona del grupo que comprende sales minerales, emulsiones, polímeros, liposomas e ISCOMs.

Para facilitar la comprensión de los siguientes argumentos, me permito incluir a continuación una breve descripción de los vehículos descritos en las referencias citadas.

- Los **liposomas** son generalmente de forma esférica y están formados por moléculas de lípidos anfifílicos. Las moléculas anfifílicas lipídicas tienen partes hidrofóbicas e hidrofílicas. Cuando estas moléculas anfifílicas se disuelven en agua, tienden a auto-ensamblarse, de manera que las partes hidrófobas se agrupan junto con las porciones hidrofílicas que se extienden hacia fuera y se exponen al agua. El liposoma formado por lípidos puede además incorporar moléculas lipófilas tales como el colesterol. Los liposomas también pueden incorporar otras moléculas tales como saponinas.
- Los **ISCOMS** son conocidos en la técnica como complejos inmunoestimulantes que son partículas esféricas de tamaño uniforme con un diámetro de 30-40nm. Se forman por la interacción de saponinas, lípidos y colesterol.
- Las **emulsiones** se forman cuando se mezcla aceite con agua y un emulsionante, y se somete a una fuerza de cizallamiento alto. Como resultado de la fuerza de cizalla aplicada, el aceite se dispersa uniformemente en la fase acuosa y las moléculas anfifílicas emulsionantes estabilizan las partículas diminutas del aceite resultantes.

ii) Específicamente, en cuanto al arte previo citado por el Despacho y las objeciones de novedad relacionadas, llamo respetuosamente la atención sobre lo siguiente:

D1 describe una composición de vacuna que comprende una saponina (QS21), un esterol (colesterol) y un antígeno. Las composiciones preferidas de D1 forman liposomas o ISCOMs, y además pueden contener un vehículo emulsionado O/W. El Ejemplo 1 de D1 establece que *"los liposomas tienen un tamaño definido de 100nm y se conocen como SUV (pequeñas vesículas unilamelares). Si esta solución se congela y se descongela repetidamente, las vesículas se fusionan para formar grandes estructuras multilamelares (MLV) de tamaños que van desde 500nm a 15µm"* Debido a la muy fuerte afinidad del colesterol por los lípidos, el colesterol no estaría disponible para la formación de micelas helicoidales, como en la presente invención.

D1 también describe el orden de adición de los componentes cuando se combinan la saponina y colesterol (Ejemplo 1.1). Siguiendo la secuencia de pasos de este ejemplo, necesariamente se obtiene la formación de liposomas (opcionalmente con lípido A monofosforil 3-desacilado (MPL o 3D MPL), presente en las membranas de los liposomas. Posteriormente en el Ejemplo 1.2, la saponina (QS21 en solución acuosa) se añade a los liposomas. A continuación, la mezcla liposoma/saponina se añade a una solución de antígeno (opcionalmente también en la presencia de MPL). La formulación de liposomas de acuerdo con los Ejemplos 1.1 y 1.2 es estándar a través de los ejemplos restantes de D1.

Como se describe en el Ejemplo 1.1, la saponina se agrega a los liposomas (o ISCOMs) que contienen colesterol, preparados por adición de una solución acuosa a una mezcla de lípidos y colesterol. Los liposomas, ISCOMs y partículas de una emulsión O/W son generalmente de forma esférica y están formados por moléculas de lípidos anfífilos. Éstas **NO forman micelas helicoidales porque el colesterol ya está ligado en los liposomas**. Además, debido a la afinidad muy fuerte del colesterol por los lípidos, el colesterol no estaría disponible para la formación de micelas helicoidales como en la presente invención. Como se ha indicado en D1 (página 7, líneas 16-18), *"... El colesterol es insoluble en soluciones acuosas y no forma una suspensión estable ... en la presencia de fosfolípidos el colesterol reside dentro de la bicapa de fosfolípidos que puede formar una suspensión estable de las vesículas llamadas liposomas ... "*

Una distinción importante entre los liposomas y las micelas (helicoidales o no), es que los liposomas tienen una bicapa lipídica, mientras que las micelas tienen solamente una **monocapa**.

La evidencia de que el proceso de D1 requiere de liposomas esféricos, se encuentra en la sección de 1,3 de D1, donde se describe que el colesterol en liposomas es necesario para inhibir la actividad lítica de QS21. También se describe que el lípido es necesario para inhibir la actividad lítica (ver página 7, líneas 16-21). Se describe además en la sección 1.4 del D1 que la QS21 es mucho menos susceptible a la hidrólisis a un pH superior a 7, si ésta se asocia con liposomas que contienen colesterol. Una prueba adicional de que el proceso de D1 forma liposomas esféricos o ISCOMs, es que la reivindicación 1 de la patente de los Estados Unidos US6.846.489, equivalente a D1, tiene la limitación que "... *la composición adyuvante está en la forma de una ISCOM*". Por lo tanto, los lípidos son un componente esencial de la referencia D1, mientras que no hay lípidos en el procedimiento de la presente invención.

La presente invención no se obtiene de la misma manera que enseña D1, y por lo tanto la microestructura de la composición es diferente. El proceso de la presente invención corresponde a la adición lenta de una pequeña cantidad de una solución madre de colesterol en alcohol (Ejemplo 21) al agua ya que contiene saponinas. El colesterol y las saponinas interactúan entre sí para formar las micelas helicoidales de la Reivindicación 1. El resultado del procedimiento de los solicitantes es la obtención de micelas helicoidales. No hay lípidos en el procedimiento de la invención. Por el contrario el procedimiento añade esteroides al agua que contiene saponina, lo cual es diferente del proceso de D1 para hacer liposomas con colesterol y fosfatidilcolina y añadiendo luego la saponina a los liposomas que contienen colesterol.

D1 no enseña micelas helicoidales largas y enrolladas formadas por la interacción de saponinas y esteroides, tal como se presenta en la Reivindicación. D1 enseña esencialmente una combinación de colesterol esféricos/liposomas lípidos y saponinas. El Capítulo Descriptivo de la presente solicitud proporciona una descripción detallada del género de micelas helicoidales reivindicada, como el Ejemplo 21, donde se describe en detalle la composición de las micelas helicoidal y el método de fabricación de las mismas. Por tanto, no hay divulgación o sugerencia alguna en D1 sobre la formación de micelas helicoidales necesariamente por interacción entre saponinas y esteroides. D1 únicamente divulga la formación de liposomas o ISCOMs.

En conclusión, la materia objeto de la presente solicitud es novedosa en relación con D1.

iii) Ahora bien, el Despacho afirma que D2 describe composiciones de vacuna que comprenden la fracción de saponina inmunológicamente activa Quil-A, QS21 (página 1, párrafo 005; página 1, las Reivindicaciones 1 y 4), en un vehículo que comprende emulsión submicronizada O/W (página 4, párrafo 0026), que tiene un tamaño de gota de 1µm (página 1,

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Reivindicación 17), dicha composición que comprende también un antígeno viral y bacteriano (página 2, párrafo 0014, y Reivindicación 13), y un esterol - colesterol (página 3, el párrafo 0023 y 0026; Reivindicaciones 5-7).

Difícil respetuosamente de la posición del Despacho, en tanto considero que D2 describe una composición de vacuna que comprende un adyuvante basado en una saponina y una emulsión submicrónica O/W, que comprende un esterol en la fase oleosa de la emulsión O/W. Tales preparaciones de vacunas se formulan junto con un antígeno en mezcla. Las partículas o gotas de una emulsión O/W son generalmente de forma esférica y están formados por moléculas de lípidos. NO hay enseñanza alguna en D2 sobre la formación de micelas helicoidales mediante la interacción entre las saponinas y esteroleos. D2 claramente enseña que la saponina se añade al colesterol, y que las partículas están en forma de liposomas, NO de micelas helicoidales. Véase en particular el párrafo [0023] de D2, donde se afirma que *"una vacuna que contiene QS21 y una emulsión O/W, que cae dentro de la relación deseada, se formula en la presencia de un esterol..."* y el párrafo [0025], donde se afirma que *"formulación de la QS21 en liposomas que contienen colesterol"*. Así, D2 describe la adición de colesterol a la fase oleosa de la emulsión y la adición luego de la saponina a la emulsión (véanse los párrafos [0037] y [0038], y la Reivindicación 26). NO se forman micelas helicoidales porque el colesterol ya está ligado en los liposomas. Esto es contrario al orden de adición de los componentes en la presente invención, donde se añade el esterol al glicósido de saponina.

En la presente solicitud, la Reivindicación 1 reclama un glicósido saponina, un esterol y un antígeno, sin mención alguna sobre una emulsión O/W. La sección titulada "complejos formados por una saponina y un esterol" contiene una descripción de las composiciones que contienen las micelas helicoidales. En esta descripción, no hay ninguna mención de una emulsión O/W. Además, el Ejemplo 21 describe en detalle la composición de las micelas helicoidales y el método de fabricación de éstas.

D2 **no** anticipa la presente invención por varias razones:

- (1) D2 describe una microestructura de liposomas, **NO** micelas helicoidales;
- (2) las composiciones adyuvantes de la Reivindicación 1 comprenden un glicósido de saponina y un esterol, pero no una emulsión O/W;
- (3) en la presente invención, el esterol no se añade a la emulsión, se añade a la solución que contiene el glicósido de saponina; y

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(4) en aquellas modalidades que comprenden una emulsión, la emulsión de la invención no comprende un aceite metabolizable. D2 enseña el uso de escualeno, que es un aceite metabolizable, mientras que en realizaciones de la presente invención siempre comprenden un aceite no metabolizable, tal como el aceite mineral. D2 afirma que "la fase de aceite del sistema de emulsión debe comprender un aceite metabolizable" (párrafo [0008]). Esto aleja la materia de D2 de la presente invención. No hay descripción permitiendo alguna en D2 de todas las características esenciales de las reivindicaciones ni indicación a los expertos en la materia para intentar productos la presente invención con una expectativa de éxito.

En conclusión, la materia objeto de la presente invención es novedosa sobre D2.

iv) El Despacho afirma que D3 describe vacunas inmunogénicas que comprenden: a) un agente viral de VHB-1, b) un antígeno de BVDV tipo I y tipo II (página 44, Reivindicaciones 1 y 4, página 45, Reivindicación 12); c) un adyuvante seleccionado de glucósido de saponina o derivados del mismo -Quil-A/GPI-0100, colesterol (página 44, la Reivindicación 2), citoquinas, etc (página 11, líneas 12-22); y d) un vehículo veterinariamente aceptable que comprende emulsiones O/W microfluidizadas (página 11, línea 22). El examinador concluye que WO2004/017990 destruye la novedad de la reivindicación 7.

Me permito anotar respetuosamente que D3 describe una composición de vacuna que comprende un adyuvante basado en una saponina y una emulsión O/W submicronizada, que comprende un esterol en la fase oleosa de la emulsión O/W. Las partículas o gotas de una emulsión de aceite en agua son generalmente de forma esférica y están formadas por moléculas de lípidos. Sin embargo, NO hay ninguna enseñanza en D3 sobre la formación de micelas helicoidales por la interacción entre saponinas y esteroides. En la presente solicitud, la Reivindicación 1 reclama un glicósido de saponina, esterol, pero no incluye una emulsión O/W. La sección titulada "complejos formados por una saponina y un esterol" contiene una descripción de las composiciones que contienen micelas helicoidales. En esta descripción, no hay mención alguna de una emulsión O/W. Además, el Ejemplo 21 describe en detalle la composición de las micelas helicoidales y el método de fabricación de las mismas. Nada en D3 anticipa la presente invención, porque no se incluye una discusión en la técnica citada del método de fabricación de la emulsión. Las enseñanzas de D3 no son lo suficientemente detalladas para permitir a la persona calificada para usar dicha información e inequívocamente obtener la materia de la presente invención.

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En conclusión, la materia objeto de la presente invención es novedosa sobre D3.

v) En resumen, la presente invención corresponde a una composición de vacuna que comprende un glicósido de saponina y un estero (en un rango entre 1:100 y 5:1 peso a peso), en donde **dicho glicósido saponina y dicho estero se asocian entre sí para formar complejos en forma de micelas helicoidales**. No hay enseñanza alguna en las referencias citadas como arte previo sobre la formación de micelas helicoidales por la interacción entre las saponinas y esteroides. Adicionalmente, estos complejos en micelas helicoidales tienen actividad inmunoestimulante y son especialmente útiles como adyuvantes en composiciones de vacuna como la reclamada en la presente solicitud.

c- En este punto y a la luz de lo anterior, me permito llamar la atención del Despacho sobre el hecho que para fundamentar una afectación válida contra la novedad de una invención, el arte previo debe **divulgar explícitamente** todas y cada una de las características de la invención³. Es más, el Tribunal Andino de Justicia (TAJ) ha determinado que para establecer una objeción válida contra la novedad, la divulgación del arte previo **debe ser lo suficientemente detallada para que una persona versada en la materia pueda usar dicha información para reproducir y explotar la invención**⁴.

3.5. Finalmente, deseo poner bajo su conocimiento que para el concepto inventivo comprendido en la presente solicitud, el privilegio de patente ha sido otorgado hasta el momento por parte de las Oficinas de Patentes en Australia (AU20052130708), India (249232), Mexico (261163), Nueva Zelanda (550152), Filipinas (1-2006-501952), Rusia (2347586), Taiwan (I301067), Ucrania (89631), Estados Unidos (7,122,191) y Europa (1,742,659).

Si bien es cierto que las concesiones de patentes en oficinas de otros países no son vinculantes en el sentido que obliguen a la SIC a tomar una decisión en el mismo sentido, considero que dichas concesiones sugieren el cumplimiento de los requisitos de patentabilidad de la materia reivindicada, ya que los criterios de evaluación y estudio aplicados para concluir la aceptación de dichas patentes resultan equivalentes a los que la SIC debe emplear.

4. Para terminar, de conformidad con lo establecido en el artículo 59 del Código Contencioso Administrativo, solicito respetuosamente que **todos y cada uno** de los puntos

³ Manual Andino de Patentes. (2004). Sección 9.4. (disponible en <http://www.comunidadandina.org/public/patentes.pdf>).

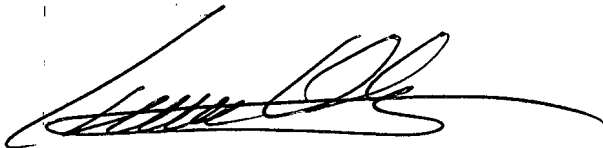
⁴ Proceso 06-IP-89 (<http://intranet.comunidadandina.org/Documentos/Procesos/209-IP-2005.doc>); y Proceso 36-IP-2004 (<http://www.notinet.com.co/serverfiles/servicios/archivos/30jul04/ca36-04.htm>)

planteados en el presente recurso sean resueltos de manera individual, completa y motivada, respecto de las razones de hecho, derecho y/o conveniencia que lleven a esa Superintendencia a adoptar la decisión que estime frente a la solicitud aquí formulada.

5. NOTIFICACIÓN

Recibiré notificaciones en mis oficinas ubicadas en la Carrera 5 No. 34-03, de Bogotá.

Atentamente,



CARLOS R. OLARTE
C.C. No. 79.782.747 de Bogotá
T.P. 74.295

Anexos:

- Capítulo Reivindicatorio Modificado
- ANEXO 1: Declaración Dr. Paul Dominowski suministrada para la contraparte europea
- ANEXO 2: Hoja de vida Dr. Paul Dominowski.
- ANEXO 3: Artículo científico: Kidambi, S, Patel, S. Sitosterolacemia: pathophysiology, clinical presentation and laboratory diagnosis.
- ANEXO 4: Artículo científico: Sarek, J. Betulines. Triterpenes.
- ANEXO 5: Artículo científico: Higuichi, R, Komori, T. Structures of compounds derived from the acyl moieties of Quillajasaponin.
- ANEXO 6: Artículo científico: Ronnberg, B. Effects of carbohydrate modification of Quillaja saponaria Molina QH-B fraction on adjuvant activity, cholesterol-binding capacity and toxicity.
- ANEXO 7: Estructura química de QS 21. www.chemicalbook.com

ANEXO 1

Pfizer

23-25 avenue du Docteur Lannelongue
F-75668 Paris Cedex 14
Direct Line: +33 1 58 07 43 52
Direct Fax: +33 1 70 70 72 37
Email: ~IPGSParis@pfizer.com



European Patent Office,
Postbus 5818
NL-2280 HV Rijswijk
THE NETHERLANDS

VIA EPOLINE

28 March 2012

Our Reference: **PC32251A-EP**

Re: European Patent Application No. 05718292.5 – 1222
Pfizer Products, Inc.
Declaration of Paul J. Dominowski, PhD

Dear Sirs,

I, Paul J. Dominowski, do declare as follows:

I hold the position of Senior Principal Scientist at Applicant's assignee, Pfizer Inc.

I received a B.S. degree in Microbiology and a B.S. degree in Public Health from the University of Wisconsin in 1991, a M.S. degree in Microbiology, Immunology and Preventive Medicine from Iowa State University in 1994, and a Ph.D. degree in Immunology from Western Michigan University in 2008.

I have been employed by Pfizer Inc. since 1997, specializing in the areas of Analytical and Chemistry and Drug Formulation. I am a co-inventor of European Patent Application No. 05718292.5 – 1222, which is the subject of the present proceedings. My research focus is drawn to the field of engaging the immune system to treat cancer and infectious diseases, either as a method of therapy or prophylaxis. My specialty has focused on developing immunomodulators and vaccine adjuvants designed to accomplish this task. I have areas of expertise that include vaccine design and formulation and vaccine adjuvant design.

The present Declaration under Oath is provided in regard of the subject matter disclosed in European Patent Application No. 05718292.5 – 1222.

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Sterols

Sterols are a family of polycyclic compounds. The nucleus of a sterol molecule, which is rigid and planar, is shown in Figure 1.

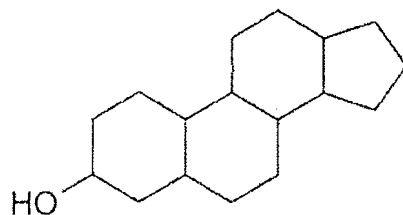


Figure 1. Sterol nucleus

The plant sterols are triterpenes with a tetracyclic cyclopenta[*a*]phenanthrene structure and a side chain at carbon 17. (http://lipidlibrary.aocs.org/lipids/plant_st/index.htm) As can be seen in Figure 2, the representative examples of sterols contain the sterol nucleus but have different hydrocarbon side chains.

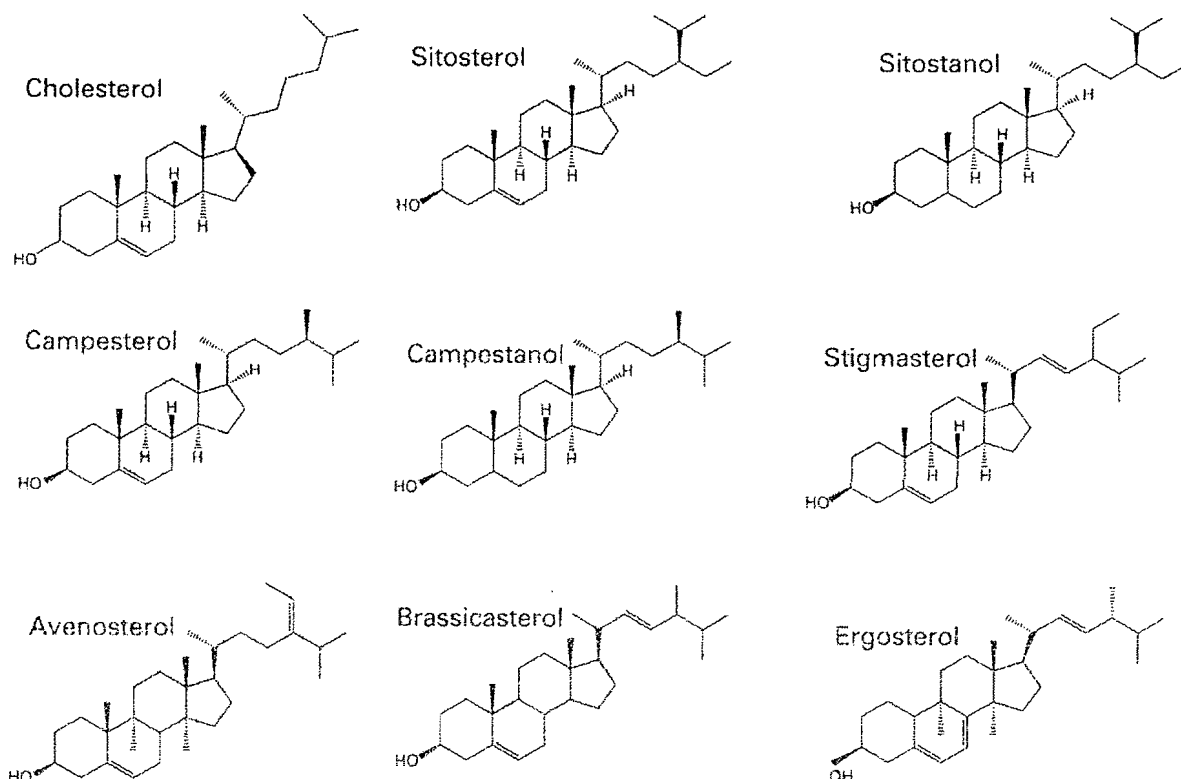


Figure 2. Structure of cholesterol and other sterols.

(From: Kidambi, *J. Clin. Pathol.*, 2008; Vol. 61:588-594)

(A)

Cholesterol is a fat-like bipolar (hydrophilic and hydrophobic) sterol (triterpene) of animal origin. It contains the four-ring sterol nucleus with a flexible hydrocarbon side chain at carbon 17. It is insoluble in water because of its hydrophobic component. It is considered a basic sterol because all steroids are made from cholesterol – hence the decision to use cholesterol as the “test sterol” in Example 21 of the present application as it would be predictable that other sterols would react with saponins in the same way.

Saponins

Saponins are a complex mixture of triterpenoids (modified triterpenes). They are glycosidic compounds that are produced as secondary metabolites in a wide variety of plant species. Structurally, saponins consist of any aglycone attached to one or more sugar chains. Saponins can be classified according to their aglycone composition: Triterpene glycosides, Steroid glycosides, and Steroid alkaloid glycosides (as per page 11, lines 8-10 of WO 2005/097181 A1).

Triterpenes are a class of terpenes consisting of six isoprene units and have the molecular formula $C_{30}H_{48}$. The isoprene units of triterpenes can be linear or rings. If in a ring structure, the rings are arranged in a four or five ring configuration of 30 carbons with several oxygens attached. Triterpenoids suitable for use in the adjuvant compositions can come from many sources, either plant-derived or synthetic equivalents, including but not limited to, *Quillaja saponaria*, tomatine, ginseng extracts, mushrooms, and alkaloid glycosides structurally similar to steroidal saponins. The structure of betulin, naturally-occurring triterpene, is shown in Figure 3.

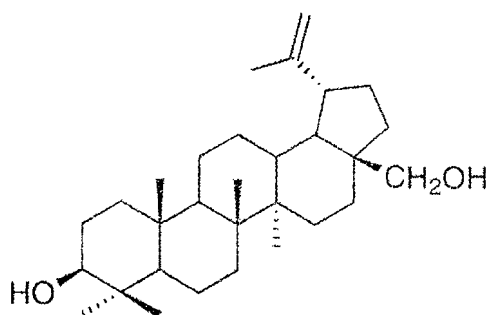


Figure 3. Structure of betulin

(From: <http://www.betulinines.com/index.php?page=triterpeny>)

Saponin (or triterpene) glycosides (or glycosidic triterpenes) reduce surface tension of water with foaming and will break down lipids. Usually triterpene saponins are designated as such by the suffix ending -side, such as ginsenoside, or astragaloside, named for the plant genera they were first discovered in. Some, such as the ginsenosides and eleutherosides are designated Rx where the suffix x = a, a1, b2, is indicative of the relative position of the saponin spots from top to bottom of a thin layer chromatogram. Another, common, example of a saponin glycoside is Quil A, the partially purified saponin fraction extracted from the bark of the South American tree

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Quillaja saponaria Molina in which the non-saponin components are removed. Quil A is a heterogeneous mixture of closely related saponins (more specifically, saponin glycosides). The components of Quil A differ mainly in the composition of their sugar chains (Higuchi and Komori, *Phytochemistry*, 1987; Vol. 26, No. 8:2357-2360). Quillaja saponins have a five-ringed quillaic acid backbone with two carbohydrate chains, consisting of two to five sugar units, attached to the 3' and 28' carbons of the quillaic acid molecule (Higuchi and Komori, 1987 and Rönnberg *et al.*, *Vaccine*, 1997; Vol. 15, No. 17/18:1820-1826; references from Demana *et al.*, *Int. J. Pharmaceutics*, 2007; Vol. 342, Issues 1-2:49-61). The structure of QS21 (a purified extract from Quil A) is shown in Figure 4.

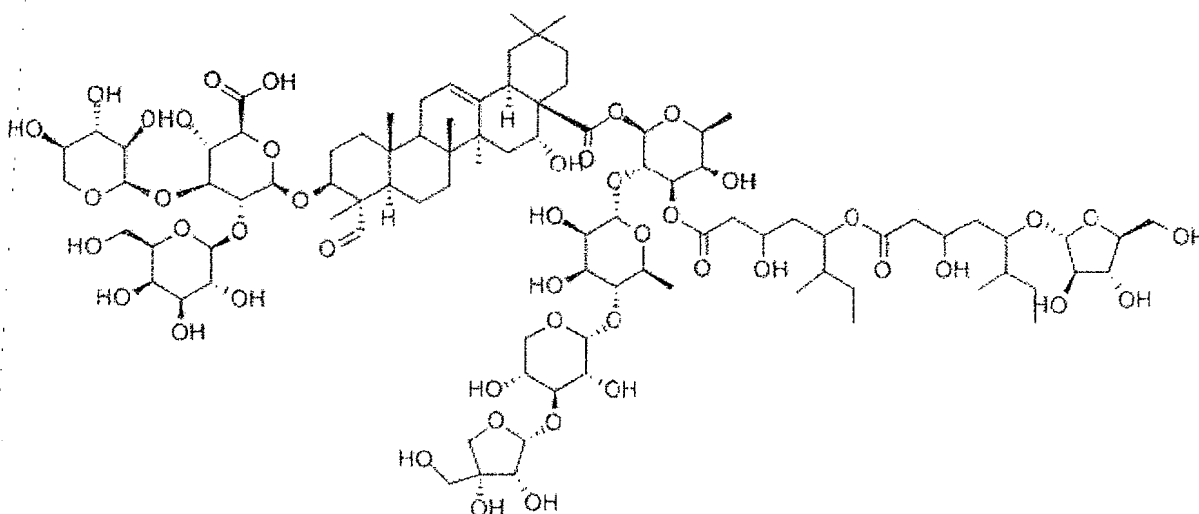


Figure 4. Structure of QS21

(From: http://www.chemicalbook.com/ChemicalProductProperty_EN_CB21396474.htm)

Helical Micelles

Saponins are noted for forming colloidal solutions in water which foam on shaking, and for precipitating sterols (e.g. cholesterol) if the sterol is already in water because sterols are hydrophobic molecules. However, if a small amount of a sterol (e.g. cholesterol; see Example 21 of the present application) is slowly add to water already containing saponins (e.g. Quil A; see Example 21), the sterol and saponins interact with one another to form the helical micelles of claim 1 of the New Main Request of the present invention.

Rationale for Use of the Terms "Sterol" and "Saponin Glycoside"

Because sterols and saponin glycosides are both triterpenes, they have the same nucleus, as seen in Figure 1 (and the Figures 2-4). Three 6-carbon rings that are part of the nucleus of the sterol molecule interact with the same structure on the saponin glycoside molecule to form the helical micelles. Moreover, by doing so, this results in the elimination of some of the less

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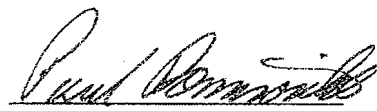
desirable effects of the saponin glycoside (such as severe local reactions, granulomas, and toxicity) without compromising its immunostimulatory capacity.

Cholesterol is a basic (simple) sterol. The side chain is uncomplicated. Saponin glycosides are available from several sources. Quil A is a heterogeneous mixture of closely related saponin glycosides and provides a rich source of the same. After discovering that Quil A and cholesterol could interact to form helical micelles, other sterols would predictably react with other saponin glycosides in the same manner. Minor differences in the structure of the sterol or the saponin glycoside will not affect the chemistry of the reaction.

I hereby certify that all statements made of my own knowledge are true and that all statements made on information and belief are believed to be true.

Dated: 28 March 2012

Respectfully submitted,



Paul J. Dominowski, PhD

ANEXO 2
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PAUL J. DOMINOWSKI

5743 Sandalwood Drive Apt. 1-B
Kalamazoo, MI 49048

pjdom2700@gmail.com
paul.j.dominowski@pfizer.com

Home: (908) 672-6539
Work: (269) 833-2775

EDUCATION

<i>Ph.D., Immunology</i> Western Michigan University, Kalamazoo, MI	2003 – 2008
<i>M.S., Microbiology, Immunology and Preventive Medicine</i> Iowa State University, Ames, IA (<i>continuing</i>)	1991 – 1994
<i>B.S., Microbiology</i> <i>B.S., Public Health</i> University of Wisconsin, Oshkosh, WI	1989 – 1991

RESEARCH FOCUS

Engaging the immune system to treat cancer and infectious diseases, either as a method of therapy or prophylaxis. My specialty has focused on developing immunomodulators and vaccine adjuvants designed to accomplish this task. Current work on animal models should be readily transferrable to human health applications. I am particularly strong in forming partnerships and collaborations toward the rapid develop and potential commercialize of drug candidates.

AREAS OF EXPERTISE

- | | |
|---|---|
| <ul style="list-style-type: none">• Vaccine design and formulation• Vaccine adjuvant design• Bioprocess engineering• Biopharmaceutical development• Cell culture and microbiology• Downstream Processing• Amorphous solids technology | <ul style="list-style-type: none">• Analytical Chemistry of metabolic functions• Manufacturing facility design• Leadership and team development• Establishment of strong national and international collaborations• Demonstrated ability to secure research funding |
|---|---|

PROFESSIONAL EXPERIENCE

PFIZER, INC. 1997 – Present

Senior Principle Scientist Analytical and Chemistry and Drug Formulation

- Responsible for the successful transfer of new products from development to production, and development and implementation of new processes and technologies to improve production.
- Vaccine research and development specifically pertaining to antigen selection, adjuvants and immunomodulators for use as therapeutic or prophylactic commercial products.
- Currently developing new vaccine formulations, stabilizers, cryopreservatives, and technology that will break the cold chain for vaccine storage. Additional work on nanotechnology drug delivery and microencapsulation and alternative dosage administration methods.
- Primary inventor for Pfizer leading vaccine adjuvant platforms such as “PreZent-A™”, “Procision™”, “Procision-DC™”, “Procision-RT™” and “Immulast™”; inventor of the Q-series configurable immunomodulator platform.

- Manage and perform all aspects of vaccine formulations; consultant for bioreactor cell culture, fermentation, downstream processing, process development, lyophilization, master seed production and operation of filling, stability, labeling, stability and scaling line for parenterals for USDA, EU, JP and FDA projects.
- Global Health Fellow 2010. Director of vaccine development and investment for Malawi, Kenya, Ethiopia, and ad hoc support to Republic of South Africa.
- Strong leadership skills: Served as senior member whose responsibilities were to serve as a key resource for adjuvant and immunomodulators selection, product assembly and sometimes as clinical study coordinator for project teams. Core member of the Adjuvant and Immunomodulator Research Team.
- Led the process development teams for such products as recombinant subunit vaccines, parasite vaccines, eradication, and marker vaccines.
- All project teams operated within a matrix organization structure and required significant coordination of clinical trials, research activities as well as governance bodies. Project team could be internal to the company or external with other research organizations.
- Strong collaborative skills: numerous projects underway with leading research universities, other pharmaceutical companies, the United States Government, and private research laboratories.

WESTERN MICHIGAN UNIVERSITY

2011 – Present

Adjunct Professor Vaccinology and Principles of Bioprocess Engineering

- Founder of Vaccinology and Principles of Bioprocess Engineering program.
- Instructor of graduate level classes in vaccinology and bioprocess engineering.
- Mentor of students at the Western Michigan University, and internal employees of Pfizer taking coursework or helping students seeking to learn additional information on vaccine development.
- Chairperson and organizer of the annual "Global Vaccinology Seminar Series."

MALLINCKRODT VETERINARY, INC., Mundelein, IL

1995 – 1997

Senior Associate Research Scientist

- Performed research and development of controlled release live viral vaccines and recombinant vectored vaccines.
- Company liaison responsible for process development and corporate technology exchange issues.
- Key company contact during the design, construction and validation of a new multi-use GMP manufacturing site specifically designed for the production of biological products. Ensured that the facility met global regulatory standards and was primarily responsible for equipment and design process issues.
- Successful experience in small and large-scale cell culture and fermentation to include upstream and downstream processing of biological products, the extraction and purification of membrane proteins and lyophilization and chemistry and bioprocess engineering.
- Exceptional skills in bioreactor/fermenter system design and operation to include bioprocess control strategies.
- Team member on MAFF (DEFRA) inspections of European pharmaceutical manufacturing facilities.

WYETH

1992 – 1995

Process Development Specialist

- Performed production strategy optimization and process design for biological manufacturing.
- Extensive use of skills in yield improvement, quality assurance (GMP and GLP standards), HPLC and spectroscopy, bioengineering, system design and chemistry.
- Research and implement new technology to the manufacturing process to reduce costs and improve production standards.
- Wrote standard operating procedures and manufacturing directions for new and existing products. Also wrote machine/instrument design specifications.

ABEL MANUFACTURING

1989 – 1992

Process Engineer

- Developed production processes for a global company involved in the fabrication of material handling machinery for the chemical, food, and agricultural industries.

- Designed new manufacturing facilities and redesigned existing facilities to meet the ever-changing needs of the company and the development of new products.

WR GRACE

1985 – 1989

Process Chemist

- Responsible for development and manufacturing of the solid phase used in HPLC on analytical and preparative scale systems.
- Developed a column to separate oligonucleotides, and cation and anion exchange columns. These were reverse phase columns for separating small molecules.
- Developed RP columns in the microbore range for very precision measurements and separations.

GRANTS

Cysticercosis - Provided an active drug development strategy for GALVmed and the Bill and Melinda Gates Foundation grant for introducing oxfendazole. This organization is working with a network of partners to develop and make available the tools which could eventually help to eradicate the diseases caused by *Taenia solium*, a tapeworm which can be transmitted between pigs and people in South America, India and South Asia.

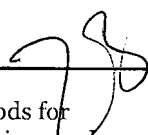
Capacity Building and Recommending Investment – Serving as a consultant and Director of Vaccine Development for GALVmed on a Bill and Melinda Gates Foundation grant designed to build biotechnology development and commercialization capacity as well as investment in biotechnology in African nations.

Bioprocess Expert for Rift Valley Vaccine Bank – Currently serving as a consultant to assist an NGO to establish a strategic stock of a newly commercialized monovalent Rift Valley fever C13 live vaccine that can be rapidly made available to and used in the event of an RVF outbreak, in partnership with Onderstepoort Biological Products, South Africa. This work is being funded by Bill and Melinda Gates Foundation.

Filarial Disease River Blindness - Assisting Michigan State and McGill Universities in developing the active drug flubendazole to treat this neglected disease in humans in rural African regions. My responsibility is researching and developing the proper formulation methods to render the active drug substance useable as either a patch or tablet. The success of my research led to the approval of the grant. This work is being funded by Bill and Melinda Gates Foundation and the project is being managed in Switzerland.

PATENTS

1. US20070196384 – Novel glycolipid adjuvant compositions. Compositions and methods of preparing stable adjuvant diluent stock solutions and final adjuvant solutions comprising glycolipids, weak acids, alcohols, nonionic.
2. US20060251674 – Formulations and process for production of *Bordetella bronchiseptica* P68 antigen and vaccines. Comprises new formulations and a process for making such formulations for vaccine compositions comprising a *Bordetella bronchiseptica* p68 antigen.
3. US20040258701 – Microfluidized oil-in-water emulsions and vaccine compositions. Provides submicron oil-in-water emulsions useful as a vaccine adjuvant for enhancing the immunogenicity of antigens; also provides vaccine compositions.
4. US20040081666 – Cattle reproductive disease vaccines. Relates to combination vaccines and methods for treating or preventing diseases or disorders in an animal caused by infection with Bovine Viral Diarrhea Virus (BVDV) Types 1 and 2.
5. US20050220814 – Microfluidized oil-in-water emulsions and vaccine compositions. Provides submicron oil-in-water emulsions useful as a vaccine adjuvant for enhancing the immunogenicity of antigens; also provides vaccine compositions.
6. US20040158195 – Needle-free mass injection device. Needle-free injection device including: a housing having a striker channel, a dose chamber, and a jet orifice; a syringe plunger movable in the dose chamber between reload and ejection positions.

- 
7. US20040185062 – Canine vaccines against Bordetella bronchiseptica. Relates to vaccines and methods for protecting dogs against disease caused by Bordetella bronchiseptica; also relates to combination vaccines and methods.
 8. US20070298053 – Cattle reproductive disease vaccines. Relates to combination vaccines and methods for treating or preventing diseases or disorders in an animal caused by infection with Bovine Viral Diarrhea Virus Types 1 and 2.
 9. US20080226670 – Canine combination vaccines. Relates to vaccines and methods for protecting dogs against disease caused by Bordetella bronchiseptica; also relates to combination vaccines and methods.
 10. 7122191 – Microfluidized oil-in-water emulsions and vaccine compositions. Provides submicron oil-in-water emulsions useful as a vaccine adjuvant for enhancing the immunogenicity of antigens; also provides vaccine compositions.
 11. (WO/2009/156960) Novel adjuvant compositions. Relates to adjuvant formulations comprising various combinations of triterpenoids, sterols, immunomodulators, polymers, and Th2 stimulators; methods for making the adjuvant compositions; the use of the adjuvant formulations in immunogenic and vaccine compositions with different antigens; and the use of the formulations in the treatment of animals.
 12. Application number as filed: 61349244. Antigen and immunomodulatory vaccines and cholesterol and uses thereof. Cholesterol can potentiate the activity of modulatory molecules and therefore the combination of cholesterol and immunomodulatory molecules can be used in the treatment and/or prevention of human and animal disorders. Vaccines comprising one or more antigens and cholesterol and vaccines comprising one or more antigens, one or more immunomodulatory molecules and cholesterol are described.
-

AWARDS AND HONORS

2000 – Pfizer Global Innovation Achievement Award for the development of a needle-free high duty-cycle injection device.

2009 – Individual Achievement award for the development of a globally deployed comprehensive vaccine adjuvant and immunomodulator database.

2009 – Pharmaceutical Discovery Team Award for adjuvant and immunomodulation research achievements.

2009 – Individual Performance Award for resolving problems with vaccine safety.

2010 – Individual Achievement award for the creative reformulation of the first Pfizer Animal Health biopharmaceutical product.

2012 –WHO Advisory Committee on Global Vaccine Safety Concerns (GVS) and Immunization, Vaccines and Biologicals (IBV)

PUBLICATIONS

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LANGUAGES

I have a working knowledge of the following languages:

- French
- Amharic
- Swahili
- Mandarin

25

Sitosterolaemia: pathophysiology, clinical presentation and laboratory diagnosis

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Accepted 7 December 2007

ABSTRACT

Sitosterolaemia is an extremely rare autosomal recessive disease, the key feature of which is the impairment of pathways that normally prevent absorption and retention of non-cholesterol sterols, for example plant sterols and shellfish sterols. The clinical manifestations are akin to familial hypercholesterolaemia (such as presence of tendon xanthomas and premature atherosclerosis), but with "normal to moderately elevated" cholesterol levels. The gene(s) causing sitosterolaemia was mapped to the STSL locus on human chromosome 2p21, and mutations in either of the two genes that comprise this locus, ABCG5 or ABCG8, cause this disease. Exact prevalence is unknown, but there are estimated to be 80–100 cases around the world. This rare disease has shed light into the molecular mechanisms that control sterol trafficking in the enterocyte and hepatocyte; ABCG5 and ABCG8 heterodimerise to form a sterol efflux transporter in the liver and intestine. In this review the pathophysiology, clinical manifestations and approach to clinical and laboratory diagnosis of this disease are described.

different from that of the vertebrates. In plants, two separate pathways can be used to synthesise the C5 isoprenes (isopentenyl diphosphate and dimethylallyl diphosphate) that are used for sterol (and isoprenoid) synthesis, the cytosolic mevalonate (MVA) pathway or the plastidal 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway (vertebrates use only the MVA pathway). It appears that there are multiple genes encoding each enzyme; the significance of this is not clear.⁸ Post-lanosterol (with the characteristic A, B, C and D rings), the sterol synthesis pathway differs from vertebrates in that the "R" tail contains more than 8 carbon atoms, resulting in sterols that have more than 27 carbon atoms, with varying levels of reduced and oxidised C–H bonds. This results in a panoply of sterols species (>20) in plants. Each species of plant may have varying levels of each of these different species. In vertebrates, cholesterol is the major sterol synthesised.

Bhattacharyya and Connor reported a new disease in two Caucasian sisters who were referred to them for tendon xanthomas dating back to their childhood. Surprisingly, plasma cholesterol in these individuals was in the normal range, unlike in familial hypercholesterolaemia—the most common cause of tendon xanthomas. Astuteness of these physicians led to the discovery of high levels of plant sterols (specifically β -sitosterol) in these sisters, and to the description of a new disease named " β -sitosterolaemia".¹

PATHOPHYSIOLOGY

Familiarity with the normal metabolism of plant sterols is essential before understanding the disease of sitosterolaemia.

Chemistry of plant sterols

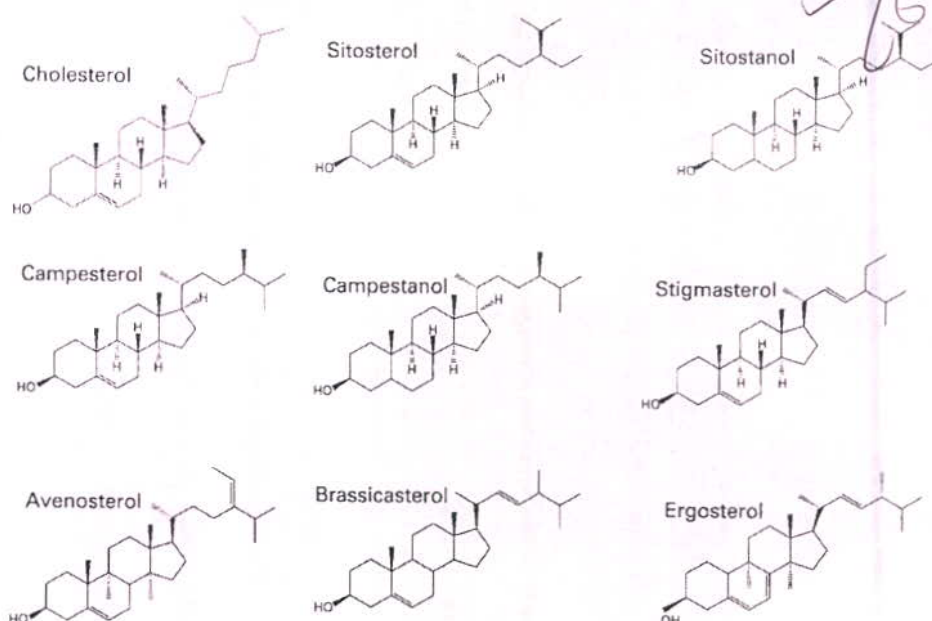
The term plant sterol constitutes both phytosterols and phytostanols. Phytosterols are closely related in structure to cholesterol (which is not found in any abundance in plants), differing mainly in the side-chain configuration (fig 1). The most common plant sterols include sitosterol, campesterol and stigmasterol, but more than 20 other non-cholesterol sterols occur naturally.⁷ Human beings cannot synthesise plant sterols; hence the only source of plant sterols present in them comes from the diet. Phytostanols have a saturated B ring (the mammalian body can convert sterols to stanols, but usually can not metabolise these plant sterols any further). To produce a wide variety of sterols, plants have evolved a biosynthetic pathway

Plant sterol metabolism

A typical western diet contains 150–400 mg of plant sterols per day, which is in the same range as dietary cholesterol intake.^{9–11} Usually the net absorption of plant sterols is <5%, compared to an average of 55% for cholesterol.^{10–15} Each sterol species has its own rate of absorption; in general, the closer the structural similarity to the cholesterol, the higher the absorption and the greater the desaturation of the structure (the ring or the R tail), the poorer the absorption. In addition to the "first-pass" poor absorption in the intestine, the liver rapidly and preferentially excretes these sterols into the bile. Both these processes ensure a very low level of net retention of non-cholesterol sterols,¹⁶ resulting in total plasma plant sterol levels of <1 mg/dl^{17–19} compared to 200 mg/dl for cholesterol. It should also be pointed out that non-cholesterol sterols can come from non-plant sources, such as from shellfish etc, and these are also excluded in a similar manner.³

It was unclear until recently how the vertebrate gastrointestinal tract and liver differentiated structurally similar compounds such as cholesterol and plant sterols, although it was known that plant sterols were poorly absorbed and their presence decreased intestinal cholesterol uptake, suggesting that their pathways did intersect.^{19–21} Earlier proposed mechanisms focused on understanding how plant sterols interfered with cholesterol uptake. Plant sterols, being more hydrophobic than cholesterol, were thought to compete with cholesterol for micelle formation, thus resulting in reduced cholesterol solubilisation and intestinal uptake.^{22–25} In addition, the small amounts of plant

Figure 1 Structures of plant sterols and stanols compared to cholesterol. Plant sterols have the same squalene ring nucleus as cholesterol, but differ mainly in the side-chain configuration. All plant sterols have a side-chain of variable length at C24. In addition, sitostanol and campestanol lack a double bond between C5 and C6 compared to their unsaturated counterparts; stigmasterol has a double bond between C22 and C23; avenosterol has a double bond between C24 and C28; brassicasterol has a double bond between C22 and C23; and ergosterol differs by the presence of a double bond between C7 and C8.



sterols that were absorbed did not seem to be subjected to the same metabolism as cholesterol (esterification, storage, or metabolised to bile salts or steroid hormones), but were preferentially excreted into bile by the liver.²⁶ They are transported via the same lipoproteins and there is no evidence at this time if the presence of higher amounts of plant sterols in these lipoproteins alters their function in any fashion. Non-cholesterol sterols can be hydroxylated at the 5 α position (leading to the formation of 5 α stanols). The majority of the absorbed non-cholesterol sterols are largely excreted unchanged in the bile.²⁷

One of the early mechanisms attributed to this poor absorption of plant sterols was the high specificity of the esterification enzyme, ACAT-2, in the enterocyte for cholesterol, thus leaving phytosterols unesterified and barring their entry into the chylomicrons.²⁸

The observation that patients with sitosterolaemia had higher absorption and circulating levels of non-cholesterol sterols suggested that these individuals lack the natural ability to diminish the uptake of non-cholesterol sterols in the gut and fail to excrete non-cholesterol sterols into their bile.¹ This genetic disorder pointed towards the existence of a gene that was critical for intestinal sterol absorption and discrimination and for biliary sterol excretion. In 1998, the sitosterolaemia locus, *STSL*, was mapped by Patel *et al* to human chromosome 2p21.²⁹ This locus comprises two adjacent and highly homologous genes, *ABCG5* and *ABCG8*, encoding two intestinal sterol half-transporters, sterolin-1 and sterolin-2; mutations in either lead to the disease.⁴⁻⁶ *ABCG5* and *ABCG8* are thought to act as an obligate heterodimers, based on the fact that complete mutations on either gene can cause sitosterolaemia; this is supported by in vitro data.^{4-6,30-31}

Structure and function of ABCG5 and ABCG8

Sterolin-1 (*ABCG5*) and sterolin-2 (*ABCG8*) belong to the ATP-binding cassette (ABC) transporter superfamily and the "C" sub-family. They localise to the apical membrane of the enterocyte and the canalicular membrane of the biliary tract.³⁰⁻³² Each *ABCG5* and *ABCG8* gene is composed of 13 exons; they are arranged in a head-to-head configuration on

chromosome 2p21, with about 140 bases separating their first exons³³ (fig 2). Their genomic organisation suggests a gene duplication event during evolution. There is a higher prevalence of polymorphisms in *ABCG8*, compared to *ABCG5*, despite its close proximity and homology with *ABCG5*. A number of studies have implicated this locus in disease or physiological processes, other than sitosterolaemia, ranging from gallstone formation, lipoprotein kinetics, cholesterol absorption and obesity to response to drug therapy.³⁴⁻⁴⁵ Indeed, the remarkable conservation of the *STSL* locus between species as diverse as fish, amphibians, rodents and humans seems to indicate that the polymorphic changes may have a more dramatic effect on function.⁴⁶ Sitosterolaemia as well as other human studies have confirmed that sterol absorption is genetically regulated.^{35-39,40,47}

ABCG5 and *ABCG8* proteins contain six transmembrane domains and one ATP-binding domain each. Once expressed, they travel as heterodimers to the apical membrane where they form full, active transporters and require ATP to function. On the cell surface, they likely promote flopping of sterols from the inner to the outer leaflets of the plasma membrane.⁴⁸

Animal and human studies have demonstrated the role of *ABCG5* and *ABCG8* in regulating net plant sterol and cholesterol absorption and elimination from the body.⁴⁹ In the enterocyte, they promote efflux of plant sterols back into the intestine, resulting in very low net absorption (<5%), and to some extent may play a role in excreting cholesterol.^{2,50,51} In the liver, they promote the excretion of sterols (cholesterol and plant sterols) into the bile.⁵²⁻⁵⁵ In mice deficient in *Abcg5* or *Abcg8*, biliary sterols are very low and do not increase, despite forced excretion.^{52,56,57} In man, it appears that the liver may be a more important organ than the gut for keeping the levels of plant sterols low; a patient with sitosterolaemia underwent a liver transplantation, and following this, his plant sterol levels almost normalised despite his genetic defect in the intestine.⁵⁸ Thus, *ABCG5* and *ABCG8* may selectively "pump" non-cholesterol sterols out of the intestinal cells as a first-line defence to dietary input, and the sterols that escape the enterocyte and get absorbed are pumped out by hepatic canalicular *ABCG5* and *ABCG8*. Moreover, in the fasting state, the liver can continually pump sterols (cholesterol and

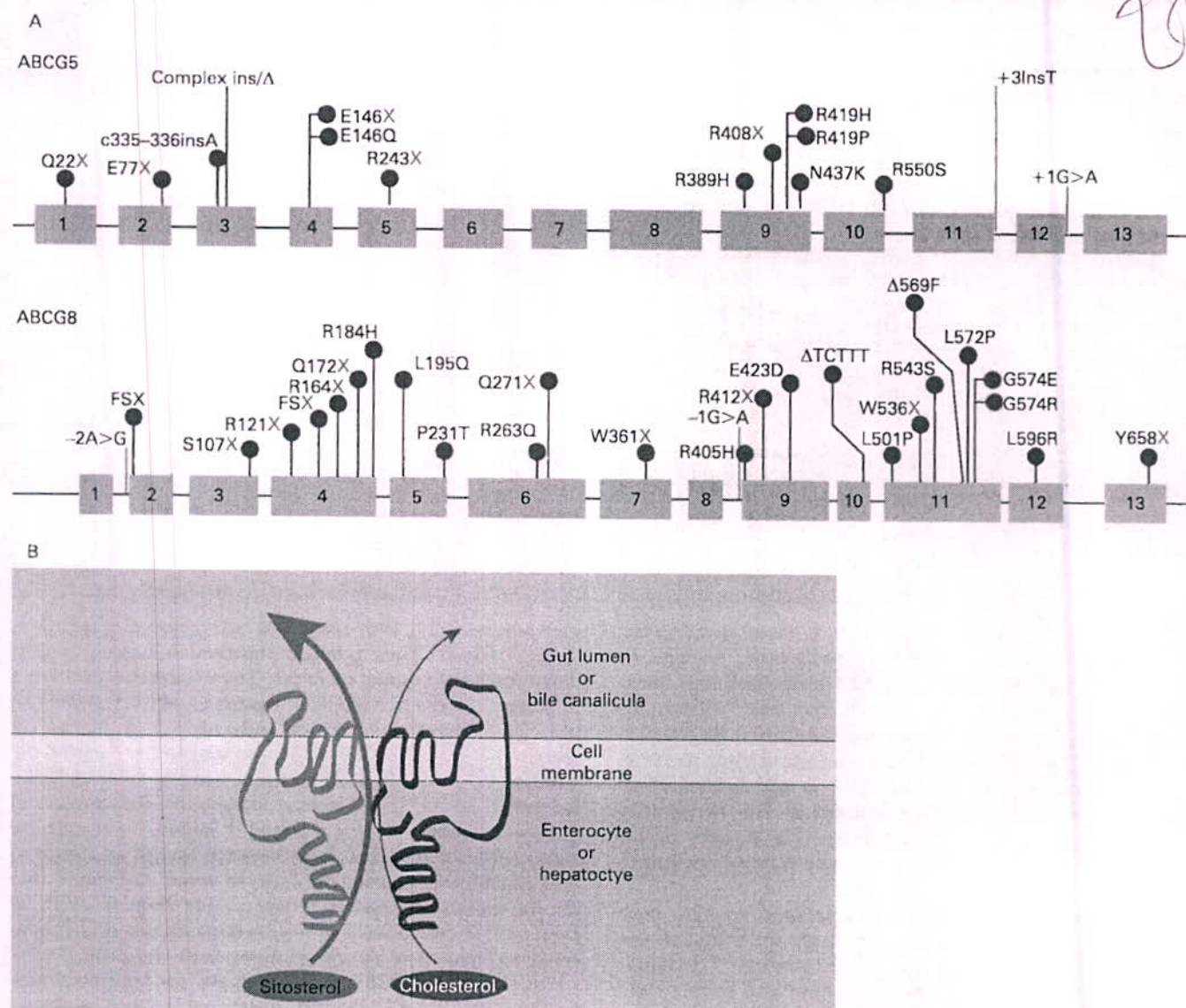


Figure 2 Gene structure and putative model for action for ABCG5 and ABCG8. (A) ABCG5 and ABCG8 gene structure. Each comprise 13 exons (rectangles) separated by 12 introns. Introns and exons are not shown to exact size. Known mutations are indicated on the top of each gene structure. (B) Model of how ABCG5 and ABCG8 heterodimer may function at the luminal surface; current evidence indicates that these pumps show a preference for non-cholesterol sterols, but in their absence they will "flip" cholesterol into the lumen of the intestine or biliary channels for ultimate excretion through the stools.

non-cholesterol sterols) into bile and thus maintain a low non-cholesterol sterol level in the body.⁵⁰

Regulation of ABCG5 and ABCG8

Liver X receptor (LXR) is a transcriptional factor that increases ABCG5 and ABCG8 gene expression on the enterocyte border, thus decreasing the intestinal cholesterol absorption.⁵⁹ LXR activation restores sterol secretion in ABCG5 knockout mice which normally produce a sterol-poor bile,⁵⁷ suggesting again an important role of LXR. More recently, ABCG5 and ABCG8 expression has been shown to be regulated by HNF-4.⁶⁰

Shortly after identification of ABCG5 and ABCG8, Nieman Pick C1-like-1 protein (NPC1L1) was identified to be the sterol transporter that is responsible for uptake of all sterols (cholesterol and plant sterols) into the enterocyte in the proximal small intestine.⁶¹⁻⁶² However, they differ in their post-uptake path, with plant sterols predominantly being expelled back into the

enterocyte lumen via sterolin pumps, whereas dietary cholesterol is esterified and incorporated into chylomicrons for secretion into the lymph at the basolateral surface.

There remain several gaps in our understanding of these pumps; for example, the exact mechanism of how these pumps recognise plant sterols and secrete them back into the lumen is still not known. One hypothesis argues that ABCG5/ABCG8 may act as "extruders", exposing sterols in the outer leaflet of the membrane for facilitated extraction into the lumen by sterol acceptors,⁶³ such as bile acid:phospholipid complexes, whereas others have proposed that sterolins may act as "flippases", akin to the flipping of phospholipids from the inner to the outer leaflet of the apical membranes.⁶⁴ It is not known under what circumstances sterolins start excreting cholesterol and which transcriptional factors, other than LXR and HNF4α, control this process. In addition, are sterols the only substrate for sterolins, and if not, what are the other substrates that interact with

these pumps? Studies in mice deficient in ABCG5 and ABCG8 show that some sterol secretion continues and suggest that genes other than ABCG5/ABCG8 can control the absorption of cholesterol and plasma plant sterol levels.⁶⁵

Clinical features

The genetic disease of sitosterolaemia attests to the role of ABCG5 and ABCG8 in keeping non-cholesterol sterols (plant sterols as well as shellfish sterols, etc) out of the human body. Studies on sitosterolaemic patients reveal a 3–12-fold increase in plant sterol absorption, a 30–100-fold increase in plasma plant sterol levels, and a 13–100-fold increase in body plant sterol pool size due to chronic increase in absorption and decrease in excretion.^{66–68} In the plasma, these sterols are transported, much like cholesterol, in all the lipoprotein sub-fractions, including high density lipoprotein (HDL), low density lipoprotein (LDL), and very low density lipoprotein (VLDL) cholesterol.¹

Clinical presentation may vary from the presence of tendon xanthomas, atherosclerosis and its complications to a milder phenotype with very few specific symptoms or signs. In their original description of the disease, Bhattacharyya and Connor highlighted the presence of tendon and tuberous xanthomas due to increased absorption of sitosterol from the diet, resulting in increased plant sterols in the plasma, lipoproteins, red blood cells, xanthomas, adipose tissue and skin surface.¹ Subsequently, several new clinical features have been described (box 1).^{69–74}

Tendon xanthomatosis is similar to that observed in patients with homozygous familial hypercholesterolaemia. Xanthomas in sitosterolaemia have been reported as early as 8 years of age and usually involve the Achilles tendon, though any tendon can be involved.

Premature atherosclerosis, and early onset myocardial infarction and requirement for coronary revascularisation procedures^{17, 68} as early as 16 years of age have been reported.^{72–75–78} The reason for premature cardiovascular disease is unclear in patients with sitosterolaemia. It could be because of elevated cholesterol levels (cholesterol absorption is also high and its excretion in bile is low, similar to plant sterol levels), or it might be a direct result of elevated plant sterol concentrations. It is not clear whether mildly elevated plant sterols, as seen in some normal subjects, are responsible for cardiovascular disease, with some studies showing neutral effect, some showing elevated risk and some even suggesting a protective effect; the jury is still out on this issue.^{79–83} It seems entirely plausible that plant sterols are associated with cardiovascular disease. Pathological examination of the xanthomas and arteries of sitosterolaemia patients shows that they contain plant sterols.⁷² Plant sterols seem to form xanthomas, even at lower plasma levels (30–40 mg/dl), whereas cholesterol levels >400 mg/dl are needed to induce tendon xanthomas in familial hypercholesterolaemia. Perhaps some species of non-cholesterol sterols (or their metabolites) are more effective in formation of foam cells and plaque formation.⁶² These concepts need more investigation.

Box 1: Symptoms and signs in sitosterolaemia

- ▶ Arthritis
- ▶ Arthralgias
- ▶ Premature atherosclerosis and cardiovascular disease
- ▶ Haemolysis
- ▶ Thrombocytopenia
- ▶ Hypersplenism

The full clinical spectrum of this disease is probably not known due to under diagnosis of the condition. It is important to recognise that there may be few or no clinical signs to suggest a person has sitosterolaemia; sometimes haemolysis may be the only presenting feature.⁷⁹ In addition, patients may just present with atherosclerotic disease (a common clinical finding) but no other clues to suggest one should consider sitosterolaemia as a diagnosis. Thus alerting the medical community may lead to an "increase" in prevalence.

Moreover, sitosterolaemia may be mistaken for familial hypercholesterolaemia (FH) or cerebrotendinous xanthomatosis (CTX). Sitosterolaemia is differentiated from FH merely due to the presence of normal cholesterol levels. CTX may also present with tendon xanthomas and premature cardiovascular disease; however, other clinical features of CTX, such as premature cataracts and neurological symptoms, will help to differentiate it from sitosterolaemia. The key test is the analysis of blood by GC or HPLC for different sterol species.

GENETIC ASPECTS OF SITOSTEROLAEMIA

Since it is an autosomal recessive disease, it is necessary to have mutations in both the alleles of either ABCG5 or ABCG8. Parents of sitosterolaemic patients (heterozygotes) have no biochemical phenotype under physiological conditions, although they have half-normal functioning sterolins. However, serum plant sterol concentrations are increased minimally when a diet enriched in plant sterols is consumed, indicating that the capacity to prevent accumulation of non-cholesterol sterols may not be rate-limiting.⁸⁴ Several mutations have been reported and provide important insights in to the nature of the inheritance. Founder effects for mutations underlie many of the cases, suggesting that this disease has been present for many generations, perhaps more than 4000 years.^{85–86} Caucasians seem to carry mutations in ABCG8, whereas Chinese, Japanese and Indian (20% of known cases) patients seem to have mutations in ABCG5.⁵ We do not know the prevalence of heterozygous mutations in the community, and whether this would confer any selective advantage.

LABORATORY DIAGNOSIS

The plant sterols that are elevated in sitosterolaemia are mainly sitosterol and campesterol (since these are the most abundant plant sterols in the diet), but stigmasterol, avenasterol, brassicasterol, campestanol, sitostanol, and shellfish sterols can also be increased, based on dietary intake.^{1, 3, 87–90} Cholesterol forms only 80% of the total plasma sterols in patients with sitosterolaemia.¹⁷ Other notable findings include variable cholesterol levels (normal or mild to moderately elevated plasma cholesterol levels), thrombocytopenia, chronic haemolytic anaemia and elevated liver enzymes in some patients.

Disease is suspected in patients with either tendon or tuberous xanthomas, premature cardiovascular disease associated with normal cholesterol levels or in those with unexplained haemolysis. Plant sterol levels in normal or heterozygotes should not be >1 mg/dl as opposed to >20–30 mg/dl in the patients with sitosterolaemia. Some heterozygotes were found to have higher than normal levels of plant sterols; but levels were still substantially lower than in homozygotes.⁹¹

Measurement of phytosterols

Usually enzymatic or colorimetric methods are used to quantify sterols. The colorimetric assay depends on the double bond between carbons 5 and 6; the enzymatic method uses

"cholesterol" oxidase that detects the 3β hydroxyl group. These methods cannot differentiate between cholesterol and plant sterols¹⁷⁻²² (fig 1). To detect plant sterols and their stanols, gas chromatography (GC) or high performance liquid chromatography (HPLC) are needed. However, to detect normal physiological concentrations (0.05–0.5 mg/dl range), even more sensitive analytical methods are necessary. Although gas chromatography–mass spectrometry (GC–MS) and HPLC remain the mainstay, more rapid throughput technologies are now available, such as electron-spray liquid chromatography–mass spectrometry (LC–MS), or atmospheric pressure photo-ionisation combined with liquid chromatography–tandem mass spectrometry (LC–MS/MS) which could make it possible to rapidly screen a much larger population for this condition.⁹³ It should also be pointed out that sterols are very stable and can be measured from serum stored for a long time.

This condition is active from birth. However, it is not clear whether early neonatal screening for this disease will be effective. Plant sterol levels in the plasma are highly dependent on diet, and if the diet does not contain plant sterols, the levels in the plasma can be predicted to be low in neonates and infants. Presumably, plant sterols may only accumulate after the child has been exposed to a diet containing plant sterols (either as artificial milk or semi-solid food). Screening is still possible in a sibling of a patient with sitosterolaemia, but should be undertaken much later than the neonatal period.

The key element in the diagnosis for this disease is, therefore, ordering the correct test. To date, no other medical conditions result in elevation of plant sterols; thus detection of high levels of plant sterols is pathognomonic for this disease. Genetic testing can also be attempted after a biochemical diagnosis has been established.

TREATMENT

Even though there is impairment of both intestinal and hepatic elimination pathways in patients with sitosterolaemia, it is sufficient to interrupt one of the pathways to decrease the plant sterol levels.⁹⁴ Ezetimibe binds to NPC1L1, which is a sterol transporter in the proximal intestine, and blocks uptake and reuptake of sterols, resulting in lower concentrations of both cholesterol and plant sterols. Ezetimibe leads to a marked improvement in plasma sterol concentrations, regression of xanthomatosis and resolution of cardiovascular disease in patients with sitosterolaemia.⁶⁹ Recently, hepatic transplantation in a sitosterolaemic patient resulted in complete normalisation of plant sterol levels. Thus even if intestinal sterol uptake goes unchecked, the absorbed plant sterols can be eliminated completely by the restored ABCG5 and ABCG8 in the liver.⁹⁵ Before the discovery of ezetimibe, sitosterolaemia was treated with bile-acid binding resins,⁹⁵ with fair results.

Restriction of dietary plant sterols may also lower the plasma plant sterol levels. However, studies on the efficacy of this treatment are based on a few patients and not in universal agreement, in part because it is very difficult to not consume plant-based foods.⁹⁶⁻⁹⁷

PLANT STEROLS/STANOLS AS TREATMENT FOR HYPERCHOLESTEROLAEMIA

The hypocholesterolaemic effect of plant sterols have been known for more than 50 years and they have been used as an add-on therapy.⁹⁸⁻¹⁰⁰ ATP III guidelines have also recommended the addition of plant sterols/stanols (2 g/day) as a part of the initial therapeutic lifestyle change for hypercholesterolaemia.¹⁰¹

Take-home messages

- ▶ Sitosterolaemia is a rare genetic disease associated with high plant sterol levels; it manifests with tendon xanthomas, premature cardiovascular disease, arthralgias and occasionally unexplained haemolysis.
- ▶ ABCG5 and ABCG8 encode two half-transporters which normally expel plant sterols into the gut and biliary lumen; homozygous mutations in either one of these two genes result in impairment of normal mechanisms that keep plant sterol levels low in the human body.
- ▶ Elevated plant sterol levels in the range 25–30 mg/dl (normal <1 mg/dl) is pathognomonic for diagnosis of sitosterolaemia.
- ▶ The usual methods employed to estimate cholesterol levels cannot differentiate between cholesterol and plant sterols. GC–MS or HPLC are necessary to measure plant sterol levels.

As already indicated, plant sterols decrease plasma cholesterol by interfering with their absorption. However, we need to keep in mind that this universal recommendation cannot be applied to patients with sitosterolaemia due to high plant sterol absorption and their potential pathogenicity. Additionally, in subjects with heterozygous mutations (such as the obligate carriers), such diets also led to an increase in plant sterols. Given the rarity of this condition, the use of plant sterol fortified foods is unlikely to be harmful to the majority of people.

Even though several questions remain unanswered, sitosterolaemia is one of the monogenic diseases, study of which has not only helped the patients who suffer from it but also contributed to the greater understanding of sterol metabolism. Greater awareness among cardiologists, to whom the patients with premature coronary atherosclerosis present, haematologists, to whom patients with unexplained haemolysis present, and general internists and pathologists who help in their initial evaluation and diagnosis, is essential for prompt diagnosis and effective treatment.

Competing interests: None declared.

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ANEXA.



Intro Products Services Demand Triterpenes About us Workroom Contact

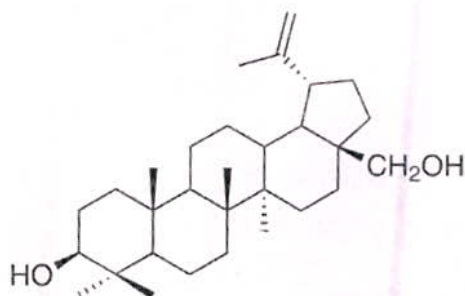
Triterpenes

Triterpenes as well as mono-, sesqui-, di-, sester- a and tetra- are part of extensive group of natural compounds - terpenoids, which are wide spread not only in plants but in few cases in marine organisms, moulds, microorganisms or rarely in fungi.^{1,2} Terpenoids with steroids constitute very wide area of isoprenoids. Basic building block of all isoprenoids is isoprene (2-methylbuta-1,3-diene). Biological precursor of triterpenes is squalene. Monoterpenes (two building blocks) - eg. camphor, borneol, menthol, pinene, limonene, carvone, citroneol, iasmon, terpinene, terpineole, geraniol and in part also sesquiterpenes (3 building blocks) - eg. ionones, methylionones, irone, nerolidole, farnesole, cedrole, caryophyllen are natural fragrant principles,³ they constitute major part of etheric oils and resins. Sester-, tetra- and triterpenes on the other hand are mostly solids in natures either free or as esters or glycosides.

Until now more than 30 000 terpenoids was isolated from which there are 4000 triterpenoids derived from more than 40 skeletons^{1,2,4}. Range of biological activities of triterpenoids is incredibly broad eg. antimicrobial (fusidic and helvolic acid), antifeedant (radermasinin), antimycotic (trianthenol), antiviral (derivatives of betulinic acid), antiinflammatory (derivatives of bosswellic acid, platycodin D, betulin, lupeol), antitumor effects (betulinic, bosswellic, pomolic acid), anticariogenic (oleanolic, glycyrrhetic acid), antiangiogenic, antiulcerogenic (glycyrrhetic, ursolic and oleanolic acid), antiallergic (oleanolic acid), hepatoprotective (lupeol, ursolic, oleanolic acid), tonizing activity (24-hydroxytormentic and bosswellic acid) and many other.

Some of the before mentioned are well known for long time from their usage in the oriental or folk medicine⁵ (for example bark of Brazilian tree *Ocotea suaveolens* is used in ingenious medicine for pain relief, as tonicum and for asthma treatment)⁶, so their pharmaceutic potential is used from very small part.

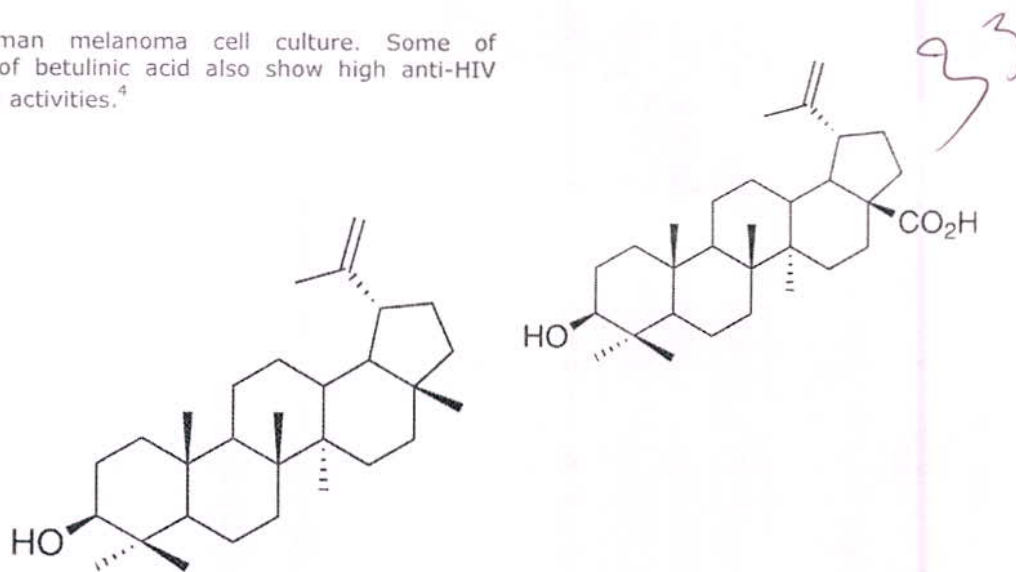
Betulin is natural triterpenic alcohol (lup-20(29)-en-3 β ,28-diol), which is widespread in nature, mainly in the birch bark (*Betula sp.*)⁷, from which comes its name. Betuline was one of the first triterpenoids to be isolated from natural source by Löwitz in 1788 by sublimation from birch bark as pure chemical substance.⁷ Then it was obtained by extraction of the outer layers of birch bark by ethanol. I took several decades before its structure could be determined. White colour of birch bark is mainly caused by betulin, which gives birch bark specific properties. For example birch bark never rot or mould which is caused by the anti-mould and antibacterial properties of betulin. Big question is why birch synthesises betulin and deposit it into its bark. Probably it is natural agent against pests and microorganisms. Betulin and its derivatives however posses other biological effects, eg. antiinflammatory, antiviral, anti-HIV, hepatoprotective and others.⁴



Betulinic acid is natural pentacyclic acid (3 β -hydroxylup-20(29)-en-28-oic acid), also widespread in nature. First isolated from *Vauquelinia corymbosa*,⁸ and also from african bush *Ziziphus mauritiana* Lam. (Rhamnaceae)⁹, also present in Plate bark (*Platanus hispanica*)¹⁰. Betulinic acid was studied in more detail than betulin, mainly due to its selective antitumor activity

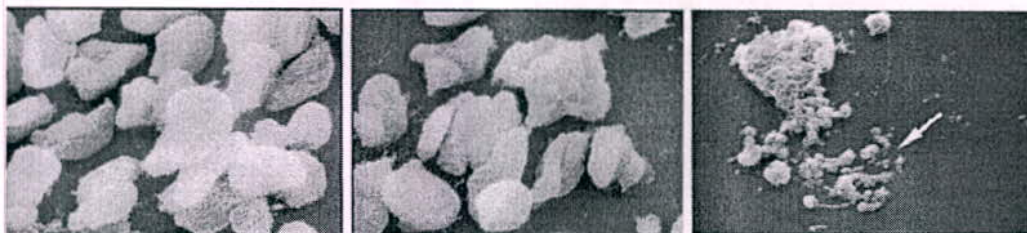
against human melanoma cell culture. Some of derivatives of betulinic acid also show high anti-HIV and antiviral activities.⁴

Lupeol
is natural



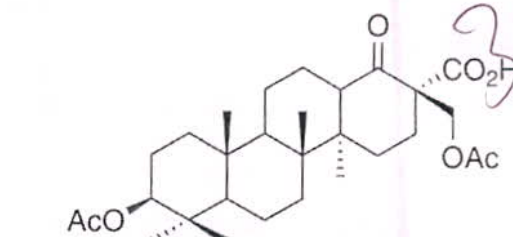
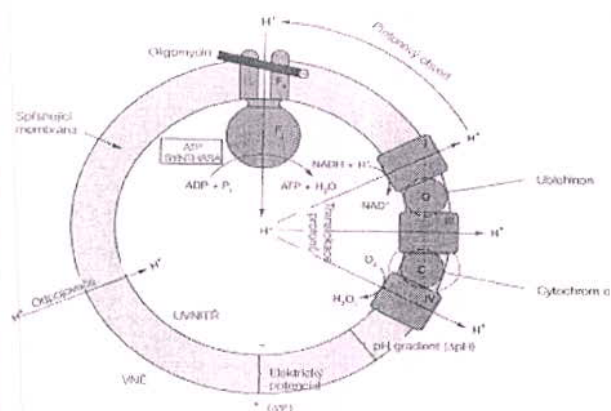
pentacyclic triterpene alcohol (lup-20(29)-en-3 β -ol), present in the number of herbs⁴ or fruit (eg. apples, mango), and also accompanying betulin. Lupeol shows some chemopreventive, cytotoxic and antifeedant properties⁴, and also direct antioxidant effects and is potential drug against free radical inflicted diseases⁴. Among other activities are antiangiogenic effect (from 30 μ g/ml)⁴.

Betulinines¹¹ represents group of triterpenoids prepared by Jan Šarek in 1999-2001 in the Natural compounds research group, Department of organic chemistry, Faculty of science, Charles university. The compounds, derived from lupane, des-E-lupane and 18 α -oleanane skeletons, possess, between many others, significant cytotoxic activity ($IC_{50} < 5 \mu$ mol/l). In contrast to longest known cytotoxic triterpenoid - betulinic acid - antitumor activity is not restricted to cell lines of melanoma (meL-2). Betulinines possess broad range of antitumor activity against many cancer cell lines of different histogenetic origin including multidrug resistant¹¹, eg. A549, Saos2, BT549, DU145, OVCAR-3, K562, U2OS, CEM and so on. This effectivity reaches as far as p53 negative cells, pRb negative cells regardless of their histogenetic origin, with low toxicity against non-cancer cells.¹¹ From the morphologic analysis tissue cell culture with most effective compound - betaketoacid (3 β ,28-diacetoxy-19,20,21,29,30-pentanorlupan-22-oic acid), which show on the most cell lines cytotoxicity lower than 1 μ mol/l, arose conclusion that mechanism of action is apoptosis (programmed cell death), see next picture.¹¹



Study of apoptosis induction with electron microscopy on leukemia cell line CEM: from left - control sample, incubated with betulinic acid and betaketoacid. Typical morphology of the cells after 24 hour of incubation. Arrow points to the morphologic evidence of apoptosis: blebbing of the cytoplasmic membrane, fragmentation of cells and formation of apoptotic corpuscle (zoom 3.600x).

Flow cytometry studies shown that betaketoacid induce very fast apoptosis, even faster than with paclitaxel, one of the most effective conventional cytostatics. According to current knowledge apoptosis is induced by activation of caspase cascade by release of cytochrome c or AIF (apoptosis inducing factor). Betaketoacid is causing block in G1/S phase of cell cycle. According to latest experiments this compound possesses unique ability to block last step of respiratory chain and subsequently start production of peroxide radical (see picture). Blocking is probably caused due to direct conjugation of betaketoacid with cytochrome c. Primary molecular target is therefore localised in mitochondria.¹²



Principle of chemiosmotic theory of oxidative phosphorylation. F1, F0 - subdomain complexes involved in phosphorylation; proton cycle is closed by oxidation and translocation of proton from within membrane by the respiratory complexes I, III, IV, which works as proton pump; attack of betaketoacid into respiratory chain and production of peroxide is in red.

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STRUCTURES OF COMPOUNDS DERIVED FROM THE ACYL MOIETIES OF QUILLAJASAPONIN

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(Received 5 December 1986)

Key Word Index—*Quillaja saponaria*; Rosaceae; Quillaja Bark; Quillajasaponin; 3,5-dihydroxy-6-methyl-octanoic acid; 3,5-dihydroxy-6-methyl-octanoic acid 5-*O*-glycosides.

Abstracts—Three compounds, an acid and two glycosidic acids, derived from the acyl moieties of quillajasaponin were obtained from the alkaline hydrolysate of the saponin mixture. On the basis of chemical and spectral evidence, they were identified as 3,5-dihydroxy-6-methyl-octanoic acid, 3,5-dihydroxy-6-methyl-octanoic acid 5-*O*- α -L-arabinofuranoside and 5-*O*- α -L-rhamnopyranosyl-(1 $\rightarrow$ 2)- α -L-arabinofuranoside. The first compound was, in fact, isolated and characterized as its lactone form. FDMS was useful in providing information on the *M_r* of the unique acid and glycosidic acids.

INTRODUCTION

A physiologically active triterpenoid saponin mixture (so-called quillajasaponin) obtained from the bark of *Quillaja saponaria* is a mixture of acylated triterpenoid oligoglycosides (acylated saponins). The isolation and the structure elucidation has been reported [1] of the two desacylsaponins (quillaic acid 3,28-*O*-bisglycosides) obtained, together with less polar compounds (eliminated acyl groups), by mild alkaline hydrolysis of the quillajasaponin. In this paper we wish to report the isolation and structure determination of the eliminated acyl groups, compounds 1, 2 and 3, originating from the acyl moieties of quillajasaponin.

RESULTS AND DISCUSSIONS

A mixture of the less polar compounds obtained as described in the preceding paper [1], showing three spots on thin-layer chromatography due to the eliminated acyl groups (compounds 1, 2 and 3), was separated by normal phase (silica gel) column chromatography as shown in the experimental section to give compounds 2, 3 and 4. Compound 1 was converted into 4 during the separation by the column chromatography.

Compound 4 revealed the IR absorptions due to hydroxy and carbonyl groups and was assigned the molecular formulae $C_9H_{16}O_3$ by taking the FD and EI mass spectral data into account, while the 1H and ^{13}C NMR (Table 1) spectra suggested the presence of one primary methyl, one secondary methyl, three methylenes, one methine, two oxygen-bearing methines and one estercarbonyl group.

Compound 4 was acetylated to afford the monoacetate 5. Since 4 contained two degrees of unsaturation, these data suggested 4 to be a monocyclic compound possessing hydroxy and ester functionalities. The characteristic fragment peak at m/z 57 [C_4H_9] $^+$ in the EI mass spectrum and

Table 1. ^{13}C NMR spectral data (C_2D_2N) for compounds 2, 3 and 4

C	4*	2	3	Reference compounds
1	171.9 s	177.7 s	175.4 s	
2	39.6 t ^a	44.8 t ^b	44.3 t ^c	
3	63.8 d	66.0 d	65.7 d	
4	34.8 t ^a	39.3 t ^b	39.4 t ^c	
5	80.8 d	78.6 d	78.7 d	
6	38.9 d	38.6 d	38.7 d	
7	25.0 t	24.9 t	24.9 t	
8	11.6 q	12.3 q	12.4 q	
6-Me	13.9 q	14.9 q	15.1 q	
1'		108.8 d	106.5 d	[A] 110.5 d
2'		83.4 d	88.3 d	83.4 d
3'		78.2 d	77.1 d	78.6 d
4'		85.7 d	85.0 d	85.5 d
5'		62.9 t	62.2 t	62.7 t
1''			100.9 d	[R] 102.6 d
2''			72.4 d ^d	72.1 d
3''			72.5 d ^d	72.7 d
4''			73.8 d	73.8 d
5''			70.1 d	69.5 d
6''			18.5 q	18.6 q

*in $CDCl_3$, ^{a,b,c,d}: Assignments may be reversed in each vertical column.

[A]: Methyl α -L-arabinofuranoside.

[R]: Methyl α -L-rhamnopyranoside.

the ^{13}C NMR signals (δ 11.6, 13.9, 25.0, 38.9) [2] of 4 indicated the existence of partial structure A (1-methyl-propyl group).

Detailed analysis of the 1H NMR spectrum of the acetate 5, including spin-decoupling experiments, implied

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the presence of the partial structure B in 4. Irradiation of H-3 (proton next to acetoxy group) at $\delta 5.19$, which was assigned by comparison of the ^1H NMR spectra of 4 and 5, collapsed two dd (H_2 -2) at $\delta 2.54$ and 2.95 to two d (each $J = 17$ Hz) indicating C-2 was adjacent to a quaternary carbon(carbonyl group), and also collapsed two ddd (H_2 -4) at $\delta 1.68$ and 2.30 to two dd . Irradiation of H-5 (proton next to ester group) at $\delta 4.14$ collapsed two ddd , due to H_2 -4, to two dd . The partial structure B was also supported by a fragment peak at m/z 115 [$\text{C}_5\text{H}_7\text{O}_3$] $^+$ in the EI mass spectrum of 4. These data established the structure of compound 4 as a monohydroxy δ -lactone.

Since 4 was an artefact of 1 and it gave 1 by alkaline treatment, compound 1, the genuine eliminated acyl group, must be 3,5-dihydroxy-6-methyloctanoic acid though it was not isolated as its acid form.

Compound 2 was hydrolysed with acid to yield compounds 1, 4 and arabinose and revealed in the FD mass spectrum the molecular ion peak as a cationized cluster ion, [$\text{M}(\text{C}_{14}\text{H}_{26}\text{O}_8) + \text{Na}$] $^+$ at m/z 345. In the ^{13}C NMR spectrum the signals due to one anomeric carbon and a carboxylic acid were observed at $\delta 108.8$ and 177.7 , respectively. The above data indicate that compound 2 is the monoarabinoside of 1. Comparison of the ^{13}C NMR spectrum of 2 with those of 4 (artefact of 1) and methyl arabinosides (Table 1) indicated the existence of an α -arabinofuranosyl moiety in 2. Consideration of the glycosylation [3, 4] and esterification [5, 6] shifts revealed that the 5-hydroxy group was glycosylated. The location and the configuration of the arabinose was confirmed as follows.

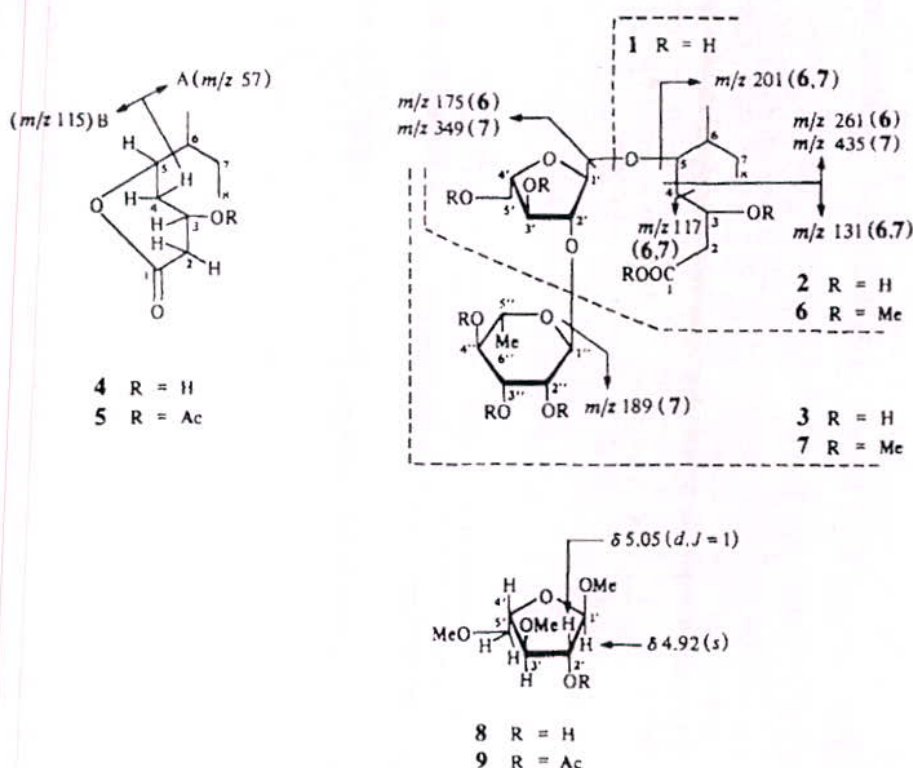
When 2 was methylated by the method of ref. [7] it produced the permethylate 6 which afforded methyl 2,3,5-

tri-*O*-methyl-arabinofuranoside upon methanolysis. The EI mass spectrum of 6 gave characteristic fragment peaks (m/z 261, 131, 117) due to the cleavage shown in Scheme 1, thus indicating the site of linkage of the arabinose together with the peaks originating from the terminal permethylated pentose (m/z 175) [8] and aglycone residue (m/z 201). The α -configuration was verified by comparison of the anomeric proton signal ($\delta 5.08$, s) in the ^1H NMR spectrum of 6 with those of methyl furanosides of 2,3,5-tri-*O*-methyl- α - and β -arabinose [9].

Consequently, if arabinose is assumed to be the most commonly found L-series, compound 2 is 3,5-dihydroxy-6-methyl-octanoic acid 5-*O*- α -L-arabinofuranoside.

Compound 3 gave, on acid hydrolysis, 1, 4, arabinose and rhamnose, and showed in the FD mass spectrum the molecular ion peaks at m/z 469 and 491 as a cationized cluster ion, [$\text{M}(\text{C}_{20}\text{H}_{36}\text{O}_{12}) + \text{H}$] $^+$ and [$\text{M} + \text{Na}$] $^+$. In the ^{13}C NMR spectrum two anomeric carbon signals were observed at $\delta 106.5$ and 100.9 . Therefore, compound 3 is regarded to consist of one mol. each of 1, arabinose and rhamnose. The FD mass spectrum of 3, further, revealed the peak at m/z 345, [$\text{M} + \text{Na} - 146$] $^+$, derived from the elimination of a terminal rhamnose residue [10]. In the ^{13}C NMR spectrum signals due to aglycone moieties of 2 and 3 showed good agreement (Table 1). The above data indicated 3 was the 5-*O*-rhamnosyl-arabinoside of 1, and the structure of the sugar moiety was determined as follows.

When the ^{13}C NMR spectrum of 3 was compared with those of 2 and methyl rhamnosides, the glycosylation shift was observed on the signals at C-1', C-2' and C-3' of α -arabinofuranose in 3 and the α -pyranose form of a rhamnose moiety in 3 was also indicated (Table 1).



Permethylation of **3** followed by methanolysis of the product (**7**) afforded methyl 2,3,4-tri-*O*-methyl-rhamnopyranoside and **8**. Compound **8** was assigned by the ^1H NMR spectrum of its acetate (**9**) as methyl 3,5-di-*O*-methyl-arabinofuranoside. Since the α -configuration of rhamnose and arabinose units was confirmed by their anomeric proton signals, δ 4.94 (doublet, $J = 2\text{ Hz}$) and 5.12 (singlet) [9, 11], in the ^1H NMR spectrum of **7**, the sugar moiety of **3** was assignable to be the α -rhamnopyranosyl-(1 $\rightarrow$ 2)- α -arabinofuranoside.

Accordingly, compound **3** is 3,5-dihydroxy-6-methyl-octanoic acid 5-*O*- α -L-rhamnopyranosyl-(1 $\rightarrow$ 2)- α -L-arabinofuranoside because rhamnose is also usually found as the L-series in natural products.

Investigations concerning the stereochemistry of **4** and the aglycone moiety of **2** and **3** are in progress, but, **2** and **3** are, to the authors' knowledge, novel natural glycosides. For example the aglycone moiety is especially unique in that it is the first of C_9 natural carboxylic acid and the sugar moiety of **3** is also unique in that arabinofuranose, which is usually located at a terminal, in this case is located at the internal position of the glycoside.

It is assumed that compounds **1**, **2** and **3** are combined with desacylsaponins which were described in the preceding paper [1] to form the respective parent acylated saponin, quillajasaponin.

EXPERIMENTAL

For general methods, except for those described below, refer to the preceding paper [1]. Solvent systems for TLC (silica gel and Avicel): (a) CHCl_3 -MeOH- H_2O (7:3:0.3), (b) *n*-hexane-EtOAc (1:1), (c) upper layer of *n*-BuOH-pyridine- H_2O (6:2:3) + pyridine (1). Conditions of GLC (FID mode): glass column (1.2 m $\times$ 3 mm) packed with 10% 1,4-butanediol succinate on shimalite W (60-80 mesh), column temp. 135°. IR spectra were run as CCl_4 soln.

Isolation of compounds 4, 2 and 3. A mixture of less polar compounds (eliminated acyl groups) (2.2 g), obtained from quillajasaponin by mild alkaline hydrolysis [1] and showing three major spots (R_f 0.39, 0.23, 0.10) on TLC (silica gel; solvent a), was chromatographed on silica gel (eluant; CHCl_3 -MeOH- H_2O , 8:2:0.2 $\rightarrow$ 7:3:0.3) to give three fractions, fraction 1 (R_f 0.90), 2 (R_f 0.23) and 3 (R_f 0.10). Each fraction was passed through a Sephadex LH-20 column (eluant; MeOH) to give compound **4** (105 mg), **2** (107 mg) and **3** (169 mg), respectively.

Compound 4. Colourless syrup, $[\alpha]_D^{25} + 39.5^\circ$ (MeOH; c 1.15). IR $\nu_{\text{max}}^{\text{CCl}_4}$ cm^{-1} : 3600 (OH), 1725 (ester). FDMS m/z : 173 [$\text{M}(\text{C}_9\text{H}_{16}\text{O}_3) + \text{H}$] $^+$. EIMS m/z : 154.1009 [$\text{M} - \text{H}_2\text{O}$] $^+$, calcd for $\text{C}_9\text{H}_{14}\text{O}_2$: 154.0994 (16%), 115.0391 [$\text{C}_4\text{H}_9\text{O}_3$] $^+$, calcd for $\text{C}_5\text{H}_9\text{O}_3$: 115.0393 (100%), 57 [C_4H_9] $^+$ (45%). ^1H NMR (CDCl_3): δ 0.92 (3H, t , $J = 6\text{ Hz}$, Me), 0.97 (3H, d , $J = 6\text{ Hz}$, Me), 4.16 (2H, m , H-3 and H-5). ^{13}C NMR (CDCl_3): Table 1.

Monoacetate 5 of compound 4. Compound **4** (21 mg) was acetylated with Ac_2O (1.5 ml) pyridine (1.5 ml) at 70° for 30 min and the reaction mixture was diluted with H_2O and extracted with CHCl_3 . The CHCl_3 layer was washed, dried and evaporated. The residue was passed through a silica gel column (eluant *n*-hexane-EtOAc, 1:1) to give an acetate (**6**) (15 mg) as a colourless syrup. IR $\nu_{\text{max}}^{\text{CCl}_4}$ cm^{-1} : no OH, 1740 (ester). ^1H NMR (CDCl_3): δ 0.93 (3H, t , $J = 6\text{ Hz}$, Me), 0.98 (3H, d , $J = 6\text{ Hz}$, Me), 2.08 (3H, s , OAc), 1.68 (1H, ddd , $J = 14, 12, 9\text{ Hz}$, H-4), 2.30 (1H, ddd , $J = 14, 7, 3\text{ Hz}$, H-4), 2.54 (1H, dd , $J = 17, 7\text{ Hz}$, H-2), 2.95 (1H, dd , $J = 17, 7\text{ Hz}$, H-2), 4.14 (1H, ddd , $J = 12, 4, 3\text{ Hz}$, H-5), 5.19 (1H, $dddd$, $J = 9, 7, 7, 7\text{ Hz}$, H-3). Irradiation of δ 5.19: δ 1.68 (dd , $J = 14, 12\text{ Hz}$), 2.30 (dd , $J = 14, 3\text{ Hz}$), 2.54 (d , $J = 17\text{ Hz}$), 2.95 (d , J

$= 17\text{ Hz}$). Irradiation of δ 4.14: δ 1.68 (dd , $J = 14, 9\text{ Hz}$), 2.30 (dd , $J = 14, 7\text{ Hz}$).

Compound 2. Amorphous hygroscopic powder, $[\alpha]_D^{25} - 82^\circ$ (MeOH; c 0.87). FDMS m/z : 345 [$\text{M}(\text{C}_{14}\text{H}_{26}\text{O}_8) + \text{Na}$] $^+$. ^{13}C NMR ($\text{C}_5\text{D}_5\text{N}$): Table 1. Compound **2** was heated with 1 N H_2SO_4 for 1 hr at 70° and diluted with H_2O . The reaction mixture was extracted with Et_2O and the Et_2O layer was washed, dried and evaporated to give crude aglycone fraction in which **4** and **1** were detected by TLC (silica gel, solvent a). The H_2O layer was neutralized with $\text{Ba}(\text{OH})_2$ soln, filtered and the filtrate was concentrated. The residue was examined by TLC (Avicel, solvent b), and arabinose was detected.

Permethylate 6 of compound 2 [7] and its methanolysis. Compound **2** (47 mg) was treated with NaH (250 mg) and MeI (7 ml) in DMSO (10 ml). The reaction mixture was diluted with H_2O and extracted with CHCl_3 , and the CHCl_3 layer was washed, dried and evapd. The residue was chromatographed on silica gel (eluant, *n*-hexane-EtOAc, 2:1) to give **6** (23 mg) as a colourless syrup. IR $\nu_{\text{max}}^{\text{CCl}_4}$ cm^{-1} : no OH, 1740 (ester). FDMS m/z : 392 [$\text{M}(\text{C}_{19}\text{H}_{36}\text{O}_8) + \text{H}$] $^+$. EIMS m/z (rel. int.): 261 (7), 201 (66), 175 (100), 131 (12), 117 (17). ^1H NMR (CDCl_3): δ 0.8-1.0 (6H, m , Me $\times$ 2), 1.4-1.9 (5H, m , H-4, H-6, H-7), 2.53 (2H, d , $J = 6\text{ Hz}$, H-2), 3.38 (12H, s , OMe $\times$ 4), 3.68 (3H, s , COOMe), 5.08 (1H, s , anomeric H of arabinofuranose). Compound **6** was heated with 7% HCl in MeOH for 2 hr, the mixture was treated with Ag_2CO_3 and filtered. The filtrate was evapd and the residue (methanolysate) was examined by TLC (silica gel, solvent b) and GLC and methyl 2,3,5-tri-*O*-methyl-arabinofuranoside was detected (α -anomer, R_f 0.56, R_t 10.6 min; β -anomer, R_f 0.39, R_t 14.0 min).

Compound 3. Amorphous hygroscopic powder, $[\alpha]_D^{25} - 98.3^\circ$ (MeOH; c 0.58). FDMS m/z : 469 [$\text{M}(\text{C}_{20}\text{H}_{36}\text{O}_{12}) + \text{H}$] $^+$, 491 [$\text{M} + \text{Na}$] $^+$, 345 [$\text{M} + \text{Na} - 146$] $^+$. ^{13}C NMR ($\text{C}_5\text{D}_5\text{N}$): Table 1. On hydrolysis with acid under the same conditions as for **2**, compound **3** gave **4** and **1** (detected by TLC; silica gel, solvent a) and a sugar mixture. The sugar mixture was found to consist of arabinose and rhamnose (TLC; Avicel, solvent b).

Preparation of permethylate 7. Methylation of **3** (88 mg) in the same manner as for **2** afforded **7** (61 mg) as a colourless syrup. IR $\nu_{\text{max}}^{\text{CCl}_4}$ cm^{-1} : no OH, 1740 (ester). FDMS m/z : 567 [$\text{M}(\text{C}_{27}\text{H}_{50}\text{O}_{12}) + \text{H}$] $^+$. EIMS m/z (rel. int.): 435 (1), 349 (62), 201 (79), 189 (100), 131 (10), 117 (11). ^1H NMR (CDCl_3): δ 0.8-1.0 (6H, m , Me $\times$ 2), 1.27 (3H, d , $J = 6\text{ Hz}$, H-3'), 1.4-1.9 (5H, m , H-4, H-6, H-7), 2.53 (2H, d , $J = 6\text{ Hz}$, H-2), 3.37, 3.39, 3.42, 3.48, 3.50, 3.53 (each s , 3H, OMe $\times$ 6), 3.67 (3H, s , COOMe), 4.94 (1H, d , $J = 2\text{ Hz}$, anomeric H of rhamnose), 5.12 (1H, s , anomeric H of arabinofuranose).

Methanolysis of compound 7. Permethylate **7** (40 mg) was boiled with 10% HCl in MeOH for 2 hr and treated in the same manner as for **6**. The methanolysate was examined by TLC (silica gel, solvent b) and GLC and methyl 2,3,4-tri-*O*-methyl-rhamnopyranoside was detected (α -anomer, R_f 0.54, R_t 8.6 min). It was then chromatographed on silica gel (eluant, *n*-hexane-EtOAc, 1:1) to give **8** (colourless syrup) (R_f 0.20, silica gel, solvent b). Compound **8** yielded, by usual acetylation, a monoacetate **9** (colourless syrup). ^1H NMR (CDCl_3): δ 2.10 (3H, s , OAc), 3.40 (3H, s , OMe), 3.44 (6H, s , OMe $\times$ 2), 3.6 (3H, m , H-3', H-5'), 4.16 (1H, m , H-4'), 4.92 (1H, s , H-1'), 5.05 (1H, d , $J = 1\text{ Hz}$, H-2'). Irradiation of δ 5.05 simplified the signal at δ 3.6.

Acknowledgements—The authors are grateful to Emeritus Prof. T. Kawasaki of Kyushu University for helpful discussion and suggestion and to Mrs E. Onae (née Uemura) for technical assistance. Thanks are also due to Mr. I. Maetani, Mr A. Tanaka and Miss K. Soeda of the Faculty of Pharmaceutical Sciences, Kyushu University, for FD- and EIMS, ^{13}C NMR and ^1H NMR data, respectively.

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Effects of carbohydrate modification of *Quillaja saponaria* Molina QH-B fraction on adjuvant activity, cholesterol-binding capacity and toxicity

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The iscom is an efficient antigen-presenting system for various antigens inducing both MHC class I and class II restricted immune responses. Protective immunity has been evoked against a variety of infectious agents. The saponin adjuvant Quil A, which was originally used to form iscoms, is composed of a mixture of structurally similar triterpenoids from *Quillaja saponaria* Molina having different biological activities. A purified, toxic *Quillaja* triterpenoid fraction with strong adjuvant activity, designated QH-B, was used to study whether modification of the carbohydrate moiety with sodium periodate would alter the toxicity without harming adjuvant activity and cholesterol-binding capacity. Most sugars, and in particular Api, Gal and Xyl, were modified by periodate treatment with only minor changes of the molecular weights indicating no loss of sugar residues. The adjuvant activity of QH-B was reduced in a dose-related manner, and at a concentration of 25 mM sodium periodate a significant reduction in toxicity was observed. The differences in both toxicity and adjuvant activity of the periodate-treated QH-B could be derived from alterations in the structure of the sugars Gal and Xyl, while modification of Api may influence adjuvant activity but not toxicity in vivo. The cholesterol-binding capacity, a prerequisite for iscom formation, was not affected by periodate oxidation at the doses tested. However, the use of modified QH-B as described in the present study for iscom-matrix formation resulted in "saponin-lipid complexes" which, to a various degree or totally, deviated from the characteristic iscom morphology. © 1997 Elsevier Science Ltd.

Keywords: Quil A; *Quillaja saponaria*; carbohydrate modification; adjuvant activity; cholesterol binding activity; toxicity

A number of potentially interesting substances for use as adjuvants are saponins of various kinds. However, the toxicity of these substances has been the limiting factor for their use in vaccines for man and animals. To circumvent these obstacles, non-toxic saponins have to be selected fulfilling certain criteria. Alternatively,

identified saponins which are toxic, but otherwise desired, have to be modified to render them non-toxic. Saponins have been identified with strong immuno-enhancing capacity and low general toxicity, but they cause in free form strong local reactions at the site of injection mainly due to their lytic properties¹⁻³. We have developed a technique, based on the incorporation of the triterpenoid saponins into iscoms^{4,5}, which to a great extent blocks the lytic effect and thereby reduces the local reactions.

The iscom was created to combine an optimal immunogenic/physical presentation of antigens in a multimeric small particular form^{6,7} with a build-in adjuvant⁴. In a number of publications it has been shown that the iscom is an efficient adjuvant formulation (for a review see Cox and Coulter⁸, Höglund *et al.*⁹ and Morein *et al.*¹⁰). The unique part of the iscom is composed of triterpenoids from the bark of the tree

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Quillaja saponaria Molina. They consist of a triterpene, two branched carbohydrate chains with eight to ten monosaccharides in total¹¹. One important prerequisite for the iscom formation is the ability of the triterpenoids to bind cholesterol. The iscom particle is formed between triterpenoids firmly bound to cholesterol and an additional lipid, e.g. phosphatidylcholine and the amphipathic antigen⁵. If the antigen is omitted the particle is named iscom-matrix. A number of *Quillaja saponaria* Molina triterpenoids have been isolated² in the search for defined adjuvants for human use. However, for their use in iscom formulations three criteria have to be fulfilled: (i) the triterpenoids should bind cholesterol; (ii) have potent adjuvant activity; and (iii) they should be non-toxic. We have access to three chemically and functionally defined triterpenoid fractions named QH-A, QH-B, and QH-C¹. These fractions form iscom-matrix and iscoms in various combinations. A combination of QH-A and QH-C (ISCOPREP™ 703) in an influenza iscom vaccine formulation is currently under Phase 1 and 2 studies in man. QH-A has little adjuvant activity while QH-B and QH-C both have strong adjuvant activity. In mice QH-B is toxic at doses ranging between 50 and 200 µg, while QH-C, like QH-A, seems to be virtually non-toxic in these doses¹. Another purified *Quillaja* saponin designated QS21 was found to be tolerated at a dose of 100 µg when used as an immunological adjuvant in free form in a Phase 1 trial with a GM2 ganglioside-keyhole limpet haemocyanin conjugate vaccine in patients with malignant melanoma¹².

QH-B was selected because it is the most toxic of the triterpenoid fractions, which limits its use in man and animals. We formulated the present study to explore whether its toxicity could be altered by chemical modification with sodium periodate¹³ without harming the cholesterol-binding capacity and adjuvant activity. However, while the cholesterol-binding capacity was retained the adjuvant activity and toxicity decreased by sodium periodate treatment.

MATERIALS AND METHODS

Materials

Quillaja saponaria Molina triterpenoid QH-B¹ was obtained from Iscotec AB (Uppsala, Sweden). [³H]-cholesterol was purchased from Amersham. All other chemicals and reagents were obtained from Sigma, unless otherwise specified.

Cells

Seed stocks for WEHI 164 cells clone 13¹⁴ used for assaying intracellular dehydrogenase activity were the generous gift of Hans Wigzell, Karolinska Institute, Stockholm, Sweden. Cells were maintained in 75 cm² T-flasks (Costar) with the medium and serum supplement used by Espevik and Nissen-Meyer¹⁴.

Animals

Female mice of the NMRI strain, 18–20 g, were obtained from the National Veterinary Institute, Uppsala, Sweden and used for the adjuvant activity studies. Female ICR mice, 17–20 g (5 to 6 weeks old),

used for the toxicity studies, were obtained from Centers for Disease Control, Atlanta, GA, USA.

Periodate oxidation

QH-B (5 mg ml⁻¹) was treated with sodium periodate in 50 mM sodium acetate buffer, pH 4.5, at varying concentrations, ranging from 2.5 to 50 mM, by incubation for 1 h in the dark at 4°C. The oxidation was stopped by the addition of ethylene glycol. The samples were dialyzed against H₂O and kept lyophilized until used.

Thin layer chromatography

The effects of periodate oxidation on QH-B were demonstrated by thin layer chromatography on silica gel using the solvent system chloroform-methanol-water containing 0.2% CaCl₂ (4:4:1 by volume). 2% H₂SO₄ and 1% anisaldehyde in acetic acid with heating (5 min at 120°C) were used for detection.

Sugar analysis

Samples (2 mg) were hydrolyzed with 2 M trifluoroacetic acid at 120°C for 2 h, evaporated to dryness, dissolved in 1 ml 0.1 M ammonia and treated with 5 mg sodium borohydride for 2 h. The samples were treated with Dowex 50 (H⁺) ion exchange resin and the solutions were filtered and evaporated to dryness. To remove the formed boric acid, 2 ml of methanol was added and the solutions were concentrated to dryness; this procedure was repeated twice. The products were acetylated with acetic anhydride (0.5 ml) in pyridine (0.5 ml) at 100°C for 30 min, the solvents evaporated and the formed alditol acetates were analyzed by gas liquid chromatography-mass spectrometry (GLC-MS) using a HP-1 glass capillary column and a Hewlett Packard 5890 chromatograph connected to a Hewlett Packard 5970 mass selective detector. The alditol acetates were compared with authentic samples.

For analysis of apiose the samples were hydrolyzed with 2 M trifluoroacetic acid at 90°C for 30 min and then analyzed as described above.

NMR spectroscopy and mass spectrometry

For 400 MHz ¹H NMR spectra the samples (5 mg) were dissolved in deuteriomethanol and analyzed at 30°C on a Varian VXR 400 spectrometer. Matrix-assisted laser-desorption-ionization mass spectrometry (MALDI-TOF) was performed in the positive mode on an LDI-1700XS time-of-flight instrument using 2,5-dihydroxybenzoic acid as matrix.

Virus and virus antigen preparations

The influenza virus strain used was A/PR/8/34 (H1N1) (PR-8). The virus was propagated in 11-day-old embryonated hen eggs. The allantoic fluid was clarified by centrifugation and the virus was purified by density gradient centrifugation in sucrose¹⁵.

Preparation of micelles

Micelles were prepared to contain the haemagglutinin (HA) and neuraminidase (NA) proteins from

influenza virus as described previously¹⁵. Briefly, 10 mg of the influenza virus PR-8 was solubilized in 2% MEGA for 30 min at room temperature. The solubilized viral envelope proteins, HA and NA, were isolated by ultracentrifugation (30 min, 200 000 g, 20°C) into a sucrose layer (20% w/v sucrose containing 0.5% MEGA). The gradient was fractionated and the fractions were analyzed for protein content according to Bradford¹⁶. Micelles were prepared by removal of the detergent by dialysis in PBS.

Cholesterol-binding capacity

The capacity of periodate-modified QH-B to bind cholesterol was assessed by a dialysis method for preparation of iscom-matrix and subsequent analysis by sucrose gradient centrifugation and electron microscopy⁵. Briefly, 1 mg of cholesterol (containing tracer amounts of [³H]-cholesterol) and 1 mg of phosphatidylcholine in 2% MEGA were mixed with 5 mg of QH-B. The mixture was dialyzed extensively against PBS, first overnight at room temperature and then at 4°C. The mixture was purified by sedimentation through a sucrose gradient (10–50% sucrose in PBS) (TST 41.14 rotor, for 18 h at 39 000 rpm and 10°C). The gradient was collected in 15 fractions starting from the bottom of the tube. Each fraction was analyzed for radioactivity (cholesterol). The fractions containing the iscom-matrix were extensively dialyzed against PBS at 4°C and characterized by electron microscopy.

Hemolytic activity

The hemolytic activity of periodate-modified QH-B was measured in chicken red blood cells. The cells were washed with PBS and diluted to 0.5% in PBS. To 100 µl of this suspension, 100 µl of sample solution in PBS was added. The mixture was incubated at 37°C for 1 h and then centrifuged. The absorbance of the supernatant was measured at 414 nm in a Labsystems Multiskan MCC/340 microtiter plate reader.

Assay for intracellular dehydrogenase activity

The effect of periodate-modified QH-B on the intracellular dehydrogenase activity was determined by the MTT method¹⁷. MTT is a tetrazolium salt that is cleaved by mitochondrial dehydrogenases in living cells only to give a dark-blue product. Cells were seeded in 96-well tissue culture plates (Nunc) and incubated for 24 h. The cells (50 000 cells per well) were incubated for 2 h with different concentrations of modified QH-B. MTT solution (5 mg ml⁻¹ cell culture medium) was added, and the plates were incubated for another 90 min. The color was measured in a Labsystems Multiskan MCC/340 microtiter plate reader.

Toxicity in mice

To determine the maximum amount of periodate-modified QH-B tolerated by mice without local or general reactions, groups of 10 ICR female mice were inoculated subcutaneously with varying doses of modified QH-B dissolved in PBS. Inoculated mice were observed daily for 3 weeks for signs of sickness and possible development of local reactions at the site of inoculation. Time and number of deaths were

recorded and the lethal dose (LD₅₀) was calculated by the method of Reed and Muench¹⁸.

Statistical analysis

The differences between time to death among treatment groups were examined using Kaplan–Meier survival analysis¹⁹. Overall and in pairs, comparison was made for survival functions using the log rank test.

Immunization of mice

Groups of 10 NMRI mice were each immunized subcutaneously twice, 4 weeks apart, with 1 µg PR-8 envelope proteins in micelles supplemented with 10 µg QH-B treated with the sodium periodate concentrations indicated. One group of 10 mice received 1 µg PR-8 envelope proteins in micelles only and served as a non-adjuvanted control. Primary bleeds were at Day 21 and secondary bleeds at Day 42. Serum samples were analyzed for total antibody response in an ELISA.

ELISA

PR-8 micelles (100 µl) at 3 µg ml⁻¹ in 0.05 M sodium carbonate buffer, pH 9.6, were added to wells of microtiter plates (Nunc). Following overnight incubation at 4°C and washing with PBS containing 0.05% Tween-20 (PBS-T), the plates were incubated sequentially in the following reagents with intermediate washings in PBS-T: 100 µl of serial three-fold dilutions (commencing at 1:30) of sample mouse sera for 1 h at 37°C, 100 µl of 1:3000 dilutions of horseradish peroxidase-conjugated rabbit anti-mouse Ig (Dakopatts) for 1 h at 37°C, 200 µl substrate for 10 min at room temperature, followed by 50 µl 4.5 M H₂SO₄ to stop the enzyme reaction. The absorbance of plate wells was measured at 450 nm in a Labsystems Multiskan MCC/340 microtiter plate reader.

RESULTS

Initial experiments analyzed the effects of increasing sodium periodate concentrations on the chromatographic behavior of QH-B, indicating its suitability for a dose-dependent modification. The modified QH-B was analyzed for various parameters, i.e. degree of modification, cholesterol-binding capacity, hemolytic activity, cytotoxicity, *in vivo* toxicity, and immunoenhancing capacity.

Modification of QH-B with sodium periodate

Figure 1 shows the change in chromatographic behavior of modified QH-B, as determined by thin layer chromatography, following treatment with increasing molarities of sodium periodate. At a concentration of 2.5 mM sodium periodate an increased number of bands was observed, indicating chemical modifications of the QH-B structures. Excessive modification of QH-B was apparent at sodium periodate concentrations higher than 10 mM.

Chemical and spectroscopic characterization of periodate-modified QH-B

Treatment of a sugar with periodate will cleave the carbon–carbon bond between vicinal hydroxyl groups

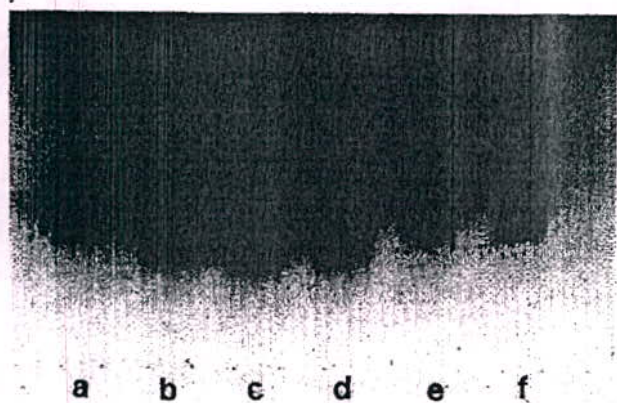


Figure 1 Thin layer chromatography pattern of QH-B after treatment with increasing concentrations of sodium periodate. QH-B was treated with (a) 0 mM, (b) 2.5 mM, (c) 5 mM, (d) 10 mM, (e) 25 mM, and (f) 50 mM sodium periodate

with the subsequent formation of two aldehyde groups. The latter can be involved in hemiacetal formation with adjacent hydroxyl groups and thus form new rings, but different from the original sugar. Sugars with vicinal hydroxyl groups are terminal or 1,2-, 1,4- and 1,6-linked sugars.

In order to identify the modifications of QH-B achieved by the periodate treatment, sugar analyses of the native and the periodate-treated saponins were performed. Samples with inositol as an internal standard were hydrolysed with aqueous 2 M trifluoroacetic acid, the released sugars converted to their alditol acetates by reduction with sodium borohydride and then acetylation. The alditol acetates were analyzed by GLC-MS and the amounts estimated relative to the internal standard (Figure 2). The results showed that Fuc and Ara were only marginally affected, Rha and Glc to approximately 60%, whereas most of the Xyl and Gal were destroyed. Apiose was rapidly oxidized and the oxidation was total even with the lowest concentration (2.5 mM) of sodium periodate (data not shown).

The modified QH-B was also analyzed by MALDI-TOF. This technique allows determination of the molecular weights of the saponins. The spectrum of the

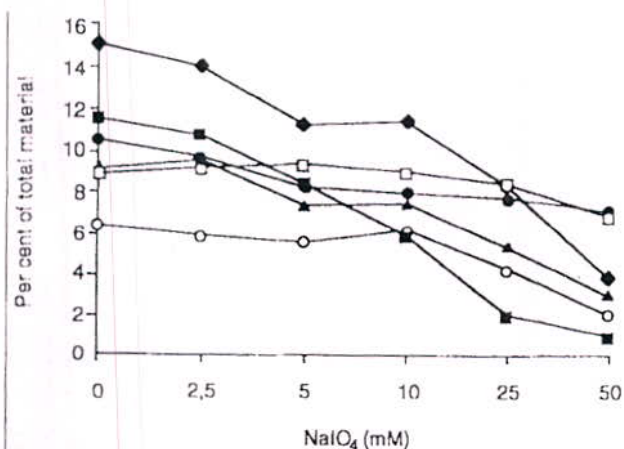


Figure 2 Sugar composition of native and periodate-treated QH-B expressed as a percentage of total material. The sugars were determined as their alditol acetates by GLC-MS. Gal (■); Glc (○); Fuc (□); Rha (▲); Ara (●); and Xyl (◆)

original saponin showed a mixture of components, with a different number of sugar residues in the oligosaccharide part of the saponin, as indicated from the ions around m/z 2170, 2040 and 1906 (Figure 3). Each of these three peaks consisted of several peaks separated by 14 Da, indicating a mixture of saponins with a different number of pentoses and 6-deoxyhexoses (a molecular weight difference of 14).

In the spectra of the periodate-treated materials the peaks were broader, showing an even more complex mixture of modified saponins. However, no loss of sugar residues or any other part of the molecule was observed since the peaks in the spectra were observed at approximately the same masses.

The ¹H NMR spectra of the samples showed several signals for anomeric protons (data not shown). These signals decreased in spectra of the periodate-treated samples showing that the ring for several of the sugars was opened due to the oxidation of vicinal hydroxyl groups.

Cholesterol-binding capacity

An important property of *Quillaja* triterpenoids is the binding to cholesterol for the formation of iscomatrix and iscom. The ability of periodate-modified QH-B to bind cholesterol was analyzed by sucrose gradient centrifugation and electron microscopy. Non-bound cholesterol will remain at the top of the gradient, while cholesterol bound to the *Quillaja* triterpenoids sediments to the lower part of the gradient⁵. QH-B modified with all five sodium periodate concentrations were able to bind cholesterol (Figure 4) but did not form characteristic iscom-matrix (data not shown). Instead, aggregated "saponin-lipid complexes" were formed.

Hemolytic activity

The hemolytic activity of periodate-modified QH-B was studied. A decrease of the hemolytic activity was seen with QH-B, following treatment with increasing molarities of sodium periodate (Figure 5). A concentration of approximately 5 $\mu\text{g ml}^{-1}$ of untreated QH-B is required to obtain an absorbance of 0.5 at 414 nm, while as much as 40 $\mu\text{g ml}^{-1}$ is needed for QH-B treated with 50 mM sodium periodate.

Effect on intracellular dehydrogenase activity

The effect of periodate-modified QH-B on the viability of WEHI cells was studied using the MTT method. This assay is based on mitochondrial dehydrogenase conversion of the yellow dye MTT to a purple formazan product as a measurement of the metabolic activity of living cells. As can be seen in Figure 6, a decrease in cytotoxicity of QH-B is found in WEHI cells, following treatment of QH-B with increasing molarities of sodium periodate, compared with untreated control cells. Unmodified QH-B causes a 50% inhibition of enzyme activity at a concentration of approximately 5 $\mu\text{g ml}^{-1}$. QH-B treated with 2.5 or 5 mM sodium periodate inhibits the enzyme activity at a concentration of approximately 10 $\mu\text{g ml}^{-1}$, while QH-B treated with the highest sodium periodate concentration tested, i.e. 50 mM, is required at a more than 10 times higher concentration to inhibit enzyme activity by 50%.

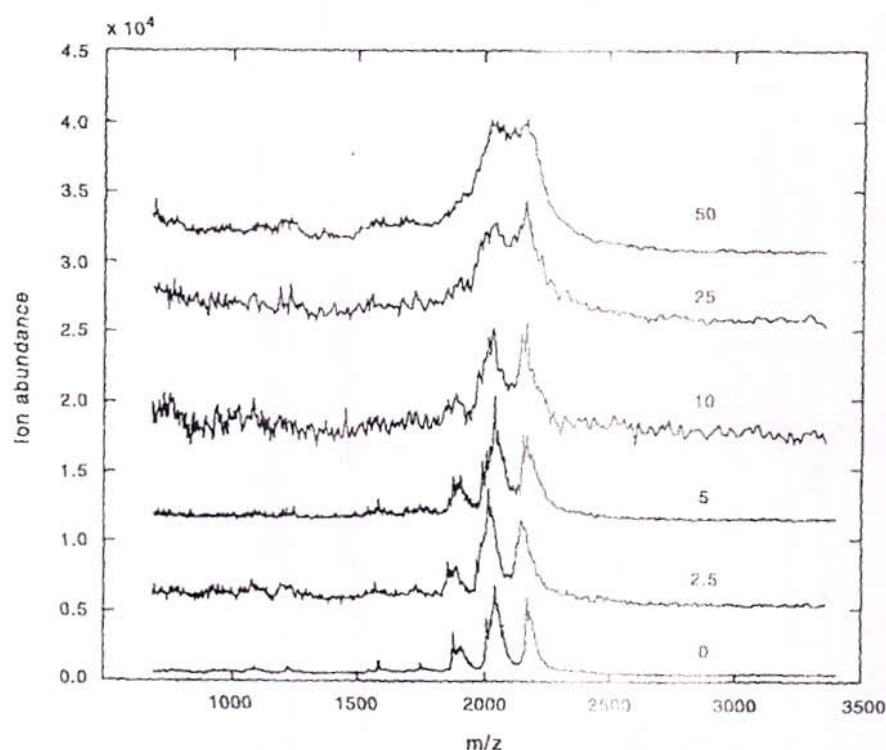


Figure 3 Molecular weight distribution of native and periodate-treated, QH-B. The mass spectra were obtained by MALDI-TOF using 2,5-dihydroxybenzoic acid as matrix

Toxicity in mice

All mice that were inoculated subcutaneously with more than 100 μg of QH-B, regardless of the QH-B preparation, developed local reactions ranging from a hard swelling that did not decline during the observation period of 3 weeks to large (2–6 cm in diameter) soft edematous swelling. Most of the mice that developed either lesions died within 3 days after inoculation, and those mice that survived developed a large necrotic scar at the site of inoculation. The LD_{50} of QH-B is shown in Table 1. Non-treated QH-B and

QH-B treated with 2.5, 5 and 10 mM sodium periodate caused about the same mortality expressed as LD_{50} , i.e. ranging from 29 to 70 μg . After treatment of QH-B with 25 and 50 mM sodium periodate, the LD_{50} increased to 197 and 132 μg , respectively.

Analysis of survival function by Kaplan–Meir survival analysis¹⁹ indicated significant differences in the distribution time to death among treatment groups ($P = <0.001$; log rank statistic 53.4). Mice treated with 25 and 50 mM sodium periodate were indistinguishable from each other ($P = 0.32$), but were significantly different from mice treated with 2.5, 5 and 10 mM ($P < 0.001$ for all in pairs comparison). Mice treated

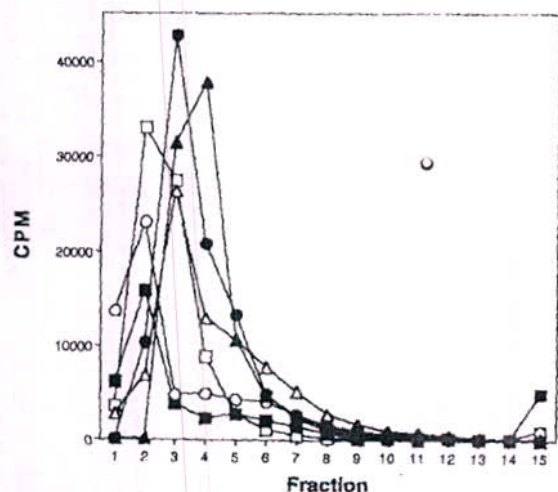


Figure 4 The capacity of QH-B to bind cholesterol after treatment with increasing concentrations of sodium periodate as assessed by sucrose gradient centrifugation. Fractions were collected from the bottom of the tube. Symbols indicate radioactive cholesterol. QH-B was treated with 0 mM ($\square$); 2.5 mM ($\blacksquare$); 5 mM ($\circ$); 10 mM ($\bullet$); 25 mM (Δ); and 50 mM ($\blacktriangle$) sodium periodate

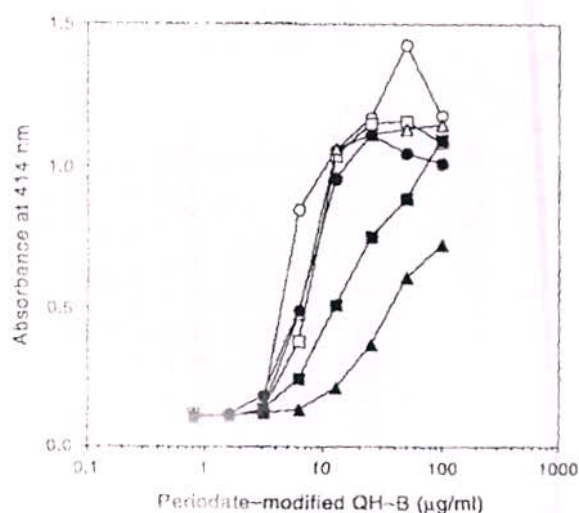


Figure 5 Influence of periodate oxidation on the hemolytic activity of QH-B. QH-B was treated with ($\circ$) 0 mM, ($\square$) 2.5 mM, (Δ) 5 mM, ($\bullet$) 10 mM, ($\blacksquare$) 25 mM, and ($\blacktriangle$) 50 mM sodium periodate

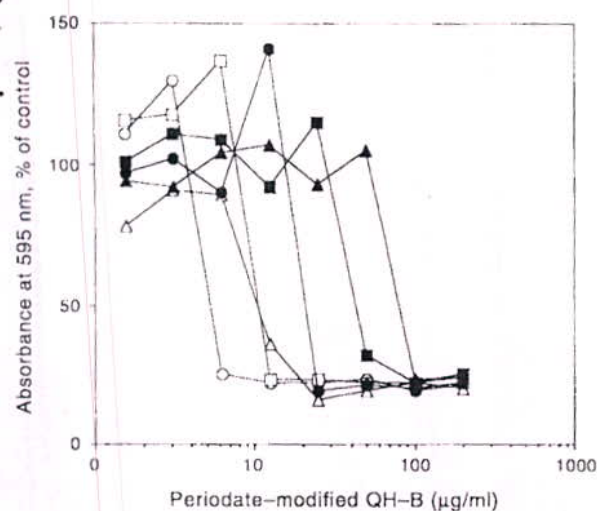


Figure 6 Toxicity of periodate-treated QH-B as measured by the MTT method. QH-B was treated with (○) 0 mM, (□) 2.5 mM, (△) 5 mM, (●) 10 mM, (■) 25 mM, and (▲) 50 mM sodium periodate

with 2.5, 5 and 10 mM sodium periodate were not statistically different from each other.

Immunoenhancing capacity

The adjuvant activity of modified QH-B was evaluated in a system using the envelope proteins of influenza virus in micelle form as the antigen and the serum antibody response was tested in a mouse model. After the primary immunization all animals responded with clearcut antibody titers which after boost increased about ten-fold. The effect of increasing concentrations of periodate on the adjuvant activity of QH-B is shown after the second immunization in *Figure 7*. A concentration-dependent loss of adjuvant activity occurred. Mice immunized with micelles supplemented with QH-B treated with the highest concentrations of sodium periodate, i.e. 25 and 50 mM, showed little or no increase of the serum antibody response compared with the non-adjuvanted control. QH-B modified with either 2.5, 5 or 10 mM sodium periodate enhanced the serum antibody response, with 10 mM showing the lowest enhancing capacity and 2.5 mM the highest.

DISCUSSION

Triterpenoid saponins from *Quillaja saponaria* Molina consist of an aglycone with two carbohydrate chains¹¹. Most likely the aglycone skeleton is fairly well conserved among these triterpenoids and the major differences are found in the carbohydrate residues²⁰. It

Table 1 Lethality of periodate-treated QH-B to ICR mice

Sodium periodate (mM)	LD ₅₀ (µg) ^a
0	52
2.5	70
5	29
10	55
25	197
50	132

^aLD₅₀ is the dose (µg) that kills 50% of the inoculated mice as calculated by the method of Reed and Muench¹⁸

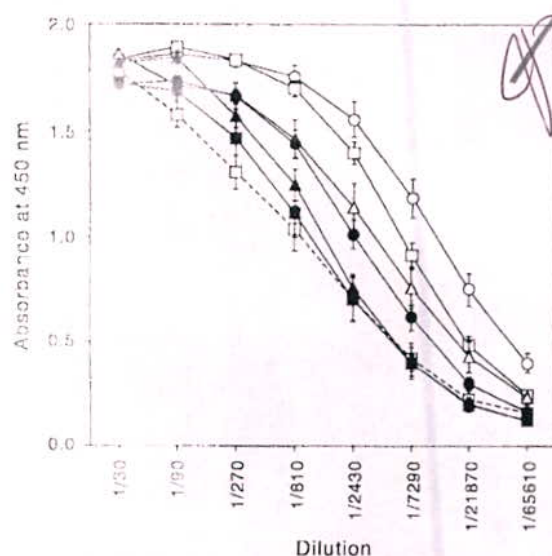


Figure 7 Serum antibody response in mice immunized twice subcutaneously (Day 0 and Day 28) with 1 µg PR-8 micelles and periodate-modified QH-B. 1 µg PR-8 micelles and 10 µg QH-B treated with (○) 0 mM, (□) 2.5 mM, (△) 5 mM, (●) 10 mM, (■) 25 mM, and (▲) 50 mM sodium periodate and (—□—) 1 µg PR-8 micelles only. Symbols indicate mean values and vertical bars the SE

is possible that toxic, as well as adjuvant properties, are found in the carbohydrate part of the molecule. A key question is whether modification of the carbohydrates is altering the toxic properties or the immunoenhancing capacity or both. Other biological functions of particular interest and previously not dealt with are cholesterol-binding capacity and iscom formation and their relation to adjuvant activity. It is also important to design methods to monitor the effects of modification of saponins and to correlate these to biological functions.

Mild periodate oxidation at acidic pH has been shown to cleave carbohydrate vicinal hydroxyl groups¹³. This method was used to modify the carbohydrate chains of QH-B, a *Quillaja saponaria* Molina triterpenoid saponin defined as the most toxic fraction but also exhibiting strong adjuvant activity¹. The present study demonstrates that sodium periodate oxidation at acidic pH is a reproducible method to modify QH-B in a dose-related manner, which was readily monitored and visualized by thin layer chromatography. Using increasing concentrations of sodium periodate the lytic activity as well as the cell toxic activity measured by the MTT method declined and so did the immunoenhancing activity. In contrast, the toxicity observed *in vivo* decreased in a stepwise manner when increasing the concentration of sodium periodate from 10 to 25 mM. However, the ability to bind cholesterol was retained at all concentrations of sodium periodate. The correlation between loss of hemolytic activity and loss of adjuvant activity has also been observed in a recent study by Soltysik *et al.*²⁰ using the *Quillaja* saponin QS-21 and derivatives thereof. They showed that QS-21 modified at the aldehyde at carbon 4 on the triterpene skeleton lost its adjuvant activity, suggesting that this group may be involved in the adjuvant mechanism. It has also been reported by Kensil *et al.*²¹ that periodate oxidation of a toxic *Quillaja* saponin

designated QS-18 eliminates the adjuvant activity associated with this saponin. However, the evaluation of the effect of this treatment was limited to adjuvant activity and no attempts were made to correlate this activity to other biological functions or chemical effects on the molecule as was done in this study. The present study demonstrates that, although periodate-modified QH-B binds cholesterol, it does not form characteristic iscom-matrix. However, the integrity of the iscom-matrix is not only determined by the cholesterol-binding capacity, but also by electrostatic interactions³. It has been proposed that the glucuronic acid, a charged sugar present in most *Quillaja* saponins, prevents aggregation of iscom-matrix. "Saponin-lipid complexes" have been reported to be formed by other saponins than from *Quillaja saponaria* Molina, indicating a wide range of saponins with the ability to bind cholesterol²².

Under the milder conditions (2.5 and 5 mM sodium periodate) used in this study, only the most accessible sugars were oxidized, whereas at higher concentrations of periodate all of the sugars with vicinal hydroxyl groups were affected. Since no further treatment of the oxidised material was done, the modified sugars should still be present. This was confirmed by mass spectroscopy where only minor changes in the molecular weights were found and no new components of lower molecular weight occurred for the periodate-treated samples. At lower concentrations of sodium periodate the apiose, the galactose and the xylose residues are preferentially oxidized. These three sugars are found as terminal groups in the structures presented on similar saponins¹¹. Thus, most of the differences in properties of the periodate-treated material could be derived from changes in the structure of these sugars, except that modification of apiose apparently did not affect toxicity *in vivo*.

In conclusion, this study shows that the toxicity of QH-B can be reduced by periodate oxidation, but is also accompanied with a reduction in adjuvant activity. The use of modified QH-B as described herein for iscom-matrix formation resulted in "saponin-lipid complexes" which deviate from the characteristic morphology, although the capacity to interact with cholesterol remains.

ACKNOWLEDGEMENTS

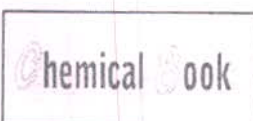
This work was supported by grants from the Bank of Sweden Tercentenary Foundation (92-0350) and the Swedish Council of Forestry and Agricultural Research. S.B. holds a scholarship from the Ministry of Culture and Higher Education of Iran. Thanks are due to Å. Ohagen for the electron microscopy analysis. The excellent technical assistance of J.H. Shaddock is greatly acknowledged.

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ANEX 7



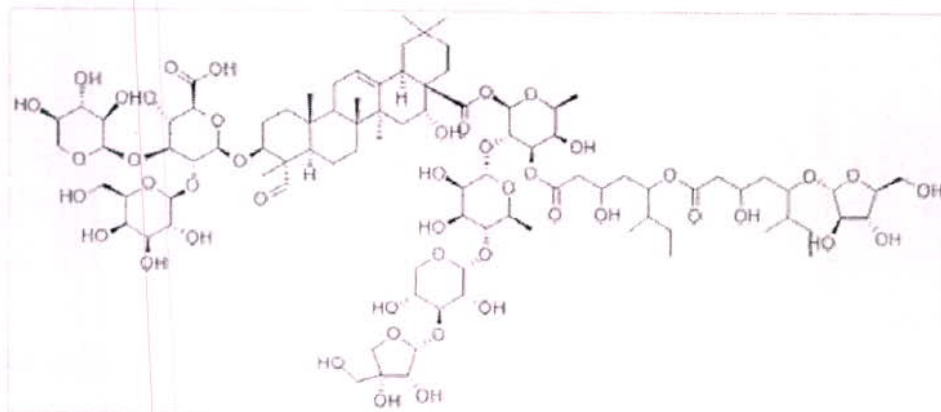
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