

P-2017/14361

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

DIRECCIÓN DE NUEVAS CREACIONES

E. S. D.

Re: Expediente No. **NC2017/0010807**
Solicitud de patente a nombre de **GIVAUDAN SA**

Yo, CAROLINA DAZA MONTALVO identificada como aparece al pie de mi firma, domiciliada en la Carrera 11 No. 86-53, obrando como apoderada de la sociedad GIVAUDAN SA, domiciliada en Vernier, Suiza, solicitante de la patente de la referencia, en respuesta al oficio No. **1859**, notificado electrónicamente el 11 de febrero de 2020, relacionado con los requerimientos de fondo de la solicitud, atentamente me permito presentar la siguiente información:

1. Modificación a las Reivindicaciones y Claridad

Con fundamento en la facultad que confiere el artículo 34 de la Decisión 486 al solicitante para modificar la solicitud de patente en cualquier momento del trámite, presento junto con este memorial nuevo capítulo reivindicatorio, el cual consta de diez (10) reivindicaciones y reemplaza completamente el capítulo presentado anteriormente. Específicamente, se realizaron las siguientes modificaciones:

- 1.1** El examinador ha indicado que *"Sea lo primero advertir por esta Dirección que esta solicitud reclama la misma materia contenida en la solicitud NC2017/0010720 de acuerdo a la siguiente tabla comparativa (...) Si bien el objeto de la solicitud NC2017/0010807 se refiere al proceso de síntesis de Ambrox y la solicitud NC2017/0010720 refiere el método de purificación de Ambrox, se evidencia que en ambas solicitudes comprenden las mismas características esenciales, las cuales fueron resaltadas en la tabla anterior.*

Por lo tanto, esta Superintendencia no puede conceder dos patentes para el mismo objeto de la invención.”. En este respecto, el solicitante respetuosamente desea manifestar que las invenciones descritas en la solicitud NC2017/0010807 y en la solicitud NC2017/0010720 corresponden a invenciones diferentes (como lo demuestran las diferentes secuencias reclamadas - SEQ ID N° 21 y SEQ ID N° 1, 2 y 4 respectivamente). Sin embargo, y con el fin de agilizar el trámite, se han eliminado las antiguas reivindicaciones 7 a 13, con el fin de superar la objeción emitida.

- 1.2** Se ha modificado la antigua reivindicación 14 (nueva reivindicación 7) eliminando toda referencia al isómero [(3Z, 7E), (3E, 7Z)], también designado como el isómero [EZ:ZE], porque, como se discute más abajo, estos isómeros de homofarnesol no producen (-)-Ambrox después de la ciclación con el biocatalizador SHC reclamado. La referencia al rango de umbral de olor también se ha eliminado de esta reivindicación. Por tanto, se ve superada dicha objeción.

Como se discutió anteriormente, la reivindicación 14 anterior (nueva reivindicación 7) se ha modificado para eliminar toda referencia al isómero [(3Z, 7E), (3E, 7Z)], también designado como isómero [EZ: ZE] como lo hace esta combinación de isómeros no produce compuestos (-)-Ambrox después de la ciclación con un biocatalizador SHC. A este respecto, el solicitante ha demostrado por primera vez que el isómero homofarnesol EE produce (-)-Ambrox (compuesto I) y un isómero de (-)-Ambrox (compuesto IV) mientras que el isómero EZ produce 9-epi-Ambrox (compuesto III) y un nuevo compuesto de éter macrocíclico (compuesto II) pero no (-)-Ambrox (como se resume en la Figura 12 de la solicitud PCT). Como el compuesto macrocíclico II es nuevo (ver Tabla 21 de la solicitud PCT) y su existencia no se ha descrito previamente, el producto de reacción que comprende el compuesto (-)-Ambrox obtenible por el proceso reclamado utilizando [(EE:EZ)] o la mezcla de isómeros de homofarnesol [(EE: EZ: ZE)], la cual también es nueva, incluso si solo contiene trazas de compuesto II.

El Ejemplo 22 de la solicitud PCT indica que:

"El por ciento total de cada uno de los 3 subproductos (compuestos II, III y IV) en el extracto "crudo" es aproximadamente 3%." mientras que, "el por ciento total de

cada uno de los 3 subproductos (compuestos II, III y IV) en el extracto "cristalizado" es aproximadamente 1% (escala de laboratorio)".

Como la antigua reivindicación 14 (nueva reivindicación 7) es nueva, las antiguas reivindicaciones 15-17 (nuevas reivindicaciones 8-10) que dependen de la antigua reivindicación 14 (nueva reivindicación 7) también son novedosas (e inventivas como se discute más adelante) y deben mantenerse en el pliego reivindicatorio.

1.3 Respecto a las objeciones a las antiguas reivindicaciones 16 y 17 (nuevas reivindicaciones 9 y 10), el solicitante desea manifestar respetuosamente su desacuerdo ya que la inventiva del método reivindicado no se dirige a condiciones técnicas particulares de los pasos. Además, de acuerdo con la legislación colombiana, hay dos tipos de reclamaciones, reclamaciones de productos y reclamaciones de procesos, en las cuales estas deben definir el invento de manera clara y concisa, es decir, que un experto en la materia puede entender y reproducir, pero es claro que ninguno de los documentos de la legislación vigente y aplicable en Colombia establece que una reclamación debe ser tan limitada y específica al punto de incluir todas las condiciones técnicas, ya que está claro, y cualquier persona con un nivel medio de conocimiento en el tema, sabe y comprende que muchas veces tales condiciones son totalmente **irrelevantes** para un proceso, y su inclusión sería simplemente una limitación no deseada que llevaría a obtener una patente que no tendría uso debido a su alcance limitado. Por tanto, se solicita sea retirada la objeción.

1.4 Las reivindicaciones restantes, así como sus dependencias, fueron reenumeradas.

En cuanto a estas modificaciones, me permito decir que las mismas no amplían de ninguna manera la materia objeto de la presente solicitud y se realizaron con el ánimo de superar las objeciones de claridad y patentabilidad planteadas por el examinador en su concepto técnico.

En consecuencia, nos permitimos indicar que el presente capítulo reivindicatorio, que incluye diez (10) reivindicaciones, se ajusta a las disposiciones del artículo 30 de la Decisión 486/00.

2. Pago de tasas

En relación con el número de reivindicaciones, atentamente nos permitimos decir que no se adjunta tasa por concepto de reivindicaciones adicionales, toda vez que el nuevo capítulo reivindicatorio, aunque cuenta con un menor número de reivindicaciones a las presentadas anteriormente, y en esencia es el mismo objeto, pero definido con mayor claridad.

3. Novedad

El examinador establece que las reivindicaciones 14 y 15 carecen de novedad a la luz de las enseñanzas descritas en el documento US8759043 (D2). Las reivindicaciones 16 y 17 carecen de novedad a la luz de las enseñanzas descritas en el documento EP0165458 (D1).

A este respecto, me permito decir que el artículo 16 de la Decisión 486 de la Comisión de la Comunidad Andina establece que la invención se considera nueva cuando no está comprendida en el estado de la técnica. Si de acuerdo con el espíritu de la norma legal indicada, la invención no se considera como nueva si está "*comprendida*" en el estado de la técnica, dicha norma se está refiriendo, naturalmente, a la totalidad del invento y no a alguno o a algunos de los componentes.

Aplicando lo anterior al caso concreto, para que las razones que aduce ese Despacho para desvirtuar la novedad fuesen ciertas, sería forzoso que se demostrara que la invención está comprendida en el estado de la técnica, específicamente en forma individual en los documentos D1 y D2 de forma TOTAL, lo cual no sucede con la materia reclamada en el nuevo capítulo reivindicatorio.

Por lo tanto y teniendo en cuenta lo anteriormente expuesto, como mínimo la novedad debe contemplar los siguientes aspectos:

- Que la invención en estudio esté contenida de forma TOTAL y ABSOLUTA en cada uno de los documentos que se mencionan como anterioridades; y

- Que la invención en cuestión puede partir de conocimientos conocidos, caso en el cual habrá que hacer la comparación no de lo conocido sino de lo inédito y novedoso, teniendo que analizarse el valor agregado que aporta la invención con sus nuevas características al campo técnico al cual pertenece la solicitud en estudio.

De acuerdo con lo anterior, el solicitante desea manifestar respetuosamente su desacuerdo, ya que teniendo en cuenta las modificaciones presentadas en el nuevo capítulo reivindicatorio, se superan las objeciones realizadas, por cuanto **dichas características no se encuentran divulgadas o sugeridas en los documentos D1, D2 y D3 citados por el examinador**. Por lo tanto, las reivindicaciones modificadas son novedosas e inventivas a la luz del estado del arte.

D2 (US8759043B2)

La reivindicación 1 es nueva sobre D2 porque D2 solo describe el uso de un isómero de homofarnesol ((3Z, 7E) como se describe en el Ejemplo 3a) y no dos o tres isómeros de homofarnesol como se reclam.

La reivindicación 1 también es nueva sobre D1 ya que no proporciona una divulgación habilitante para la producción de (-)-Ambrox. En este sentido, se indica incorrectamente en la columna 2, líneas 18-23 de D2 que (con énfasis en **negrita subrayada**):

*"La síntesis biocatalítica del compuesto ((-)-2 ha sido descrita en la literatura [Neumann et al; Biol. Chem. Hoppe Seyler (1986), 367; 723]. **Aquí** la molécula se obtiene del homofarnesol (compuesto (1), (3Z, 7E)-4, 8, 12-trimetiltrideca-3,7, 11-trien-1-ol)".*

El párrafo correspondiente en el documento WO2010/139719 (D1-DE-BASF) establece que (con énfasis en **negrita y subrayado**):

*"Die biokatalytische Synthese von Verbindung ((-)-2) ist in der Literatur beschrieben [Neumann et al; Biol Chem Hoppe Seyler (1986) 367:723]. **Dabei** wird das Molekuel aus Homofarnesol (Verbindung (1), (3Z, 7E)-4, 8, 12-Trimethyltrideca-3, 7, 11-trien-1-ol) gewonnen"*

*"La síntesis biocatalítica del compuesto ((-)-2 ha sido descrita en la literatura [Neumann et al; Biol. Chem. Hoppe Seyler (1986), 367; 723]. **Aquí** la molécula se obtiene del homofarnesol (compuesto (1), (3Z, 7E)-4, 8, 12-trimetiltrideca-3,7, 11-trien-1-ol)".*

No está claro en el primer párrafo en cursiva proporcionado anteriormente si la palabra "Aquí" al comienzo de la segunda oración se refiere a la publicación de Neumann et al (1986) o a la divulgación en D2. Sin embargo, la oración correspondiente en el segundo párrafo en cursiva en el idioma alemán tal como se presenta (D2 (DE-BASF)) usa el término "Dabei", lo que aclara que D2 es: (i) refiriéndose a la divulgación de la publicación Neumann et al (1986); y (ii) declarar incorrectamente que la publicación de Neumann et al (1986) divulga un proceso para producir (-)-Ambrox usando un isómero de homofarnesol (3Z, 7E) ya que este no es el caso y es una interpretación errónea de la divulgación en la publicación de Neumann y Simon (1986).

Para el registro, Neumann y Simon (1986) revelan que: (i) Ambroxan se puede producir a partir de una mezcla de cuatro posibles isómeros de homofarnesol (EE, ZE, ZZ y EZ) usando una enzima SHC; y (ii) concluyen de sus estudios que solo el isómero EE-homofarnesol (de una mezcla de cuatro posibles isómeros de homofarnesol (EE, ZE, ZZ y EZ)) es ciclado por SHC para producir Ambroxan, lo que implica que no tiene lugar la ciclación del isómero (3Z, 7E)-homofarnesol. Por lo tanto, el análisis de la técnica anterior relevante en la columna 2, líneas 18-23 de D1 es: (i) objetivamente incorrecta porque Neumann y Simon (1986) no revelan ni sugieren ni demuestran la producción de (-)-Ambrox a partir del isómero (3Z, 7E)-homofarnesol; y (ii) experimentalmente imposible como lo demostró el solicitante y se analiza con más detalle a continuación porque la biocatálisis de un isómero (3Z, 7E)-homofarnesol que usa una enzima SHC no da como resultado la producción de (-)-Ambrox.

A este respecto, el Ejemplo 3a de D2 establece que se usó el isómero de homofarnesol (3Z, 7E) y usando los análisis de GC descritos en el Ejemplo 4, se obtuvo una tasa de conversión del 41% usando una homofarnesol ciclasa recombinante (SEQ ID N° 2) de *E. coli* (LU15568). Si bien el producto final real resultante de la conversión SHC del isómero (3Z, 7E) no se identifica, se implica que es (-)-Ambrox. Sin embargo, si los productos de conversión del Ejemplo 3a se analizaron utilizando la metodología GC descrita en el Ejemplo 4, entonces no se encontrarían picos GC correspondientes a (-)-Ambrox (lo que

explica la ausencia total de datos de perfil GC en D2 debido a la falta total de evidencia de apoyo para la producción de (-)-Ambrox).

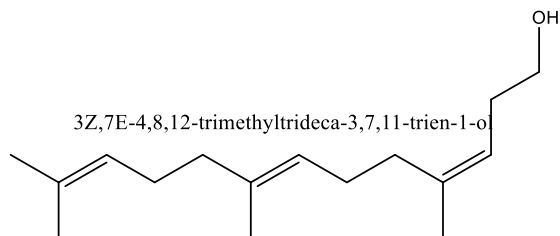
El solicitante ahora ha demostrado claramente que el isómero (3Z, 7E) (también designado como isómero "EZ" o "EZH") produce un nuevo éter macrocíclico (compuesto II) y 9-epi-Ambrox (compuesto III) pero no (-)-Ambrox después del tratamiento con una enzima SHC (ver el Ejemplo 22 y la Figura 12 de la solicitud PCT).

Dado que el isómero de homofarnesol (EE) necesario para la producción de (-)-Ambrox no se usó para la ciclación en el Ejemplo 3a de D1, no se pudo haber producido (-)-Ambrox. En consecuencia, la divulgación en D2 no está habilitada para la producción de (-)-Ambrox usando una enzima SHC debido a la falla en la selección del isómero de homofarnesol correcto requerido para la producción de (-)-Ambrox.

Estado de la técnica adicional (para demostrar la divulgación no habilitante en D2)

Antes de la primera fecha de presentación de la solicitud presentada, era bien sabido en la técnica que el isómero de homofarnesol (3Z, 7E) descrito en D1 no produce (-)-Ambrox. - Se hace referencia a algunos documentos que demuestran lo anterior, los cuales se adjuntan a este memorial para su revisión.

En este sentido, Schaefer (2011) (Chemie Unserer Zeit 45, 374-388), enseña que la configuración estructural del isómero (7E, 3Z) de:

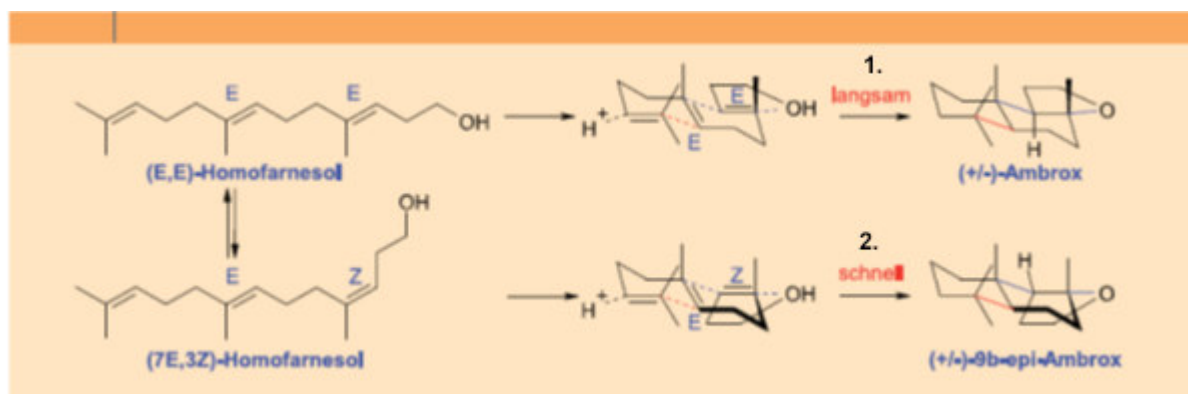


es tal que solo se puede convertir a 9b-epi-Ambrox a diferencia del isómero de homofarnesol (E, E) cuya configuración estructural permite que se convierta a (-)-Ambrox (ver Figura 21 de Schaefer (2011)) independientemente de la naturaleza del proceso de

conversión (por ejemplo, químico o enzimático). Se proporciona una referencia a la divulgación relevante en Schaefer (2011) en la página 10, líneas 10-15 de la solicitud PCT con la aclaración de que *"una referencia al homofarnesol (3Z, 7E) es una referencia a E, Z-homofarnesol, que también se designa como EZH"*.

Para facilitar la referencia, se adjunta una copia de una traducción (del alemán al inglés) de la referencia de Schaefer (2011).

FIG.21 CONSIDERACIONES MECANISTAS EN LA CICLIZACIÓN DE POLIENO



Por lo tanto, Schaefer ya estableció la falta de divulgación habilitadora en D2 en 2011 (independientemente de la naturaleza del proceso de conversión (por ejemplo, químico o enzimático)) y simplemente se confirmó en la presente solicitud (página 10, líneas 10-15 de la solicitud PCT) y en una publicación posterior de revisión por pares, escrita por los inventores de la presente (Eichhorn et al 2018 Adv. Synth.Catal 360: 2339-2351).

Además, la Figura 21 de Schaefer (2011) sólo enseña que es posible producir el compuesto de la fórmula I (es decir, (-)-Ambrox) a partir del isómero EEH y el compuesto de la fórmula III (es decir, 9-epi-Ambrox) a partir del isómero (EZH). En cambio, la Figura 12 (página 133, líneas 19-24 de la solicitud PCT) muestra por primera vez que los productos de reacción producidos ((-)-Ambrox (I) y producto (IV)) cuando se utiliza el EEH como material de partida (para la bioconversión con WT SHC y/o un derivado de SHC/HAC); y los productos de reacción producidos ((-)-Ambrox (I) y productos (II), (IV) y (III) (véase la Tabla 21) cuando se utiliza el EE:EZ se utiliza como material de partida) aclarando así que el SHC convierte el isómero EEH en compuestos de fórmulas I y IV y el isómero EZH

en compuestos de fórmulas II y III. Este hallazgo fue completamente inesperado, especialmente en vista de la enseñanza en Schaefer (2011) (como se discutió anteriormente) y Escher *et al* (1990), donde en el párrafo de "Introducción" se afirma que: *"Las consideraciones de conformación hacen improbable la existencia del altamente forzado cuarto diastereoisómero 3"*. Dado que el altamente forzado cuarto diastereoisómero 3 es en realidad el compuesto IV, la información que figura tanto en Escher *et al* (1990) como en Schaefer (2011), tanto por sí sola como en combinación, apunta a la producción de compuestos distintos de los compuestos de las fórmulas I y III.

Para facilitar la referencia, los compuestos I-IV pueden identificarse de la siguiente manera:

I: (3aR;5aS,9aS,9bR)-3a,6,6,9a-tetrametildodecahidronafto [2,1-b]furano (-)-Ambrox

II: (7aS,11aS;Z),5,8,8,11a-tetrametil-2,3,6,7,7a,8,-9,10,11,11a-decahidrobenzo[b]oxonina

IV: (3aS,5aS,9aS,9bS)-3a,6,6,9a-tetrametildodecahidronafto[2,1-b]furano

III: (3aRS,5aSR-9aSR,9bSR)-3a,6,6,9a-tetrametildodecahidronafto[2,1-b]furano 9-epi-Ambrox

Así pues, D1 (BASF) no anticipa el proceso de la reivindicación 1 ni ninguna reivindicación que dependiente de la reivindicación 1.

Como la antigua reivindicación 14 (nueva reivindicación 7) es novedosa respecto del documento D2, todas las reivindicaciones dependientes de la antigua reivindicación 14 (nueva reivindicación 7) son también novedosas respecto del documento D1.

D1 (EP0165458) – Hasegawa

La antigua reivindicación 14 (nueva reivindicación 7) también es novedosa respecto al documento D1 porque este se refiere a un método de síntesis química para producir un isómero ópticamente inactivo (+/-)-Ambrox a partir de un sustrato distinto del supuesto sustrato de homofarnesol. Por consiguiente, el proceso de D1 no da lugar a un producto de reacción que comprenda el (-)-Ambrox y el nuevo compuesto II. Por lo tanto, la antigua reivindicación 14 (nueva reivindicación 7) es novedosa respecto del documento D1 y todas

las reivindicaciones dependientes de la antigua reivindicación 14 (nueva reivindicación 7) son nuevas respecto del documento D1.

4. Nivel Inventivo

El examinador establece que las reivindicaciones 1 a 12 carecen de nivel inventivo a la luz de las enseñanzas descritas en los documentos US8759043 (D2) y US2015/0104851 (D3).

Respecto al nivel inventivo, me permito decir que el artículo 18 de la Decisión 486 establece que *"Se considerará que una invención tiene nivel inventivo, si para una persona del oficio normalmente versada en la materia técnica correspondiente, esa invención no hubiese resultado obvia ni se hubiese derivado de manera evidente del estado de la técnica"*.

Lo anterior significa que el análisis de nivel inventivo **NO** es una comparación de los objetos de las anterioridades y la solicitud en estudio. El análisis de nivel inventivo **debe demostrar** la obviedad de la invención y establecer cómo ésta no contiene un salto cualitativo, que demuestre su altura inventiva, teniendo en cuenta que se hace **necesario demostrar** que la invención analizada carece de nivel inventivo y hubiese sido obvia para una persona versada en la materia.

En este sentido, el solicitante desea manifestar respetuosamente que, el estado de la técnica citado (D2) no revela ni sugiere que una enzima SHC (por ejemplo, SEQ ID No 21) cicle no solo el isómero EE-homofarnesol para producir (-)-Ambrox sino que también cicle otros isómeros de homofarnesol para producir compuestos no (-)-Ambrox, incluido el nuevo compuesto de éter macrocíclico II. El solicitante ha demostrado por primera vez que el isómero de homofarnesol EE produce (-)-Ambrox (compuesto 1) y un isómero de (-)-Ambrox (compuesto IV) mientras que el isómero de homofarnesol EZ produce 9-epi-Ambrox (compuesto III) y un compuesto de éter macrocíclico (compuesto II) pero no (-)-Ambrox (como se resume en la Figura 12 de la solicitud PCT). De esta manera, el solicitante ha demostrado con éxito que al menos la combinación de isómero de homofarnesol (EE/EZ) es una combinación viable como material de partida en un método para producir (-)-Ambrox usando una enzima SHC y productos secundarios como 9-epi-ambrox producido por el isómero EZ no interfiere sustancialmente con el proceso de producción, aislamiento y recuperación de (-)-Ambrox.

Como se considera que el proceso reclamado deja una huella distintiva en el producto resultante que lo distingue de los productos divulgados en el arte citado, la antigua reivindicación 14 (nueva reivindicación 7) es inventiva y cualquier reivindicación que dependa de la antigua reivindicación 14 (nueva reivindicación 7) también es inventiva.

En cuanto al documento D3 también a nombre de BASF, tiene reivindicaciones dirigidas a los mutantes con actividad ciclásica y su uso en un método de ciclización de terpenos, en particular la producción de isopulegol mediante la ciclización del citronelal (véase el resumen y el párrafo [0013] en la columna 1 de D3). Aparte de la referencia al WO2010/0139719A2 y su completa revelación relativa a los polipéptidos propuestos como biocatalizadores para la ciclización del homofarnesol al ambroxán en el párrafo [0010] de la columna 1 de D3, no hay ninguna revelación de la demostración de la producción de (-)-Ambrox en D3. Más bien, D3 simplemente revela biocatalizadores adecuados útiles para la ciclización de terpenos como el citronelal con la formación de isopulegol. Específicamente las mutaciones reveladas en los párrafos [0046-006] son mutaciones dirigidas a la ciclización de un objetivo distinto del objeto reclamado del homofarnesol. Por lo tanto, no habría ningún incentivo para que la persona experta en la materia combine lo divulgado en D3 con D1. Incluso si lo hicieran, no llegarían a la invención reclamada porque, como se ha discutido anteriormente, D1 (US8759043B2) que se fragmentó de WO2010/0139719A2 es una divulgación no habilitada ya que no se produce (-)-Ambrox.

De acuerdo con lo anterior, y teniendo en cuenta que los documentos D1, D2 y D3 **no mencionan, describen o sugieren**, en forma individual o en combinación, todas las características técnicas reclamadas en el nuevo capítulo reivindicatorio presentado por el solicitante, **las nuevas reivindicaciones presentadas son novedosas, e inventivas sobre el estado del arte.**

En este sentido, y siguiendo el Manual Andino de Patentes, la presente invención tal y como se encuentra definida en las reivindicaciones 1 a 10, tiene diferencias con el estado del arte e implicó un nivel inventivo durante su desarrollo, por lo cual debe ser considerada como novedosa e inventiva según las disposiciones de los artículos 16 y 18 de la Decisión 486.

5. Conclusión

Con las modificaciones introducidas y los argumentos presentados puede concluirse que:

- i) La presente solicitud es clara, concisa y está debidamente soportada en la descripción, cumpliendo con los requerimientos del Artículo 30 de la Decisión 486; y
- ii) Las reivindicaciones actuales son patentables sobre D1, D2 y D3, cumpliendo así con los requerimientos del Artículo 18 de la Decisión 486.

Por tanto, atentamente me permito solicitar a ese Despacho otorgar el privilegio de patente solicitado a la solicitud en estudio, toda vez que cumple a cabalidad con los requerimientos de la Decisión 486/00.

En consecuencia, solicitamos a ese Despacho pronunciarse en relación con todos los argumentos presentados en este memorial dando estricta aplicación al principio del debido proceso consagrado en el artículo 29 de la Constitución Nacional.

6. Anexos

- Nuevo capítulo reivindicatorio conformado por 10 reivindicaciones.
- Neumann and Simon (1986)
- Eichhorn et al 2018 Adv. Synth.Catal 360: 2339-2351
- Schaefer (2011) (Chemie Unserer Zeit 45, 374-388)) (original and traducción al inglés)
- Escher et al (1990)

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REIVINDICACIONES MODIFICADAS

1. Un proceso para preparar (-)-Ambrox o una mezcla que comprende (-)-Ambrox, caracterizado porque una mezcla de isómeros que comprende EEH se convierte enzimáticamente a (-)-Ambrox o una mezcla que comprende (-)-Ambrox, en donde la conversión enzimática se lleva a cabo usando una enzima escualeno-hopeno-ciclasa/homofarnesol-Ambrox-ciclasa (SHC/HAC) que tiene una secuencia de polipéptido de SEQ ID No. 21 para la producción de (-)-Ambrox y en donde la mezcla de isómeros que comprende EEH es seleccionada de uno o más de los siguientes grupos que consisten de [(3E,7E) y [(3Z,7E)] y/o [(3E,7E) y (3E,7Z)] y/o [(3Z,7E), (3E,7E) y (3E,7Z)] también designados como [EE:EZ], [EE:ZE] y [EE:EZ:ZE] respectivamente, en donde la conversión de homofarnesol a (-)-Ambrox toma lugar a una temperatura en el intervalo de 30°C a 60°C, a un pH en el intervalo de 4-8.

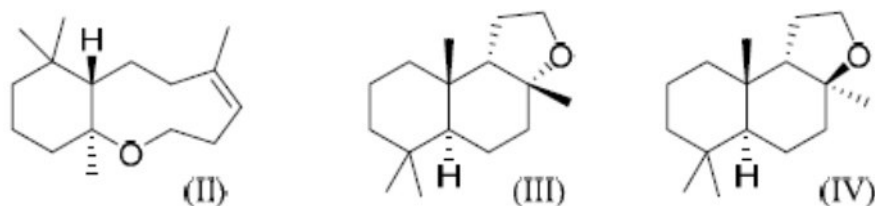
2. El proceso de conformidad con la reivindicación 1, caracterizado porque usa células hospedadoras recombinantes que producen la enzima SHC/HAC.

3. El proceso de conformidad con la reivindicación 1 o reivindicación 2, caracterizado porque la conversión de homofarnesol a (-)-Ambrox toma lugar en un intervalo de pH de 5.0 a 6.2.

4. El proceso de conformidad con la reivindicación 1 o reivindicación 2, caracterizado porque la conversión toma lugar a una temperatura de 35°C.

5. El proceso de conformidad con cualquiera de las reivindicaciones 1-4, caracterizado porque los pasos de reacción de bioconversión y crecimiento celular se llevan a cabo en el mismo recipiente de reacción.

6. El proceso de conformidad con cualquiera de las reivindicaciones 1-5, caracterizado porque se produce (-)- Ambrox en mezcla con como un mínimo de uno o más de los subproductos (II), (IV) y/o (III)



7. Un producto de reacción que comprende (-)-Ambrox que se obtiene por el método de una cualquiera de las reivindicaciones 1-6, caracterizado porque la mezcla de isómeros que comprende EEH es seleccionada de uno o más de los siguientes grupos que consisten de [(3E,7E) y [(3Z,7E)] y/o [(3Z,7E), (3E,7E) y (3E,7Z)] también designados como [EE:EZ], y [EE:EZ:ZE] respectivamente.

8. El producto de reacción que comprende (-)-Ambrox de conformidad con la reivindicación 7 está en una forma sólida, en una forma amorfa o cristalina.

9. Un proceso para producir un producto que contiene (-)-Ambrox, en donde el producto es un producto de fragancia, un cosmético, un producto de limpieza, un producto detergente o un producto de jabón caracterizado porque comprende incorporar el (-)-Ambrox de conformidad con la reivindicación 7 u 8 en el producto.

P-2017/14361

Reivindicaciones Modificadas para respuesta Tercer fondo

28 de mayo de 2020

10. Una fragancia o cosmético o producto de cuidado de consumidor o composición de cuidado de consumidor, caracterizada porque comprende el (-)-Ambrox de conformidad con la reivindicación 7 u 8 y uno o más componentes adicionales.

Purification, Partial Characterization and Substrate Specificity of a Squalene Cyclase from *Bacillus acidocaldarius*

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Dedicated to Prof. Dr. Günther Kreße on the occasion of his 65th birthday

Summary: From the thermophilic *Bacillus acidocaldarius*, a membrane bound cyclase catalysing the formation of 22(29)-hopene (diploptene) and 22-hopanol (diplopterol) from squalene was enriched 670-fold to a purity of 95% as judged by gel chromatography. The specific activity of the purified enzyme in the presence of Triton X-100 is 0.22 μmol product formation per min and mg protein at 54 °C. The molecular mass is 150 kDa and that of the subunits 80 kDa as determined by FPLC gel chromatography and sodium dodecyl sulfate gel electrophoresis, respectively.

Not only squalene but also *E,E*-homofarnesol, homogeraniol and homofarnesyl (1,5,9-trimethyl-4,8-decadienyl) ether are substrates. The ether bond of the latter substrate is split by the enzyme. The products of the aforementioned three additional substrates are ambroxan (dodecahydro-3a,6,6,9a-tetramethylnaphtho[2,1-*b*]furan), octahydro-4,4,7a-trimethylbenzofuran and ambroxan together with 1,5,9-trimethyl-4,8-decadienol. The cyclization rates of these substrates compared to squalene are 3%, 0.05% and 0.3%, respectively. The half-life of the enzyme activity is 80 h at 45 °C and 18 h at 54 °C.

Reinigung, partielle Charakterisierung und Substratspezifität einer Squalene Cyclase aus Bacillus acidocaldarius

Zusammenfassung: Aus dem thermophilen *Bacillus acidocaldarius* wurde eine Membran-gebundene Cyclase 670-fach angereichert. Nach Gelchromatographie war das Material zu mehr als 95% rein. Die Cyclase katalysiert die Bildung von 22(29)-Hopen (Diploptene) und 22-Hopanol (Diplopterol) aus Squalen. Die spezifische Aktivität des gereinigten Enzyms beträgt in Gegenwart von Triton X-100 0.22 μmol Produkte pro min und mg Protein bei 54 °C. Die beiden Hopanoide entstehen im Verhältnis von ca. 6:1. Die apparente Molmasse der Cyclase, bestimmt durch FPLC-Gelchromatographie, ist 150 kDa und die durch Natriumdodecylsulfat-Polyacrylamidgel-Elektrophorese bestimmte Molmasse der Untereinheiten beträgt 80 kDa.

Nicht nur Squalen ist Substrat, sondern auch *E,E*-Homofarnesol, Homogeraniol und Homofarnesyl-(1,5,9-trimethyl-4,8-decadienyl)-ether werden cyclisiert. Dabei wird die Etherbindung des letztgenannten Substrats durch das Enzym gespalten. Die Produkte dieser Substrate sind Ambroxan (Dodecahydro-3a,6,6,9a-tetramethylnaphtho[2,1-*b*]furan), Octahydro-4,4,7a-trimethylbenzofuran sowie Ambroxan zusammen mit 1,5,9-Trimethyl-4,8-decadienol. Die Cyclisierungsgeschwindigkeiten dieser Substrate sind 3%, 0.05% und 0.3% der des Squalens. Die Halbwertszeit der Enzymaktivität beträgt 80 h bei 45 °C und 18 h bei 54 °C.

Abbreviations:

FPLC, Fast protein liquid chromatography; GLC, gas liquid chromatography; HPLC, high performance liquid chromatography; SDS, sodium dodecyl sulfate; TLC, thin-layer chromatography.

Trivial names:

Ambroxan, dodecahydro-3a,6,6,9a-tetramethylnaphtho[2,1-*b*]furan; diploptene, 22(29)hopene; diplopterol, 22-hopanol; Polidocanol, (polyoxyethylene-9) lauryl ether.

Key words: Cyclase, squalene cyclase, substrate specificity, *Bacillus acidocaldarius*.

Hopanoids belong to a family of triterpenes formed by many microorganisms as well as by plants such as lichens and ferns^[1,2]. They are ubiquitous in mineral oil, slate, peat and other sediments^[3,4]. It is generally accepted that the complicated structures containing five rings such as 22(29)-hopene (2a) or 22-hopanol (2'a) (see scheme) and the many derivatives found in sediments are of biological origin. The wide-spread occurrence of hopanoids in microorganisms^[5] raised the question of their physiological role. There seems to be no doubt that they strengthen the rigidity of microbial membranes. The interaction of hopanoids with other membrane components and their cholesterol-like function has been described by Poralla et al.^[6-8]. Bringer et al.^[9] showed that higher steady state ethanol concentrations of continuous cultures of *Zymomonas mobilis* increased the hopanoid content of the cells. Squalene is the precursor of the hopanoid ring system and the similarity of hopanoid and cholesterol formation is evident. However, in hopanoid formation oxygen is not involved^[10]. Nevertheless, in cell-free systems of *Acetobacter pasteurianum*^[11], *Methylococcus capsulatus*^[12] and in the protozoon *Tetrahymena pyriformis*^[13] squalene as well as (R,S)-2,3-epoxy-2,3-dihydrosqualene have been cyclized.

After the completion of studies described here, we became aware of results of Renoux and Rohmer^[14] who showed that by a cell-free system of *T. pyriformis* all-trans-pentaprenyl methyl ether is cyclized to a sclarane-type sesterterpene and all-trans-hexaprenyl methyl ether to a mixture of bi-, tri-, tetra- and pentacyclo-hexaprenyl methyl ethers. The authors assume that all these polycyclic ethers were formed by the hitherto not isolated squalene cyclase of the ciliate *T. pyriformis*.

So far no enzyme catalysing the formation of hopanoids has been purified and characterized. Seckler and Poralla^[15] described a partial purification of this enzyme. We report on the purification, partial characterization and the substrate specificity of the cyclase from *Bacillus acidocaldarius*.

Materials and Methods

[³H]Squalene nominally labelled in positions 4, 8, 12, 13, 17 and 21 was a product from New England Nuclear. Its specific activity was 19 Ci/mmol. The material contained 10% of an impurity as judged by thin-layer chromatog-

raphy and tritium analysis by an Automatic TLC-Linear Analyser LB 2832 — Fa. Prof. Berthold, (D-7547 Wildbad). This contamination was neither enzymatically nor otherwise converted. It stayed constant and did not disturb the substrate and product analysis.

Authentic dodecahydro-3a,6,6,9a-tetramethylnaphtho-[2,1-b]furan (2b) derived from sclareol was a gift from Henkel KGaA, D-4000 Düsseldorf and 22(29)-hopene from Hoechst AG, D-6000 Frankfurt (Dr. Vertesy). Geraniol and nerol were bought from Aldrich-Chemie, D-7924 Steinheim. All other substrates tested were gifts from Consortium für Elektrochemische Industrie GmbH, München. Authentic *E,E*-homofarnesol (1b) and a diastereomeric mixture of octahydro-4,4,7a-trimethylbenzofuran (4) were also obtained from this institution. The detergent Polidocanol was a product from Sigma, D-8024 Deisenhofen and Amberlite XAD-2 from Serva, D-6900 Heidelberg.

Bacillus acidocaldarius DSM 446 was grown as described by Darland and Brock^[16] at 55 °C and at a pH of 3.4 in volumes up to 150 l. This volume was purged with 3.5 m³ air/h after inoculation with 1 l of a freshly grown cell suspension. The cells were harvested when an optical density of 2.8 was reached and stored without washing at -18 °C. They did not loose enzyme activity for several months.

Protein was determined according to Bradford in the modified form of Read and Northcote^[17].

Enzyme assay

The test is based on a procedure described by Rohmer et al.^[11] and was carried out according to Seckler and Poralla^[15]. A total volume of 1.0 ml containing 100mM sodium citrate buffer pH 6.0, 50 μ M [³H]squalene (1×10^5 dpm), and 0.1 mg Triton X-100 was incubated in the presence of approximately 0.25mU cyclase for 60–120 min at 54 °C and then stopped by extracting the chilled vial with 2 ml hexane/2-propanol 3:2 (v/v). After concentrating the organic phase to about 100 μ l it was separated on thin-layer plates covered with 0.25 mm silicagel (Merck 5553) with chloroform. The chromatography was stopped when the solvent front reached 1/3 of the distance to the upper edge. Afterwards the dried plate was further developed in n-hexane. The *R_F* values of squalene, 22(29)-hopene and a second product turned out to be 0.35, 0.60 and 0.10, respectively.

For testing other substrates a total volume of 2.0 ml contained 100mM sodium citrate buffer pH 5.0, 1.0mM substrate i.e. homofarnesol, 0.8 mg Triton X-100 and resolubilized sediment of the membrane fraction of *B. acidocaldarius* (vide infra) corresponding to 4.0 mg protein. The assays were incubated for 14 h at 54 °C. The aqueous phase was once extracted with 4 ml of the aforementioned mixture of organic solvents. These were evaporated, the residue dissolved in 0.1 ml carbon tetrachloride and a 1- μ l fraction injected on a 20-m capillary column Carbowax 20 M, Fa. Hewlett Packard.

The column temperature was raised 4°/min from 160 to 200 °C. *E,E*-Homofarnesol showed a retention time of 10.5 min, three other isomers of homofarnesol were eluted within 8.5–10 min. The product 2b appeared after 5 min. Other 20-m capillary columns used were OV 1 and OV 1701. The GLC-mass spectroscopy analysis of 2b was conducted on an OV 1 column coupled to a Varian MAT 112 S. The pH-dependence of the cyclase was studied with homofarnesol in 100mM citrate and 100mM phosphate buffer in the range of pH 5.0 to 7.0.

Enzyme purification

Wet-packed cells (15 g) suspended in 100 ml 10mM Tris/HCl pH 8.0 were broken by ultrasonic treatment and the cell debris spun down at 5000 × g for 10 min. The resulting supernatant was centrifuged for 3 h at 80000 × g and the sediment washed twice with 60 ml 10mM citrate buffer pH 6.0. The suspensions obtained by the washing procedure were centrifuged each time for 1.5 h at 80000 × g. The sediments from the last centrifugation were resuspended in 25 ml citrate buffer and the cyclase solubilized by incubation with 0.4 mg Triton X-100/mg of membrane protein for 30 min at 54 °C. The detergent was eliminated from the supernatant with 6 g Amberlite XAD-2 by gentle shaking, the solution placed on a 1.5 × 17 cm column filled with 30 ml phenyl-Sepharose CL-4B equilibrated with 100mM citrate buffer pH 6.0 and eluted with a linear gradient from 0–70% 1,2-propanediol in a total volume of 160 ml. The cyclase appeared at a concentration of 60% 1,2-propanediol. The combined main fractions were applied on a DEAE-Cellulose DE-52 column (1.5 × 14 cm) equilibrated with 0.05% Polidocanol in 10mM citrate buffer pH 6.5 and eluted with the same detergent containing buffer by a linear gradient from 0 – 800mM KCl in a total volume of 200 ml. The enzyme was in a total volume of 21.6 ml in the range from 70 to 130mM KCl. After concentration to 5.4 ml by an ultrafiltration through an Amicon YM-10 membrane the residual detergent was eliminated with 0.1 g Amberlite XAD-2. The enzyme-containing solution was chromatographed in aliquot parts of 1.5 ml on a Mono-Q-prepacked column HR 5/5 of an HPLC apparatus (Pharmacia). The column was equilibrated with 20mM phosphate buffer pH 6.5. The cyclase was eluted by applying a linear gradient from 0–1.0M KCl at a KCl concentration of 450mM.

Determination of the molecular mass and criteria of purity

The enzyme obtained from the Mono-Q column of the HPLC apparatus was chromatographed in 20mM phosphate buffer pH 6.5 containing 0.05% Polidocanol on a calibrated FPLC-gel column (Superose 6 HR 10/30, Pharmacia). The cyclase was eluted as a sharp single peak. For the calibration of the gel column the following proteins with their molecular masses given in parenthesis were applied: Cytochrome *c* (12.5 kDa), bovine serum albumin (67 kDa), aldolase (148 kDa), catalase (242 kDa) and ferritin (440 kDa). The disc electrophoresis was conducted according to Maurer^[18] in the presence of 0.2% Polidocanol and the SDS electrophoresis as described by King and Lämml^[19].

Results and Discussion

Assay

All enzyme assays and substrate studies had to be conducted in the presence of Triton X-100 or Polidocanol. Without a detergent the solubility in an aqueous system of squalene (1a), homofarnesol (1b) and the other substances checked for cyclization is too low. A complete conversion to 22(29)-hopene (2a) and a second product can be achieved with a 50μM solution of squalene. It is assumed that the second product is 22-hopanol (2'a) since it shows an *R_F*-value identical with that of 22-hopanol found in *Bacillus acidocaldarius*^[15]. Furthermore, Seckler and Poralla^[15] found for 22(29)-hopene and 22-hopanol a ratio of 83 : 17 and we observed with the purified enzyme a ratio of 86 : 14. For analytical purposes only a partial conversion was conducted. The amounts of products formed were calculated on the basis of the known specific radioactivity of squalene applied in the test and the tritium activity found on the plate for squalene and the products. The other products were determined by calibration of the gas-chromatography procedure with authentic references of 2b, 2c and 4.

Enzyme purification

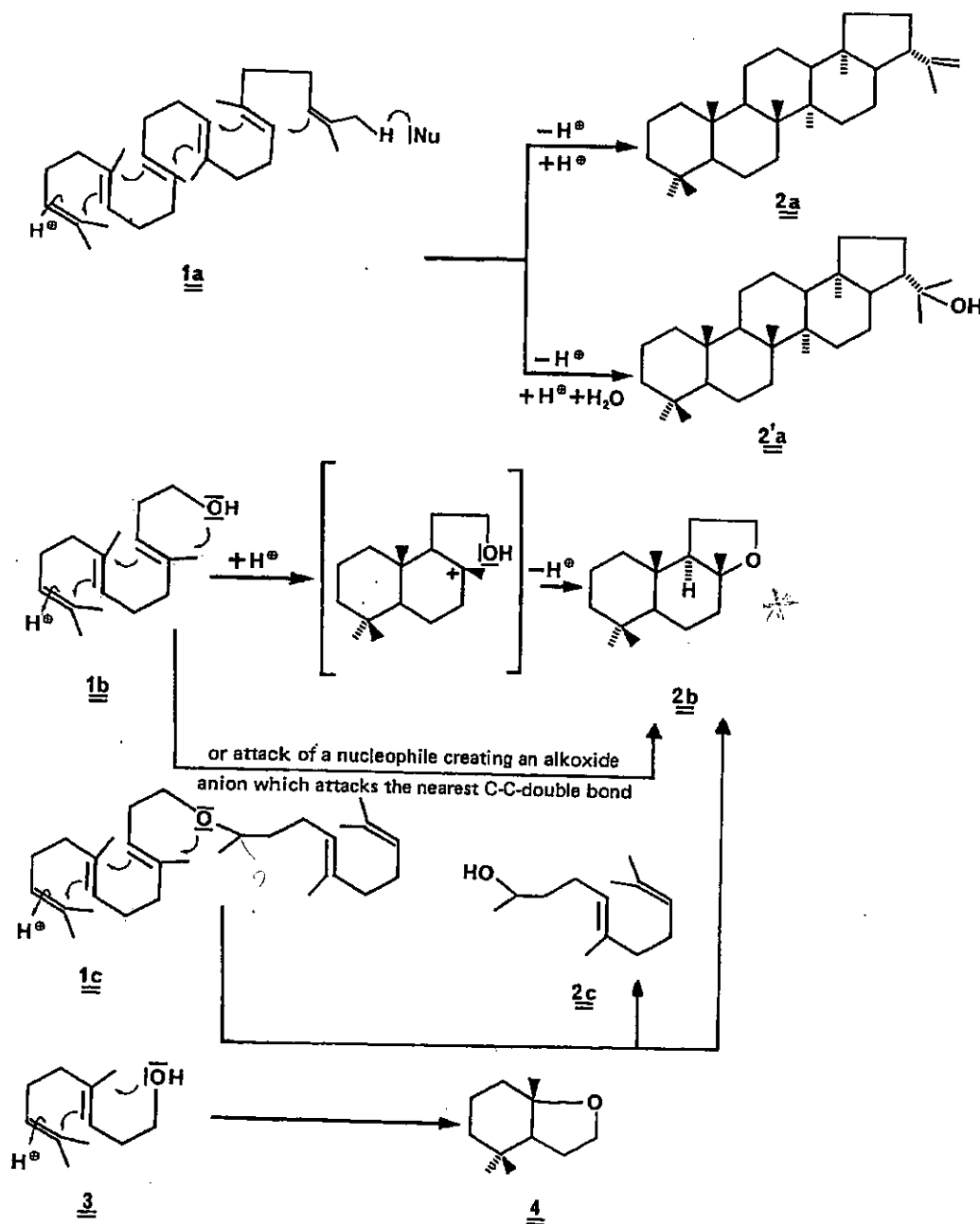
The squalene cyclase is a membrane-bound enzyme. The purification procedure described in Materials and Methods is the result of many tests especially with respect to the absence or presence as well as the kind and concentration of detergent.

The cyclase could be purified to homogeneity (> 95%) with about 22% yield. A typical protocol is depicted in Table 1. All chromatographic separations were carried out at room temperature.

The enzyme binds very tightly to phenyl-Sepharose and cannot be eluted even with 70% glycol. However, 1,2-propanediol elutes the cyclase with satisfying yield and about 8-fold enrichment. The propanediol-containing solution of the phenyl-Sepharose CL-4B column can be put directly onto the DEAE cellulose DE-52 column. This step leads to the separation of the enzyme from propanediol and to an almost three-fold enrichment. The detergent Polidocanol does not interfere with the measurement of protein absorption. The elution peak from the DEAE cellulose column is rather broad. Fractions containing smaller amounts of enzyme were discarded. Altogether about 65–70% of the activity put on the column is eluted.

Table 1. Purification of squalene cyclase (activity measured with [^3H]squalene).

Purification step	Volume [ml]	Protein concentration [mg/ml]	Activity [mU]	Specific activity [mU]	Enrichment [<i>n</i> -fold]	Yield [%]
Crude extract	100	13	442	0.34	—	100
Resolubilized enzyme	25	4.1	350	3.4	10	79
Phenyl-Sepharose CL-4B	43	0.23	264	27	79	60
DEAE-Cellulose DE-52	21.6	0.088	148	78	229	33
Mono-Q	19.6	0.022	99	228	671	22



Scheme: Reactions catalysed by squalene cyclase of *Bacillus acidocaldarius*. H^+ is a proton delivered from the enzyme, Nu a nucleophile of the enzyme.

The chromatography on the strong basic anion exchange resin Mono Q needs the complete removal of the detergent. Because Amberlite XAD-2 also binds the enzyme to some extent most of the Polidocanol was eliminated by ultrafiltration and only the residual small amount of detergent was adsorbed on Amberlite XAD-2. The chromatography on the Mono Q-column led to rather sharp peaks containing the enzyme in 4 ml. However, some of the enzyme seems to bind unspecifically and irreversibly.

The purity of the so obtained enzyme can be seen in Fig. 1. The apparent molecular mass is 150 kDa. Omission of the detergent during the gel chromatography causes the elution of the enzyme in the void volume of the column. That means the molecular mass of aggregates is much higher than 600 kDa. Tested in vitro the purified enzyme exerts a specific activity of 230 mU for the cyclization of squalene. This corresponds to a turnover number of about 33 mol of product/(mol enzyme $\times$ min).

The question arises whether this small activity accounts for the hopanoid formation in *B. acidocaldarius*? According to ref.^[5,10] the organism contains 1.1 mg (2.7 μ mol) hopanoids per gram dry weight if grown under the conditions described here. The doubling time was about 1 h. That means 500 mg of protein (50% of the dry mass) should be able to produce about 3 μ mol hopanoid/h. The protein of the crude extract showed a specific activity of 0.34 mU, which means 500 mg protein produce per hour = $500 \times 0.00034 \times 60 = 10.2 \mu$ mol hopanoids. This indicates clearly, that the low turnover number is sufficient for the formation of the observed hopanoid.

The stability of the cyclase under operational conditions is shown in Fig. 2.

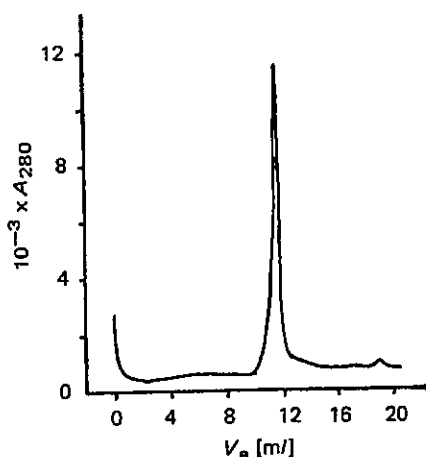


Fig. 1. Elution profile of cyclase purified according Table 1 from a FPLC-gel column.

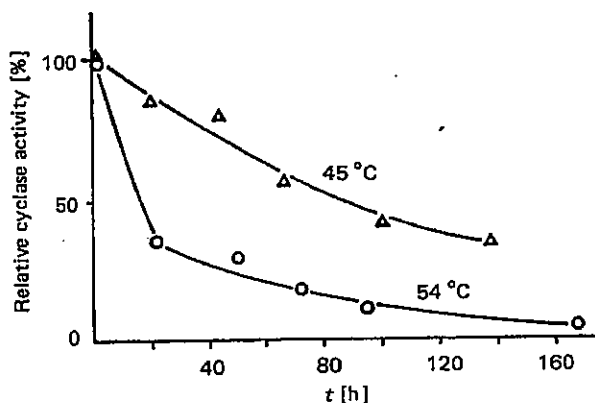


Fig. 2. Time course of the enzyme activity of resolubilized cyclase (see Table 1) at two temperatures.

Electrophoresis

The cyclase could not be analysed by disc electrophoresis. Without detergent, the protein did not move even into a 3.75% acrylamide gel. In the presence of 0.1% and 0.2% Polidocanol in the enzyme solution and the buffer the cyclase entered the gel and led to a single band. However, above this band a continuous weak smear was observable. SDS gel electrophoresis showed a clear band from which the molecular mass of 80 kDa for the subunits could be calculated. As reference proteins chymotrypsinogen, ovalbumin and catalase were used.

Substrate specificity, structure of the products and kinetic parameters

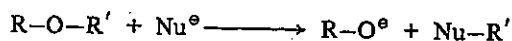
Crude extract as well as purified enzyme delivered about the same ratio of 22(29)-hopene (2a) and 22-hopanol (2'a). Using a crude extract the ratio of the radioactivity of 2a to that of 2'a on the TLC-plate was 6.3 and the ratio obtained by purified enzyme was 6.1.

The enzyme cyclizes besides squalene also *E,E*-homofarnesol (1b), homofarnesol (1,5,9-trimethyl-4,8-decadienyl) ether (1c) and homogeraniol (3). The rates of the cyclization of these substrates are 3%, 0.3% and 0.05% of that of squalene. The conclusion that from a mixture of different homofarnesol isomers only *E,E*-homofarnesol is cyclized comes from the observation that the treatment of a mixture of all four possible homofarnesol isomers (6,7-*E*, 10,11-*E*; 6,7-*Z*, 10,11-*E*; 6,7-*Z*, 10,11-*Z*; and 6,7-*E*, 10,11-*Z*) with the enzyme followed by capillary GLC analysis resulted in unchanged amounts of three of the four isomers and an obvious decrease of one isomer which was *E,E*-homofarnesol. It could be identified by comparison with authentic material by GLC analysis.

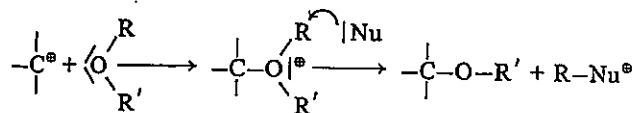
The structure of 2b is assumed from the following facts and observations: The product formed by the enzyme and the authentic material depicted by formula 2b cannot be differentiated by capillary GLC analysis, coupled GLC/mass spectroscopy and by their characteristic odor. Furthermore, corresponding positions of the carbon skeleton of 2b and the hopanoids have identical configurations. For the determination of the structure and the configuration of the hopanoids see Ourisson et al.^[20]. The configuration of authentic 2b is established by the oxidation of sclareol to 2b. For the structural relationships see l.c.^[21,22]. The two products formed from 1c were 2b and 1,5,9-trimethyl-4,8-decadienol (2c). The latter could not be differentiated from authentic 2c by capillary GLC analysis. Finally the furan derivative 4 could again be identified by the comparison with a diastereomeric mixture of octahydro-4,4,7a-trimethylbenzofuran. One of the two peaks obtained by GLC analysis co-eluted with the enzymic product.

It is assumed that hopanoid cyclases catalyse their reaction by the combination of proton attack and proton abstraction by a nucleophile^[1,4,20]. The formation of 2b and 4 can be understood if it is assumed that in the cyclization process a nucleophile from the enzyme deprotonates the hydroxy group of 1b or 3 leading to a strong nucleophile which plays the role of a pair of π electrons from the squalene. In the case of the cyclization of 1b to 2b an electron pair of the oxyanion corresponds to the π electron pair of the double bond between the carbons 14 and 15 of squalene and in the case of formation of 4 an electron pair of the oxyanion would correspond to the double bond between carbon atoms 10 and 11. Another possibility would be that the OH-groups of 1b or 3 capture intermediate carbonium ions.

The formation of 2b from 1c is also interesting. The GLC analysis showed clearly the presence of 1b and 2b as products when 1c was incubated with cyclase. A further product observed after the incubation of 1c with the enzyme is probably 2c. By GLC no other products have been observed. The products 1b, 2b and 2c can be explained by the fission of the ether bond of 1c by the enzyme. Ether bond fission by strong nucleophiles is a well known phenomenon in organic chemistry. However, if this is correct the enzyme becomes intermediately alkylated:



Again an intermediate carbonium could interact with the ether function. The oxonium ion could be split by a nucleophile of the enzyme:



The question remains how the enzyme splits off R?

Homofarnesylcarboxylate, homofarnesyl methyl ether, farnesol, geraniol and nerol were not substrates of the cyclase. That homofarnesyl methyl ether, in contrast to homofarnesyl (1,5,9-trimethyl-4,8-decadienyl) ether, does not react may be explained by the assumption that the former is too small and the probability for a "correct" position in the active center of the enzyme is unlikely. Farnesol or geraniol would lead to four membered rings, which is highly improbable. However, one can predict that a bishomofarnesol might be a substrate.

The low probability of substrates such as the C_{16} compound 1b to be correctly bound by an active site of an enzyme which has a natural substrate of 30 carbon atoms may be the explanation that the rate of conversion of 1b to 2b is only 3% of that of the hopanoid formation from squalene. In the reaction of the C_{11} -substrate 3 this effect is even more marked.

Since we have been mainly interested in the formation of 2b from 1b we studied the pH-dependence of this reaction rate and the product inhibition of 2b. Tested in 0.1M phosphate buffer and 0.1M citrate buffer the cyclization rate decreased from 0.056 mU at pH 5.0 to 0.014 mU at pH 7.0.

The rate of the formation of 2b increased with the concentration of 1b from 0.25mM to 2.0mM and declined slightly in the presence of 4mM 1b. The product inhibition of 2b is shown in Table 2. Only 25 μ M 2b inhibit the cyclization by more than 50%. This is an extremely strong product inhibition.

This work was partially supported by the Bundesministerium für Forschung und Technologie. We are grateful to Dr. H. Günther for advice and establishing the GLC

Table 2. Inhibition of the cyclization of homofarnesol 1b by the cyclization product 2b.

The concentration of substrate in all experiments was 1mM.

Concentration of 2b [μ M]	Cyclase activity [mU]	Inhibition [%]
0	0.02	0
25	0.009	54
50	0.006	67
75	0.003	83

analysis and Dr. Staiger, Consortium für Elektrochemische Industrie GmbH, München for substrates and discussion. We acknowledge especially that Prof. K. Poralla called our attention to the Ph.D. thesis of B. Seckler (cf. [15]).

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Biocatalytic Process for (–)-Ambrox Production Using Squalene Hopene Cyclase

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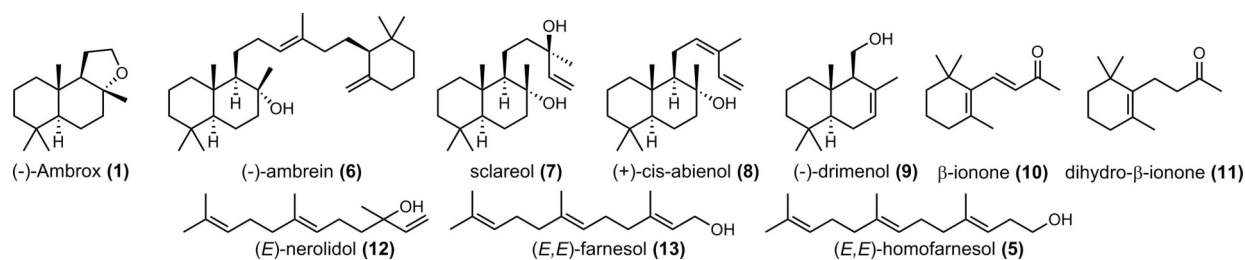
Abstract: *Alicyclobacillus acidocaldarius* Squalene Hopene Cyclase was evolved to a biocatalyst suitable for (–)-Ambrox production at industrial scale. One round of random mutagenesis led to the identification of three variants with (*E,E*)-homofarnesol conversion properties improved about 1.5- to 10-fold over that of the wild type enzyme. Eight distinct amino acid mutations were identified overall; only one mutation was at the active site of the enzyme. Each of the three variants contained only two or three mutations over the 631 amino acids of the *Alicyclobacillus acidocaldarius* Squalene Hopene Cyclase polypeptide chain. Mutations responsible for improved (*E,E*)-homofarnesol conversion were identified. Investigations on reaction conditions led to the selection of one variant, with which reaction parameters were optimized towards process-relevant conditions. A whole cell biotransformation process is presented in which *Escherichia coli* cells producing an improved Squalene Hopene Cyclase variant allows the conversion of 125 g/L (*E,E*)-homofarnesol in ≤ 72 hours. The developed process for the production of the fragrance ingredient (–)-Ambrox as Ambrofix[®] expands the biocatalysis toolbox by setting out a general basis for biocatalytic Squalene Hopene Cyclase cyclization reactions at industrial scale.

Keywords: (–)-Ambrox; Biocatalysis; Fragrances; Protein engineering; Isomerases; Squalene Hopene Cyclase

1 Introduction

Ambergris prized for centuries for its qualities and use in fine perfumes or for medicinal purposes,^[1] is an excretion of the sperm whale *Physeter macrocephalus*. It is thought to be produced in response to irritation of its intestine caused by beak-like mouthparts, eye lenses and pen of squid, the major part of its diet. Living and feeding in the ocean's depths, the sperm whale's life remains a mystery as is the development of ambergris' odor properties. Having a nauseating odor when it is expelled, as it ages under the action of sea water and air, ambergris develops a characteristic pleasant complex odor profile. This is the result of the oxidative degradation of (–)-ambrein (**6**) (Scheme 1), the most abundant but odorless component of amber-

gris. The smell of ambergris is described as well balanced with soft musky, animalic, marine, sweet, earthy, and woody facets. When having the privilege to smell an authentic ambergris alcoholic tincture, one's eyes close and one gets transported on an old wooden raft floating on an open sea, being caressed by a salty breeze. Used in trace amounts in perfumes, it serves like musks as a fixative and for exhaling a perfume's most delicate fragrant notes. Ambergris, a rare and extremely valuable material was at certain times worth more than gold.^[2] (–)-Ambrox (**1**) (Scheme 1), the olfactory most important component of ambergris, is recognized as the prototype of all ambergris odorants. It shows a characteristic and memorable ambery and woody smell, historically linked to ambergris, and reminiscent of tobacco and



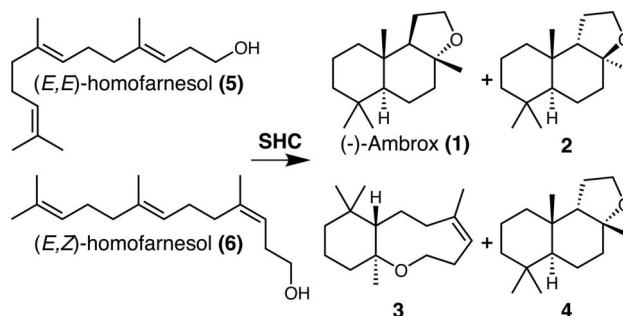
Scheme 1. (–)-Ambrox and compounds for (–)-Ambrox synthesis.

clary sage. The odour of (–)-Ambrox is most often described as triggering a soft, powdery, and almost mineral smoothness wrapping oneself in a pleasant feeling. Its first semi-synthesis starting from the diterpene sclareol (**7**) (Scheme 1) was reported in 1950;^[3] this major industrial resource for (–)-Ambrox production is currently isolated from clary sage (*Salvia sclarea*). A biosynthetic route to sclareol was however recently described.^[4] Several synthetic and biocatalytic routes to (–)-Ambrox and (±)-Ambrox starting from a variety of other compounds including (E,E)-homofarnesol (EEH) (**5**) (Scheme 1) have been reviewed.^[5]

The yearly use of (–)-Ambrox as one of the most valuable perfumery ingredients is estimated at above 100 tons. It benefits from good ecotoxicological properties, and is biodegradable. It is used in all types of applications ranging from fabric and personal care to fine fragrances. One of its advantages over its low odor threshold and good substantivity is its softness compared to other pungent ambery notes like e.g. Ambermax[®] or Amberketal. (–)-Ambrox appears currently in about 30% of perfume creations at a mean dosage of 0.35%, but reaching towards 1% sometimes in fine fragrance applications. Its use appears to be in constant progression. (–)-Ambrox is available from several manufacturers e.g. as Ambrox[®] or Ambroxan[®].^[6] and is produced by Givaudan SA as Ambrofix[®].

In 1986 it was reported for the first time that (–)-Ambrox was the product of the enzymatic cyclization of EEH by a Squalene Hopene Cyclase^[7] (SHC, EC 5.4.99.17, Swissprot P33247, PDB 1SQC), a member of the triterpene cyclase family class II^[8] isolated from *Alicyclobacillus acidocaldarius*. Only a few examples of EEH cyclization to (–)-Ambrox by SHC enzymes have been reported;^[7,9] the ability of these enzymes to produce (–)-Ambrox has attracted the attention of industry.^[10] The first characterization of the enzyme reported a 3% conversion at a substrate concentration of approx. 0.24 g/L using a mixture of the four possible homofarnesol diastereomers.^[7] A method for producing (–)-Ambrox from a mixture of (E,E)-, (E,Z)-, (Z,E)-, and (Z,Z)-homofarnesol isomers with approx. 60% conversion but at only 2 g/L substrate was also described^[10a] where it was stated that (–)-Ambrox and

9-epi-Ambrox (**4**) (Scheme 2) were detected as products, and that (–)-Ambrox was produced from the EEH isomer. Whilst SHC enzymes from *Zymomonas mobilis* and *Bradyrhizobium japonicum* were described as allowing approx. 40 and 22% conversion respectively at approx. 2.4 g/L of substrate, compared to only 1.2% conversion with *A. acidocaldarius* SHC, the homofarnesol isomer necessary for the production of (–)-Ambrox was not used for cyclization.^[10b,c] When homofarnesol was used as a substrate and as reported so far, the concentration ranges used and the conversions achieved were very low. To our knowledge no reports are available showing either a naturally high activity of SHC towards EEH, or on activity improvement studies towards this substrate.



Scheme 2. Homofarnesol cyclization from a mixture of isomers. (E,E):(E,Z) ratio: 90:10 or 80:20.

SHC enzymes catalyze the most remarkable step in steroid and triterpenoid synthesis. They have attracted the attention of chemists and biochemists for several decades because unlike other terpene cyclases they do not require a phosphorylated precursor, but proceed via a Brønsted acid catalysis-driven mechanism.^[11] *A. acidocaldarius* SHC has served as a model for detailed mechanistic studies. Structure determination^[12] and mutagenesis studies^[13] not only elucidated the mechanism of C–C, C–N, and C–O bond formation by these enzymes, but also showed their plasticity and ability to be evolved to promiscuous biocatalysts that could serve as a tool in chemical synthesis.^[14] We evolved *A. acidocaldarius* SHC for (–)-Ambrox pro-

Table 1. Amino acid mutations in Squalene Hopene Cyclase variants.

SHC variant	Amino acid mutation							
	T77A	I92V	F129L	M132R	A224V	I432T	Q579H	F601Y
101A10							x	x
111C8	x	x	x					
215G2				x	x	x		
3								x
10			x					
26				x		x		
30			x					x
31			x	x		x		
32				x		x		x
33			x	x		x		x

duction under process-relevant conditions using a substrate consisting of a mixture of only two isomers: (*E,E*)-homofarnesol (**5**) and (*E,Z*)-homofarnesol (EZH) (**6**) (Scheme 2).

2 Results and Discussion

2.1 Evolution of Squalene Hopene Cyclase

EvoSight™, a proprietary method of Protéus SA was applied to create SHC variants. It determines both the optimal number of random mutations to be introduced for obtaining an enzyme library with high proportion of improved variants and the minimum number of variants to screen to ensure that all possible independent variants are screened.^[15]

Libraries were created with increasing mutational load; about 100 clones of each library were assayed for EEH conversion to (–)-Ambrox. The average number of mutations per gene in the 7 libraries was 1.9, 4.5, 5.5, 8.1, 9.2, 13.6, and 17.6. The processing of the relationship between the fraction of active variants and the number of mutations indicated that the optimally mutated library was a library of variants with 4 mutations. Consequently the corresponding library of 10350 variants was screened. 90 hits were selected from microtiter plate assays for validation at milliliter scale. Three improved variants (101A10, 111C8, and 215G2) were identified showing a 1.6- to 3.5-fold better EEH conversion at 24 h of reaction. Their initial rate of (–)-Ambrox production was improved by a factor of 4.6 to 19. The sequencing of the corresponding *shc* genes indicated a total of 8 distinct amino acid mutations (Table 1).

Surprisingly only one of the mutations was located at the active site (F601Y). The other mutations identified in the three improved variants were all but one (Q579H) located in domain 2 of the SHC crystal structure.^[12] Further mutations identified during screening, but not leading to any activity improvement were spread equally over the two domains of the enzyme (Figure 1).

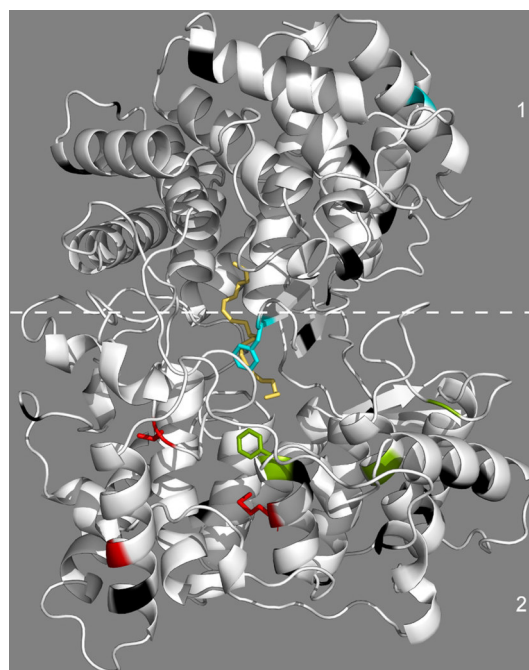


Figure 1. Location of amino acid mutations in the Squalene Hopene Cyclase crystal structure (PDB 1SQC). In blue, green, and red are the positions mutated in 101A10, 111C8, and 215G2, respectively. The highlighted side chains indicate the positions where the mutations have been identified as beneficial: F601Y, F129L, M132R, and I432T. Other positions with mutations but showing no improvement on activity are in black. In yellow is the co-crystallized substrate analogue lauryl dimethylamine-*N*-oxide,^[12] (prepared using PyMOL, The PyMOL Molecular Graphics System, version 2.0, Schrödinger, LLC).

2.2 Mutations Study

28 variants were constructed in total based on the mutations present in the 3 parent variants (as single mutations or in combinations) for identifying mutations responsible for activity improvement, and for evaluating the possibility of further activity improve-

ment when combining beneficial mutations. Combination of beneficial mutations (Figure 1) did not improve further EEH cyclization to (–)-Ambrox. Activity assay under validation reaction conditions (55 °C, pH 6.0, 0.05% (w/v) SDS (Sodium Dodecyl Sulfate)) allowed for the selection of 7 new variants (Table 1) showing improved initial rates over wild type SHC (Figure 2A).

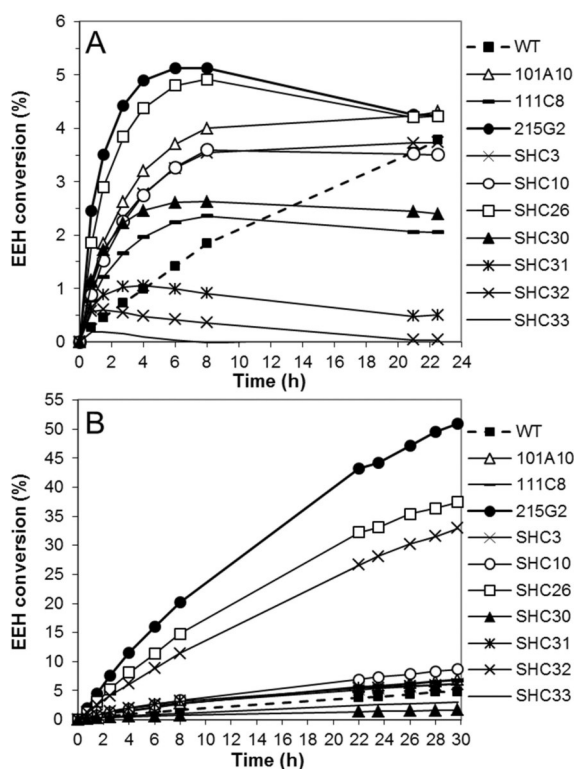


Figure 2. Influence of reaction conditions on activity of variant and wild type Squalene Hopene Cyclases. 4 g/L (*E,E*)-homofarnesol was cyclized at pH 6.0 and 55 °C in presence of 0.05% (w/v) SDS (A), or at individually optimized temperature, pH, and [SDS] (B).

2.3 Characterization and Ranking of SHC Variants

The EEH conversion profiles obtained under validation assay conditions (Figure 2A) strongly suggested that setting the reaction parameters to optimum individually for each SHC variant would allow for a first step in activity improvement. Providing optimal reaction conditions to each of the wild type and variant enzymes was a prerequisite for the selection of the best available SHC variant. EEH conversion at 4 g/L was investigated at constant cell concentration (OD_{650nm} of 10, approx. 12.2 g/L wet weight of cells) with wild type and 10 variant SHC enzymes (Table 1) at various pH, temperature, and [SDS] conditions.

Studied ranges and resulting optimal reaction conditions are detailed in Table S1 (Supporting information). Significant variations in optimal values for the 3 parameters were noted among the variants. Wild type SHC tolerated broad variations while the variants showed rather a distinct optimum for the 3 parameters. All variants showed in particular a temperature optimum between 13 to 23 °C lower than that of wild type SHC. This observation confirmed the hypothesis that the reaction profiles obtained under validation assay conditions (Figure 2A) were due to a pronounced sensitivity of the SHC variants towards reaction conditions, and towards temperature in particular. The high temperature (55 °C) led to enzyme inactivation, and, due to its high volatility, promoted (–)-Ambrox evaporation. This explained the apparent decrease in conversion observed with some of the variants. This was in contrast not observed with wild type SHC, which retains its activity at 55 °C: enzyme activity compensated then for (–)-Ambrox evaporation.

The 10 SHC variants were finally ranked with regard to their performance in EEH cyclization to (–)-Ambrox using *E. coli* cells that had been grown and induced under strictly the same conditions. (*E,E*)-homofarnesol cyclization was tested in the standard activity test applying the reaction conditions defined as optimal for each of the variant and wild type enzymes (Figure 2B). The SHC content of the cells was analyzed by SDS-PAGE using a sample of the reactions. A very consistent amount of SHC in the *E. coli* cells producing the wild type and variant SHC enzymes was observed (Figure 3). It was concluded that the differences in activity observed were not biased by strong variations in SHC production levels. The individual optimization of reaction conditions by itself resulted in a ~10-fold activity improvement with variant 215G2 (Figure 2B).

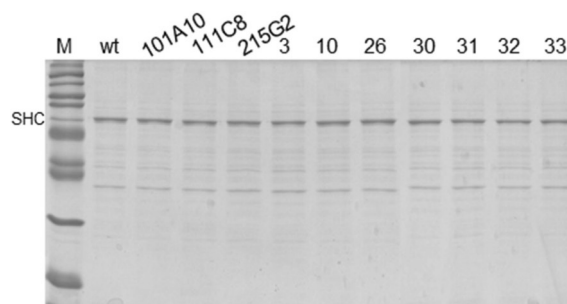


Figure 3. SDS-PAGE analysis of the SHC content of *E. coli* cells producing wild type and variant SHC enzymes. Reaction samples were loaded equivalent to an OD_{650nm} of cells of 2.0 (M: BioRAD broad range marker).

The chemical purity of the homofarnesol stock influenced the velocity of EEH conversion. Depending both on the homofarnesol stock used and the reaction time applied, conversion increased overall and reached completion with variant 215G2.

Variant 215G2 was selected as best variant and chosen for developing process-relevant reaction conditions for (–)-Ambrox production. This variant was the result of a single round of evolution; the introduction of only very few mutations (0.3% of amino acid chain length) was responsible for activity improvement, and very surprisingly the mutations were not located in the active site of the enzyme.

A. acidocaldarius SHC accommodates rather large compounds as substrates and shows decreasing activity with substrates decreasing in size. It is still able to cyclize homogeraniol (C_{11})^[7] but does not cyclize geraniol (C_{10}).^[11a] A *Zymomonas mobilis* SHC enzyme was recently characterized that accepts (*S*)-citronellal (C_{10}) and EEH (C_{16}) as substrates.^[9] It shows a preference for short-chain substrates and an improved activity towards EEH compared to the *A. acidocaldarius* enzyme: 23% vs. 3.5% conversion at approximately 2.4 g/L EEH in 5 h.

The enzyme evolution program we conducted changed dramatically the substrate scope of *A. acidocaldarius* SHC and created the ability for that enzyme to efficiently cyclize the short-chain substrate EEH. This is an additional example that the biocatalysis toolbox can be expanded not only by discovering new biocatalysts (strains or enzymes) but also by enzyme engineering to improve the performance of natural enzymes, which are often reported as not suited for most industrial processes. Recent reviews outlined the achievements obtained in various industrial applications when evolution approaches were considered to address enzyme stability, substrate specificity or activity.^[16]

2.4 Biocatalyst Production

Wild type and variant SHC enzymes were produced from plasmids where the *shc* gene was under the control of an IPTG-inducible promoter (T7 in pET-24a(+) and pET-28a(+), T5 in pJExpress441). The influence on the specific activity of the composition of the cultivation medium, of the isopropyl- β -D-1-thiogalactopyranoside (IPTG) concentration, and of the temperature for induction of SHC production was investigated (Supporting information, Figure S1).

E. coli strains producing the variants were grown and induced both in complex medium (LB-Lennox, Terrific broth (TB)), and in minimal media such as M9 or M63.^[17] The cultivation medium affected not only the growth profile but also the amount of SHC enzyme produced, and the specific activity of the resulting biocatalyst. The minimal medium used for

biocatalyst production in fermenters was the best medium for biocatalyst production also at small scale.

IPTG for induction was applied in the range of 25 μ M to 1 mM at temperatures between 23 and 46 °C. SHC production increased with increasing IPTG concentration and increasing temperature. The optimal IPTG concentration was at 300–1000 μ M; the optimal induction temperature was 37–40 °C. The relatively high temperature optimum for induction of SHC production may be explained by the mesophilic origin of the parent microorganism *A. acidocaldarius*. Temperatures higher than 41 °C impaired growth and reduced the specific activity of the cells. The enzyme was equally well produced from T7 and T5 promoters, with or without *N*-terminal HisTag.

2.5 (*E,E*)-Homofarnesol Bioconversion

2.5.1 Role of the Surfactant

To evaluate if solubilizing agents other than SDS could be used, various surfactants and detergents were tested for EEH cyclization with variant 215G2 (standard activity assay). These included the non-ionic surfactant Triton X-100 mostly used in SHC studies (Supporting information, Table S2).

SDS was confirmed as the only surfactant allowing for EEH cyclization to (–)-Ambrox and it was found that Triton X-100 did not promote EEH cyclization. Triton X-100 and the other compounds tested (Table S2) allowed for the conversion, which was obtained in a reaction in the absence of any solubilizing agent. No activity improvement was obtained when combining SDS with any of the other compounds.

In addition to the compounds listed in Table S2 some deep eutectic solvents and ionic liquids^[18] were tested as well, but with no positive result. This confirmed that under the conditions applied, SDS and only SDS could be used for EEH cyclization.

The influence of SDS was investigated at 4 g/L EEH and constant cell concentration (OD_{650nm} of 5.0, approx. 6.1 g/L wet weight of cells) at SDS concentrations from 0.010 to 0.070% (w/v). It was recognized that maximal activity was obtained only when the SDS concentration was properly set. The importance of this parameter was investigated further and confirmed during the optimization of reaction conditions towards process-relevant conditions.

One of the commonly cited and prominent disadvantages of whole cell biocatalysts is limitation in mass transfer due to the membrane.^[19] The use of a whole cell biocatalyst in the present process together with SDS as an emulsifying agent facilitates EEH dissolution into the membrane of the cells and access to the non-polar channel connecting the active site to the membrane-immersed region of the enzyme.^[12,20]

The benefits, recent advances, and ways to overcome the limitations of whole cell biocatalysis were recently reviewed.^[19] Productivity metrics often suffer from low substrate load and product titer, reactions being either tested at low substrate concentration or in aqueous systems where many synthetic hydrophobic substrates show low solubility. In the present case the use of a whole cell system in combination with SDS as a surfactant adequately addresses these limitations: it allows a high conversion of the water-immiscible EEH at high concentration in an aqueous system.

2.5.2 Optimization of Bioconversion Conditions

The screening for the identification of an SHC variant with improved EEH conversion properties was carried out at a low substrate concentration of only 4 g/L. Preliminary studies were run to evaluate the possibility of evolving the reaction conditions towards process-relevant conditions. They showed that the parameters “[EEH]”, “[cells]”, and “[SDS]” were interconnected, higher substrate concentrations requiring apparently higher [SDS] and [cells]. The sensitivity of the reaction towards changes in pH was recognized; acetic acid/sodium acetate buffers were most appropriate for running reactions in small volumes without requirement for pH regulation. A reaction with EEH and cells both at approx. 66 g/L suggested that the probability for full conversion of at least 50 g/L EEH was high. These parameters, including temperature and pH were studied through design of experiment to define a response surface. Because the operating ranges were originally not clearly defined, a Doehlert matrix was chosen for the possibilities it offers for an easy extension (adaptation of parameters, change of design space).

In a first set of experiments (DOE1) only the 3 parameters “[cells]”, “[EEH]”, and “[SDS]” in the range 40–80 g/L, 40–80 g/L, and 0.1–0.5% (w/v), respectively, were studied. Temperature and pH were fixed at their central points (35 °C and 5.0). The discontinuous performance of the reaction in the chosen operating range did not allow for modelling of the reaction. This was explained by a strong interaction between “[cells]” and “[SDS]”. Maximal activity was obtained only when the ratio [SDS]:[cells] was properly set. When [SDS] was too low, the system underperformed, when it was too high, activity was impaired (Figure 4). This result confirmed that the ratio between [cells] and [SDS] must be carefully set to ensure maximal activity.

The analysis of DOE1 results indicated a strong effect of the concentration of the cells, whereas the effect of the EEH concentration was weaker. Consequently a new series of experiments (DOE2) was designed according to the Doehlert matrix by expanding the operating range, replacing the parameter

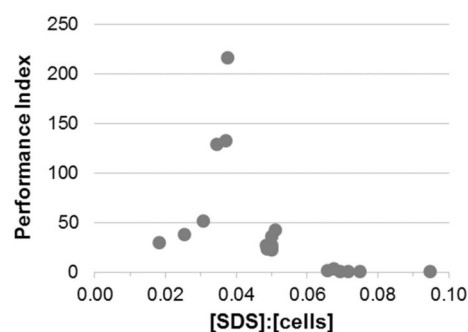


Figure 4. Performance index as a function of [SDS]:[cells].

“[SDS]” by “[SDS]:[cells]”, and adding pH and temperature as new parameters. [cells], [SDS]:[cells], and [EEH] were in the range 40–120 g/L, 0.018–0.054, and 30–90 g/L, pH at 4.8–5.2 and temperature at 30–40 °C. Data processing allowed calculation of a polynomial model, which correlated well with experimental data. This model was validated with experimental data belonging to the first set of DOE experiments.

Desirable features were defined for optimizing reaction conditions in the studied space towards process-relevant conditions. Minimal achievement and optimal target for EEH conversion, (–)-Ambrox titer, and biocatalyst productivity were set to find a compromise suited for industrialization purposes. Optimal reaction conditions were chosen at 50 g/L EEH, 110 g/L cells, [SDS]:[cells] of 0.041, at pH 5.1 and 37 °C. Some experiments were implemented at this optimum and their performance compared to the benchmark conditions 66 g/L cells, 65.7 g/L EEH, [SDS]:[cells] of 0.050, pH 5.2, 33 °C. The calculated model allowed for a very good prediction (Figure 5).

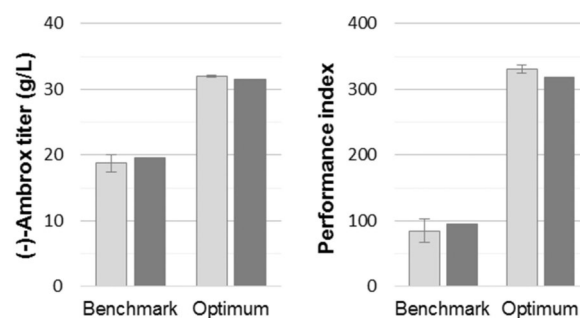


Figure 5. Comparison of experimental (light grey) and calculated (dark grey) data. Reaction time: 18 h.

A thorough analysis of the model allowed for a better understanding of the behaviour of the parameters and their interactions. Some rules were determined and extrapolation was done outside the operating range studied. Successive 2³ factorial designs were run in order to increase the EEH load with the

following approach: (i) target the proper relative amounts of cells and EEH, which were identified as the most impacting parameters, (ii) adjust optimal [SDS]:[cells], (iii) keep pH at 5.1 and temperature at 37 °C. A new optimum was identified at 180 g/L cells, 90 g/L EEH, [SDS]:[cells] of 0.048, pH 5.1, 37 °C. Analyzing the result of successive sets of experiments showed that best results were obtained at a ratio of cells to EEH of approximately 2.

A last step of extrapolation outside of the design space was done in an additional set of experiments by drastically increasing [EEH] to 125 and 150 g/L at a ratio of cells to EEH of 2. Two [SDS]:[cells] ratios per concentration of cells were tested as well as two levels of pH (4.9 and 5.1); the temperature was slightly increased to 38 °C. The best performance was obtained at the lowest pH value and highest [SDS]:[cells], which still suggested the possibility for further optimization. Figure 6 shows the performance achieved during the whole optimization procedure.

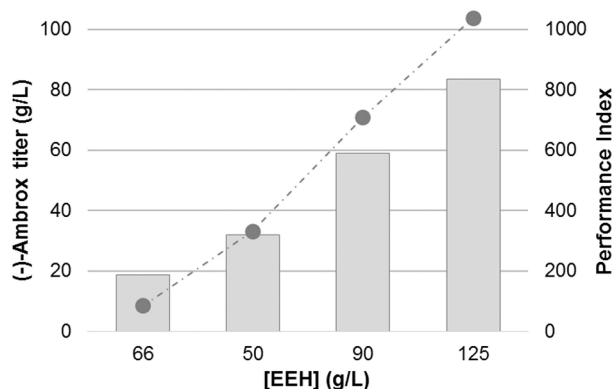


Figure 6. (–)-Ambrox titer (columns) and performance index (closed circles). 66 g/L EEH: benchmark, 50 g/L EEH: response surface optimum, 90 g/L EEH: intermediate optimum, 125 g/L EEH: final condition. Reaction time: 18 h.

2.5.3 Confirmation and Scale up of Bioconversion Conditions

Upscaling of the optimized reaction conditions was tested at 150 mL-scale in 0.1 M acetic acid/sodium acetate buffer with 125 g/L EEH, 250 g/L cells, 1.55% (w/v) SDS, at pH 4.9 and 35 °C. The biotransformation conditions resulted in high conversion at high substrate load (Figure 7). Crystalline (–)-Ambrox was produced, which is an advantage for product recovery.

EEH cyclization at 125 g/L substrate and 250 g/L cells was compared between best variant 215G2 and wild type SHC enzymes. Figure 7 shows the time course of EEH cyclization and the improvement in activity as a result of engineering both enzyme and

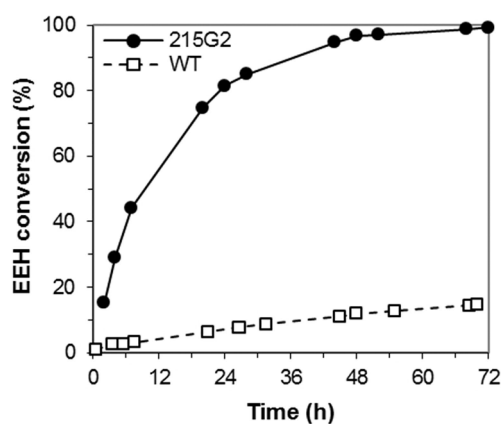


Figure 7. (*E,E*)-homofarnesol cyclization with wild type and 215G2 SHC. Reactions were run at 125 g/L EEH and 250 g/L cells, at pH 5.4 and 35 °C with variant 215G2, and at pH 6.0 and 55 °C with wild type SHC.

reaction conditions. The maximal productivity observed was about $10 \text{ g} \cdot \text{L}^{-1} \cdot \text{h}^{-1}$, with an approx. 10-fold improvement for the variant vs. parent wild type enzyme. An 11-fold increase in specific activity (initial rate) and a 7-fold increase at full substrate conversion with the variant were achieved over the wild type enzyme.

2.5.4 Robustness of Bioconversion Conditions

For the setting of process-relevant EEH bioconversion conditions were used acetic acid/sodium acetate buffers in which the pH of the reaction was stable, and no pH regulation was required. The robustness of the developed bioconversion conditions at high concentrations of substrate and cells was characterized in terms of the requirement for a buffering medium, the pH of the reaction, the sensitivity towards reaction medium composition, and the concentration of SDS. Reactions were run at 125 g/L EEH with 250 g/L cells in either a buffer (0.1 M citric acid/sodium citrate, acetic acid/sodium acetate, succinic acid/NaOH) with pH ranging from 4.6 to 5.8, or at pH 5.4 in water (tap or deionized) or in NaCl solutions (0.5–1.8% (w/v)) (Figure 8).

The medium/pH combination influenced EEH conversion (Figure 8A). The biotransformation did not depend on a buffering agent as it ran equally well in various buffers at a pH value of as low as 4.6 (acetic acid buffer), or at pH 5.4 in tap or deionized water supplemented with or without NaCl (Figure 8B). The bioconversion tolerated NaCl concentrations of up to 1.8% (w/v). When using a succinic acid/NaOH buffer at pH 5.4, the reaction ran equally well at 50, 75, and 100 mM succinic acid.

The biotransformation tolerated some variation in SDS concentration but the importance of carefully

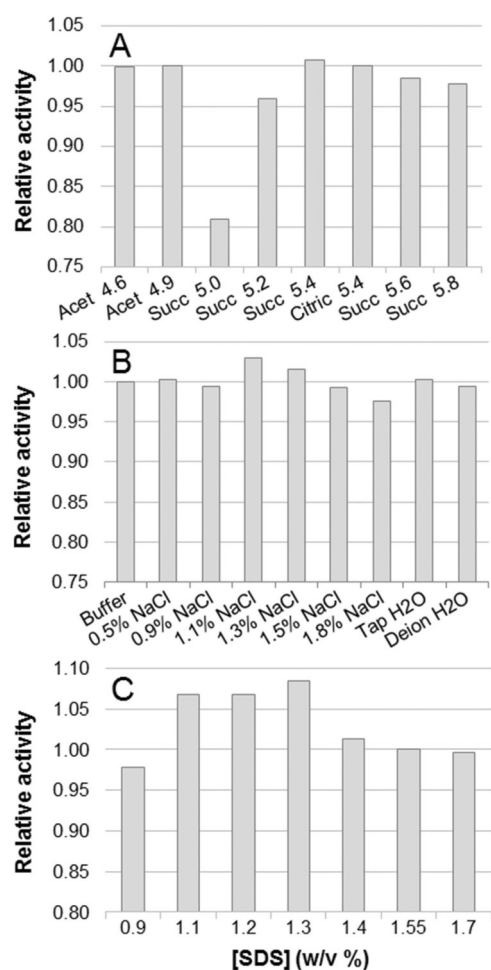


Figure 8. Robustness of (*E,E*)-homofarnesol bioconversion conditions (125 g/L EEH, 250 g/L cells). Conversion at 72 h is indicated with reference to a reaction run at pH 5.4 in citric acid buffer (between 92 and 99% EEH conversion). A: buffer and pH (Acet: acetic acid/sodium acetate, Citric: citric acid/sodium citrate, Succ: succinic acid/NaOH), B: reaction medium (pH 5.4), C: SDS concentration (pH 5.4).

setting the [SDS]:[cells] ratio was confirmed. A too low SDS concentration does not enable maximal performance whereas a too high SDS concentration is detrimental to the bioconversion (Figure 8C).

As an alternative to harvesting cells and running EEH cyclization in a defined medium, the biotransformation was run as efficiently directly in the fermentation medium containing the required amount of biocatalyst (250 g/L wet weight of cells) by adjusting the pH to 5.4, and adding the required amount of SDS and of homofarnesol stock for 125 g/L EEH to start the reaction.

2.6 Volumetric Productivity

To increase further the volumetric productivity, a 1.5-fold concentrated bioconversion was run at 188 g/L

EEH with 375 g/L cells. A control bioconversion was run at 125 g/L EEH, 250 g/L cells. 72 h after the start was EEH conversion 88% in the 1.5-fold bioconversion vs. 95% in the control. 96 h after the start was conversion 93% in the 1.5-fold bioconversion vs. 97% in the control reaction (Figure 9).

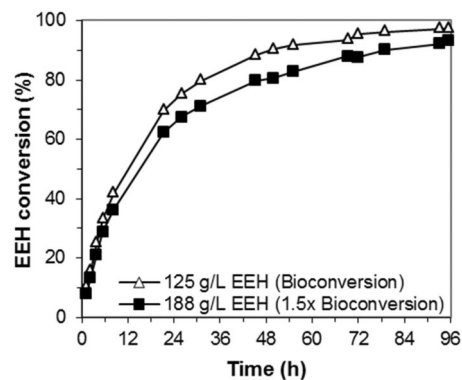


Figure 9. Volumetric productivity.

The stirring in the 1.5-fold concentrated bioconversion became more difficult over time as the oily EEH was converted to crystalline (–)-Ambrox. Stirring efficiency appeared as the only limitation of the system. The physical property *per se* of the 1.5-fold concentrated reaction may be responsible for the slightly lower conversion; a setup allowing for a better mixing would further improve EEH conversion in the concentrated reaction. The results clearly indicated the possibility of running bioconversions at 188 g/L EEH or higher provided efficient mixing is achieved. Volumetric productivity improvement was in the range of 18–30% (Table 2).

Table 2. Volumetric productivity.

Reaction time (h)	Productivity (g · L ⁻¹ · h ⁻¹)	
	125 g/L EEH ^[a]	188 g/L EEH ^[b]
1.25	10.3	12.2
8.25	6.3	8.2

^[a] 250 g/L cells; ^[b] 375 g/L cells.

The whole cell system we developed for (–)-Ambrox production as Ambrofix[®] allows conversion of almost 200 g/L substrate in approx. 96 h. It is well positioned among biocatalytic processes in terms of productivity.^[16,19]

Cost contribution of the biocatalyst to the final process is an important milestone. Guidelines for suitable biocatalysts have been developed. Minimum

productivities for feasibility in different types of processes and products have been suggested.^[21] General rules for cost analysis have been established for biocatalyst production based on biocatalyst format (whole cells, soluble or immobilized enzyme), product cost, market size and value, and the requirements for the implementation of biocatalytic processes have been carefully analyzed.^[22] The applicability of the biotransformation presented here was not limited by its high biocatalyst load.

Enzymes still are the exception rather than the rule in the synthesis of fine chemicals and pharmaceuticals; biocatalysis is often regarded as second generation process.^[23] Although it holds great promise as a powerful tool for application in process chemistry, the number of applications at commercial scale is still disproportionately low. Enzymes are not naturally evolved to work with substrates of interest or not adapted to process-relevant conditions, and are unable to meet the needs of synthetic industry.^[21] By means of both enzyme and reaction engineering we developed a cost-effective process for the production of a fragrance ingredient.

2.7 (–)-Ambrox Recovery

Crystalline (–)-Ambrox, which was present in the biotransformation at high EEH conversion was collected from the reaction broth by means of solvent extraction or filtration (solid/liquid separation).

Hexane, xylene, and toluene allowed efficient extraction of (–)-Ambrox in 3 successive extractions (0.5 vol. each). 85% of (–)-Ambrox was recovered in the first extraction. Hexane performed slightly less well as after the third extraction the reaction broth still contained 2.3% of total (–)-Ambrox vs. 0.6% and 0.9% when using toluene or xylene.

(–)-Ambrox was extracted from the solid phase of the reaction broth using toluene or water miscible ethanol. After 6 consecutive extractions with 1/2 vol. of the solid phase with toluene (1.35 vol. of the initial reaction broth volume), >99.5% (–)-Ambrox was recovered. 99% of (–)-Ambrox was recovered in 4 extractions (3.2 vol. of initial reaction broth) when ethanol was used.

2.8 Reaction Products

EEH was cyclized for the first time from a mixture of the two isomers (*E,E*)-homofarnesol (**5**) and (*E,Z*)-homofarnesol (**6**). Gas chromatography analysis of reaction mixtures identified four reaction products (Scheme 2). They were always observed when cyclizing a mixture of (*E,E*)- and (*E,Z*)-homofarnesol with either wild type or variant SHC enzymes. Conversion of neat (*E,E*)- or (*E,Z*)-homofarnesol allowed assigning the products to the corresponding substrate. NMR

analysis elucidated their structure (details and NMR spectra in Supporting information).

For the first time it was shown that EEH (**5**) was cyclized to (–)-Ambrox (**1**) and **2** ((3a*S*,5a*S*,9a*S*,9b*S*)-3a,6,6,9a-tetramethyldodeca-hydronaphtho[2,1-*b*]furan) in a ratio of approx. 97:3. **2** was not disclosed in the original work on enzymatic cyclization of EEH.^[7] This highly constrained Ambrox stereoisomer was also postulated as unlikely to exist.^[24] Cyclization of (*E,Z*)-homofarnesol (**6**) produced **3** ((7a*S*,11a*S,Z*)-5,8,8,11a-tetramethyl-2,3,6,7,7a,8,9,-10,11,11a-decahydrobenzo[*b*]oxonine), which we describe here for the first time, and (–)-9b-epi-Ambrox (**4**) ((3a*R*,5a-*S*,9a*S*,9b*S*)-3a,6,6,9a-tetramethyldodeca-hydronaphtho[2,1-*b*]furan)) in a ratio of approx. 30:70.

1: ¹H NMR (400 MHz, CDCl₃) δ 3.94–3.88 (m, 1H), 3.82 (q, *J* = 8.2 Hz, 1H), 1.94 (dt, *J* = 11.5, 3.0 Hz, 1H), 1.77–1.66 (m, 4H), 1.49–1.37 (m, 5H), 1.35–1.14 (m, 2H), 1.09–0.95 (m, 2H), 1.09 (s, 3H), 0.88 (s, 3H), 0.84 (s, 3H), 0.83 (s, 3H). ¹³C NMR (100 MHz, CDCl₃) δ 80.0, 65.1, 60.3, 57.4, 42.6, 40.1, 39.9, 36.3, 33.7, 33.2, 22.8, 21.3, 21.3, 20.8, 18.5, 15.2.

2: ¹H NMR (600 MHz, C₆D₆) δ 3.83–3.80 (m, 2H), 2.04–2.01 (m, 1H), 1.78–1.74 (m, 1H), 1.65–1.60 (m, 2H) 1.52–1.48 (m, 2H), 1.45–1.36 (m, 5H), 1.22 (s, 3H), 1.20–1.18 (m, 2H), 1.10–1.05 (m, 1H), 0.90 (s, 3H), 0.82 (s, 3H), 0.82 (s, 3H). ¹³C NMR (150 MHz, C₆D₆, ¹³C-shifts extracted from the HSQC-spectrum) δ 80.0, 65.2, 55.8, 48.4, 41.9, 37.1, 34.6, 34.7, 34.0, 33.6, 25.4, 23.4, 22.6, 21.7, 20.1, 17.9.

3: ¹H NMR (400 MHz, CDCl₃) δ 5.53 (t, *J* = 7.5 Hz, 1H), 3.48 (ddd, *J* = 11, 4.5, 1.5 Hz, 1H), 3.37 (t, *J* = 10.5 Hz, 1H), 3.14–3.06 (m, 1H), 2.34–2.25 (m, 1H), 1.86 (ddd, *J* = 13.4, 8.8, 4.4 Hz, 1H), 1.77 (dt, *J* = 12.0, 4.0 Hz, 1H), 1.66 (s, 3H), 1.61–1.54 (m, 3H), 1.50–1.38 (m, 3H), 1.37–1.24 (m, 2H), 1.24 (s, 3H), 1.14 (td, *J* = 13.0, 4.0 Hz, 1H), 0.92 (s, 3H), 0.83 (s, 3H). ¹³C NMR (100 MHz, CDCl₃) δ 138.3, 124.4, 78.4, 62.1, 44.2, 42.3, 37.1, 34.6, 33.4, 31.7, 29.1, 24.2, 23.1, 22.8, 21.9, 20.2.

4: ¹H NMR (400 MHz, CDCl₃) δ 3.85 (td, *J* = 8.3, 3.2 Hz, 1H), 3.77 (q, *J* = 8.3 Hz, 1H), 2.04 (tt, *J* = 12.2, 9.1 Hz, 1H), 1.90 (dtd, *J* = 12, 8.1, 3.2 Hz, 1H), 1.67–1.53 (m, 5H), 1.43–1.37 (m, 2H), 1.37 (s, 3H), 1.29–1.08 (m, 5H), 1.10 (s, 3H), 0.89 (s, 3H), 0.82 (s, 3H). ¹³C NMR (100 MHz, CDCl₃) δ 80.9, 64.1, 59.1, 46.8, 42.4, 38.8, 36.1, 35.9, 33.7, 33.0, 28.9, 27.8, 22.9, 21.9, 20.5, 18.6.

The NMR data of **1** and **4** are consistent with literature values.^[25]

EEH conversion profiles in reactions run at constant 4 g/L homofarnesol but with varying (*E,E*):(*E,Z*) ratio showed that (*E,Z*)-homofarnesol entered in competition with EEH for cyclization (Figure 10). At identical concentration (4 g/L) (*E,Z*)-homofarnesol cyclization was 3 to 3.5 times slower than that of EEH.

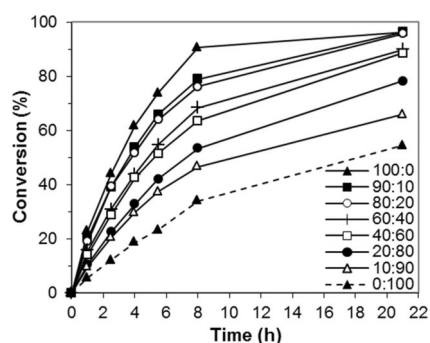


Figure 10. Cyclization of homofarnesol. Reactions were run at constant 4 g/L homofarnesol (EEH + EZH) with (E,E):(E,Z) ratios between 100:0 and 0:100. Conversion refers to EEH except for the curve in dotted line (EZH conversion), where neat (E,Z)-homofarnesol was used.

3 Conclusion

SHC enzymes have attracted the interest of the scientific community decades ago but no reports are so far available on industrial processes based on these enzymes. The work presented here is the very first example of an SHC reaction that was brought to process-relevant and viable conditions by engineering both enzyme and reaction conditions. Ongoing studies indicate that this process can be scaled-up for the production of (–)-Ambrox as Ambrofix® at industrial scale. Together with the evidence published over the past years that SHC enzymes are highly evolvable through enzyme engineering, we set out with the described process^[26] a general basis for biocatalytic SHC cyclization reactions at industrial scale, and demonstrate that these enzymes are amenable to industrial scale biocatalysts.

Experimental Section

Chemicals

All reagents except homofarnesol^[27] were from commercial sources and used without additional purification. Homofarnesol used in this study was a mixture of (E,E)- and (E,Z)-homofarnesol in a ratio 80:20 or 90:10. Any reference to EEH in the various activity assays or bioconversions in this study is a reference to the final (E,E)-homofarnesol concentration in the reaction. Depending on the chemical purity and on the (E,E):(E,Z) ratio of the homofarnesol stock used, the total homofarnesol concentration (EEH + EZH) varied slightly between the reactions.

Strains and Plasmids

The wild type and variant SHC enzymes were produced in *E. coli* MC1061(DE3)^[28] for library screening, otherwise in *E. coli* BL21(DE3)^[29] with or without N-terminal HisTag. The *shc* gene (*NdeI*-*HindIII* insert) was under the control of

a T7 promoter in plasmids pET-28a(+) or pET-24a(+) (Novagen). Alternatively the *shc* gene was under the control of a T5 promoter in pJExpress441 (ATUM).

SDS-Polyacrylamide Gel Electrophoresis

SDS-PAGE was performed on a Mini-PROTEAN II system (Bio-Rad) with 10% polyacrylamide gels under denaturing conditions.^[17a]

Enzyme Evolution

EvoSightTM^[15] was applied to the *A. acidocaldarius shc* gene (Genbank accession number AB007002.1).

High Throughput Production and Screening of SHC Variants, Validation of Selected Hits, Validation Assay

The production of SHC variants was carried out in Luria-Bertani (LB) medium in 96-wells deep well plates. 1 mL cultures were incubated at 37 °C under constant agitation (orbital shaking, 180 rpm). Protein production was induced for 15 h by the addition of IPTG to 0.1 mM when the cultures had reached an OD_{600nm} of 0.2.

SHC activity was assayed in microtiter plates with whole cells that had produced the variants (validation assay). The reactions contained cells to an OD_{600nm} of 10 (approximately 10.3 g/L wet weight of cells), 0.05% (w/v) SDS, and 4 g/L EEH in 0.1 M citric acid/sodium citrate buffer pH 6.0. The reactions were incubated at 55 °C under constant agitation (200 rpm, orbital shaking) for 24 h. Formation of (–)-Ambrox at 24 h was quantified by sampling 100 µL of reaction mixture, extraction with 200 µL hexane:2-propanol (2:1), phase separation, and GC-FID analysis of the organic phase.

Hits selected from the screening step were cultivated in Erlenmeyer flasks (LB medium) and validated at 1 mL scale with whole cells to an OD_{600nm} of 10, and under constant magnetic stirring (all other reaction conditions unchanged). Reactions were incubated for 24 h and sampled over time. Samples were solvent-extracted as above for GC-FID analysis.

Production of Variants, Standard Activity Assay

SHC variants were produced in 300 or 350 mL LB or minimal medium cultures. A 15 mL seed culture (LB medium, 100 mL Erlenmeyer flask) supplemented with kanamycin (50 µg/mL) was inoculated from an overnight preculture to an OD_{650nm} of 0.050 and grown to an OD_{650nm} of 0.50. A 350 mL main culture was then inoculated with 3.5 mL seed culture and grown to an OD_{650nm} of approx. 0.50. Enzyme production was induced by the addition of IPTG at 300 µM and lasted for 5–6 h at 37–40 °C and 280 rpm. Cells were collected by centrifugation (8280 g, 4 °C, 15 min) and stored as pellets at 4 °C or –20 °C until further use. Up to 4–6 g wet weight of cells were obtained from total 1 liter of culture.

The standard activity assay (5 or 10 mL) was run in 0.1 M citric acid/sodium citrate or acetic acid/sodium acetate or succinic acid/NaOH buffer with 4 g/L EEH, and cells to an OD_{650nm} of 10 (approx. 12.2 g/L wet weight of cells) under constant agitation (900 rpm) on a Heidolph Synthesis 1 Liquid 16 device. Optimal temperature, pH, and SDS concentration depended on the SHC enzyme considered (Supporting information, Table S1).

Biocatalyst Production

Fermentations were run in 750 mL InforsHT reactors. To the fermentation vessel was added 168 mL of deionized water. The reaction vessel was equipped with probes (pO_2 , pH, antifoam, sampling device), carbon/nitrogen feed bottle (glucose solution: 35 g glucose + 143 mL deionized water) and sodium hydroxide bottle (10% (w/v)), and sterilized by autoclaving. To the reactor was then added: 20 mL $10 \times$ phosphate/citric acid buffer (133 g/L KH_2PO_4 , 40 g/L $(NH_4)_2HPO_4$, 17 g/L citric acid $\cdot H_2O$ with pH adjusted to 6.3), 14 mL 50% (w/v) glucose in deionized water, 0.53 mL $MgSO_4$ solution (50% (w/v) $MgSO_4 \cdot 7H_2O$ in deionized water), 2 mL $(NH_4)_2SO_4$ solution (50% (w/v) $(NH_4)_2SO_4$ in deionized water), 20 μ L trace elements solution (50 g/L $Na_2EDTA \cdot 2H_2O$, 20 g/L $FeSO_4 \cdot 7H_2O$, 3 g/L H_3BO_3 , 0.9 g/L $MnSO_4 \cdot 2H_2O$, 1.1 g/L $CoCl_2$, 80 g/L $CuCl_2$, 240 g/L $NiSO_4 \cdot 7H_2O$, 100 g/L KI, 1.4 g/L $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 1 g/L $ZnSO_4 \cdot 7H_2O$), 400 μ L thiamine solution (2.25 g/L Thiamin.HCl), and 200 μ L kanamycin stock (50 g/L). The running parameters were: pH = 6.95, pO_2 = 40%, T = 30 °C, stirring at 300 rpm. Cascade: agitation set point at 300 rpm (min. 300, max. 1000), flow set point 0.1 L/min (min. 0, max. 0.6), antifoam control: 1:9.

The fermenter was inoculated from a seed culture (LB medium supplemented with 50 mg/L kanamycin, 37 °C, 220 rpm, 8 h) to an OD_{650nm} of 0.4–0.5. The fermentation was run first in batch mode for approx. 11.5 h, where after was started the carbon/nitrogen feed. The feed consisted of a sterilized glucose solution to which had been added after sterilization: 17.5 mL $(NH_4)_2SO_4$ solution, 1.8 mL $MgSO_4$ solution, 18 μ L trace elements solution, 360 μ L thiamine solution, and 180 μ L kanamycin stock. The feed was run at a constant flow rate of approx. 4.2 mL/h. Glucose and NH_4^+ measurements were done externally to evaluate availability of the carbon and nitrogen sources in the culture using Quantofix® Ammonium (Sigma Aldrich) and MQuant™ Glucose Test (Merck) according to the manufacturer's directions.

Cultures were grown for approx. 25 h reaching typically an OD_{650nm} of 40–45. SHC production was then started by adding IPTG to a concentration of 1 mM (as pulse or over a period of 3–4 hours), setting the temperature to 40 °C and pO_2 to 20%. Induction of SHC production lasted for 16 h. At the induction end the OD_{650nm} was 50–70; cells were collected by centrifugation and stored as pellets at 4 °C or –20 °C until further use. Up to 30–40 g wet weight of cells were collected from a total fermentation volume of approx. 440 mL corresponding to 70–90 g/L of cells.

Optimization of Bioconversion Conditions

Most of the optimization steps were run through design of experiments using the NemrodW® software (NemrodW SAS, France, www.nemrodw.com). For response surface modelling, a Doehlert matrix was used. Otherwise 2^3 factorial designs were used to study parameter impacts and interactions.

Reactions (1.2–7.2 mL volume) were run in tightly sealed glass vials (screw caps) in 0.1 M acetic acid/sodium acetate buffer (pH 4.8–5.2). The biocatalyst load was between 40 and 300 g/L cells (wet weight), that of EEH varied between 30 and 150 g/L. SDS varied between 0.1 and 2.08% (w/v). The reactions were incubated with constant mechanical agitation at temperatures between 30 and 40 °C.

A performance index summarizing the achievements towards all features defined as desirable (product titre, conversion, yield, and reaction time) was defined for monitoring the progress of the bioconversion optimization process.

(E,E)-Homofarnesol Bioconversion

EEH bioconversions (150 mL volume) were run at 125 g/L EEH, 250 g/L cells (wet weight), 1.30–1.55% (w/v) SDS, 35 °C, with constant stirring (700 rpm) in 750 mL InforsHT reactors. Cells were suspended to homogeneity in reaction medium; cell concentration was determined by centrifuging an aliquot of cell suspension (17210 g, 10 min, 4 °C). Once the required amounts of cell suspension, homofarnesol stock (chemical purity, (E,E):(E,Z) ratio), and 31% (w/v) SDS solution was determined, then the required volume of reaction medium was deduced. To the reactor was added: homofarnesol stock, SDS solution, reaction medium, and cell suspension.

Reactions were run either in 0.1 M citric acid/sodium citrate, 0.1 M acetic acid/sodium acetate or 0.1 M succinic acid/NaOH buffer pH 4.6–5.8, or in 0.5, 0.9–1.8% (w/v) NaCl or water (tap or deionized) at pH 5.4. pH was carefully set to the desired value dropwise with 85% H_3PO_4 . pH in the reaction was monitored *in situ* and controlled at an external electrode. If required automatic pH regulation occurred with 10% H_3PO_4 and 20% NaOH.

Alternatively EEH bioconversions were run directly in the fermentation medium. Fermentations were carried out in 750 mL InforsHT reactors and the cell concentration (wet weight) determined as described above. The amounts of homofarnesol stock and SDS were then determined for setting the ratios [SDS]:[cells] and [cells]:[EEH] at the same values as in the standard EEH bioconversion (125 g/L EEH, 250 g/L cells, and 1.30–1.55% (w/v) SDS). SDS and EEH was added, the pH set to 5.4, and temperature to 35 °C. For running a true 125 g/L EEH bioconversion, the required amount of cells collected from several fermenters was added to one fermenter to a cell concentration of 250 g/L. The reaction was then started as above.

(–)-Ambrox Recovery

The initial (–)-Ambrox content of the reaction broth was determined after extraction of 1 g material with methyl *tert*-butyl ether (MTBE) followed by GC-FID analysis.

Whole reaction broth extraction: 200 mL reaction broth (approx. 125 g/L (–)-Ambrox) was extracted by vigorous shaking for 30 s with 100–150 mL hexane, toluene, or xylene. The solvent phase was collected by centrifugation (4225 g, 10 min, 10 °C). The cells were resuspended in the aqueous phase; (–)-Ambrox was quantified by extracting 1 g suspension with MTBE and GC-FID analysis. Extraction was repeated until (–)-Ambrox in the solvent phase was $\leq 1.0\%$ of that of the initial (–)-Ambrox content of the reaction broth.

Solid phase extraction: 200 mL reaction broth (approx. 125 g/L (–)-Ambrox) was centrifuged as above to separate 80 mL solid pellet from 120 mL liquid phase. The solid phase was extracted with 160 mL ethanol 99% (vigorous shaking), and centrifugation (as above but at room temperature). The ethanolic phase was analyzed by GC-FID for its (–)-Ambrox content. The extraction was repeated until (–)-Ambrox in the solvent phase was $\leq 1.0\%$ of the initial (–)-Ambrox content of the reaction broth. As an alternative to absolute ethanol, the extractions were done with toluene.

Sample Preparation for GC-Analysis

4 g/L EEH reactions: 100–300 μ L reaction was extracted with 200 μ L hexane:2-propanol (2:1) or 700 μ L MTBE. After centrifugation (Eppendorf centrifuge 5415C, 13000 rpm) the solvent phase was analyzed by GC-FID. Up to 16 g/L EEH: 150–300 μ L reaction mixture was extracted into 700 μ L of MTBE. After phase separation, the solvent phase was analyzed by GC-FID. 30–188 g/L EEH: 1 mL reaction mixture was extracted with 5 mL of MTBE by vigorous shaking. After decantation 1 mL of solvent phase was centrifuged; 100 μ L of the supernatant was diluted into 900 μ L MTBE for GC-FID analysis.

Gas Chromatography Analysis

High throughput library screening: 1 μ L of solvent phase was injected (split ratio 1/10) onto a 30 m $\times$ 0.32 mm $\times$ 0.25 μ m HP-5 column (Surveyor gas chromatograph, Thermo). The column was developed at constant temperature (170 °C) and pressure (85 kPa, 5.2 mL/min H_2); detector temperature: 250 °C.

Standard assay and bioconversions: 1 μ L of solvent phase was injected (split ratio 1/10) onto a 30 m $\times$ 0.32 mm $\times$ 0.25 μ m Zebron ZB-5 column (Trace Focus gas chromatograph, Thermo). The column was developed at 4 mL/min H_2 constant flow: 100 °C, 15 °C/min to 200 °C, 120 °C/min to 240 °C, 4 min at 240 °C; detector temperature: 300 °C.

Structure Elucidation of Reaction Products by NMR

^1H and ^{13}C NMR spectra were recorded on a BrukerAvance 400 MHz (^1H NMR) and 125 MHz spectrometer (^{13}C NMR)

in $CDCl_3$, if not mentioned otherwise. The chemical shifts are given in ppm relative to the TMS signal (δ 0 ppm). ^{13}C spectra were referenced to $CDCl_3$ (δ 77.16 ppm) and C_6D_6 (δ 128.06 ppm).

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Abb. 1 Pottwale (*Physeter catodon* oder *Physeter macrocephalus*) werden bis zu 18 m lang und 50 t schwer. Der Darm eines Pottwals kann eine Länge von bis zu 250 Meter erreichen. In ihm entsteht das Ausgangsmaterial für die graue Ambra. (© Scott Portelli, Wildlife Photographer und Peter Kaminski).



Den Stoffwechsel- oder Verdauungsstörungen des größten noch lebenden Säugetiers, dem Pottwal, verdanken wir einen der wertvollsten Duftstoffe der Feinparfümerie: die graue Ambra (Abbildung 1) [1].

Walfangberichten zufolge leidet etwa 1 % vor allem der männlichen Tiere an dieser Erkrankung. Die Ambra-Menge kann erheblich sein: Im Fall eines 1953 in der Antarktis erlegten Pottwals betrug sie 421 kg. Die Ambra von getöteten Tieren ist von minderer Qualität, weil sie nicht im Meerwasser gereift ist. Pottwale sind heute durch das Washingtoner Artenschutz-Abkommen geschützt und die Duftstoffe werden durch chemische Synthese gewonnen (Abbildung 2).

Es handelt sich bei der Ambra um eine zunächst wachsartige, weiche Masse im Darm des Pottwals, die dieser nach mechanischer Verletzung durch die Hornkiewer der Tintenfische (*Sepia officinalis*) und Kalmare (*Teuthida*), seiner bevorzugten Nahrung, bildet. Die Ambra dient als antibakterieller Wundverschluss, in die die unverdaulichen Nahrungsbestandteile eingebettet werden. Ins Meer gelangen die Konkremente durch Erbrechen, als Kotsteine oder durch den natürlichen Tod der Tiere.

Die wenige Gramm bis zu 100 kg schweren, zunächst nach Fäkalien riechenden, fast schwarzen Klumpen treiben aufgrund ihrer geringen Dichte von 0,78–0,93 g/cm³ auf der Meeresoberfläche und verwandeln sich durch photo-

chemischen Abbau und Oxidation im Laufe von Jahren und Jahrzehnten zu dem hochgeschätzten Parfümerierohstoff, bevor die steinartigen, hellgrauen, wohlriechenden Ambrastücke von Fischereiboote geborgen oder an den Strand gespült werden. Die Duftnote liegt zwischen holzig, trocken, balsamisch, etwas tabakartig bis bouquethaft mit aphrodisierendem Einschlag. Ambra findet man im Atlantischen und Indischen Ozean, an den Küsten von Brasilien, Afrika, Madagaskar, den Malediven, China, Japan, Neuseeland und in der Karibik.

Geschichte des Duftstoffs

Ambra war bereits in der Antike begehrter Duftstoff und wertvolle Handelsware. Die Ägypter verbrannten sie als Opfergabe und im arabischen Kulturraum war der Duftstoff ebenso bekannt wie in den Küstenregionen des Indischen Ozeans.

Sie galt vermischt mit Wein als Aphrodisiakum, diente zur Abwehr der Pest und als Medikament zur Behandlung von Kopfschmerzen, Erkältungen, Epilepsie und anderen Erkrankungen. Ludwig XV. von Frankreich soll seine Lieblings Speisen mit Ambra gewürzt haben und Elisabeth I. von

England parfümierte ihre Handschuhe damit. In Ägypten benutzt man Ambra heute noch zur Aromatisierung von Zigaretten.

Erste Hinweise für die Verwendung von Ambra in der Feinparfümerie haben wir von den Arabern in Spanien im 10. Jahrhundert. Ambra importierte man zu diesem Zweck bereits von den Sunda-Inseln und dem Maghreb. Zu Beginn der Neuzeit wurde Ambra in ganz Europa gehandelt und in vielen Fällen mit Gold aufgewogen. Al-Hasan ibn Mohammed al-Wassan (genannt: Johannes Leo Africanus; * um 1490 in Granada, † nach 1550 in Tunis) schrieb, dass der Preis von einem Pfund Ambra auf dem Markt in Fes bei 60 Dukaten liege, was dem von drei Sklaven entsprach.

Über die Herkunft der Ambra herrschte lange Unsicherheit. Die Chinesen nannten sie „lung sien hiang“ und stellten sich Drachen vor, die auf den Küstenfelsen schliefen und im Schlaf ins Meer sabberten. Aus dem Speichel entstünde dann, so glaubten sie, der Duftstoff. Im japanischen Kulturraum sprach man dagegen von „kunsurano fuu“, von Walkot. Der arabische Reisende und Historiker Al-Mas'udi († 957 in al-Fustat, Ägypten) berichtete von Händlern und Seeleuten, die glaubten, Ambra wachse wie Pilze auf dem Meeresboden und werde durch Stürme gelegentlich an Land gespült. Marco Polo (1254–1324) war der erste westliche Reisende, der berichtete, dass der Pottwal der Ambra wegen von jemenitischen Seeleuten vor der Insel Socotra gejagt würde.

Gewissheit über die Herkunft erhielt man aber erst, als mit dem Aufblühen der amerikanischen Walfangindustrie in Nantucket (Massachusetts) im 18. Jahrhundert immer wieder Pottwale erlegt wurden, in deren Därmen man Ambra-Klumpen fand.

Kulturgeschichte und etymologisch ist die Ambra mit dem Bernstein (echter, gelber oder preußischer Amber, lat. *Succinum*, griech. *Electron*) oft verwechselt worden. *Ambra* oder *Amber* ist ebenfalls die englische und altdeutsche Bezeichnung für Bernstein. Zur Unterscheidung benutzt man heute für das Ausscheidungsprodukt der Pottwale im Englischen das Wort *Ambergris*, das sich aus dem Französischen *Ambre gris* und letztlich vom arabischen Wort *anbar* ableitet.

Bestandteile der Ambra

Die ersten chemischen Analysen der Ambra gehen auf die französischen Chemiker Joseph-Bienaimé Caventou (1795–1877) und Pierre-Joseph Pelletier (1788–1842) zurück. Im Jahr 1820 isolierten und charakterisierten sie das Triterpen Ambrein [3]. Es ist praktisch geruchlos und wird in der Parfümindustrie als Fixateur benutzt. Die zweite Hauptkomponente der frischen Ambra ist 24-Methyl-5 β -cholestan-3 α -ol, das zusammen mit Ambrein etwa 70–90 % der Masse ausmacht.

Die olfaktorisch wichtigen Komponenten entstehen während der Reifezeit im Meer. Durch oxidativen Abbau des Ambreins im Bereich der zentralen Doppelbindung entstehen flüchtige mono- und bicarbocyclische Verbindun-

gen, die, bedingt durch den Salzgehalt des Meerwassers, teilweise auch chloriert sind (Abbildung 3) [4].

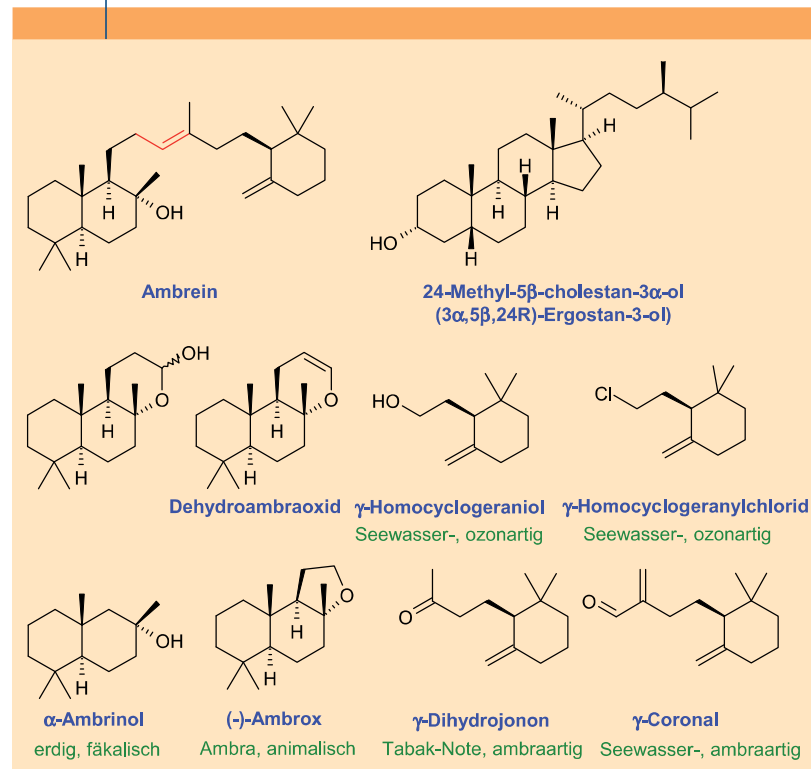
Die Schlüsselreaktion des Abbaus ist eine Photooxidation durch Singuletsauerstoff. Als Sensibilisator könnte der in Ambra nachgewiesene Porphyrinfarbstoff Hämocyanin dienen. In Modellreaktionen von reinem Ambrein mit Singuletsauerstoff wurden neben *Ambrox*[®] und γ -Coronal auch α -Ambrinol und Dehydroambroxid erhalten (Abbildung 4) [5].

Der olfaktorisch bedeutendste Inhaltsstoff in Ambra ist (-)-*Ambrox*[®] ((3a*R*,5a*S*,9a*S*,9b*R*)-3a,6,6,9a-tetramethyldecahydronaphtho[2,1-*b*]furan). Es verkörpert die ersten vier Tonalitäten einer 3-prozentigen Ambra-Tinktur in 90-prozentigem Alkohol, die man eine Weile unter gelegentlichem Schütteln reifen ließ, nach feuchtem, moosigem Waldboden, einem starken Duft nach Tabak und balsamigem San-



Abb. 2 Großer Ambra-Klumpen eines 1953 in der Antarktis erlegten Pottwals [2].

ABB. 3 | BESTANDTEILE DER FRISCHEN AMBRA



Das „Concrète“ gewinnt man meist aus Pflanzenteilen, durch Extraktion mit organischen Lösungsmitteln, die man anschließend abdampft. Das „Absolue“ ist die filtrierte ethanologische Lösung dieses Rückstands.

delholz gemischt mit einer warmen, animalischen Moschusnote.

Ambrox[®] bezeichnet die enantiomerenreine Verbindung. Das Racemat heißt *Cetalox*[®] (*cetus*: lat. Wal). Beides sind Handelsnamen der Firma Firmenich. *Ambroxan*[®] und *Amberlyn*[®] sind synonym mit *Ambrox*[®]. Es handelt sich hierbei um den Handelsnamen von Cognis (Henkel/BASF) bzw. Quest.

Die verschiedenen Diastereomere von (–)-*Ambrox*[®] haben einen ähnlichen Duft, unterscheiden sich aber teilweise in ihren Geruchsschwellen [6, 7]. Die des 3a-*epi*-(–)-*Ambrox*[®] liegt um den Faktor 100 höher als die von (–)-*Ambrox*[®]. Das 9b-*epi*-(–)-*Ambrox*[®] hat dagegen bei fast gleicher Geruchsqualität wie (–)-*Ambrox*[®] eine halb so hohe Geruchsschwelle. (+)-*Ambrox*[®] ist achtmal schwächer als das natürliche Enantiomer. Das Racemat hat eine Geruchsschwelle von 0,5 ppb und unterscheidet sich in seinen Tonalitäten kaum von dem des (–)-*Ambrox*[®] (Abbildung 5).

(–)-*Ambrox*[®] wurde nicht nur aus der natürlichen *Ambra* sondern auch aus dem Absolue des Tabaks (*Nicotiana tabacum*) isoliert und im ätherischen Öl des Muskatellersalbeis (*Salvia sclarea*), der Zistrose (*Cistus labdaniferus*) und der Trauerzypresse (*Cupressus sempervirens*) nachgewiesen (Abbildung 6).

Partialsynthesen

Die erste chemische Synthese von (–)-*Ambrox*[®] stammt von A. Hinder und A. Stoll von der Firma Firmenich aus dem Jahr 1950 [8, 9]. Das Ausgangsmaterial für diese Naturstoffsynthese war das Diterpen Sclareol, das man aus dem Muskatellersalbei (*Salvia sclarea*) gewinnt (Abbildung 7).

Die immergrüne, zweijährige bis ausdauernde krautige Pflanze erreicht Wuchshöhen von 50 bis 110 Zentimeter und wird spätestens seit dem 9. Jahrhundert kultiviert. Der Legende nach waren Weinhändler die Ersten, die den Muskatellersalbei technisch nutzten und aus ihren mäßigen Rheinweinen echten Muskatellerwein „zauberten“. Muskatellersalbeiöl findet heute Verwendung zur Herstellung von Eaux de Cologne, Eaux de Toilette, zur Aromatisierung von Kräuterlikören, als Heilpflanze und als Gewürz.

Durch Wasserdampfdistillation von 100 kg der Blütenstände und Triebspitzen gewinnt man ca. 800 Gramm Muskatellersalbeiöl (*oleum salviae sclareae*). Die Hauptinhaltsstoffe des klaren, farblosen, ätherischen Öls sind Linalylacetat, Linalool, Nerolidol, Neroliacetat und Sclareol, die jedoch je nach Anbaug Gebiet und klimatischen Bedingungen stark variieren können.

Einen höheren Anteil an Sclareol enthält das Muskatellersalbei-Concrète, das man durch Extraktion der blühenden Pflanzen mit Hexan erhält. Der Gehalt liegt bei etwa 70 %. Nach Gegenextraktion mit 87-prozentigem Methanol und Verdampfen des Lösungsmittels erhält man einen sirupösen Rückstand, der kristallisiert und einen Gehalt an Sclareol von über 90 % hat [10, 11]. Die Weltjahresproduktion beläuft sich auf 50–150 Tonnen (Abbildung 8) [12].

Schlüsselschritt der technischen Synthese ist der oxidative Abbau der Seitenkette des Sclareols entweder durch Oxidation mit Chromtrioxid oder Kaliumpermanganat gefolgt von einer Ozonolyse.

Die Reduktion des Lactons mit Lithiumaluminiumhydrid liefert das Diol, das im Säuren zum gewünschten Ether cyclisiert wird. Dieser Schritt bedarf jedoch erhöhter Sorgfalt, weil unter den Reaktionsbedingungen sehr leicht auch das thermodynamisch stabilere 9b-*epi*-*Ambrox*[®] entsteht (Abbildung 9).



Abb. 6 Lack-Zistrose (*Cistus labdaniferus*) und Trauerzypresse (*Cupressus sempervirens*). (© Carsten Niehaus und Andrea Buratti)

ABB. 4 PHOTOOXIDATIVER ABBAU VON AMBREIN

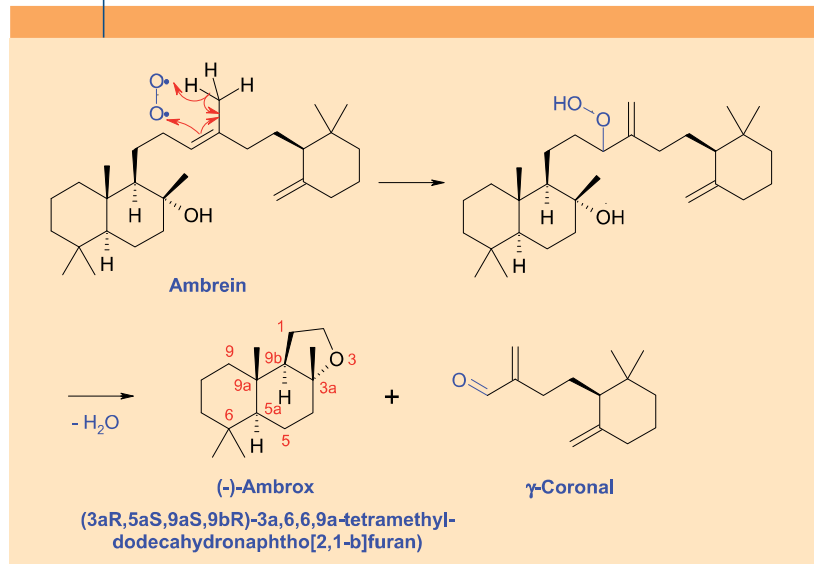


ABB. 5 DIE GERUCHSSCHWELLEN DER STEREOISOMERE DES AMBROX[®]

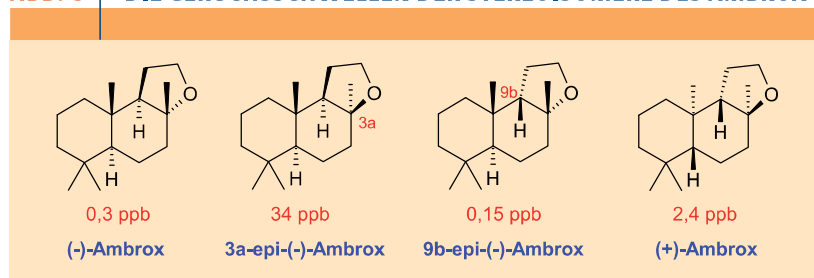




Abb. 7 Muskatelsalbei (*Salvia sclarea*, auch Muskat-Salbei, Römischer Salbei oder Scharlei) wird hauptsächlich im Mittelmeerraum, auf dem Balkan, im Gebiet der früheren UdSSR und den USA angebaut. [© Kurt Stueber]

Anfang der 80er Jahre wurde die kommerzielle Verwendung von *Ambrox*[®] patentfrei, so dass eine ganze Reihe weiterer technischer Synthesen auch bei anderen Firmen und an Hochschulen entwickelt wurden. So publizierte Sir Derek Barton eine von Quest zum Patent angemeldete Abbaureaktion des Sclareols, bei der Kaliumpermanganat durch Natriumperiodat ersetzt wurde [13, 14].

Henkel entwickelte einen sehr attraktiven Prozess zur Herstellung von Sclareolid aus Sclareol, durch Oxidation mit Natriumhypochlorit in Gegenwart von Rutheniumchlorid und eines Phasentransferkatalysators (Abbildung 10) [15].

Besonders bemerkenswert sind auch die Arbeiten bei Fritsche Dodge & Olcott sowie bei International Flavors & Fragrances, die zeigten, dass die Seitenkette auch fermentativ mit *Hyphozyma roseoniger* bzw. *Cryptococcus albidus* abgebaut werden kann [16, 17].

Abgesehen von der Oxidation mit Chromoxid, Kaliumpermanganat oder Natriumperiodat liegen die Nachteile dieser Synthese in der unzuverlässigen Versorgungslage von Sclareol und den damit oft stark fluktuierenden Preisen [6].

Alternativ zu Sclareol kann man zur Herstellung von *Ambrox*[®] auch (+)-*cis*-Abienol verwenden, das man aus Kanadabalsam gewinnt. Das Harz erhält man durch Anritzen der Rinde der im Norden der USA und in Canada wachsenden Balsamtanne (*Abies balsamea*) (Abbildung 11). Durch Wasserdampfdestillation, Extraktion und Kristallisation ist (+)-*cis*-Abienol erhältlich.

Die Ozonolyse und reduktive Aufarbeitung von (+)-*cis*-Abienol führt zu einem Diol, das mit Tosylchlorid in Pyridin in exzellenten Ausbeuten zu *Ambrox*[®] cyclisiert werden kann (Abbildung 12) [18].

Neben diesen besonders eleganten Abbaureaktionen wurde (-)-*Ambrox*[®] auch noch ausgehend von einer Reihe anderer Naturstoffe, wie (-)-Drimenol [19], (-)-Thujon [20] oder (+)-Carvon [21] synthetisiert.

Totalsynthesen

Die erste Totalsynthese von *Cetalox*[®], stammt von Masanao Matsui [22]. Ausgangsmaterial ist β -Jonon [23], dessen Doppelbindung in der Seitenkette regioselektiv an einem Kupferkatalysator hydriert werden kann. Anschließend wird sie durch die Sequenz einer Darzens-Reaktion [24] und einer Knoevenagel-Kondensation um drei C-Atome verlängert. Die (*E/Z*)-Isomere im Verhältnis von 1 : 1 trennt man destillativ nach Veresterung. Das (*E*)-Isomere wird verseift und mit Trifluoressigsäure zum (+/-)-Sclareolid cyclisiert. Die

Reduktion mit *Vitride*[®] und Etherbildung liefert dann *Cetalox*[®] (Abbildung 13).

Eine reizvolle Alternative zum Aufbau der Sclareolid-Vorstufe beinhaltet nach der Reduktion der Doppelbindung von β -Jonon mit Tributylstannan eine Grignard-Reaktion und anschließend die Umsetzung mit Dimethylformamidmethylacetal, wobei das entsprechende Carbonsäureamid durch eine [2,3]-sigmatrope Umlagerung gebildet wird. Die (*E/Z*)-Isomere können chromatographisch getrennt werden (Abbildung 14) [25, 26].

Im Jahr 1989 veröffentlichten George Büchi und Hans Wüst vom MIT eine Synthese von *Cetalox*[®], die von Firmenich in den größeren Maßstab übertragen und an entscheidenden Stellen wesentlich verbessert wurde [27]. Ausgangsmaterial ist ebenfalls Dihydro- β -jonon, das durch Umsetzung mit Dimethylcarbonat in den entsprechenden



Abb. 11 Die Balsamtanne (*Abies balsamea*) ist mit bis zu 20 Meter (selten bis 30 m) Wuchshöhe bei bis zu 50 Zentimetern Stammdurchmesser ein mittelgroßer Baum. Sie kann bis zu 200 Jahre alt werden. [© United States Fish and Wildlife Service]

ABB. 8 LABDAN

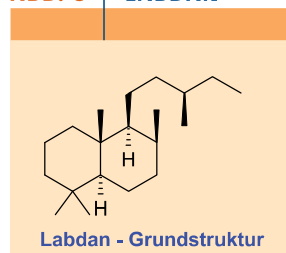


ABB. 10 SCLAREOLID

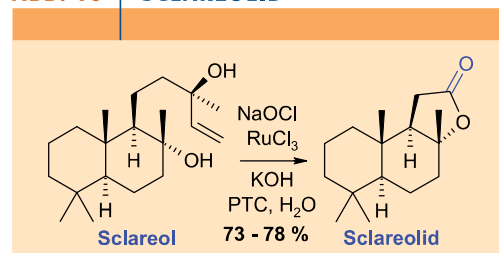
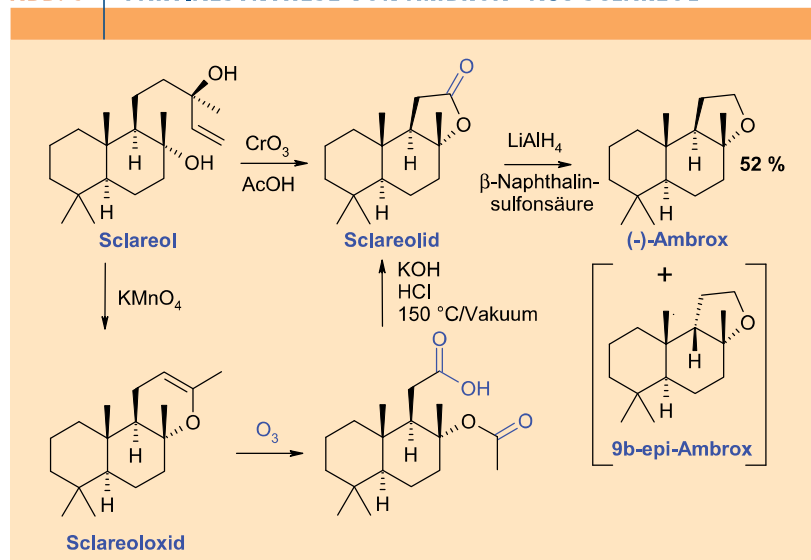


Abb. 8 Sclareol gehört zur großen Gruppe der labdanoiden Diterpene. Der Name leitet sich von Labdanum, einem Harz aus den Zistrosengewächsen (*Cistaceae*) ab, aus denen man die ersten Vertreter dieser Grundstruktur isolierte.

Abb. 10 Effiziente Synthese von Sclareolid aus Sclareol.

ABB. 9 PARTIALSYNTHESE VON AMBROX[®] AUS SCLAREOL



β -Ketoester überführt wird. Nach der Zinntetrachlorid-vermittelten Cyclisierung sollte in α -Position alkyliert werden. Dies gelang jedoch nicht. Erfolgreich war dagegen die O-Alkylierung, gefolgt von einer Claisen-Umlagerung. Die Demethoxycarbonylierung kann man in DMSO durchführen. Allerdings sind die Ausbeuten in NMP als Lösungsmittel besser. Nach der Grignard-Reaktion zur Einführung der Methylgruppe wird die Seitenkette durch Ozonolyse und Reduktion zum erforderlichen Alkohol abgebaut.

Der schwierigste Schritt der Totalsynthese war der Ringschluss zum *trans*-verknüpften Tetrahydrofuran unter kinetischer Kontrolle, da das thermodynamisch günstigere Produkt 9*b*-*epi*-Cetalox[®] ist. Unter den gewählten Bedingungen verläuft die Reaktion sehr selektiv. Der Gehalt an 9*b*-*epi*-Cetalox[®] liegt unter 1 % (Abbildung 15).

Polyencyclisierungen

Außerordentlich interessant und von industrieller Relevanz sind auch die biomimetischen Versuche Cetalox[®] und Ambrox[®] durch Polyencyclisierung zugänglich zu machen.

Die grundlegenden methodischen Arbeiten zur künstlichen Polyencyclisierung gehen auf Gilbert Stork, Albert

Eschenmoser und E. E. van Tamelen zurück, die sich in den 50er und 60er Jahren mit der säurekatalysierten Cyclisierung der Farnesylsäure und des Squalenoxids beschäftigten [28,29,30]. Weitere Pionierarbeiten aus dem Jahr 1960 betreffen dann schon die säurekatalysierte Cyclisierung von Homofarnesylsäure zu einem Diastereomergemisch des Sclareolids und stammen von Günther Lucius vom VEB Chemische Fabrik Miltitz bei Leipzig (siehe unten) [31]. In jüngerer Vergangenheit gibt es sehr elegante Beispiele für die Domino-Synthese insbesondere des Sterol-Gerüsts [32, 33]. Die biomimetischen Tetra- und Pentacyclisierungen von W. S. Johnson werden durch Lewis-Säuren oder Trifluoressigsäure initiiert (Abbildung 16).

Aber auch radikalische Polycyclisierungen sind bekannt. Zu den prominentesten Beispielen zählen die Polyencyclisierungen von Pattenden, die geradezu ein Lehrbeispiel für die Baldwin-Regeln darstellen [34, 35, 36, 37]. Die Polycyclisierung verläuft über freie Radikale, die in einer Domino-Reaktion von 6-*endo-trig*-Cyclisierungen abläuft und mit einer 5-*exo-trig*-Reaktion endet. Eine wichtige Voraussetzung zur Vermeidung von Nebenreaktionen ist die geschickte Konstruktion des Polyens, das aber dann in günstigen Fällen in enorm hohen Ausbeuten zum Steroidgerüst cyclisiert werden kann (Abbildung 17).

ABB. 12 | AMBROX[®] AUS ABIENOL

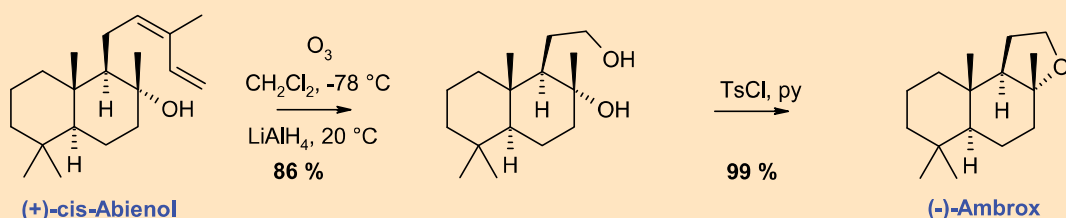
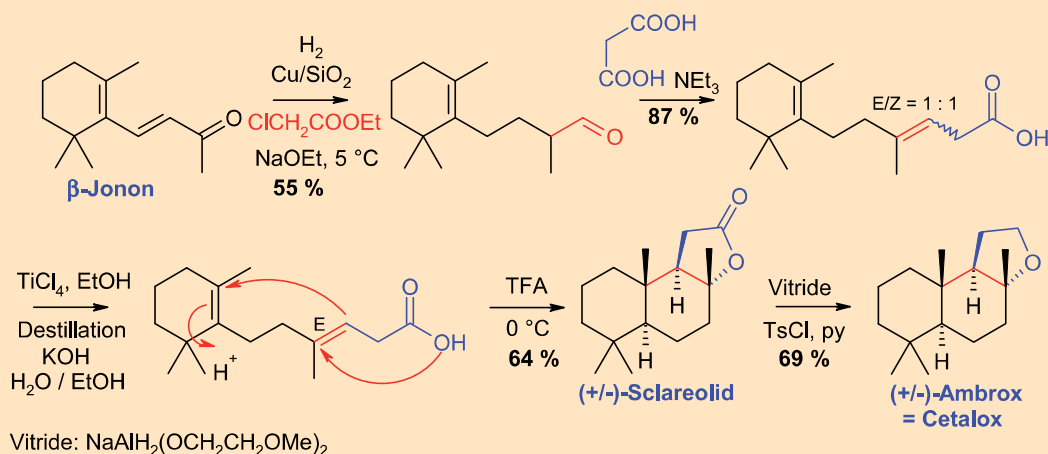


ABB. 13 | AMBROX[®] AUS β -JONON



Edukte der Polyencyclisierung

Cabreuvaöl, das durch Wasserdampfdestillation aus dem brasilianischen Tropenholz *Myrcarpus fastigiatus* gewonnen wird, ist das Ausgangsmaterial des Sesquiterpens (+)-(*E*)-Nerolidol, einem wichtigen Vorprodukt von Homofarnesyssäure und Homofarnesol.

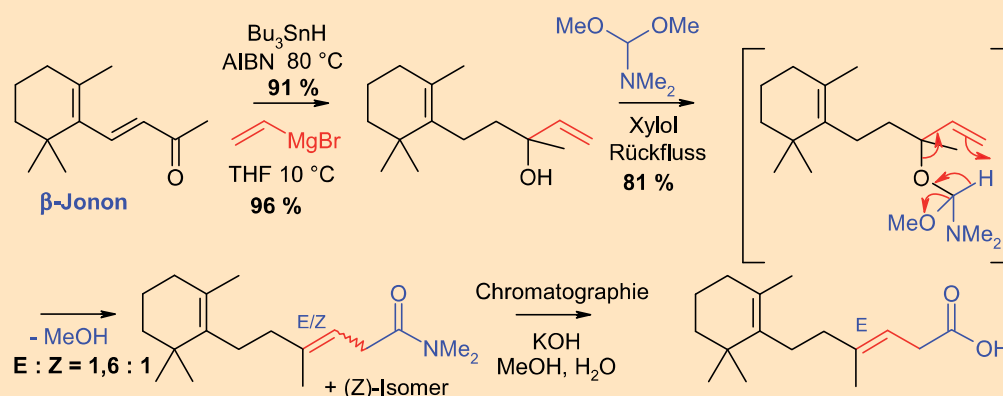
Racemisches (*E*)-Nerolidol erhält man auf vergleichsweise einfachem Weg aus Linalool durch eine Carroll-Reaktion und Umsetzung mit Vinylmagnesiumchlorid. Alternativ kann man Linalool auch mit Diketen oder Isopropenylmethylether (Saucy-Marbet-Reaktion) umsetzen und die Grignard-Reaktion durch eine Acetylenaddition gefolgt von einer Lindlar-Hydrierung ersetzen (Abbildung 18a) [38].

Die Überführung des Nerolidols in das entsprechende Bromid, Cyanid-Substitution und Hydrolyse oder dessen Carbonylierung [39] liefert ein (7*E*,3*Z*)-/(*E,E*)-Isomerengemisch der Homofarnesyssäure (Abbildung 18b).

(*E,E*)-Homofarnesol erhält man aus Nerolidol durch [2,3]-sigmatrope Umlagerung, chromatographische oder destillative Trennung des 2:1-Gemischs der Homofarnesyssäureamide und Reduktion mit Lithiumtriethylboranat (Abbildung 19a) [25].

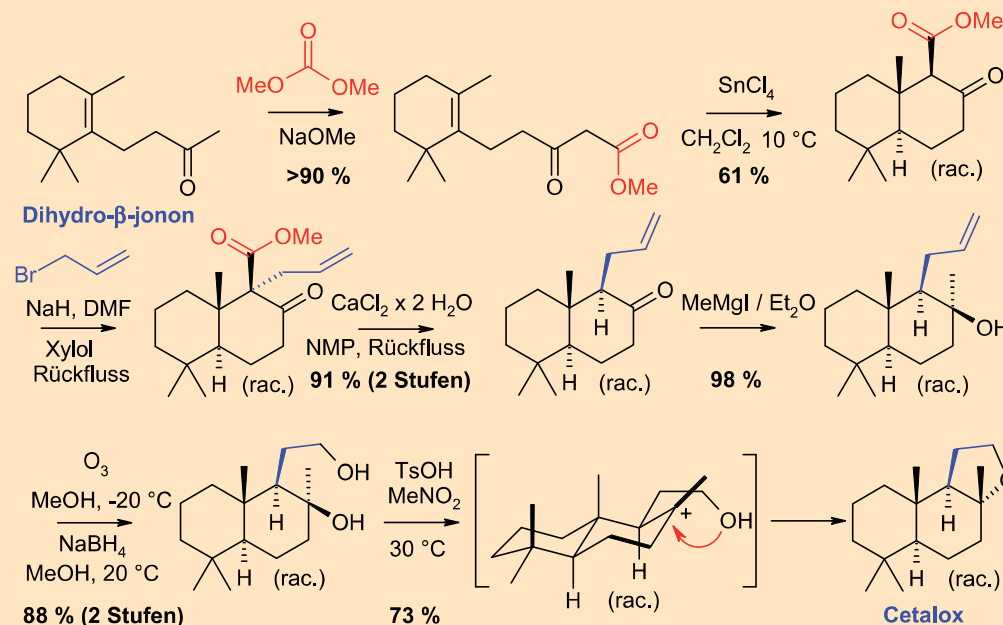
Alternativ kann Nerolidol an Wolframaten zu Farnesol, formal nach einer Metalla-Claisen-Umlagerung isomerisiert und durch Destillation gereinigt werden (Abbildung 19b) [40]. Nach der Überführung ins Farnesylchlorid wird dieses mit frisch gefälltem Barium und Kohlendioxid zu (*E,E*)-

ABB. 14 | AUFBAU DER VORSTUFE



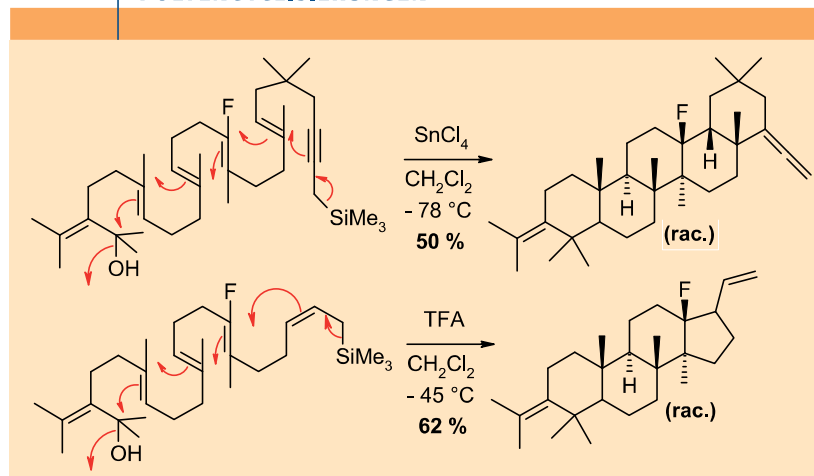
Eine reizvolle Alternative zum Aufbau der Sclareolid-Vorstufe.

ABB. 15 | AMBROX®-SYNTHESE VON FIRMINICH



Die Synthese von Cetalox® von Firmenich.

ABB. 16 | LEWIS- UND BROENSTED-SÄURE-VERMITTELTE POLYENCYCLISIERUNGEN



Homofarnesylsäure umgesetzt, die man mit Lithiumaluminiumhydrid reduziert [41, 42].

Die Oxidation von Farnesol mit Braunstein, Wittig-Reaktion und Hydroborierung mit Disiamylboran, das man *in situ* aus dem Boran-THF-Komplex und 2-Methyl-2-buten herstellt, liefert ebenfalls (*E,E*)-Homofarnesol in einer Gesamtausbeute von 70 % (Abbildung 19c) [42].

Sclareolid und Ambrox®

Günther Lucius gelang es, Homofarnesylsäure mit einer Mischung aus Ameisensäure und Schwefelsäure zu einem Diastereomergemisch des Sclareolids zu cyclisieren. Durch Kristallisation konnten die Diastereomere getrennt werden. Allerdings lag die Ausbeute nur bei 15 %. Racemisches Sclareolid war darin jedoch nicht enthalten [31].

In der Folge wurden eine Reihe anderer Brönsted- und Lewis-Säuren zur stereochemisch selektiven Cyclisierung

ABB. 17 | RADIKALISCHE POLYENCYCLISIERUNGEN

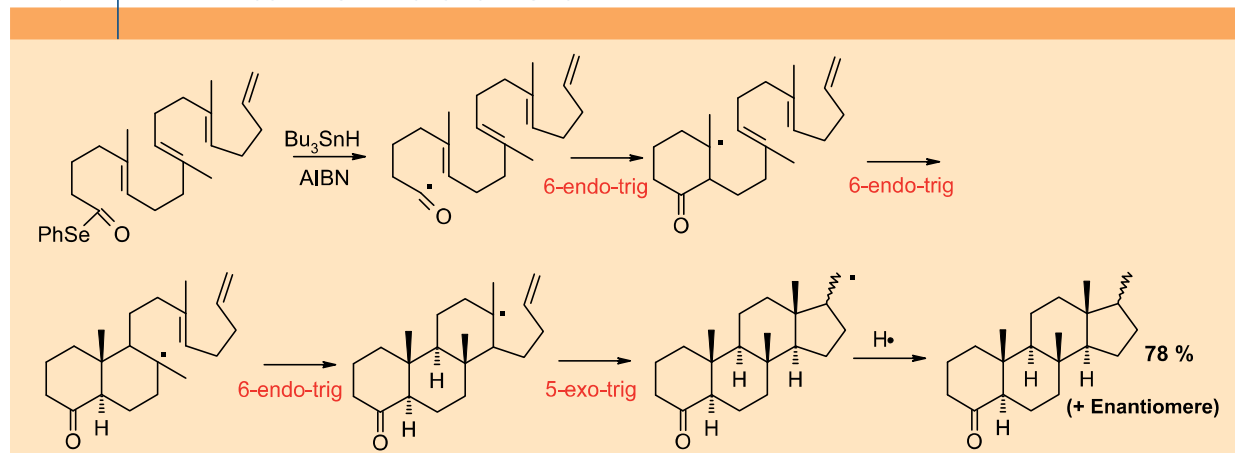
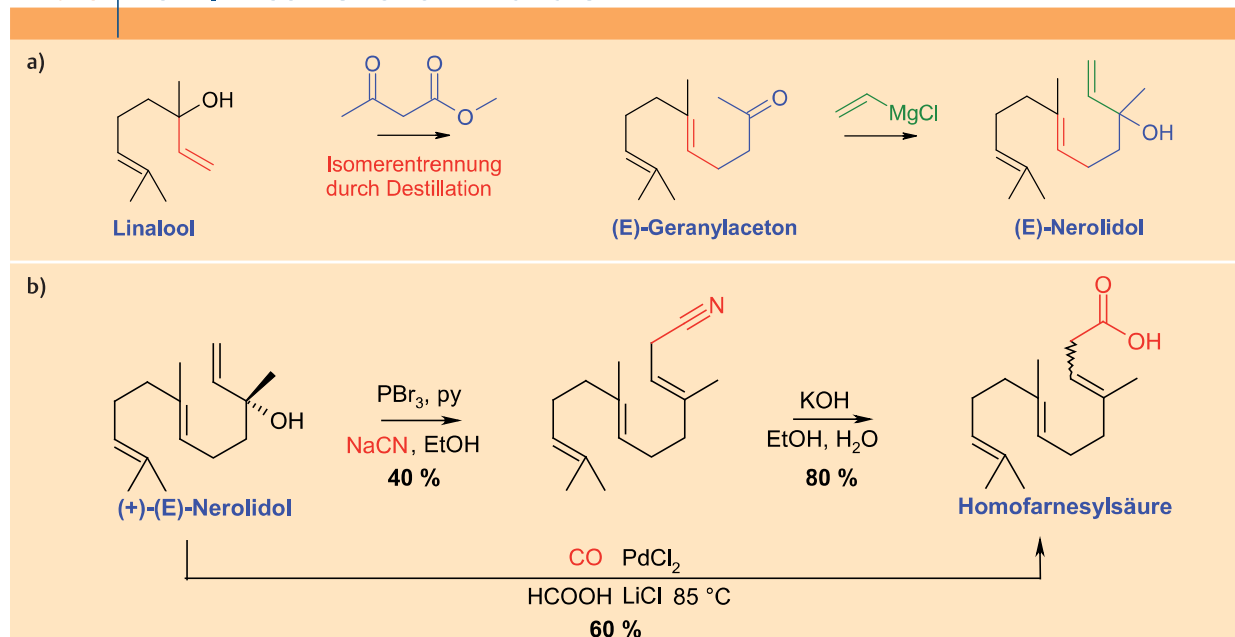


ABB. 18 | VON LINALOOL ZU HOMOFARNESYLSÄURE



Ausgehend von Linalool erhält man Nerolidol (18a), das auf unterschiedlichen Wegen weiter zu Homofarnesylsäure umgesetzt werden kann (18b)

erprobt. So erhält man aus Homofarnesylsäure die Sclareolid-Isomere in einer Ausbeute von 91 %, wenn man die Reaktion mit Zinntetrachlorid in Dichlormethan bei -78°C durchführt [43]. Verwendet man dagegen Bortrifluorid als Katalysator, so erhält man racemisches Sclareolid nur in einer Ausbeute von 38 % [44]. Die Firma Henkel patentierte ein Verfahren, bei dem man ein (*E/Z*)-Isomerengemisch der Homofarnesylsäure mit Methansulfonsäure in Dichlormethan bei einer Temperatur von -10°C umsetzt. Es wurde allerdings auch nur ein Gemisch an Sclareolid-Isomeren erhalten [45].

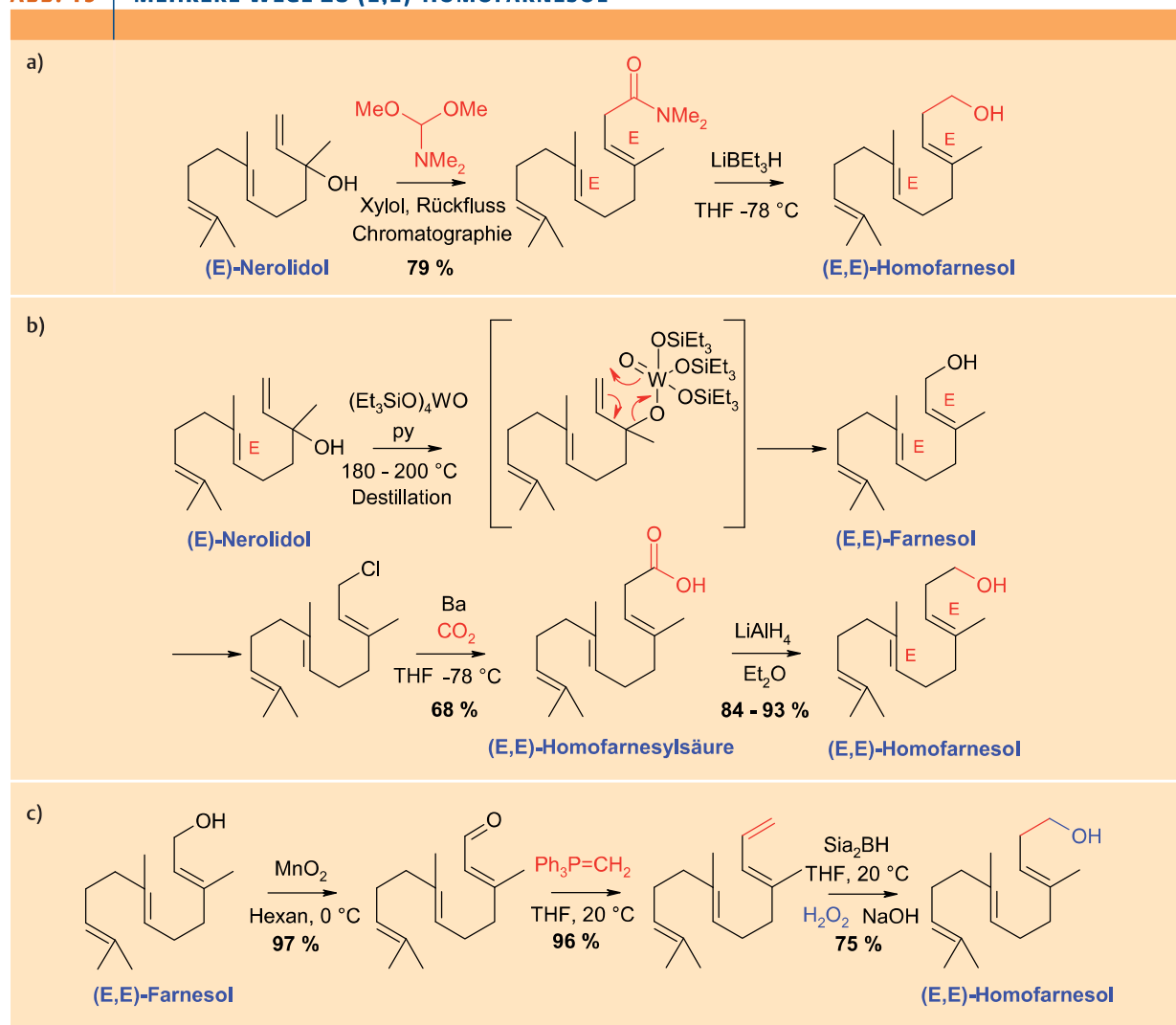
Der Durchbruch gelang schließlich P. F. Vlad am chemischen Institut der Universität von Kishinev (Moldawien) durch Verwendung von Supersäuren wie Fluorsulfonsäure in 2-Nitropropan bei niedrigen Temperaturen (Abbildung 20) [46]. Interessanterweise erhielt er unter sehr ähnlichen Reaktionsbedingungen aus (*E,E*)-Homofarnesol hoch diastereoselektiv racemisches Ambrox® in einer Ausbeute von 73 % [25, 41].

Allerdings muss kritisch angemerkt werden, dass die Ausbeuten bei Firmenich unter sehr ähnlichen Bedingungen nur bei 40 % lagen und Umsetzungen mit Chlorsulfonsäure in 1-Nitropropan ebenfalls diese Ausbeuten lieferten [25, 47].

Sehr detaillierte mechanistische Untersuchungen zur Polyencyclisierung stammen von Roger Snowden von Firmenich [48]. Eine terminale Protonierung initiiert die Cyclisierung des tricyclischen Systems. Die *anti*-Addition an den Doppelbindungen der nascenten Sechsringe erfolgt aus der Sesselkonformation. Ob die Cyclisierung nach der Stork-Eschenmoser-Hypothese synchron erfolgt, lässt sich mit den experimentellen Daten nicht belegen.

Ein sequenzieller Ablauf, bei der die rot gezeichnete Bindung zuerst geknüpft wird, ist ebenfalls denkbar. Der Ringschluss zu Ambrox® bzw. 9b-*epi*-Ambrox® erfolgt dann schneller als die Konformationsänderung des Cyclohexylcarbokations, so dass ein *trans*-Decalin-System entsteht. Die Isomerisierung der mittleren Doppelbindung in Homofar-

ABB. 19 MEHRERE WEGE ZU (*E,E*)-HOMOFARNESOL



nesol ist gegenüber der terminalen sehr langsam und entsprechend gering ist der Anteil an anderen Diastereomeren (Abbildung 21).

Totalsynthetisch ist enantiomerenreines *Ambrox*[®] zugänglich, wenn man Homofarnesylacetat mit Chlorsulfonsäure cyclisiert und das Produkt mittels einer Pseudomonas-Lipase spaltet. Die Veretherung liefert dann das gewünschte Produkt (Abbildung 22) [49].

Die unbestreitbar eleganteste Methode zur Totalsynthese von (-)-*Ambrox*[®] ist die enantioselektive Polyencyclisierung. Zur Cyclisierung benutzte Hisashi Yamamoto eine enantiomerenreine Lewis-Säure-Brönsted-Säure aus O-(o-Fluorbenzyl)-binol und Zinntetrachlorid und erhielt (-)-*Ambrox*[®] mit einem Enantiomerenüberschuss von 75 % und immerhin 54 % Ausbeute [42].

Als Ausgangsmaterial dient (*E,E*)-Homofarnesyltriethylsilylether. Hierdurch bleibt die Cyclisierung nach der

enantioselektiven Protonierung auf der Stufe des Decalinsystems stehen. Die anschließende Cyclisierung verläuft mit höherer Diastereoselektivität, wenn man sie im stark Säuren durchführt. Daher ist die Balance zwischen der Stabilität der Si-O-Bindung und der Brönsted-Acidität der enantiomerenreinen Lewis-/Brönsted-Säure von entscheidender Bedeutung für eine hohe Diastereoselektivität. Der *Si*-faciale Ringschluss des Cyclohexylkations mit äquatorialer Seitenkette führt zu *Ambrox*[®], der *Re*-faciale zu 9b-*epi-Ambrox*[®]. Der Ringschluss des Cyclohexylkations mit axialer Seitenkette liefert zwei weiteren Diastereomere, das 5a-*epi-Ambrox*[®] und das 5a-*epi*-9b-*epi-Ambrox*[®]. So gibt es als Mechanismus für die Bildung der Diastereomeren neben der (*E/Z*)-Isomerisierung auf der Stufe des Homofarnesols noch die (*Re/Si*)-faciale Cyclisierungsmöglichkeit und die Inversion der Sechsringskonformation (Abbildung 24).

ABB. 20 | POLYENCYCLISIERUNGEN MIT SUPERSÄUREN

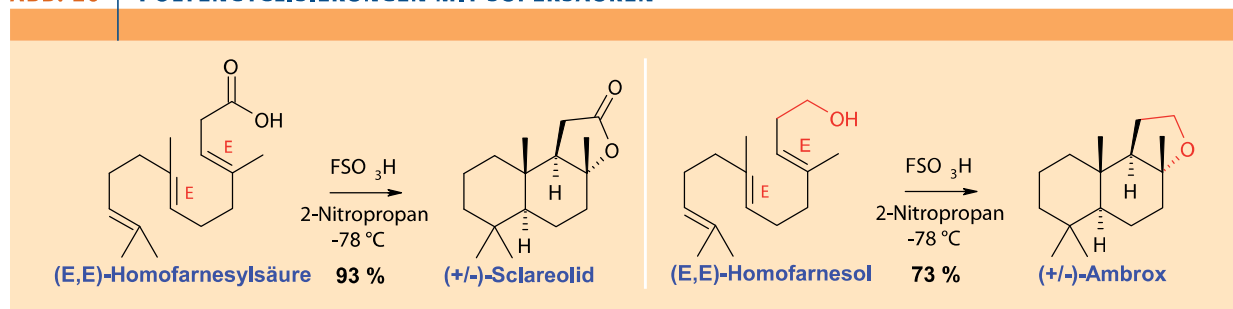


ABB. 21 | MECHANISTISCHE ÜBERLEGUNGEN ZUR POLYENCYCLISIERUNG

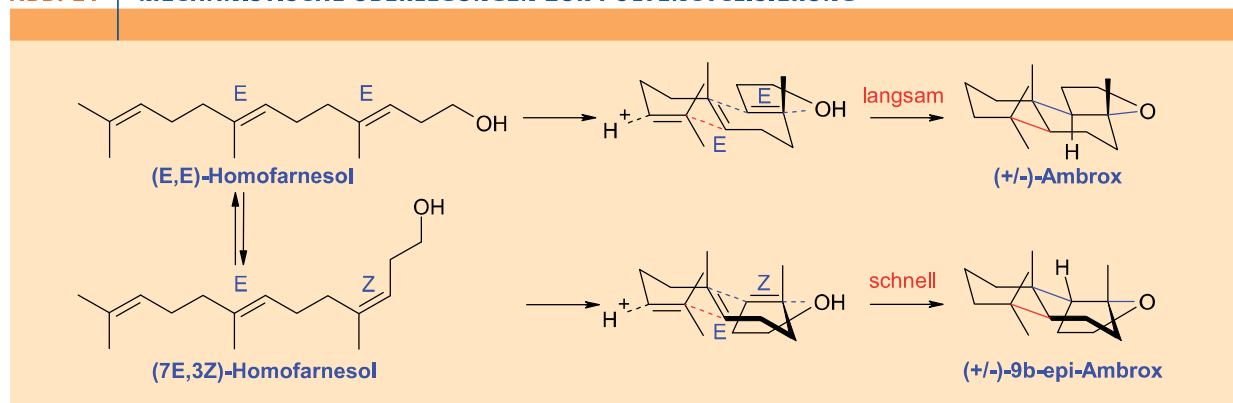
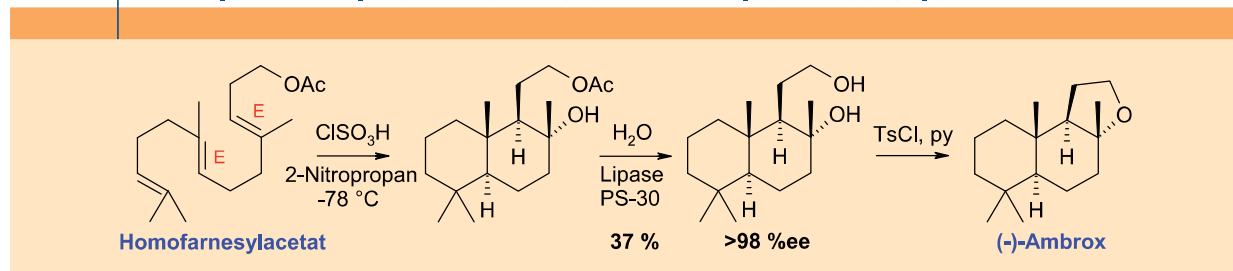


ABB. 22 | ENANTIOMERENREINES AMBROX[®] DURCH ENZYMATISCHE RESOLUTION



Unter sehr ähnlichen Bedingungen, allerdings ohne Zugabe des Trifluoressigsäure/Zinntetrachlorid-Komplexes, erhält man (+)-Sclareolid in vergleichbaren Ausbeuten mit einem Enantiomerenüberschuss von 88 % (Abbildung 23 [26]).

Enzymatische Polyencyclisierungen

Die enantioselektive Polyencyclisierung des Squalens zu dem pentacyclischen Hopan und Hopanol zählt zu den anspruchsvollsten biochemischen Reaktionen. In einem Schritt entstehen neun Asymmetriezentren. Hopan ist eines von $2^9 = 512$ möglichen Stereoisomeren.

Die Cyclisierung wird durch die Squalen-Hopen-Cyclase einiger Prokaryoten vermittelt und ist in gewisser Hin-

ABB. 23 ENANTIOMERENREINES AMBROX® DURCH ENANTIOSELEKTIVE POLYENCYCLISIERUNG

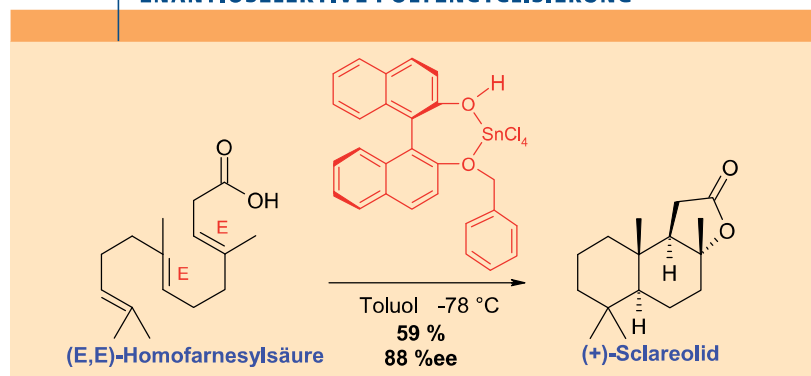


ABB. 24 ENANTIOMERENREINES SCLAREOLID DURCH ENANTIOSELEKTIVE POLYENCYCLISIERUNG

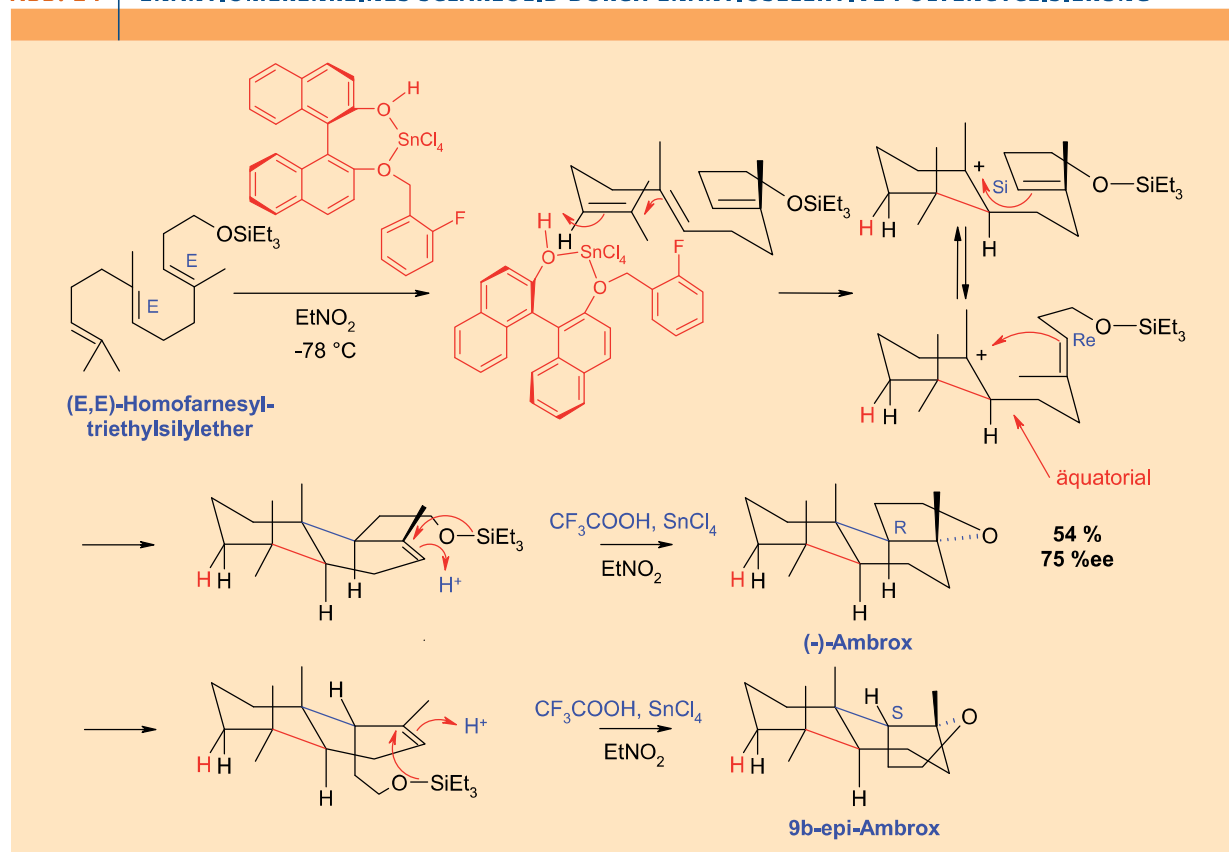
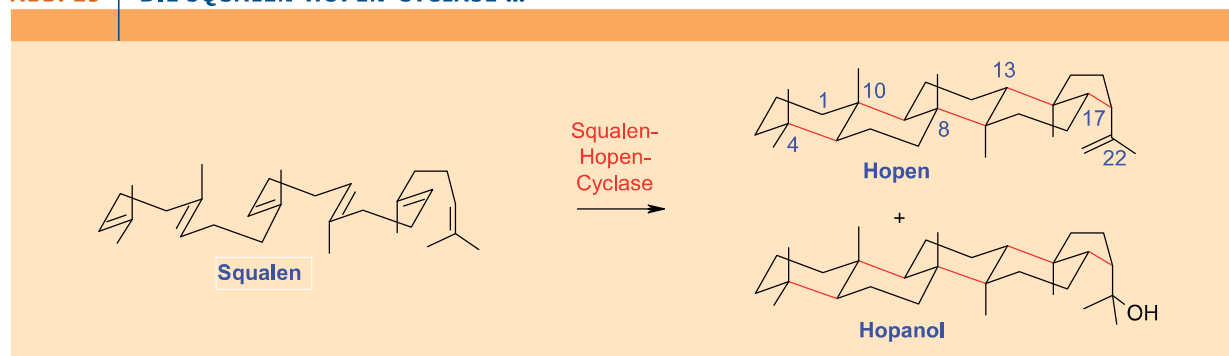


ABB. 25 DIE SQUALEN-HOPEN-CYCLASE ...



... cyclisiert Squalen zu enantiomerenreinem Hopan bzw. Hopanol. Die Hopanoide sind wichtige Bestandteile der Bakterienzellwand. Man findet sie heute in großen Mengen im bis zu 500 Millionen Jahre alten Ölschiefer. Die Menge wird auf 10^{12} t geschätzt. Damit gehören sie neben Cellulose zu den mengenmäßig bedeutendsten organischen Substanzen auf der Erde.

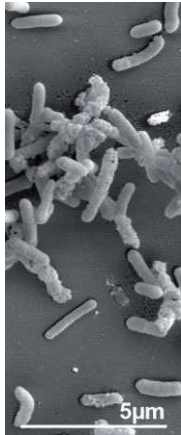


Abb. 26 *Alicyclobacillus acidocaldarius*. (© PD. Dr. Manfred Rohde, Helmholtz Zentrum für Infektionsforschung, Braunschweig)

sicht mit der Squalenoxid-Lanosterol-Cyclase der Eukaryoten verwandt (Abbildung 25) [50, 51].

Im Jahr 1997 gelang es Georg E. Schulz in Freiburg, die Struktur der membranständigen Squalen-Hopen-Cyclase aus

dem thermophilen Mikroorganismus *Alicyclobacillus acidocaldarius* aufzuklären (Abbildungen 26 und 27) [52]. Das Enzym besteht aus zwei etwa gleich großen Domänen. Das aktive Zentrum befindet sich in einer 1200 Å³ (Kubik-Ång-

ABB. 27 | **STEREOANSICHT DER SQUALEN-HOPEN-CYCLASE**

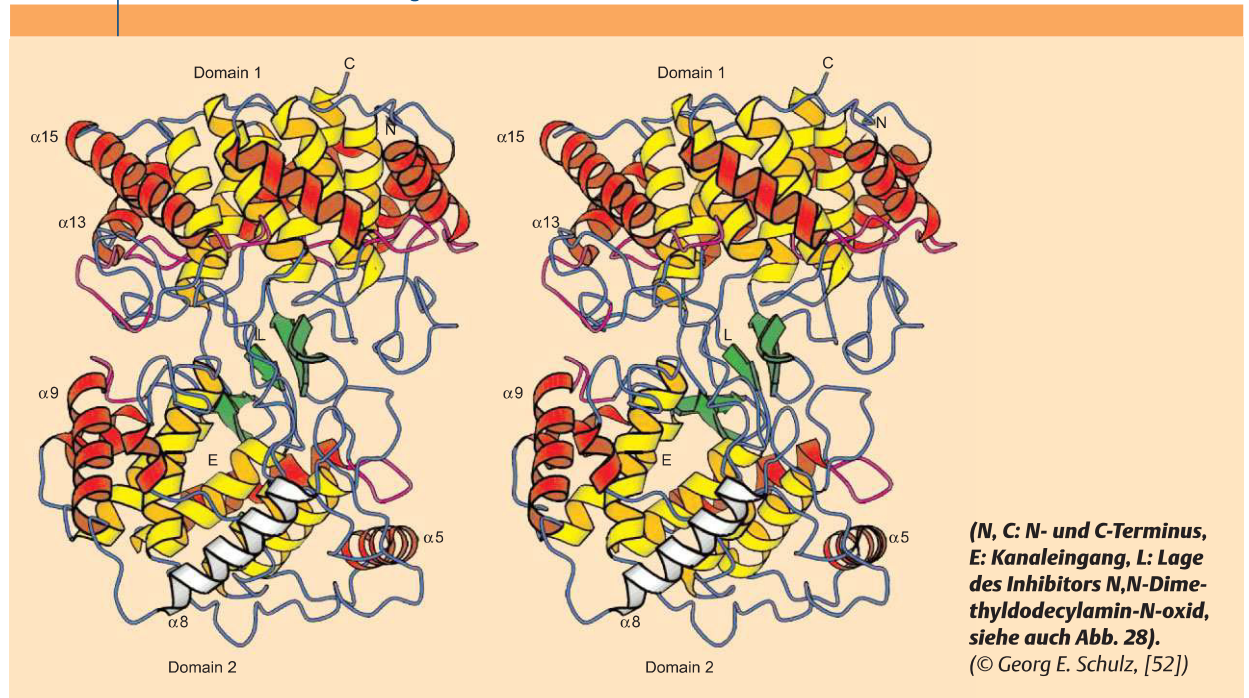
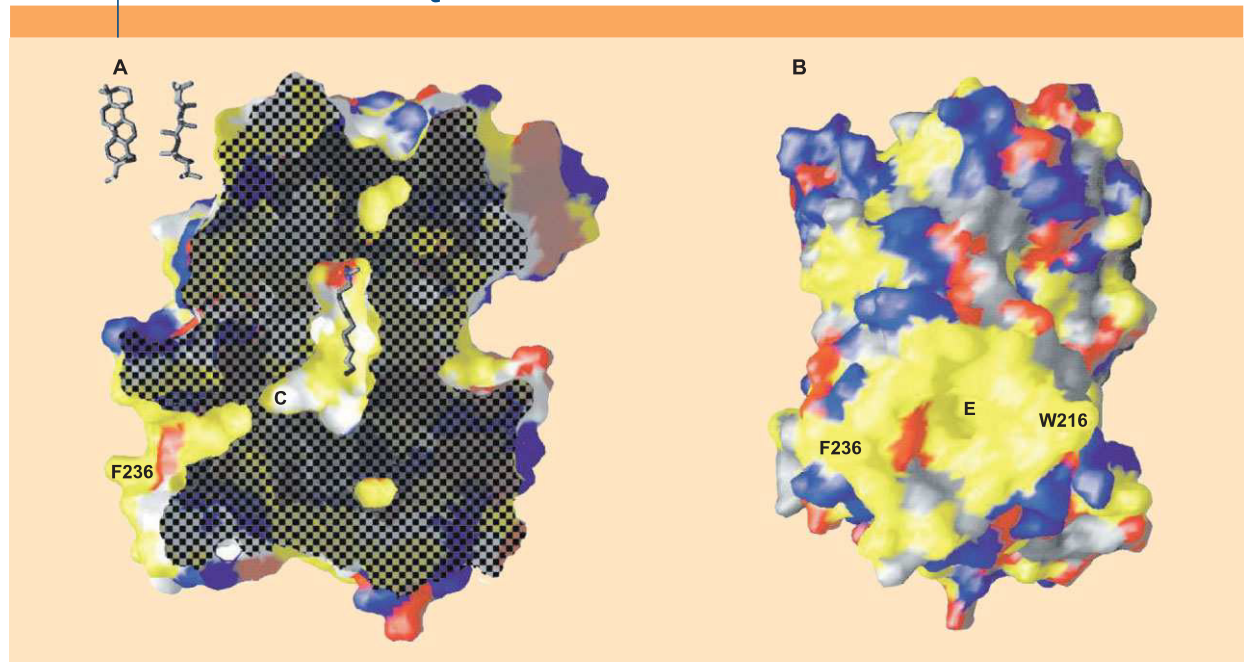
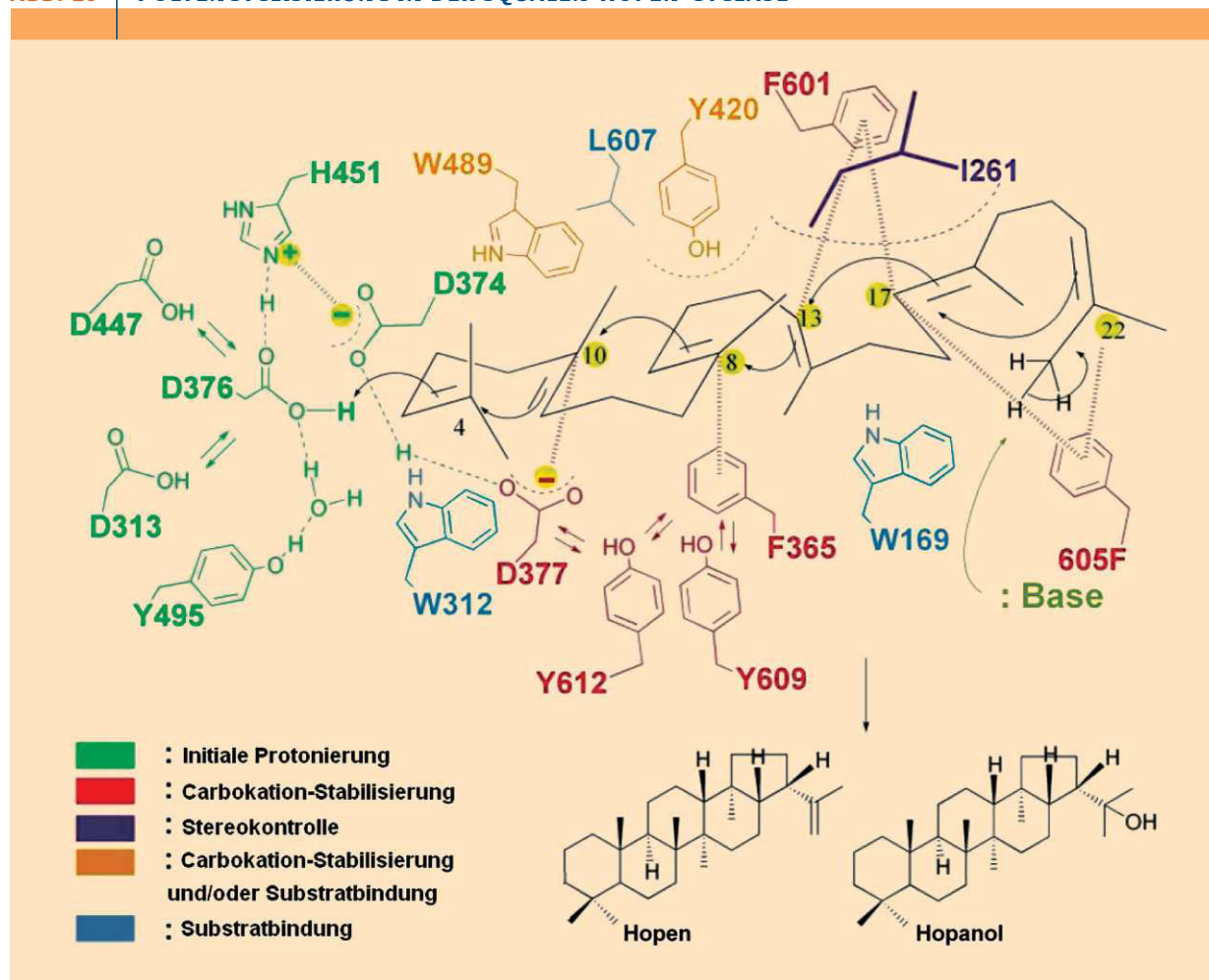


ABB. 28 | **BINDUNGSTASCHE DER SQUALEN-HOPEN-CYCLASE**



Nichtpolare Bereiche sind gelb, positive blau und negative rot dargestellt. Die Ansicht (A) zeigt das Enzym „aufgeschnitten“. Die Schnittfläche liegt im Bereich der großen Bindungstasche, in der der kompetitive Inhibitor N,N-Dimethyldodecylamin-N-oxid (LDAO) anstelle von Hopen (links oben zwei maßstabsgerechte Ansichten) gebunden ist. Ein nichtpolarer Kanal (C) führt nach links zu einer ebenfalls ungeladenen Öffnung. Die Ansicht (B) zeigt den Kanaleingang (E) des Enzyms von außen. Er ist von einer etwa 1600 Å²-großen nichtpolaren Fläche umgeben. (© Georg E. Schulz, [52])

ABB. 29 POLYENCYCLISIERUNG IN DER SQUALEN-HOPEN-CYCLASE



(D: Asparaginsäure, F: Phenylalanin, H: Histidin, L: Leucin, W: Tryptophan, Y: Tyrosin). (Abb. nach [53])

ström) großen Höhle dazwischen, die durch einen unpolaren Kanal in der Domäne 2 zugänglich ist (Abbildung 28).

Im aktiven Zentrum wird Squalen in einer Sessel-Sessel-Konformation gebunden [53]. Die Cyclisierung beginnt mit einer Protonierung am C-Atom 3 des späteren Hopens durch Asparaginsäure D376. Carbokation- π -Wechselwirkungen zu verschiedenen aromatischen Aminosäuren wie Tyrosin, Phenylalanin und Tryptophan stabilisieren die kationischen Zwischenstufen bis schließlich durch Deprotonierung Hopen bzw. Hydroxylierung Hopanol gebildet wird (Abbildung 29).

Bereits 1986 entdeckten Stefan Neumann und Helmut Simon an der technischen Universität München, dass die Squalen-Hopen-Cyclase aus *Bacillus acidocaldarius* [54], nicht nur Squalen zu Hopen und Hopanol cyclisiert, sondern auch Homofarnesol zu Ambrox [55]. Man kann davon ausgehen, dass die Cyclisierung sehr ähnlich wie die von Squalen verläuft (Abbildung 30).

Jüngst wurden Arbeiten zu diesem Thema auch von der japanischen Firma Kao Corporation aufgenommen [56]. Leider machen keine der Schriften Angaben zu Selektivität und Ausbeute.

Superambrox

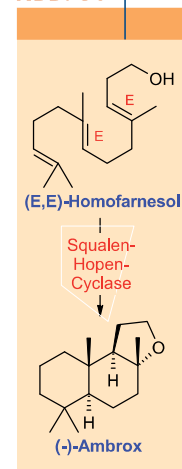
Im Jahr 1993 beschrieben Chemiker der Firmenich den Duft von Superambrox (5,5a-Dehydroambrox) [57]. Die exzellenten Duftqualitäten sind gekennzeichnet durch eine sehr kraftvolle, schöne Ambra-Tonalität mit viel Volumen und Fülle und einer starken Kopfnote.

Eine interessante Partialsynthese beschrieb Aede de Groot im Jahr 2001 [58]. Das Ausgangsmaterial ist Larixol, das man durch Extraktion und Kristallisation aus dem Terpentinöl der Lärchen gewinnt (Abbildung 31).

Die Oxidation mit Oxon (2 KHSO₅, KHSO₄, K₂SO₄) führt selektiv zur Epoxidierung der exocyclischen Doppelbindung. Das Nebenprodukt mit 19 % ist das Diepoxid. Die Reduktion mit Lithiumaluminiumhydrid liefert unter Ringöffnung das Triol. Die Seitenkette wird durch eine Periodat-Oxidation abgebaut. Reduktion und Ringschluss mit p-Toluolsulfonsäure in Nitromethan ergeben 5 α -Hydroxyambrox, aus dem man nach Mesylierung Superambrox herstellen kann (Abbildung 32).

Eine besonders elegante, stereoselektive Totalsynthese von Superambrox stammt von Charles Fehr [59]. Während Brönsted-Säure-katalysierte En-Reaktionen des racemischen

ABB. 30



Cyclisierung von Homofarnesol zu Ambrox mit Hilfe der Squalen-Hopen-Cyclase aus *Bacillus acidocaldarius*.



Abb. 31 Europäische Lärche (*Larix europaea*, *Pinus larix*, *Larix decidua*). (© Maurice Perry)

ABB. 32 PARTIALSYNTHESE VON SUPERAMBROX

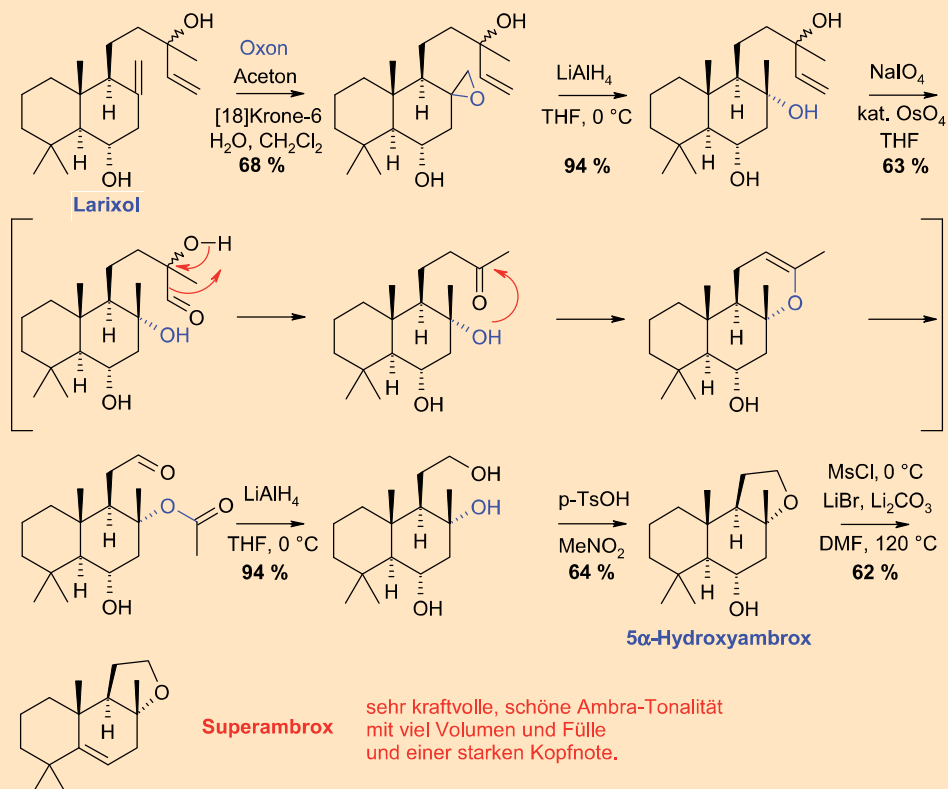
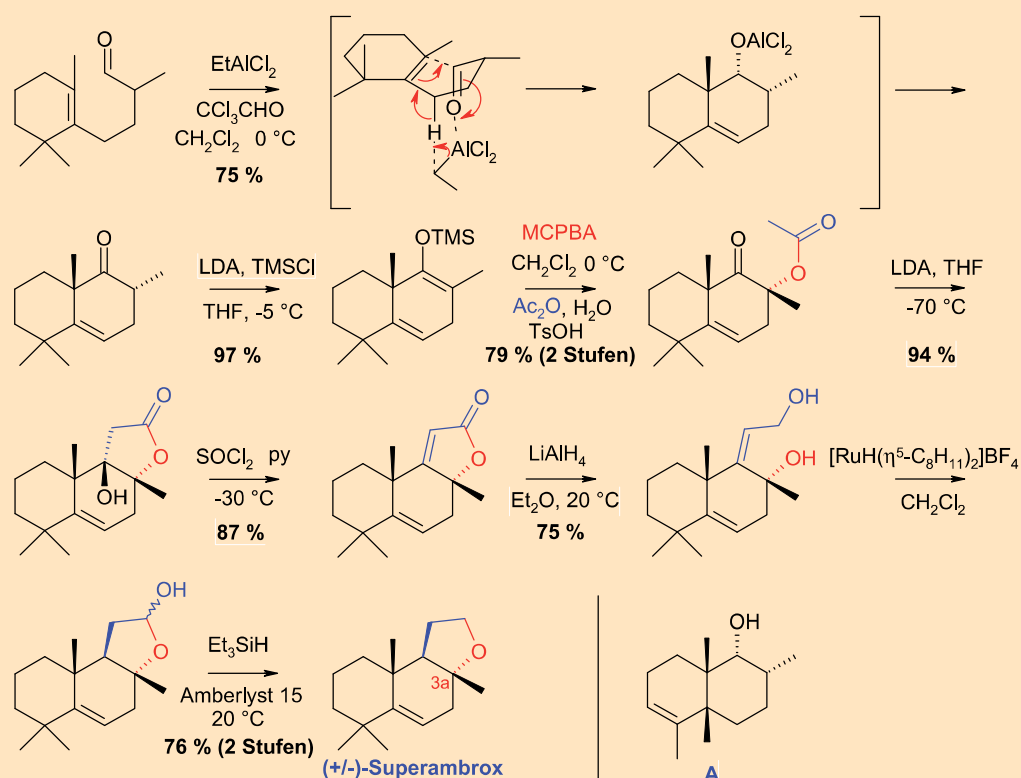


ABB. 33 TOTALSYNTHESE VON SUPERAMBROX



Dihydro-C14-Aldehyds unter Methylgruppenwanderung in Ausbeuten von ca. 40 % zum Decalinol A führen, liefert die Lewis-Säure-vermittelte En-Reaktion mit Ethylaluminiumdichlorid den gewünschten Bicyclus. Der Reaktionsverlauf ist vermutlich konzertiert und führt im Übergangszustand über ein achtgliedriges Ringsystem. Das primär erhaltene Aluminiumalkoxid kann direkt einer Oppenauer-Oxidation mit Chloral unterworfen werden und man isoliert direkt das Decalinon. Der entsprechende Silylenolether wird mit *m*-Chlorperbenzoesäure diastereoselektiv von der der axialen Methylgruppe abgewandten Seite oxidiert. Das α -Siloxyketon wird in das entsprechende Acetat überführt. Die folgende Deprotonierung mit LDA führt in einer intramolekularen Aldol-Reaktion zum Aufbau des Lactons. Nach Dehydratisierung wird dieses mit Lithiumaluminiumhydrid reduziert. In Gegenwart des Chaudret-Ruthenium-Hydrierkatalysators findet die ursprünglich beabsichtigte Hydrierung nicht statt. Es kommt vielmehr zu einer durch die Hydroxygruppe in Position 3a stereochemisch kontrollierten Doppelbindungsverschiebung. Das Lactol wird schließlich mit Triethylsilan zu racemischem Superambrox reduziert. Die Gesamtausbeute über alle Stufen beträgt 27 % (Abbildung 33).

Trennt man die Enantiomere auf der Stufe des racemischen Dihydro-C14-Aldehyds, dann ist auf diesem Weg auch enantiomerenreines Superambrox zugänglich.

Kommerzielle Verwendung

Cetalox[®] und Ambrox[®] finden Verwendung in hochwertigen Parfüms sowohl für Frauen als auch für Männer. Prominente Beispiele dieser Duftkategorie sind Chanel No. 5[®], Davidoff Cool Water[®] oder Azzaro pour Homme[®] (Abbildung 34).

Die Duftstoffe werden neben ihren hervorragenden olfaktorischen Qualitäten auch sehr gerne als Fixateur verwendet. Der Preis für Ambrox[®] beläuft sich auf ca. 1000 Dollar/kg und die Weltjahresproduktion für Ambrox[®] und Analoga liegt bei etwas mehr als 30 Tonnen [2].

Zusammenfassung

Ambra ist ein seit Jahrtausenden hoch geschätzter Duftstoff aus dem Verdauungstrakt des Pottwals. Der olfaktorisch bedeutendste Inhaltsstoff ist (–)-Ambrox[®], das durch Partialsynthese im größeren Maßstab aus dem Sclareol des Muskattellersalbeis gewonnen wird. Die Polyencyclisierungen von Homofarnesol und Homofarnesylsäure sind besonders elegante Totalsynthesen. 5,5a-Dehydroambrox zeichnet sich nicht nur durch exzellente Duftqualitäten aus, sondern auch durch die eleganten Totalsynthesen.

Summary

Ambergris, obtained from the intestinal tract of the sperm whale, is a highly esteemed fragrance since several millennia. The olfactory most important ingredient of ambergris is Ambrox[®], which is produced on large scale by partial synthesis from sclareol from clary sage. The polyene cyclisations of ho-

mofarnesol and homofarnesylic acid are highly attractive total syntheses of Ambrox[®] and sclareolide. 5,5a-Dehydroambrox is an outstanding new scent, both to its distinguished fragrance properties and its elegant total syntheses.

Schlagwörter

Riechstoffe, Partialsynthese, Polyencyclisierung, Sclareolid, Ambrox

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Abb. 34 Typische Verkaufsprodukte mit einer Ambra-Note.

(© arz, © COTY PRESTIGE, © Azzaro Parfums, www.azzaroparis.com)

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Der Autor



Bernd Schäfer hat in Kaiserslautern Chemie studiert und am Max-Planck-Institut für Kohlenforschung in Mülheim/Ruhr über stereoselektive Cycloadditionen von Methylencyclopropanen an elektronenarmen Olefinen promoviert. Er ist heute in der Forschung der BASF SE tätig. Im Verlauf seines beruflichen Werdegangs arbeitete er für die Bereiche Pflanzenschutz, Pharma und Feinchemie. Seine besonderen Forschungsinteressen liegen auf dem Gebiet der Naturstoffe, der Katalyse und der enantioselektiven Synthese. Er ist Autor des Buches „Naturstoffe der chemischen Industrie“. Seit dem Jahr 2000 hat er einen Lehrauftrag und seit 2009 eine Honorarprofessur an der Ruprecht-Karls-Universität in Heidelberg.

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Irresistible Fragrance Note – Ambrox®

Abb. 1 Pottwale (*Physeter catodon* oder *Physeter macrocephalus*) werden bis zu 18 m lang und 50 t schwer. Der Darm eines Pottwals kann eine Länge von bis zu 250 Meter erreichen. In ihm entsteht das Ausgangsmaterial für die graue Ambra. (© Scott Portelli, Wildlife Photographer und Peter Kaminski).



Den Stoffwechsel- oder Verdauungsstörungen des größten noch lebenden Säugetiers, dem Pottwal, verdanken wir einen der wertvollsten Duftstoffe der Feinparfümerie: die graue Ambra (Abbildung 1) [1].

Fig.1 Sperm whales (*Physeter cotodon* or *Physeter macrocephalus*) grow to up to 18 m length and 50 t weight. The intestine of a sperm whale can reach a length of up to 250 metres. The starting material for ambergris is formed in this. © Scott Portelli, Wildlife photographer and Peter Kaminski).

We have the metabolic or digestive disorders of the largest still living mammal, the sperm whale, to thank for one of the most valuable fragrance materials of fine perfumery: grey ambergris (Figure 1) [1].

According to whaling reports, about 1% mainly of the male animals suffer from this disease. The quantity of ambergris can be considerable: in the case of a sperm whale killed in the Antarctic in 1953, it amounted to 421 kg. The ambergris of killed animals is of lower quality, since it has not been matured in seawater. Nowadays, sperm whales are protected by the Washington Convention on the protection of endangered species and the fragrance substances are obtained by chemical synthesis (Figure 2).

Ambergris is an initially waxy, soft mass in the intestine of the sperm whale, which this forms after mechanical injury due to the horny beak of the octopus (*Sepia officinalis*) and squid

(*Teuthida*), its preferred food. The ambergris serves as an antibiotic wound closure, in which the indigestible food components become imbedded. The concretions reach the sea through vomiting, as faecal stones or through the natural death of the animals.



Fig.2 Large ambergris lump from a sperm whale killed in the Antarctic in 1953 [2].

Owing to their low density of 0.78-0.93 g/cm³, the almost black lumps, initially smelling of faeces, weighing a few grams up to 100 kg, float up on the surface of the sea and in the course of years and decades are converted by chemical degradation and oxidation into the highly valued perfumery raw material, before the stone-like, light grey, pleasant-smelling ambergris lumps are salvaged by fishing boats or washed up on the shore. The fragrance note lies between woody, dry, balsamic, somewhat tobacco-like to flowery with an aphrodisiac impact. Ambergris is found in the Atlantic and Indian oceans, on the coasts of Brazil, Africa, Madagascar, the Maldives, China, Japan, New Zealand and in the Caribbean.

History of the Fragrance Substance

In antiquity, ambergris was already a sought-after fragrance and valuable article of trade. The Egyptians used to burn it as a sacrificial offering and in the Arabian cultural region the fragrance was as well-known as in the coastal regions of the Indian Ocean.

Mixed with wine, it was regarded as an aphrodisiac, was used for protection against plague and as a medicine for the treatment of headaches, colds, epilepsy and other diseases. Louis XV of France is believed to have spiced his favourite dishes with ambergris and Elizabeth I of England perfumed her gloves with it. In Egypt, ambergris is still used today for perfuming cigarettes.

The first indications of the use of ambergris in fine perfumery we have from the Arabs in Spain in the 10th century. Ambergris was already being imported for this purpose from the Sunda Islands and the Maghreb. At the start of modern times, ambergris was being traded throughout

Europe and in many cases worth its weight in gold. Al-Hasan ibn Mohammed al-Wassan (named: Johannes Leo Africanus; b. about 1490 in Granada, d. after 1550 in Tunis) wrote that the price of one pound of ambergris on the market in Fez was about 60 ducats, which corresponded to that of three slaves.

For a long time, uncertainty reigned as to the origin of ambergris. The Chinese called it “lung sien hiang” and imagined dragons which slept on the coastal cliffs and drooled into the sea in their sleep. From the saliva, so they believed, the fragrance was formed. On the other hand, in the Japanese cultural region, they spoke of “kunsurano fu”, of whale faeces. The Arabian traveller and historian Al Mas’udi (d. 957 in al-Fustat, Egypt) reported of traders and seamen who believed that ambergris grew like fungi on the seabed and was occasionally washed up on land by storms. Marco Polo (1254-1324) was the first western traveller who reported that because of ambergris the sperm whale was hunted by Yemeni seamen off the island of Socotra.

However, certainty as to its origin was only obtained when with the flourishing of the American whaling industry in Nantucket (Massachusetts) in the 18th century, again and again sperm whales were killed in whose intestines lumps of ambergris were found.

In cultural history and etymologically, ambergris has often been confused with amber (genuine, yellow or Prussian amber, lat. *Succinum*, grk. *Electron*). *Ambra* or *amber* is also the English and old German name for amber. Nowadays, for differentiation, in English the word *ambergris*, which is derived from the French *ambre gris* and finally from the Arab word *anbar*, is used for the excretion product of the sperm whales.

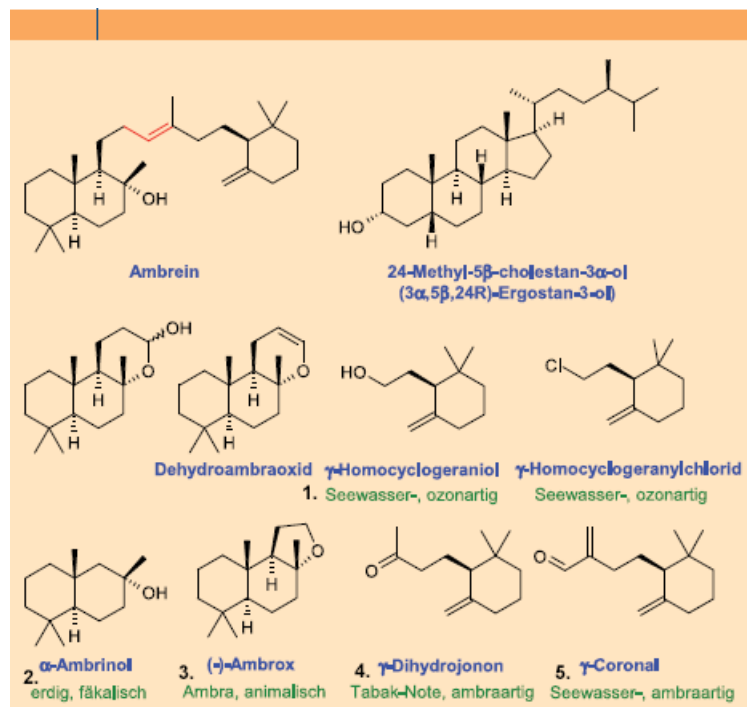
Constituents of Ambergris

The first chemical analyses of ambergris go back to the French chemists Joseph-Bienaimé Caventou (1795-1877) and Pierre-Joseph Pelletier (1788-1842). In 1820, they isolated and characterized the triterpene ambrein [3]. It is practically odourless and is used in the perfumery industry as a fixative. The second main component of fresh ambergris is 24-methyl-5 β -cholestan-3 α -ol, which together with ambrein makes up about 70-90% of the mass.

The olfactorily important components are formed during the maturing time in the sea. By oxidative degradation of ambrein in the region of the central double bond, volatile mono- and

bicarbocyclic compounds are formed, which owing to the chlorine content of seawater are to some extent also chlorinated (Figure 3) [4].

FIG.3 CONSTITUENTS OF FRESH AMBERGRIS



Key: 1. seawater- and ozone-like (x 2); 2. earthy, faecal; 3. ambergris, animal-like; 4. tobacco note, ambergris-like; 5. seawater- and ambergris-like.

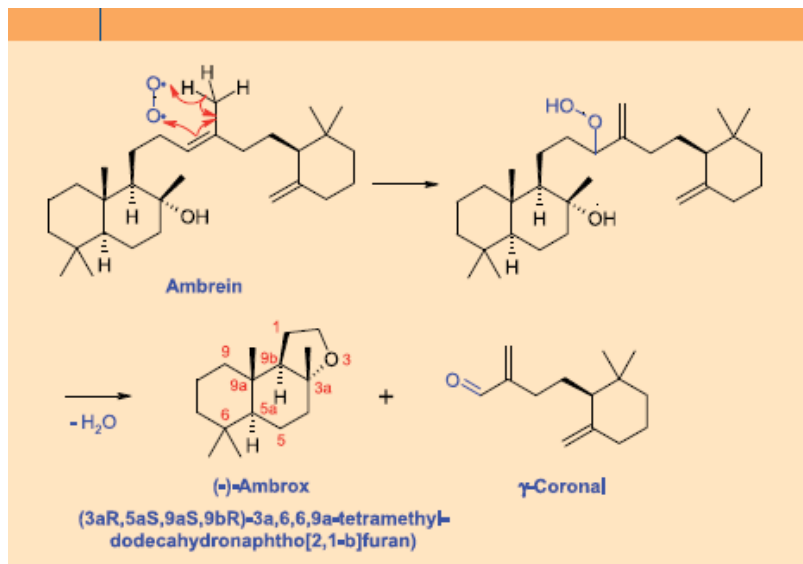
The key reaction in the degradation is a photo-oxidation by singlet oxygen. The porphyrin dye haemocyanin detected in ambergris could serve as a photosensitizer. In model reactions of pure ambrein with singlet oxygen, as well as *Ambrox*[®] and γ -coronal, α -ambriinol and dehydroambra oxide were also obtained (Figure 4) [5].

The olfactorily most important constituent in ambergris is (-)-*Ambrox*[®] ((3aR,5aS,9aS,9bR)-3a,6,6,9a-tetramethyldodecahydronaphtho[2,1-*b*]furan). It embodies the first four tonalities of a 3 percent ambergris tincture in 90% alcohol which has been allowed to mature for a while with occasional shaking, after moist, mossy forest soil, a strong fragrance of tobacco and balsamic sandalwood mixed with a warm, animal, musk note.

Ambrox[®] designates the enantiomerically pure compound. The racemate is called *Cetalox*[®] (*cetus*: lat. whale). Both are brand names of the Firmenich company. *Ambroxan*[®] and

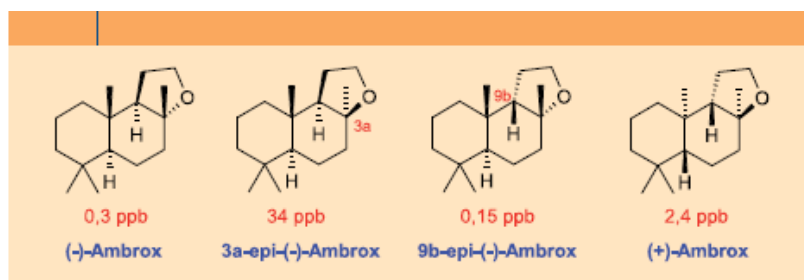
Amberlyn[®] are synonymous with *Ambrox*[®]. These are brand names of Cognis (Henkel/BASF) and Quest respectively.

FIG.4 PHOTO-OXIDATIVE DEGRADATION OF AMBREIN



The different diastereomers of (-)-*Ambrox*[®] have a similar fragrance, but to some extent differ in their odour thresholds [6, 7]. That of 3a-*epi*-(-)-*Ambrox*[®] is higher by a factor of 100 than that of (-)-*Ambrox*[®]. On the other hand, with almost identical odour quality, 9b-*epi*-(-)-*Ambrox*[®] has an odour threshold half as high. (+)-*Ambrox*[®] is eight times weaker than the natural enantiomer. The racemate has an odour threshold of 0.5 ppb and scarcely differs in its tonalities from that of (-)-*Ambrox*[®] (Figure 5).

FIG.5 THE ODOUR THRESHOLDS OF THE STEREOISOMERS OF AMBROX[®]



(-)-*Ambrox*[®] was also isolated not only from natural ambergris but also from the absolute of tobacco (*Nicotiana tabacum*), and detected in the ethereal oil of the clary sage (*Salvia sclarea*), the cistus (*Cistus labdaniferus*) and the Italian cypress (*Cupressus sempervirens*) (Figure 6).

The “concrete” is mostly obtained from plant parts, by extraction with organic solvents, which are then evaporated off. The “absolute” is the filtered ethanolic solution of this residue.



Fig.6 Gum cistus (*Cistus labdaniferus*) and Italian cypress (*Cupressus sempervirens*).

(© Carsten Niehaus and Andrea Buratti)

Partial Syntheses

The first chemical synthesis of (-)-Ambrox[®] derives from A. Hinder and A. Stoll of the Firmenich company from the year 1950 [8, 9]. The starting material for this natural substance synthesis was the diterpene sclareol, which is obtained from the clary sage (*Salvia sclarea*) (Figure 7).

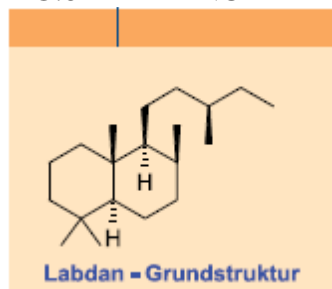
The evergreen biennial or perennial herbal plant reaches growth heights from 50 to 150 centimetres and has been cultivated since the 9th century at the latest. According to legend, wine traders were the first who industrially utilized the clary sage and from their mediocre

Rhine wine “conjured up” true Muscatel wine. Nowadays, clary sage oil finds its main use in the production of Eaux de Cologne, Eaux de Toilette for the flavouring of herbal liqueurs, as a healing plant and as seasoning.

By steam distillation of 100 kg of the inflorescence and shoot apex, ca. 800 grams of clary sage oil (*oleum salviae sclareae*) are obtained. The main constituents of the clear, colourless ethereal oil are linalyl acetate, linalool, nerolidol, neroli acetate and sclareol, which can however vary markedly depending on the cultivation region and climatic conditions.

A higher content of sclareol is contained in the clary sage concrete, which is obtained by extraction of the flowering plants with hexane. The content is about 70%. After counter-extraction with 87 percent methanol and evaporation of the solvent, a syrupy residue is obtained, which crystallizes and has a sclareol content of over 90% [10, 11]. The annual world production amounts to 50-150 tonnes (Figure 8) [12].

FIG.8 LABDANUM



Labdanum = basic structure

FIG.10 SCLAREOLIDE

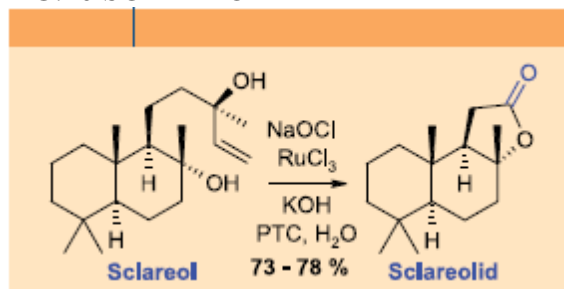
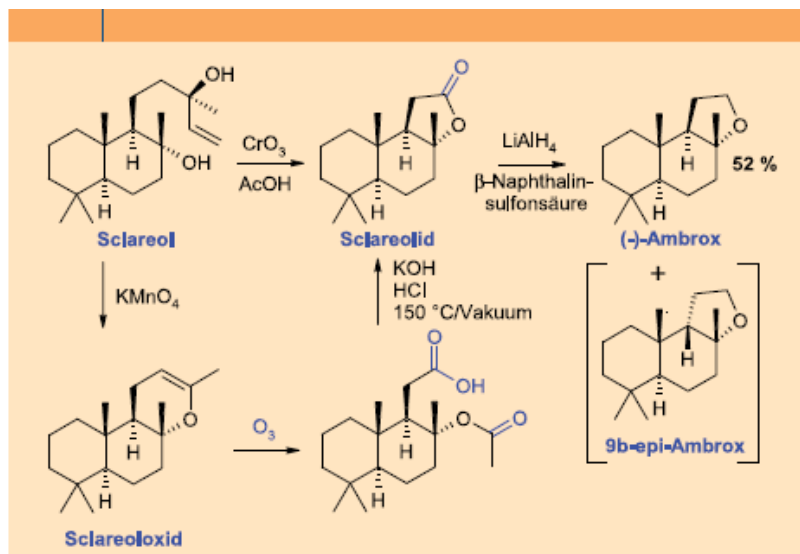


Fig.8 Sclareol belongs to the large group of the labdanoid diterpenes. The name is derived from labdanum, a resin from the cistus plants (Cistaceae), from which the first representatives of this basic structure were isolated.

Fig.10 Efficient synthesis of sclareolide from sclareol.

The key step of the industrial synthesis is the oxidative degradation of the side-chain of sclareol either by oxidation with chromium trioxide or potassium permanganate followed by an ozonolysis.

The reduction of the lactone with lithium aluminium hydride yields the diol, which in acid is cyclized to the desired ether. However, this step requires increased care, since under the reaction conditions the thermodynamically more stable 9b-*epi-Ambrox*[®] also very readily forms (Figure 9).

FIG.9 PARTIAL SYNTHESIS OF AMBROX[®] FROM SCLAREOL

Key: β -naphthalenesulphonic acid; sclareol oxide.



Fig.7 Clary sage (*Salvia sclarea*, also muscat sage, Roman sage or clary) is mainly grown in the Mediterranean region, in the Balkans, in the region of the former USSR and the USA. [© Kurt Stueber]



Fig.11 The balsam fir (*Abies balsamea*) is a medium-sized tree of growth height up to 20 metres (rarely up to 30 m) with up to 50 centimetre trunk diameter. It can be up to 200 years old. [© United States Fish and Wildlife Service]

At the start of the 1980s, the commercial use of Ambrox[®] became patent-free, so that a whole range of further industrial syntheses were also developed at other firms and in universities/technical colleges. Thus Sir Derek Barton published a degradation reaction of sclareol for which Quest applied for a patent, in which the potassium permanganate was replaced by sodium periodate [13, 14].

Henkel developed a very attractive process for the production of sclareolide from sclareol, by oxidation with sodium hypochlorite in the presence of ruthenium chloride and a phase transfer catalyst (Figure 10) [15].

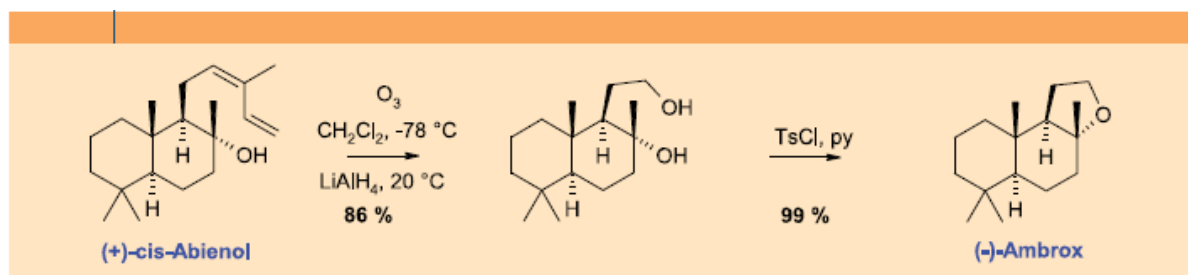
Also particularly noteworthy are the studies at Fritsche Dodge & Olcott and at International Flavors & Fragrances, which showed that the side-chain can also be degraded by fermentation with *Hyphozyma roseoniger* or *Cryptococcus albidus* [16, 17].

Apart from the oxidation with chromium oxide, potassium permanganate or sodium periodate, the disadvantages of this synthesis lie in the unreliable supply situation of sclareol and the thus often markedly fluctuating prices [6].

Alternatively to sclareol, (+)-*cis*-abienol, which is obtained from Canada balsam, can also be used for the production of (-)-*Ambrox*[®]. The resin is obtained by scoring the bark of the balsam fir (*Abies balsamea*), which grows in the North of the USA and in Canada (Figure 11). (+)-*cis*-abienol is obtainable by steam distillation, extraction and crystallization.

Ozonolysis and reductive processing of (+)-*cis*-abienol leads to a diol, which can be cyclized to *Ambrox*[®] in excellent yields with tosyl chloride in pyridine (Figure 12) [18].

FIG.12 AMBROX[®] FROM ABIENOL



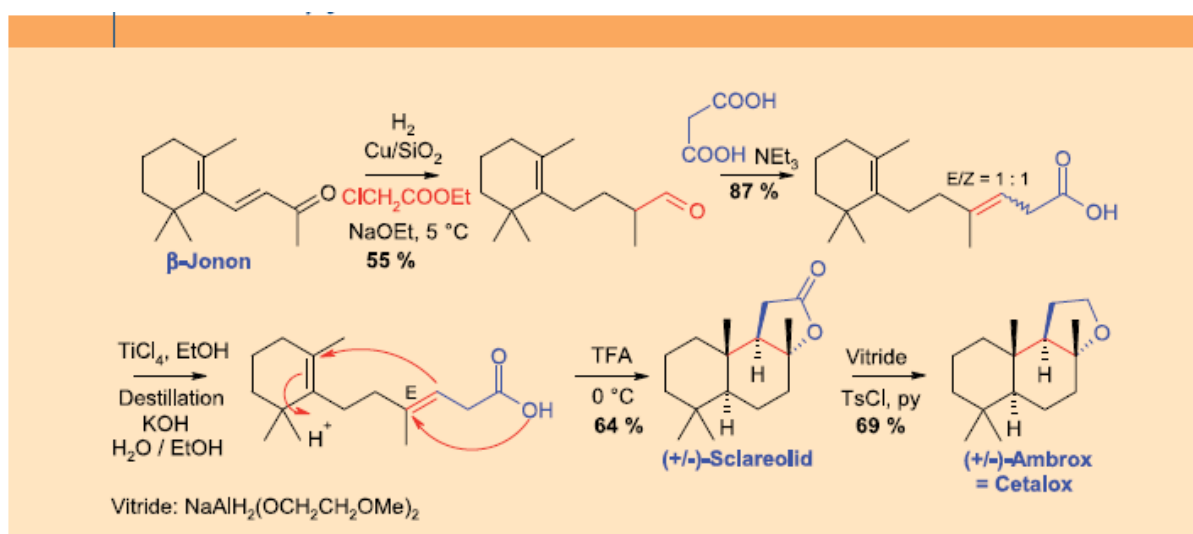
The synthesis of *Ambrox*[®] from (+)-*cis*-abienol.

As well as these particularly elegant degradation reactions, (-)-*Ambrox*[®] has also been synthesized starting from a range of other natural substances such as (-)-drimenol [19], (-)-thujone [20] or (+)-carvone [21].

Total Syntheses

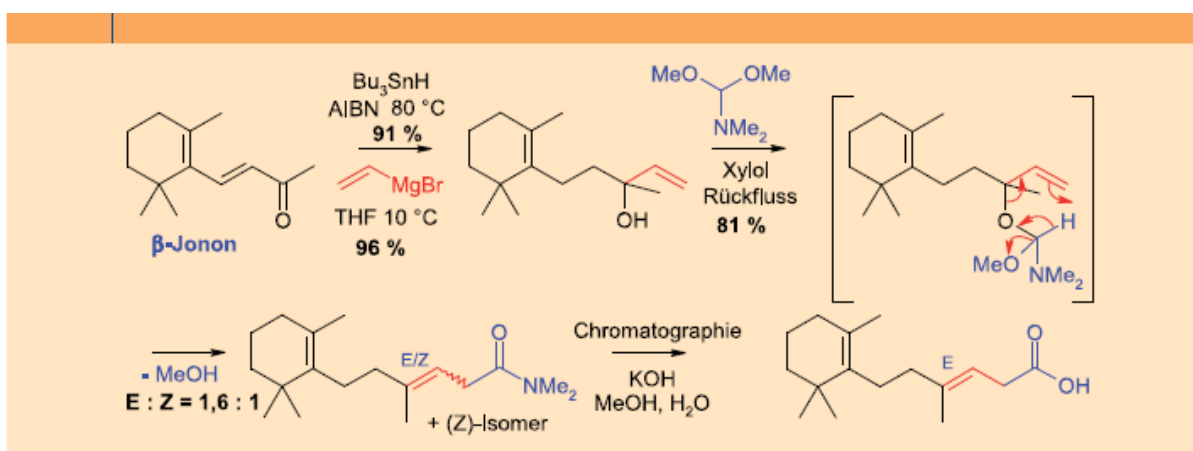
The first total synthesis of *Cetalox*[®] derives from Masanao Matsui [22]. The starting material is β -ionone [23], whose double bond in the side-chain can be regioselectively hydrogenated on a copper catalyst. Next, it is lengthened by 3 C atoms by the sequence of a Darzens reaction [24] and a Knoevenagel condensation. The (*E/Z*) isomers in the ratio of 1:1 are separated by distillation after esterification. The (*E*) isomer is saponified and cyclized to (+/-)-sclareolide with trifluoroacetic acid. Reduction with *Vitride*[®] and ether formation then yields *Cetalox*[®] (Figure 13).

FIG.13 AMBROX[®] FROM β -IONONE



The synthesis of racemic *Ambrox*[®], which is named *Cetalox*[®], starting from β -ionone.

FIG.14 DEGRADATION OF THE PRECURSOR



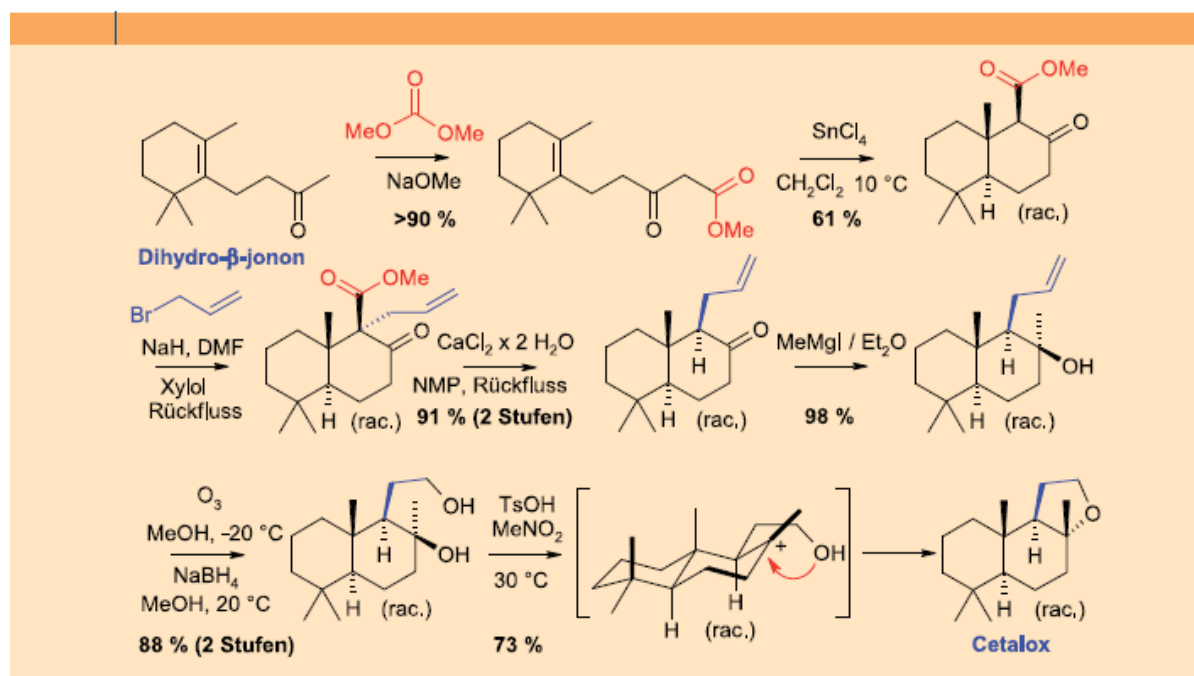
Key: xylene reflux.

An appealing alternative to the degradation of the sclareolide precursor.

An appealing alternative to the degradation of the sclareolide precursor comprises, after reduction of the double bond of β -ionone with tributylstannane, a Grignard reaction and then treatment with dimethylformamide dimethyl acetal, whereby the corresponding carboxylic acid amide is formed by a [2,3] sigmatropic rearrangement. The (*E/Z*) isomers can be separated chromatographically (Figure 14) [25, 26].

In 1989, George Büchi and Hans Wüst of the MIT published a synthesis of *Cetalox*® which was converted to the larger scale by Firmenich and considerably improved at critical points [27]. The starting material is likewise dihydro- β -ionone, which is converted into the corresponding β -keto ester by reaction with dimethyl carbonate. After the tin tetrachloride mediated cyclization, alkylation was necessary in the α position. However this was unsuccessful. On the other hand, O alkylation, followed by a Claisen rearrangement, was successful. The demethoxycarbonylation can be performed in DMSO. However, the yields are better in DMP as solvent. After the Grignard reaction for the introduction of the methyl group, the side-chain is degraded by ozonolysis and reduction to the required alcohol.

FIG.15 AMBROX® SYNTHESIS FROM FIRMENICH



Key: xylene, reflux; reflux (2 steps); (2 steps).

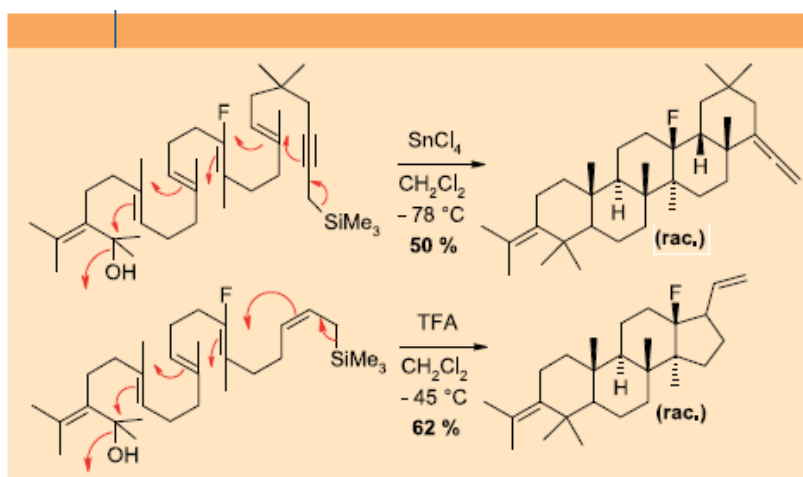
The most difficult step of the total synthesis was the ring closure to the *trans*-linked tetrahydrofuran under kinetic control, since the thermodynamically more favourable product is 9b-*epi*-*Cetalox*®. Under the chosen conditions, the reaction proceeds very selectively. The content of 9b-*epi*-*Cetalox*® is less than 1% (Figure 15).

Polyene Cyclizations

Also exceptionally interesting and of industrial importance are the biomimetic attempts to render *Cetalox*® and *Ambrox*® accessible by polyene cyclization.

The fundamental methodological studies on artificial polyene cyclization date back to Gilbert Stork, Albert Eschenmoser and E. E. van Tamelen, who in the 1950s and 1960s concerned themselves with the acid-catalysed cyclization of farnesic acid and squalene oxide [28, 29, 30]. Further pioneering studies from 1960 then already relate to the acid-catalysed cyclization of homofarnesic acid to a diastereomer mixture of sclareolide and derive from Günther Lucius of the VEB Chemical Factory Miltiz in Leipzig (see below) [31]. In the more recent past, there are very elegant examples of the domino synthesis, in particular of the steroid skeleton [32, 33]. The biomimetic tetra- and polycyclizations by W. S. Johnson are initiated by Lewis acids or trifluoroacetic acid (Figure 16).

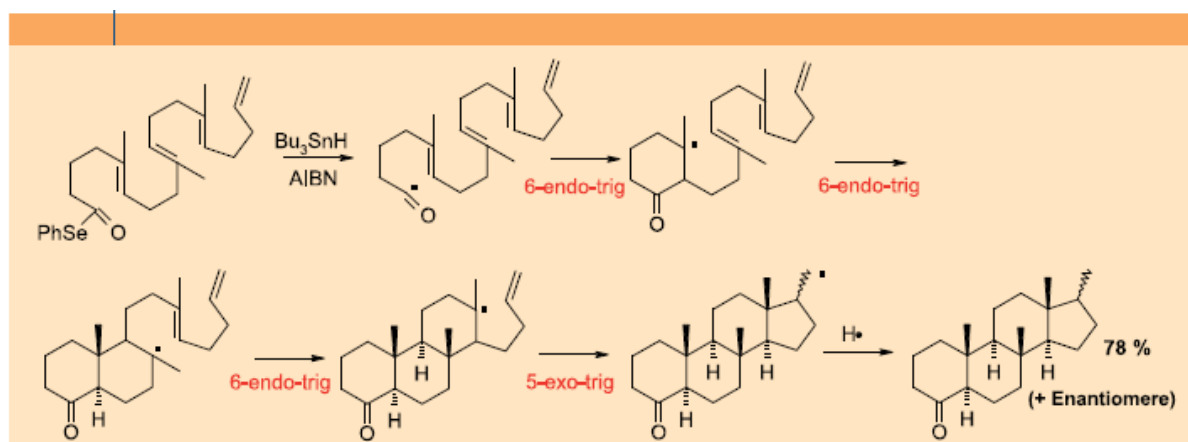
FIG.16 LEWIS AND BROENSTED ACID-MEDIATED POLYENE CYCLIZATIONS



However, radical polycyclizations are also known. Among the most prominent examples are the polyene cyclizations by Pattenden, which indeed represent a textbook example of the Baldwin rules [34, 35, 36, 37]. The polycyclization proceeds via free radicals, which proceeds

in a domino reaction of 6-*endo-trig* cyclizations and ends with a 5-*exo-trig* reaction. An important precondition for the avoidance of side reactions is the skilful construction of the polyene, which can however then in favourable cases be cyclized to the steroid skeleton in enormously high yields (Figure 17).

FIG.17 RADICAL POLYENE CYCLIZATIONS



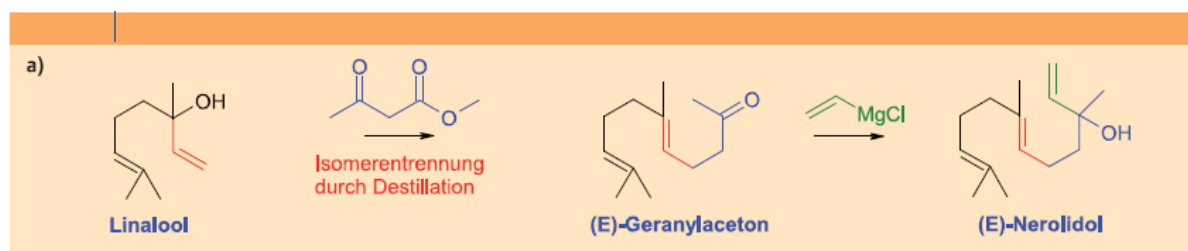
Key: (+ enantiomers).

Educts for the Polyene Cyclization

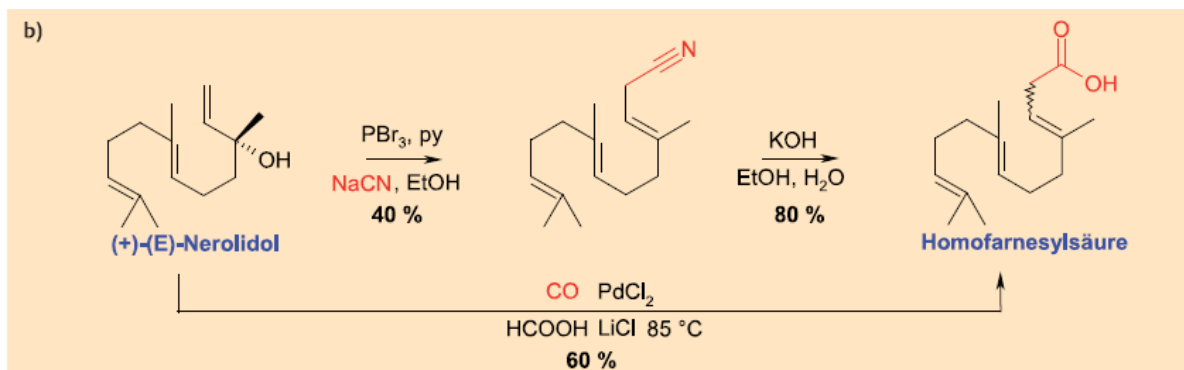
Cabreuva oil, which is obtained by steam distillation from the Brazilian tropical wood *Myrcarpus fastigiatus*, is the starting material for the sesquiterpene (+)-(*E*)-nerolidol, an important precursor of homofarnesic acid and homofarnesol.

Racemic (*E*)-nerolidol is obtained by a comparatively simple route from linalool by a Carroll reaction and treatment with vinylmagnesium chloride. Alternatively, linalool can also be treated with diketene or isopropenyl methyl ether (Saucy-Marbet reaction) and the Grignard reaction replaced by an acetylene addition followed by Lindlar hydrogenation (Figure 18a) [38].

FIG.18 FROM LINALOOL TO HOMOFARNESIC ACID



Key: isomer separation by distillation



Key: homofarnesic acid

Starting from linalool, nerolidol is obtained (18a), which can be further converted by different routes into homofarnesic acid (18b).

Conversion of the nerolidol into the corresponding bromide, cyanide substitution and hydrolysis, or its carbonylation [39] yields a (7*E*,3*Z*)-/(*E,E*) isomer mixture of homofarnesic acid (Figure 18b).

(*E,E*)-homofarnesol is obtained from nerolidol by [2,3] sigmatropic rearrangement, chromatographic or distillative separation of the 2:1 mixture of homofarnesic acid amide and reduction with lithium triethylboranate (Figure 19a) [25].

Alternatively, nerolidol can be isomerized to farnesol via tungstates, formally according to a metalla-Claisen rearrangement, and purified by distillation (Figure 19b) [40]. After conversion to farnesyl chloride, this is converted with freshly precipitated barium and carbon dioxide to (*E,E*)-homofarnesic acid, which is reduced with lithium aluminium hydride [41, 42].

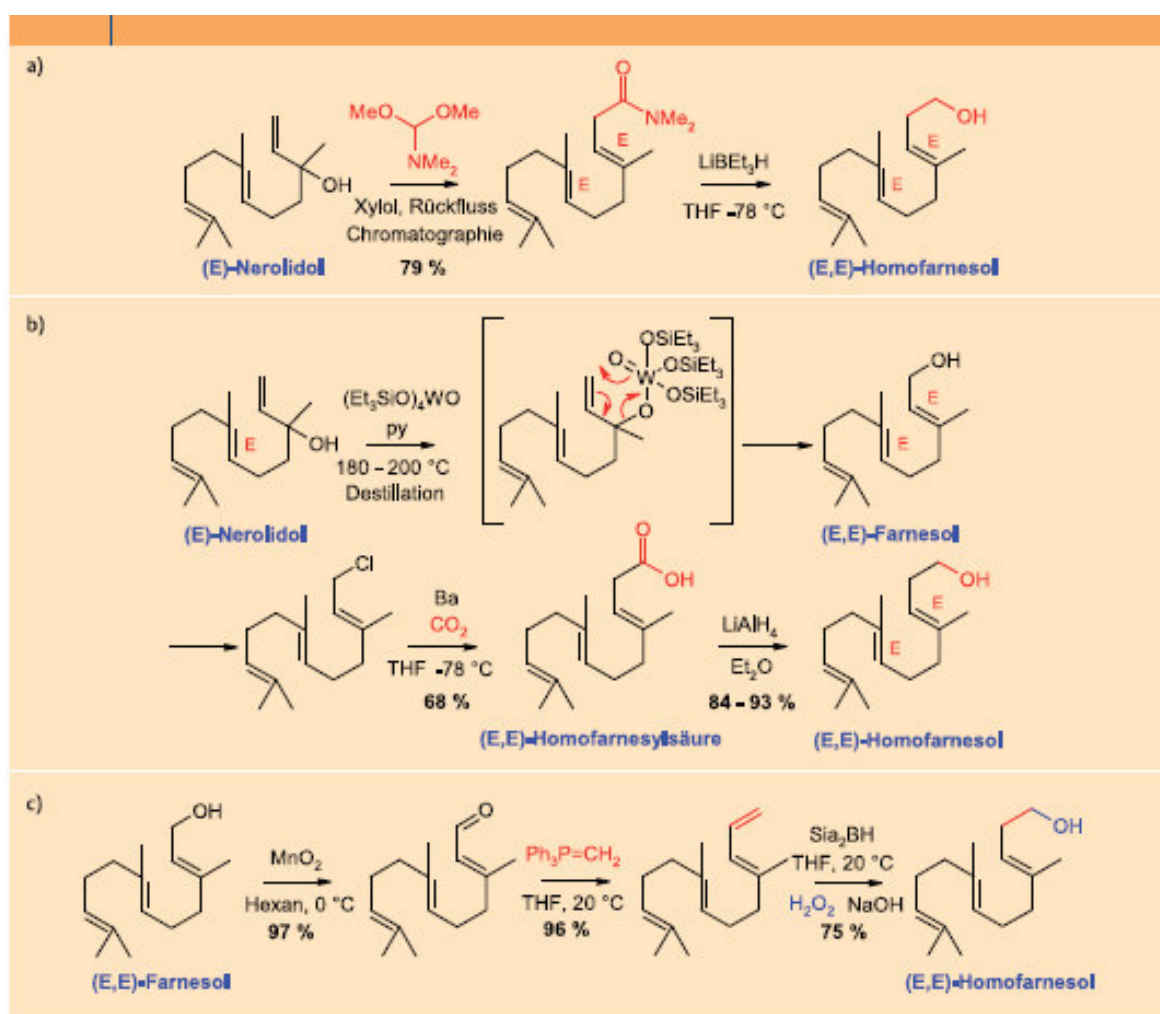
Oxidation of farnesol with manganese dioxide, Wittig reaction and hydroboration with disiamylborane, which is prepared *in situ* from the borane-THF complex and 2-methyl-2-butene, likewise yields (*E,E*)-homofarnesol in an overall yield of 70% (Figure 19c) [42].

Sclareolide and Ambrox®

Günther was able to cyclize homofarnesic acid with a mixture of formic acid and sulphuric acid to a diastereomer mixture of sclareolide. By crystallization, the diastereomers could be separated. However, the yield was only 15%. However, racemic sclareolide was not contained therein [31].

Subsequently, a range of other Brönsted and Lewis acids were tried for the stereochemically selective cyclization. Thus from homofarnesic acid, the sclareolide isomers are obtained in a yield of 91% if the reaction is performed with tin tetrachloride in dichloromethane at -78°C [43]. If on the other hand boron trifluoride is used as the catalyst, then racemic sclareolide is obtained only in a yield of 38% [44]. The Henkel company patented a process in which an (*E,Z*) isomer mixture of homofarnesic acid is treated with methanesulphonic acid in dichloromethane at a temperature of -10°C . However, also only a mixture of sclareolide isomers was obtained [45].

FIG.19 SEVERAL ROUTES TO (*E,E*)-HOMOFARNESOL

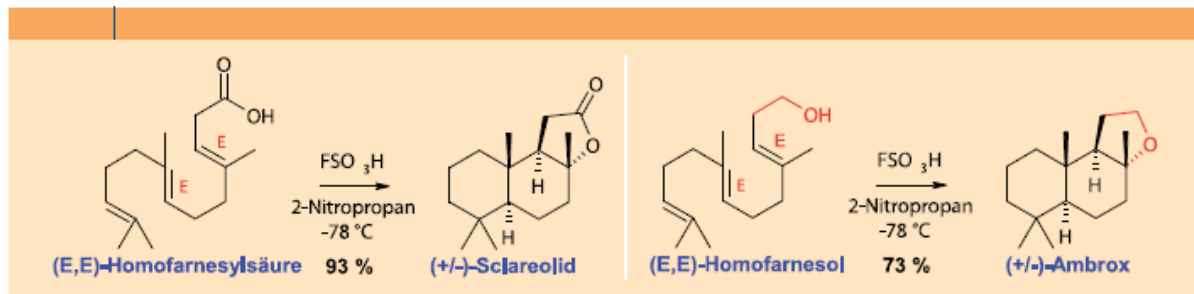


Key: xylene, reflux, chromatography; distillation; (*E,E*)-homofarnesic acid.

Finally, the breakthrough was made by P. F. Vlad at the chemical institute of the University of Kishinev (Moldavia) by use of superacids such as fluorosulphonic acid in 2-nitropropane at

low temperatures (Figure 20) [46]. Interestingly, from (*E,E*)-homofarnesol under very similar conditions he highly diastereoselectively obtained racemic *Ambrox*[®] in a yield of 73% [25, 41].

FIG.20 POLYENE CYCLIZATIONS WITH SUPERACIDS

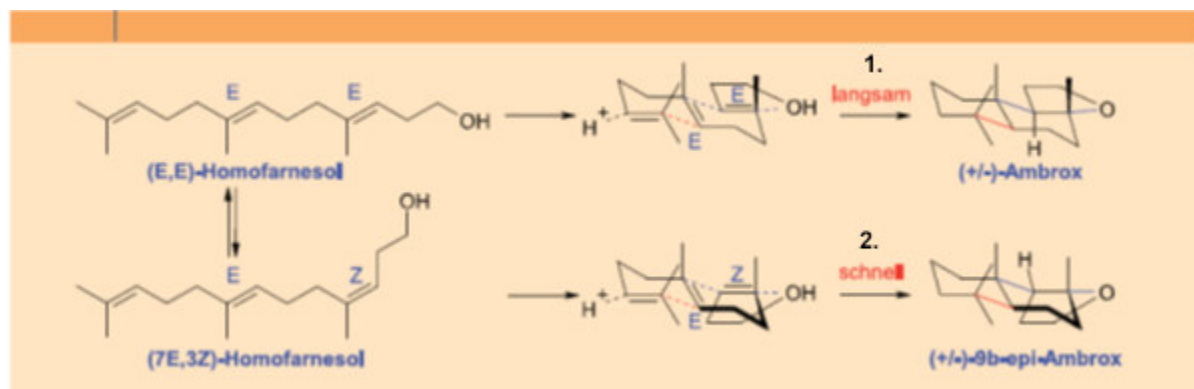


Key: (*E,E*)-homofarnesic acid

However, in criticism it must be noted that the yields at Firmenich under very similar conditions were only 40% and reactions with chlorosulphonic acid in 1-nitropropane also produced these yields [25, 47].

Very detailed mechanistic studies on polyene cyclization come from Roger Snowden of Firmenich [48]. A terminal protonation initiates the cyclization of the tricyclic system. The *anti* addition at the double bonds of the nascent six-membered ring takes place from the chair conformation. Whether the cyclization takes place synchronously in accordance with the Stork-Eschenmoser hypothesis cannot be confirmed with the experimental data.

FIG.21 MECHANISTIC CONSIDERATIONS ON THE POLYENE CYCLIZATION

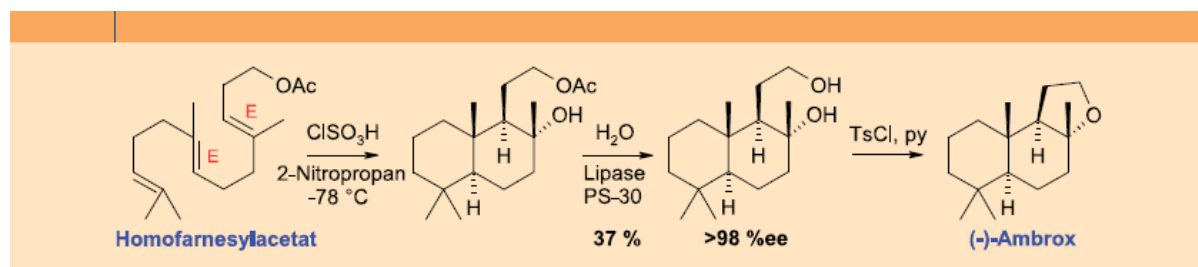


Key: 1. slow; 2. rapid.

A sequential course, in which the bond shown in red is linked at first, is also conceivable. The ring closure to *Ambrox*[®] or 9b-*epi-Ambrox*[®] then takes place more rapidly than the conformational change of the cyclohexyl carbocation, so that a *trans*-decalin system is formed. The isomerization of the middle double bond in homofarnesol is very slow compared to the terminal one and the content of other diastereomers is correspondingly low (Figure 21).

Enantiomerically pure *Ambrox*[®] is accessible by total synthesis if homofarnesyl acetate is cyclized with chlorosulphonic acid and the product cleaved by means of a *Pseudomonas* lipase. Etherification then yields the desired product (Figure 22) [49].

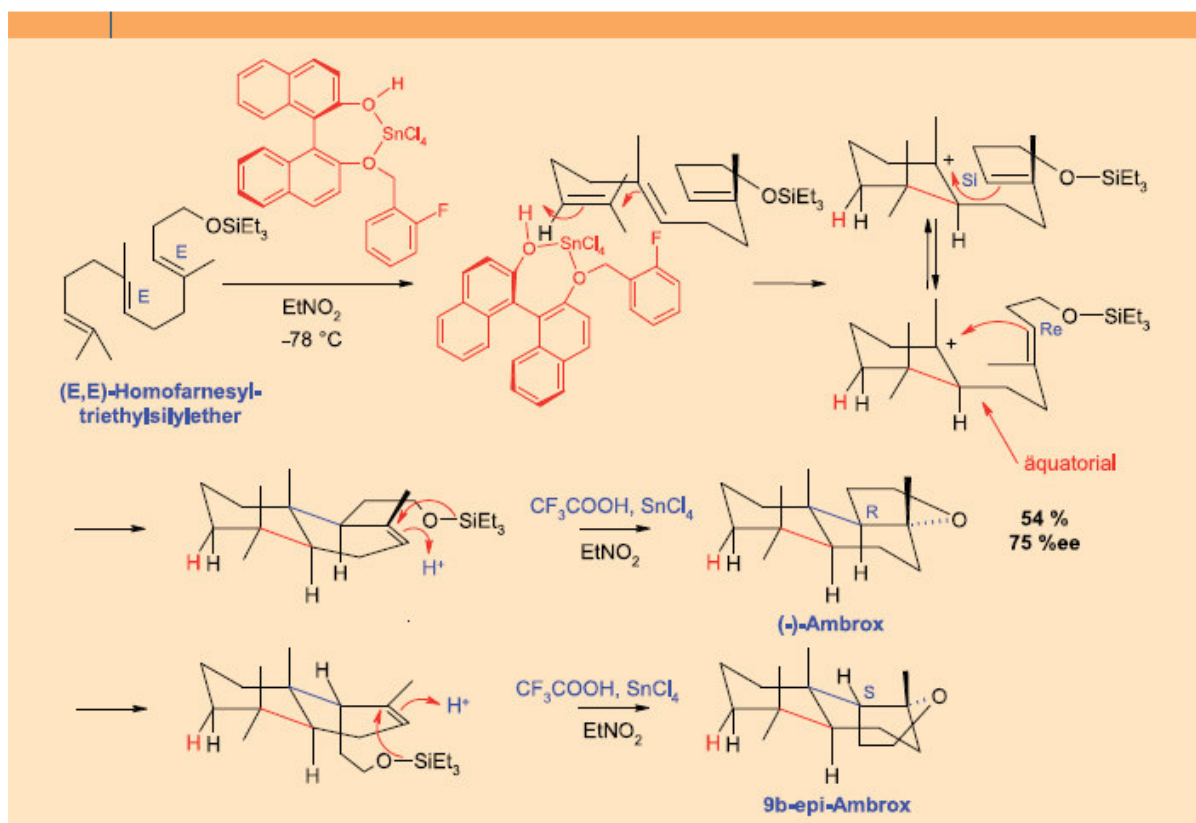
FIG.22 ENANTIOMERICALLY PURE AMBROX[®] BY ENZYMATIC RESOLUTION



The indisputably most elegant method for the total synthesis of (-)-*Ambrox*[®] is the enantioselective polyene cyclization. For the cyclization, Hisashi Yamamoto used an enantiomerically pure Lewis acid/Brönsted acid from O-(*o*-fluorobenzyl)-binol and tin tetrachloride and obtained (-)-*Ambrox*[®] with an enantiomeric excess of 75% and, even so, 54% yield [42].

As the starting material, (*E,E*)-homofarnesyl triethylsilyl ether is used. Through this, the cyclization stops at the stage of the decalin system, after the enantioselective protonation. The next cyclization proceeds with higher diastereoselectivity if it is performed in strong acid. Hence the balance between the stability of the Si-O bond and the Brönsted acidity of the enantiomerically pure Lewis/Brönsted acid is of decisive importance for high diastereoselectivity. The *Si*-facial ring closure of the cyclohexyl cation with equatorial side chain leads to *Ambrox*[®], the *Re*-facial one to 9b-*epi-Ambrox*[®]. The ring closure of the cyclohexyl cation with axial side chain yields two further diastereomers, 5a-*epi-Ambrox*[®] and 5a-*epi-9b-epi-Ambrox*[®]. Thus as the mechanism for the formation of the diastereomers, as well as the (*E/Z*) isomerization at the stage of the homofarnesol, there is also the (*Re/Si*)-facial cyclization possibility and the inversion of the six-membered ring conformation (Figure 24).

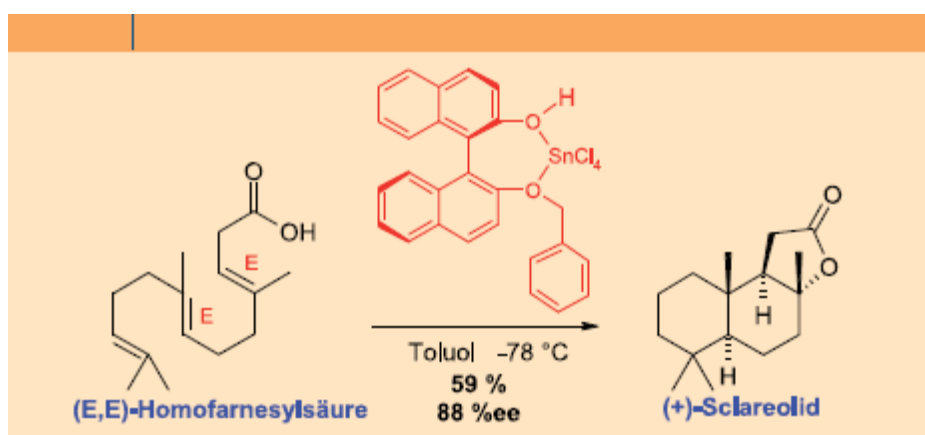
FIG.24 ENANTIOMERICALLY PURE SCLAREOLIDE BY ENANTIOSELECTIVE POLYENE CYCLIZATION



Key: equatorial

Under very similar conditions, but without addition of the trifluoroacetic acid/tin tetrachloride complex, (+)-sclareolide is obtained in comparable yields with an enantiomeric excess of 88% (Figure 23) [26].

FIG.23 ENANTIOMERICALLY PURE AMBROX[®] BY ENANTIOSELECTIVE POLYENE CYCLIZATION



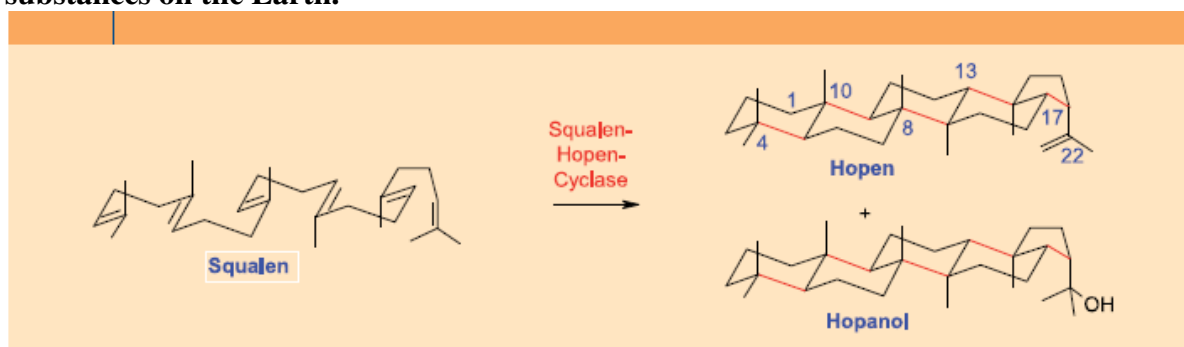
Enzymatic Polyene Cyclizations

The enantioselective polyene cyclization of squalene to the pentacyclic hopene and hopanol is among the most demanding biochemical reactions. In one step, nine asymmetric centres are formed. Hopene is one of $2^9 = 512$ possible stereoisomers.

The cyclization is mediated by the squalene-hopene cyclases of some prokaryotes and is in a way related to the squalene-lanosterol cyclase of the eukaryotes (Figure 25) [50, 51].

FIG.25 THE SQUALENE-HOPENE CYCLASE . . .

. . . cyclizes squalene to enantiomerically pure hopene or hopanol. The hopanoids are important constituents of the bacterial cell wall. They are found today in large quantities in up to 500 million year old oil shale. The quantity is estimated at 10^{12} tonnes. Thus together with cellulose they are among the quantitatively most significant organic substances on the Earth.

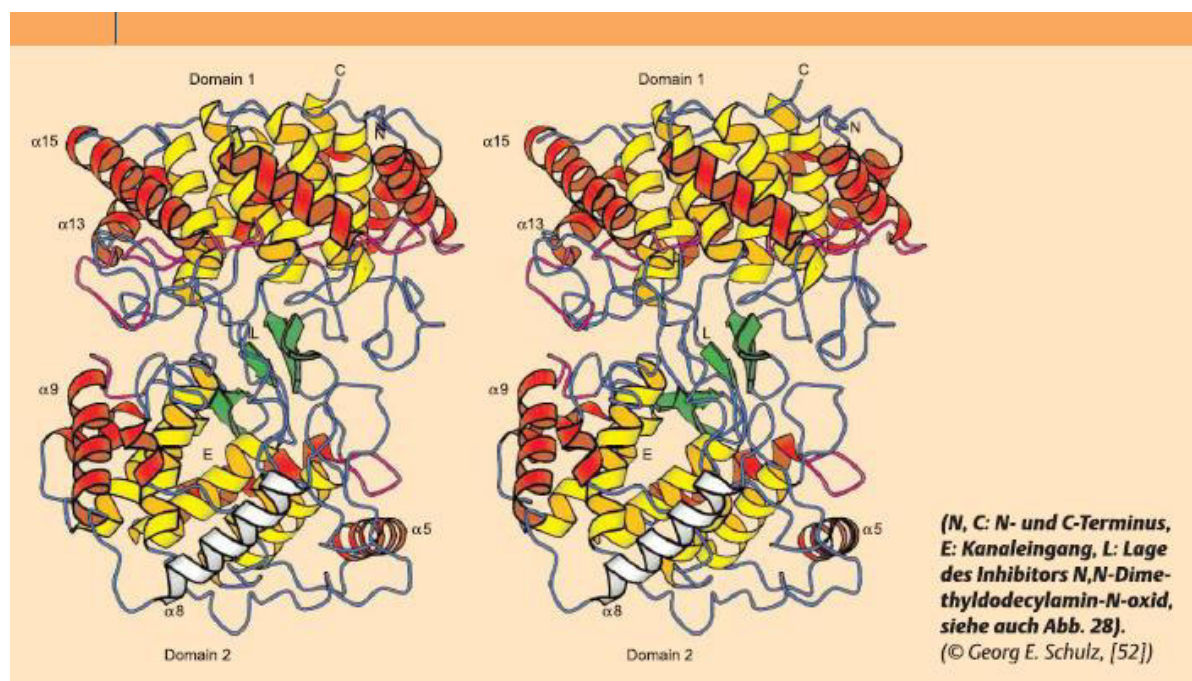


In 1997, Georg E. Schulz in Freiburg succeeded in elucidating the structure of the membrane squalene-hopene cyclase from the thermophilic microorganism *Alicyclobacillus acidocaldarius* (Figures 26 and 27) [52]. The enzyme consists of two approximately equal-sized domains. The active centre is situated in a $1200 \text{ \AA}^3$ (cubic Ångströms) sized cavity between them, which is accessible through a non-polar channel in domain 2 (Figure 28).



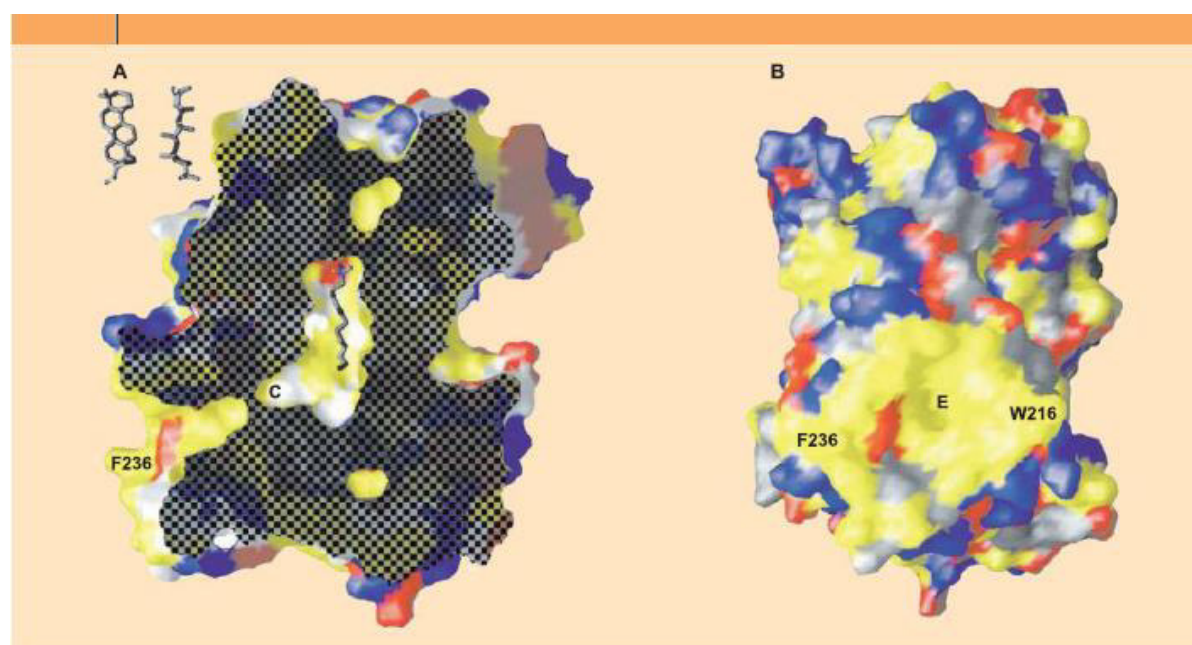
Fig.26 *Alicyclobacillus acidocaldarius*. (© PD. Dr. Manfred Rohde, Helmholtz Centre for Infection Research, Braunschweig)

FIG.27 STEREOSCOPIC VIEW OF THE SQUALENE-HOPENE CYCLASE



(N, C: N- and C-terminus, E: channel entrance, L: position of the inhibitor N,N-dimethyldodecylamine N-oxide, see also Fig.28). (© Georg E. Schulz, [52]).

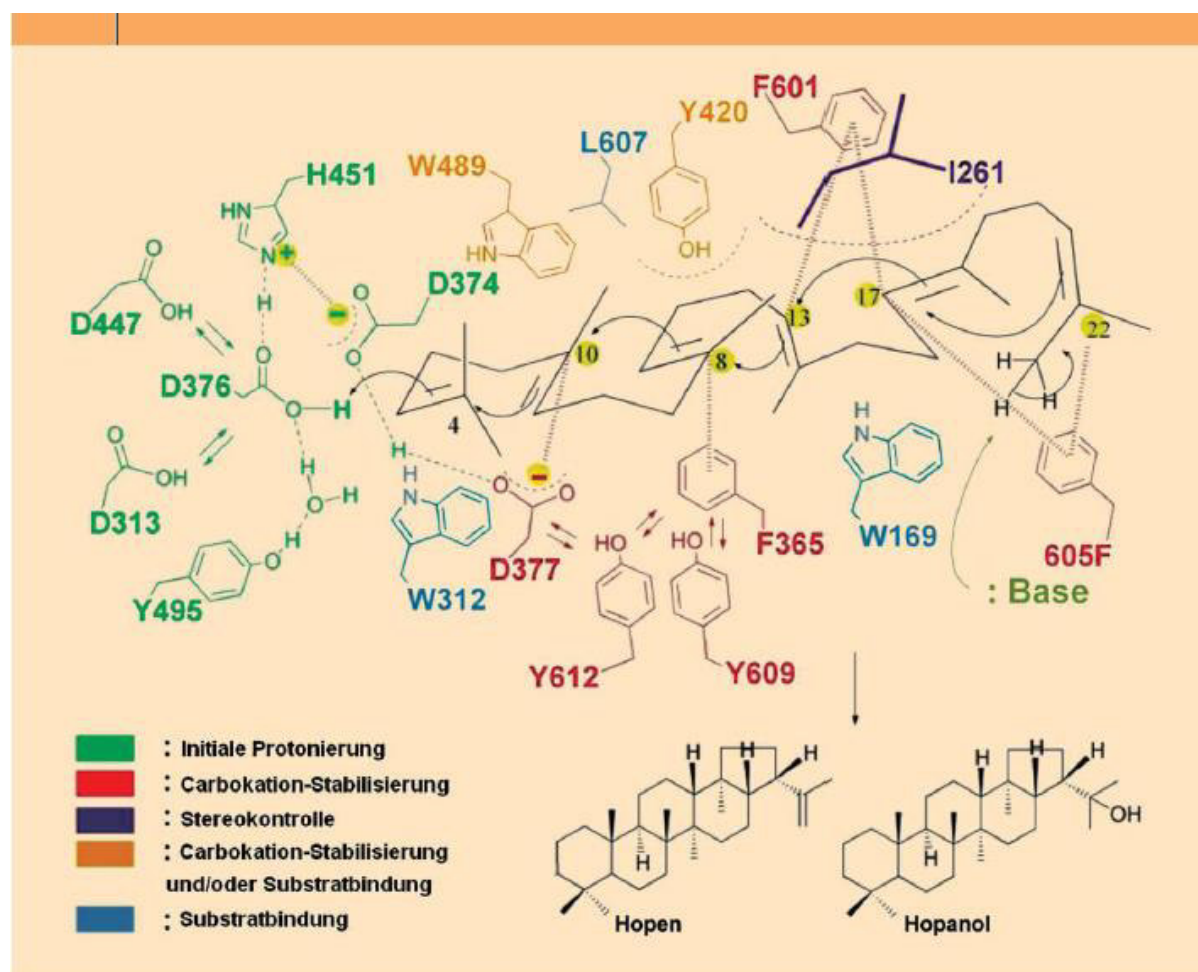
FIG.28 BINDING POCKET OF THE SQUALENE-HOPENE CYCLASE



Nonpolar regions are shown yellow, positive blue and negative red. View (A) shows the enzyme “cut open”. The cut plane lies in the region of the large binding pocket, in which the competitive inhibitor N,N-dimethyldodecylamine N-oxide (LDAO) is bound instead of hopene (on left above, two views to scale). A nonpolar channel (C) leads leftwards to a likewise uncharged opening. View (B) shows the channel entrance (E) of the enzyme from outside. It is surrounded by an approximately 1600 Å² sized nonpolar area. (© Georg E. Schulz, [52]).

In the active centre, squalene is bound in a chair-chair conformation [23]. The cyclization begins with a protonation on the C atom 3 of the future hopene by aspartic acid D376. Carbocation π interactions with various aromatic amino acids such as tyrosine, phenylalanine and tryptophan stabilize the cationic intermediate stages until finally through deprotonation or hydroxylation, hopene or hopanol respectively are formed (Figure 29).

FIG.29 POLYENE CYCLIZATION IN THE SQUALENE-HOPENE CYCLASE



(D: aspartic acid, F: phenylalanine, H: histidine, L: leucine, W: tryptophan, Y: tyrosine). (Fig. according to [53]).

Key: initial protonation; carbocation stabilization; stereochemical control; carbocation stabilization and/or substrate binding; substrate binding.

As early as 1986, Stefan Neumann and Helmut Simon at the Munich Technical University discovered that the squalene-hopene cyclase from *Bacillus acidocaldarius* [54] not only cyclizes squalene to hopene and hopanol, but also homofarnesol to Ambrox [55]. It can be assumed from this that the cyclization proceeds very similarly to that of squalene (Figure 30).

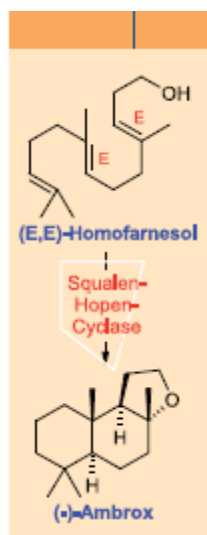


FIG.30 Cyclization of homofarnesol to Ambrox by means of the squalene-hopene cyclase from *Bacillus acidocaldarius*.

Recently, studies on this subject were also undertaken by the Japanese company Kao [56]. Unfortunately, none of the papers given information on selectivity and yield.

Superambrox

In 1993, chemists at Firmenich described the fragrance of Superambrox (5,5a-dehydroambrox) [57]. The excellent fragrance qualities are characterized by a very powerful, beautiful ambergris tonality with much volume and fullness and a strong headnote.

An interesting partial synthesis was described by Aede de Groot in the year 2001 [58]. The starting material is larixol, which is obtained by extraction and crystallization from the turpentine oil of the larch (Figure 31).

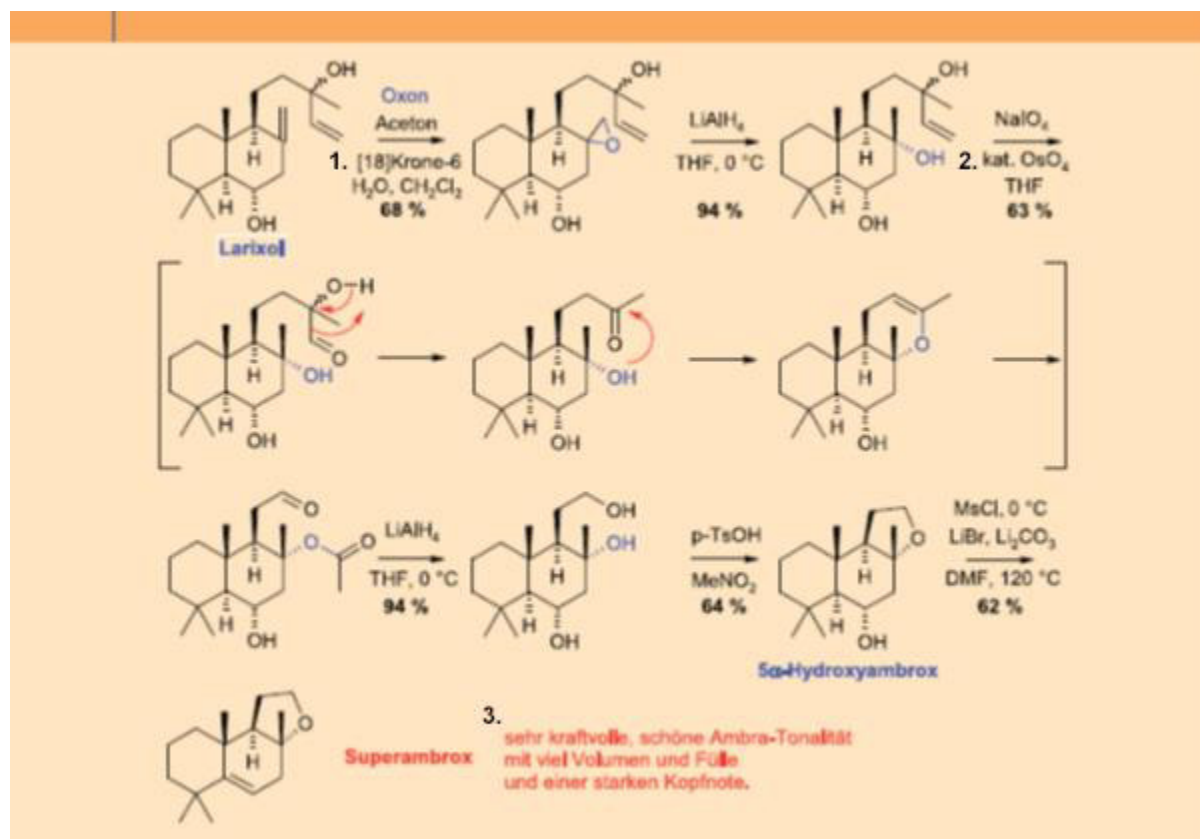


Fig.31 European larch (*Larix europaea*, *Pinus larix*, *Larix decidua*). (© Maurice Perry).

Oxidation with oxon (2KHSO_4 , KHSO_4 , K_2SO_4) leads selectively to the epoxidation of the exocyclic double bond. The side product at 19% is the diepoxide. Reduction with lithium aluminium hydride with ring opening yields the triol. The side chain is degraded by a periodate

oxidation. Reduction and ring closure with *p*-toluenesulphonic acid in nitromethane give 5 α -hydroxyambrox, from which superambrox can be produced after mesylation (Figure 32).

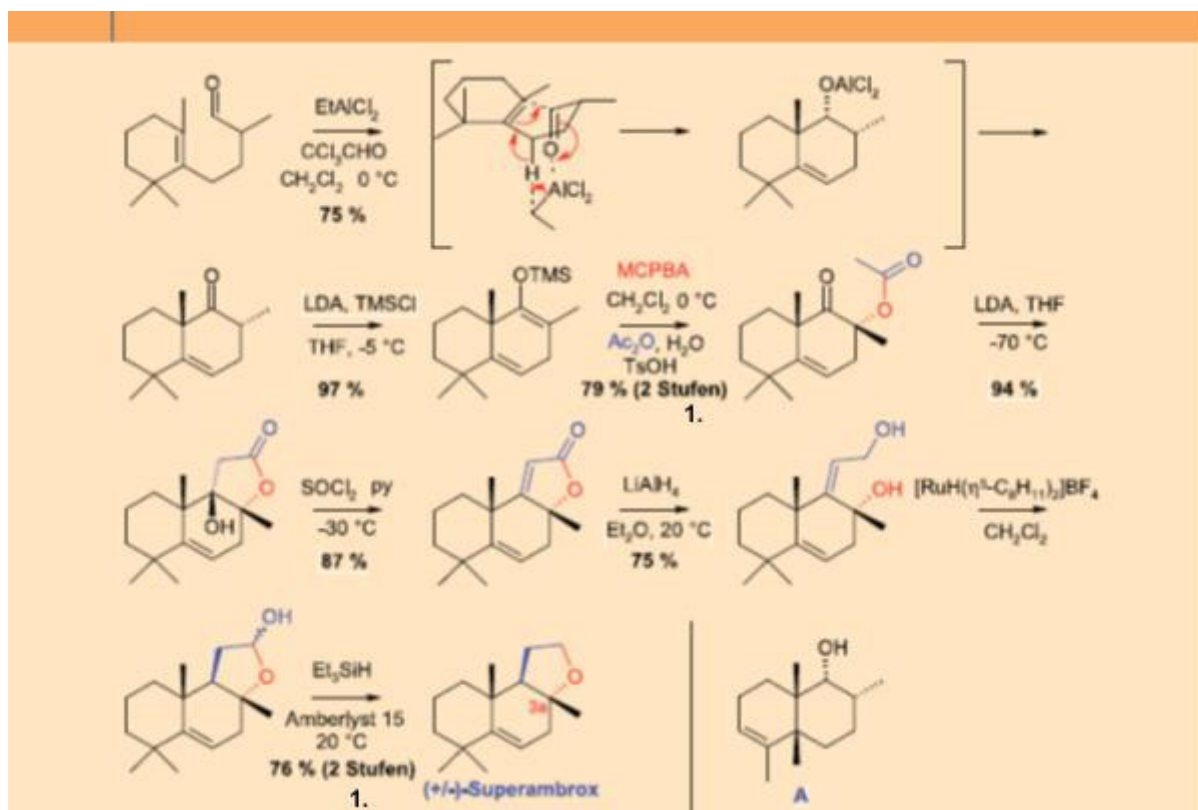
FIG.32 PARTIAL SYNTHESIS OF SUPERAMBROX



A particularly elegant stereoselective total synthesis of superambrox comes from Charles Fehr [59]. While Brönsted acid-catalysed ene reactions of the racemic dihydro-C14 aldehyde with methyl group migration lead to decalinol A in yields of ca. 40%, the Lewis acid-mediated ene reaction with ethylaluminium dichloride yields the desired bicyclic compound. The course of the reaction is presumably concerted and in the transition passes through an eight-membered ring system. The aluminium alkoxide initially obtained can be directly subjected to an Oppenauer oxidation with chloral and the decalinone is isolated directly. The corresponding silanol ether is diastereoselectively oxidized with *m*-chloroperbenzoic acid from the side facing away from the axial methyl group. The α -siloxyketone is converted to the corresponding acetate. The subsequent deprotonation with LDA leads to the degradation of the lactone in an intramolecular aldol reaction. After dehydration, this is reduced with lithium aluminium hydride. In the presence of the Chaudret ruthenium hydrogenation catalyst, the originally

intended hydrogenation does not take place. Instead, a double bond shift stereochemically controlled by the hydroxy group in position 3a takes place. Finally, the lactol is reduced to racemic superambrox with triethylsilane. The overall yield over all steps is 27% (Figure 33).

FIG.33 TOTAL SYNTHESIS OF SUPERAMBROX



Key: 1. (2 steps).

If the enantiomers are separated at the racemic dihydro-C14 aldehyde stage, then enantiomerically pure superambrox is also accessible by this route.

Commercial Use

Cetalox[®] and *Ambrox*[®] are used in high quality perfumes both for women and also for men. Prominent examples of this fragrance category are *Chanel No.5*[®], *Davidoff Cool Water*[®] or *Azzaro pour Homme*[®] (Figure 34).

Apart from their outstanding olfactory qualities, the fragrances are also very willingly used as fixatives. The price for *Ambrox*[®] amounts to ca. 1000 dollars/kg and the annual world production for *Ambrox*[®] and analogues is somewhat more than 30 tonnes [2].

Summary

Ambergris is a fragrance from the digestive tract of the sperm whale, highly valued for millennia. The olfactorily most important component is (-)-*Ambrox*[®], which is obtained on a large scale by partial synthesis from the sclareol of the clary sage. The polyene cyclizations of homofarnesol and homofarnesic acid are particularly elegant total syntheses. 5,5a-dehydroambrox is characterized not only by excellent fragrance qualities, but also by the elegant total syntheses.



Fig.34 Typical sales products with an ambergris note.

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© *Azzarro Parfums*,
www.azzaroparis.com)

Keywords

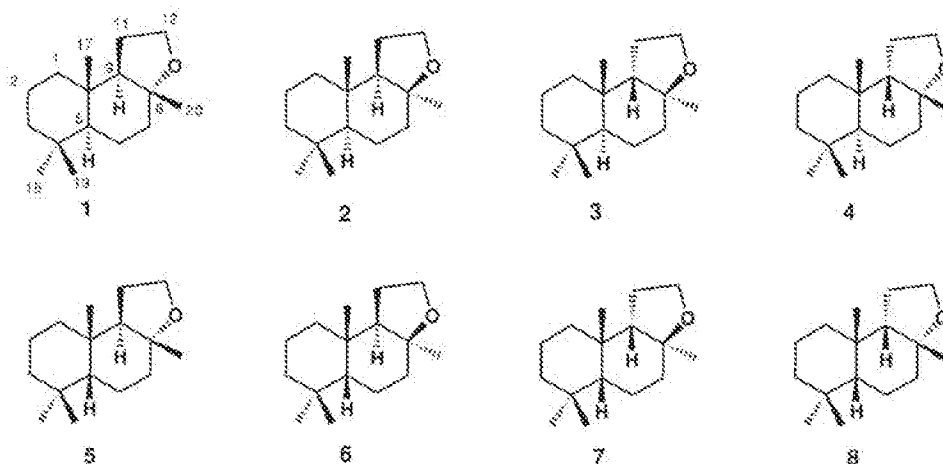
Fragrances, partial synthesis, polyene cyclization, sclareolide, ambrox.

178. Configuration-Odor Relationships in 5 β -Ambroxby Sina Escher^{a)}, Wolfgang Giersch^{b)}, Yvan Niclass^{a)}, Gérald Bernardinelli^{b)}, and Günther Ohloff^{a*)}^{a)} Firmenich SA, Research Laboratories, CH-1211 Geneva 8^{b)} Laboratory of Crystallography, University of Geneva, CH-1211 Geneva 4

(10.VIII.90)

The four possible A/B *cis*-fused diastereoisomers of *Ambrox*[®] have been synthesized and their configurations and conformations established by X-ray and NMR analysis. Only 5 β -ambrox (= 1,2,3a,4,5,5a β ,6,7,8,9,9a,9ba-dodecahydro-3a β ,6,6,9a β -tetramethylnaphtho[2,1-b]furan; **5**) has an odor quality comparable to *Ambrox*[®]. The 1,3-synperiplanar/diaxial conformation of the substituents at C(8) (= C(3a)) and C(10) (= C(9a)) has thus been confirmed to be a compulsory structure element for the particular odor.

Introduction. — *Ambrox*[®]¹⁾, the prototype of ambergris odorants, occurs in three diastereoisomeric forms **1**, **2**, and **4** with regard to the C-atoms 8 and 9. Conformational considerations make the existence of the highly strained fourth diastereoisomer **3** unlikely. All three tricyclic ethers obey the 'triaxial rule' of odor sensation [1–3]. Continuing

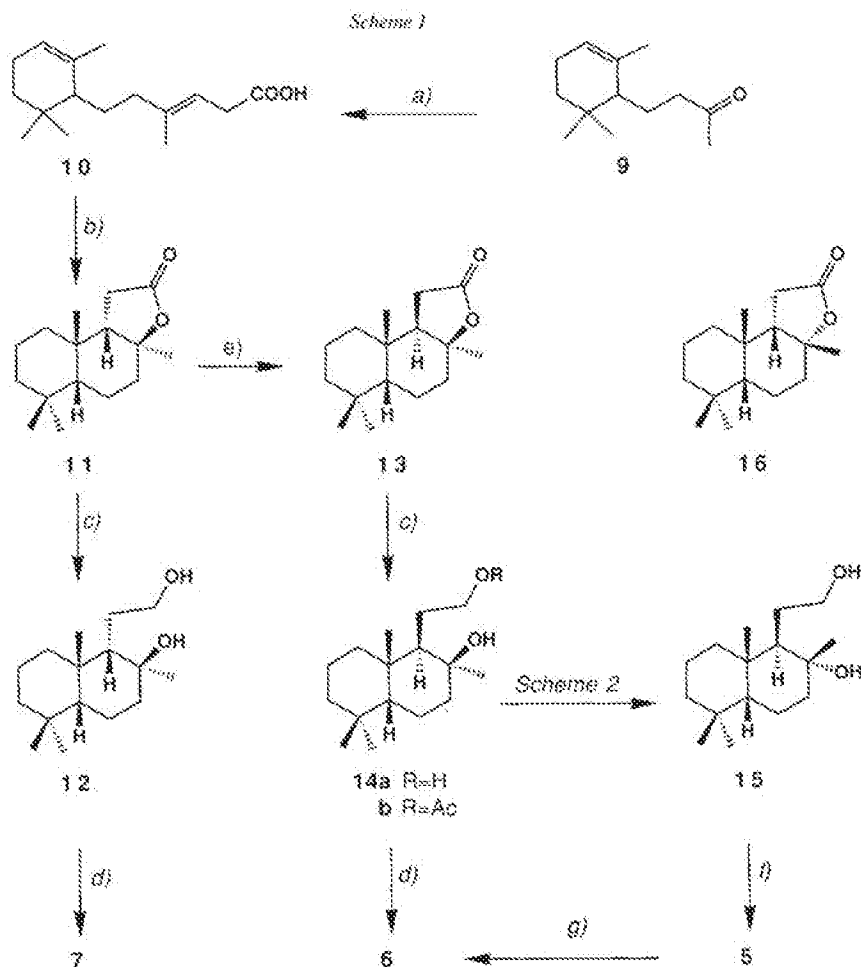


interest in this field stimulated preparation and sensory evaluation of A/B *cis*-fused *Ambrox*[®], and in this paper, we report the synthesis, conformation, and olfactory properties of the four existing 5 β -diastereoisomers **5–8**²⁾.

¹⁾ Trade name of Firmenich SA.²⁾ All compounds synthesized in this work are racemic.

Syntheses. — Racemic $5\beta,8\alpha,9\beta$ -sclareolide (**11**) represents an ideal starting material for the target molecules **5–7**. Thanks to extensive studies on cyclization of monocyclohomofarnesic acid and related compounds by *Saito* and coworkers [4] [5], lactone **11** is readily available from α -dihydroionone **9** through acid-catalyzed cyclization of (*E*)- α -monocyclohomofarnesic acid **10** (Scheme 1). The structure of **11** has been fully corroborated by X-ray diffraction analysis [4]^b). Reduction of **11** with LiAlH_4 and cyclization of the resulting diol **12** by means of POCl_3 in pyridine provided $5\beta,8\alpha,9\beta$ -ambrox (**7**).

In analogy to the well known isomerization of sclareolide [6] [7], lactone **11** was treated with $\text{HCOOH}/\text{H}_2\text{SO}_4$ at 90° for several h, whereby a new lactone was formed (Scheme 1). The ^{13}C -NMR spectrum of this lactone was identical with the spectrum of



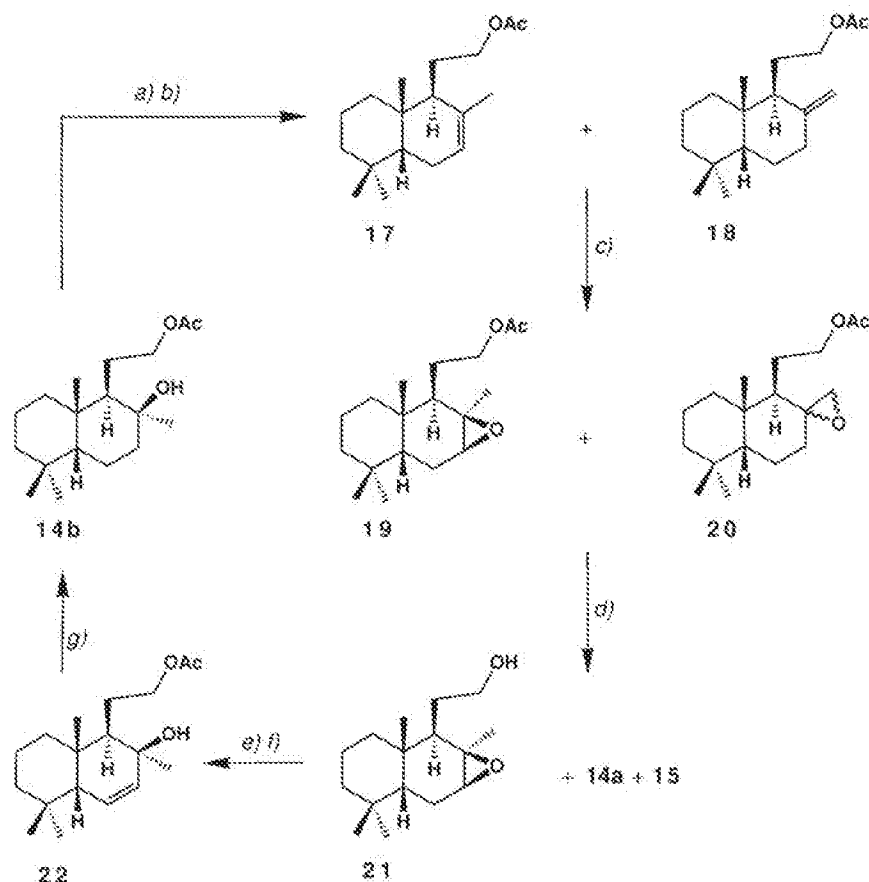
a) Horner-Wittig reaction. b) SnCl_4 , CH_2Cl_2 , -75° . c) LiAlH_4 . d) POCl_3 , Py. e) HCOOH , H_2SO_4 . f) TsCl , Py. g) TsOH , MeNO_2 .

^b) Prof. Saito kindly provided us with the X-ray diffraction data of lactone **11**.

lactone **16** reported by *Saito* and coworkers [4]. However, chemical transformations and a detailed ^1H - and ^{13}C -NMR analysis led us to revise this structure to be as in formula **13**. Lactone **13** was reduced to diol **14a** which was cyclized to noncrystalline $5\beta,8\alpha$ -ambrox (**6**).

Inversion of configuration at C(8) of diol **14a** was achieved as shown in *Scheme 2*, albeit in low yield. Thus, the monoacetate **14b** was dehydrated by treatment with *p*-toluenesulfonyl isocyanate (TsNCO) and pyrolysis of the intermediate urethane [8] to a 9:1 mixture of endo- and exocyclic olefin **17** and **18**, respectively. Other methods to bring about dehydration (POCl_3 , mesyl chloride, dinitrobenzoyl chloride) yielded the same isomeric ratio. Epoxidation of **17/18** produced one epoxide **19** derived from **17** and the diastereoisomeric mixture **20** in a ratio of 1:1 derived from **18**. Reduction of **19/20** with LiAlH_4 in refluxing THF afforded a 1:1 mixture of diols **14a** and **15** (10% of the reaction mixture), whereas the triply substituted epoxide function of compound **19**, which repre-

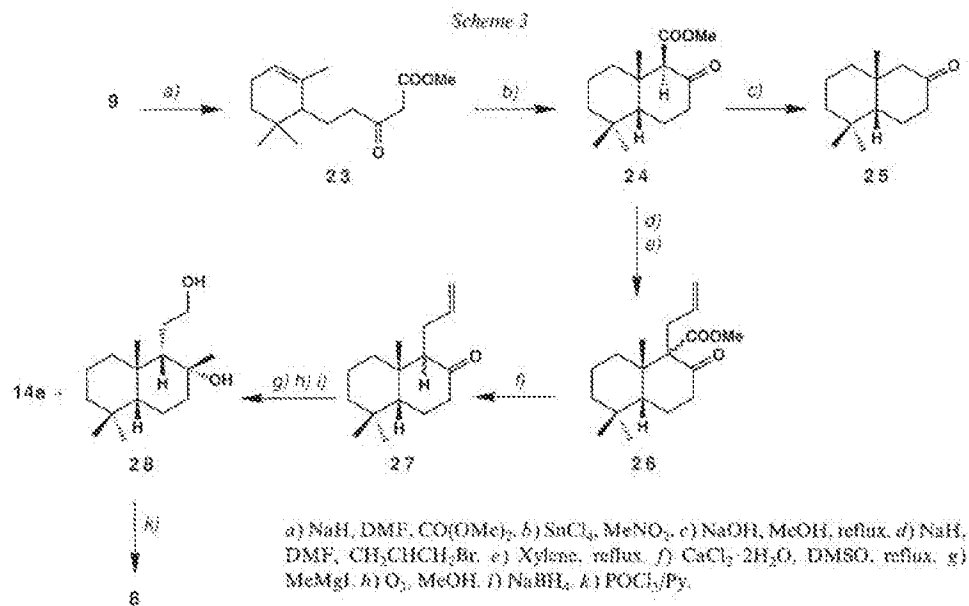
Scheme 2



a) TsNCO, *b)* Δ , *c)* $3\text{-ClC}_6\text{H}_4\text{CO}_2\text{H}$, *d)* LiAlH_4 , *e)* LDA, *f)* Ac_2O , Py, *g)* H_2 , PtO_2 .

sented some 90% of the epoxide mixture, was not reduced under these conditions, yielding only epoxy alcohol **21**. The relative configuration at C(8) of **19** was found to be unaffected; treatment of the corresponding alcohol **21** with lithium diisopropylamide (LDA) yielded, upon acetylation, allylic alcohol **22** which was hydrogenated to give the starting **14b**. Pure diol **15** was isolated from the reaction mixture **14a/15/21** by chromatography on silica gel and was cyclized in the presence TsCl in pyridine (Scheme 1). The resulting 5β -ambrox (**5**) was crystalline, and its chromatographic and spectral data ($^1\text{H-NMR}$, MS) were in full agreement with those of (–)-**5** the structure of which had been established unambiguously both by chemical correlation [9] and X-ray diffraction analysis (see below). The relative configuration of **14a** and concomitantly also of lactone **13** has thus been settled by chemical correlation with 5β -ambrox (**5**). Furthermore, **5** was isomerized to **6** when exposed to a trace of TsOH in hot nitromethane (Scheme 1).

The fourth and last of the 5β -ambrox diastereoisomers, **8**, was prepared along the lines of a recent publication by Büchi and Wüest [10] (Scheme 3). Thus, keto ester **24** which is readily available from α -dihydroionone **9** and which on demethoxycarbonylation gives the known *cis*-decalone **25** [11] was alkylated (NaH in DMF, allyl bromide). The intermediate enol ether rearranged in refluxing xylene to give **26** as a mixture of C(1) epimers.



Without separation, these keto esters were demethoxycarbonylated (CaCl₂·2H₂O in DMSO) to decalone **27** (C(1) epimeric mixture). Grignard addition of MeMgI produced two tertiary alcohols which were ozonized and reduced to a mixture of two diols, the major one being identical with **14a**. The minor diol **28** was converted (POCl₃ in pyridine) to an oily 5β -ambrox which differed from **6** and **7** and, therefore, must be **8**. In retrospect, it is rather surprising to find that Grignard addition to **27** had occurred highly stereospecifically and had given rise to only one diastereoisomer from either one of the two decalones.

It is interesting to note that lactone **13**, which is formed as a minor product by acid-catalyzed cyclization of (*E*)-monocyclohomofarnesic acid **10** [4] and of related compounds [5] or as a major product from acid-catalyzed isomerization of **11**, corresponds most probably to 'lactone XVIII' (m.p. 100°) described by *Lucius* [12] (= 'lactone XII' in [13]). It had been obtained among other lactones from **10** under vigorous cyclization conditions (HCOOH, H₂SO₄, 40–60°). The configuration of this lactone was not established at that time although it was assumed to belong to the *cis*-decalin series [12] [13] [7]. The diol which was derived from the lactone in question had m.p. 138°, the same value as we recorded for diol **14a**, whereas the reported data of the tricyclic ether (*n*_D²⁰, density, b.p.) do not permit configurational comparison with the oily compound **6**.

A second comment concerns 8 α ,9 β -sclareolide and 8 α ,9 β -ambrox described by *Ohloff* (see [14], therein *Formulae 19* and *23*, resp.). Since the two compounds had been obtained following the experimental procedure of *Lucius* [12] [13], their structures should be revised to **13** and **6**, respectively.

Conformational Analysis. – *cis*-Fused decalins may exist in either steroid or non-steroid conformation or consist of a rapidly equilibrating mixture of the two conformations. Conformation has a profound impact on the spatial arrangement of the substituents at C(8) and C(10) which play an important role in the case of ambergris odorants with respect to hydrophobic interaction with the receptor site [3]. Particular attention was thus given to establish the conformation of the four 5 β -ambrox diastereoisomers **5–8**. To this end, we were in the comfortable situation of having available three X-ray diffraction

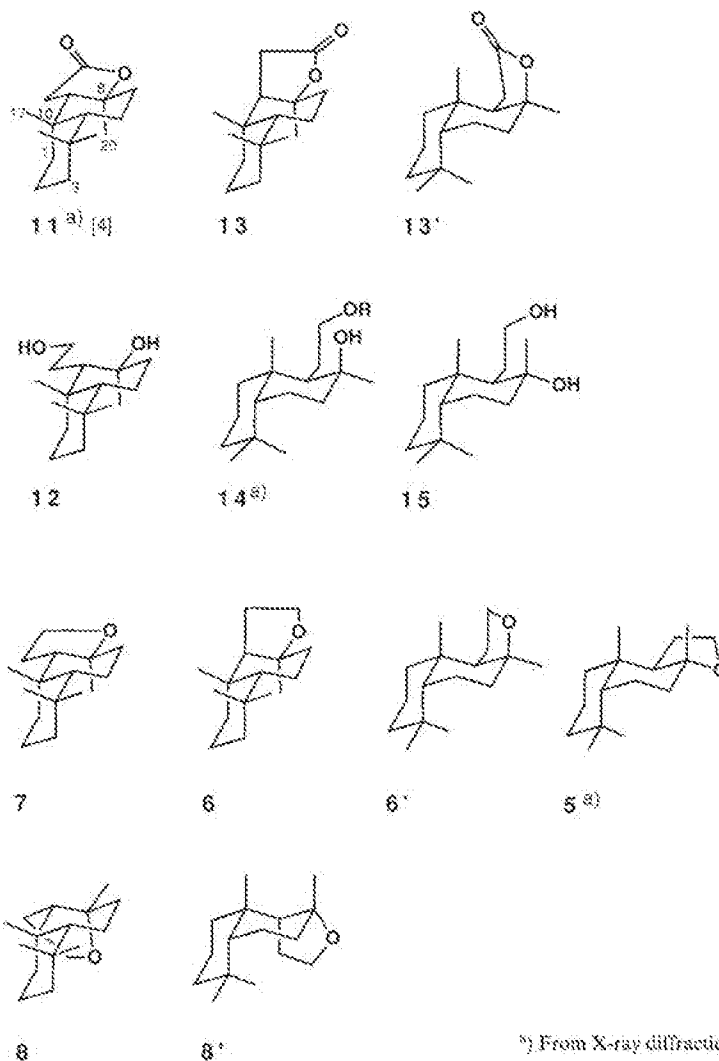
Table 1. ¹³C-NMR Chemical Shifts (ppm) and Assignments for Compounds **5–8** and **11–15**

	C(1)	C(2)	C(3)	C(4)	C(5)	C(6)	C(7)	C(8)	C(9)	C(10)	C(11)	C(12)	C(17)	C(18)	C(19)	C(20)
11	29.2	18.6	35.2	34.1	51.9	24.4	37.8	85.7	57.3	37.2	26.9	176.7	29.4	32.6	31.2	21.1
12	26.7	19.1	35.4	34.1	53.2	23.7	43.1	72.3	57.2	40.8	28.4	64.5	27.9	33.7	31.5	24.3
7	27.1	19.0	35.5	34.1	52.5	23.4	38.7	78.9	58.6	37.7	25.1	64.4	29.7	33.3	31.4	20.6
13	33.9	19.0	35.5	33.8	46.7	21.7	33.3	85.6	54.6	36.7	34.3	175.8	28.6	32.5	31.0	26.9
14a	39.8	18.8	44.5	34.8	49.7	18.1	37.3	72.7	41.7	37.7	29.4	64.8	27.9	27.3	34.0	31.8
14b	39.8	18.9	44.7	34.9	49.8	18.1	37.5	72.7	42.1	37.8	25.1	66.3	27.9	27.3	34.0	31.9
6	34.0	19.2	36.2	33.8	47.0	22.4	33.0	80.1	56.2	36.6	29.2	63.3	29.2	32.2	31.2	26.9
15	39.8	18.5	44.2	34.8	49.0	20.4	39.3	73.2	46.3	38.8	28.8	63.7	28.1	27.2	34.1	25.7
5	41.2	18.3	42.6	33.2	48.7	21.4	36.4	80.2	49.2	36.2	23.6	64.1	25.9	28.0	34.5	22.1
8	27.5	18.8	34.8	*)	52.4	19.9	35.8	80.9	55.3	*)	28.6	65.0	30.4	31.8	31.2	28.2

*) Not detectable.

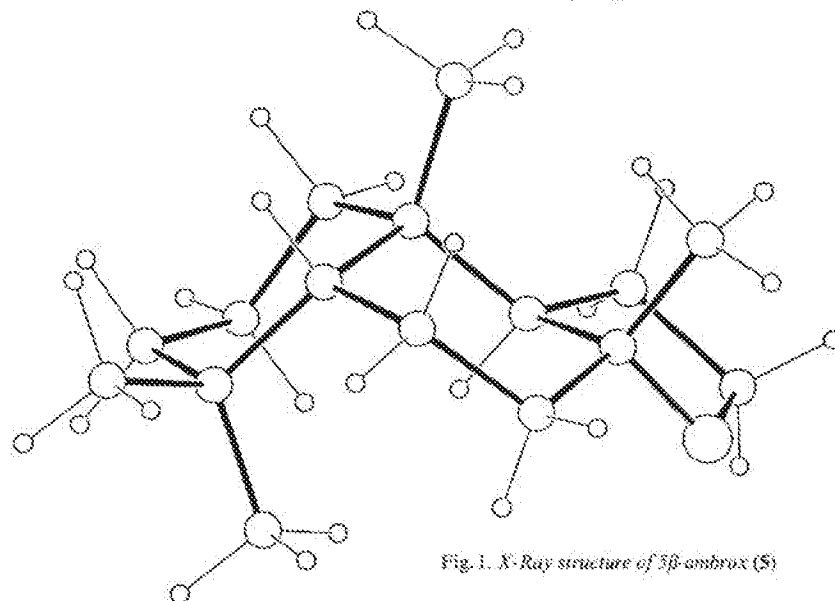
analyses and a virtually complete set of ¹³C-NMR data (Table 1). The conformation of lactone **11** had been established by X-ray diffraction analysis [4] to be steroidal (*i.e.* CH₃(17) equatorial with respect to ring B). The constancy of the ¹³C-NMR data of lactone **11**, of diol **12**, and of ether **7** clearly shows that the conformation remains steroidal throughout this series. The bonds C(8)–O and C(9)–C(11) are diequatorial, the conformation necessary to bring about *trans*-fusion of the five-membered lactone and ether moieties.

Fig. 1 shows the X-ray structure of 5 β -ambrox (**5**) as determined on (–)-**5** [9]. The non-steroid conformation (*i.e.* CH₃(17) axial with respect to ring B) of this compound equally holds for diol **15**, the ¹³C-NMR spectra of the two compounds being very similar.



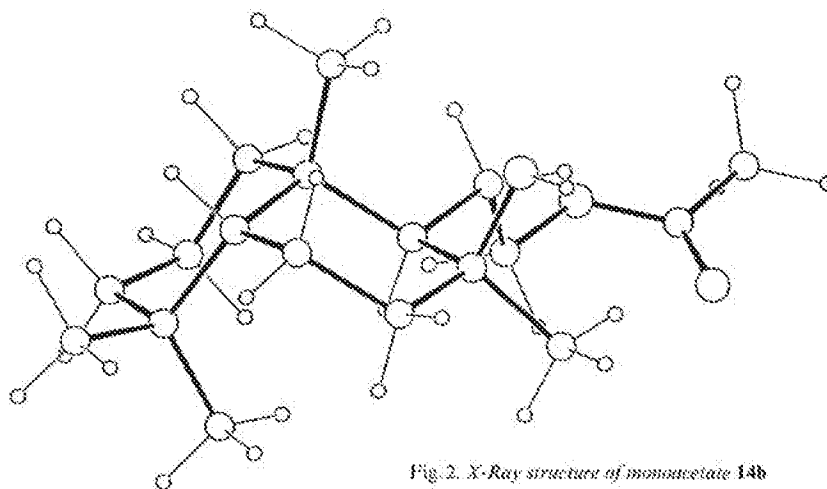
^{a)} From X-ray diffraction analysis.

The chemical shift of C(3) proved to be diagnostically useful for this type of conformational analysis: C(3) of the non-steroid-like conformers **15** and **5** resonate at *ca.* 45 ppm, the steroid ones **11**, **12**, and **7** at *ca.* 35 ppm. The difference in $\delta(\text{C}(3))$ clearly indicates that the compounds derived from 5 β ,8 α -sclareolide **13** belong to two different conformational series: $\delta(\text{C}(3))$ of **13** and **6** are observed at *ca.* 36 ppm, whereas $\delta(\text{C}(3))$ of **14** is found at 45 ppm. Lactone **13** as well as ether **6** may assume steroid or non-steroid conformation as both arrangements allow the five-membered ring to close axially-equatorially. On the basis of $\delta(\text{C}(3))$, we attributed **13** and **6** to steroid and diol **14a** to non-steroid conformation. Other C-atoms, namely C(9), C(11), and C(20), show the expected relative shifts when comparing, *e.g.*, **6** with **7** or **14a** with **15**, whereas $\Delta\delta(\text{C}(1))$

Fig. 1. X-Ray structure of 5β-ambrox (**5**)

does not follow simple additive rules [15]. The coupling constants of H-C(5) of compound **6** were calculated to be 3.6 and 12.4 Hz assuming steroid conformation and 1.3 and 4.5 Hz assuming non-steroid conformation. The former data correlate well with the observed values (*dd*, $J = 4$ and 10 Hz)⁴.

An X-ray diffraction analysis was performed on one member of the 5β,8α-series in order to have an independent control. As can be seen from Fig. 2, monoacetate **14b** and consequently also diol **14a** have non-steroid conformation. On the other hand, the steroid conformation of **13** and **6** is thus confirmed.

Fig. 2. X-Ray structure of monoacetate **14b**

⁴) We thank Dr. A. Bueching, Firmenich S.A. for the calculations [16].

The 5β -ambrox diastereoisomer **8**, for which we have no X-ray support, is very likely to be a steroid conformer, the chemical shift $\delta(\text{C}(3))$ of 34.5 ppm being the main argument. Furthermore, calculated and observed coupling constants for H–C(5) are in good agreement. We conclude that 5β -ambrox (**5**) is the only A/B *cis*-fused tricyclic ether which possesses non-steroid conformation and thus has the groups $\text{CH}_3(17)$ and $\text{CH}_3(20)$ 1,3-synperiplanar/diaxial with respect to ring B. The other 5β -diastereoisomers **6–8** have steroid-like conformations with $\text{CH}_3(17)$ equatorial with respect to ring B.

Qualitative Sensory Testing of Compounds 1, 2, and 4–8.—*Ambrox*[®] (**1**) is recognized as prototype of all ambergris odorants, both structurally and organoleptically [3]. The dominating exotic woody note of strong warm animal tonality in **1** is accompanied by an undesired earthy subnote of potato-cellar in 8α -ambrox **2**. Besides this qualitative difference between **1** and **2**, a reduction of the odor strength in **2** by a factor of one hundred has been determined [3]. The odor of the diastereoisomeric ether **4** is of similar strength than the one of **1**, and it differs only slightly from the rich and complex bouquet of **1**. Out of the four 5β -ethers **5–8**, but **5** has an odor quality comparable to prototype **1**. The odor threshold value of **5** is 11 ppb³⁾, thus *ca.* 20 times higher than the one of racemic **1** (0.6 ppb). The tricyclic ethers **6** and **8** are very weak odorants of woody-camphoraceous tonality with hardly discernable ambergris notes. In **7**, the weak ambergris-like tone is masked by a dirty-sweaty malodor. The qualitative and quantitative data of the sensory evaluation obtained are not surprising with regard to the structural features necessary for the release of an ambergris fragrance. Only the shape of 5β -ambrox (**5**) fulfills the 'triaxial rule' of odor sensation [3] perfectly well. Hence, the 1,3-synperiplanar/diaxial conformation of the substituents at C(8) and C(10) in the tricyclic ethers **1**, **2**, **4**, and **5** has been recognized as the active element for the particular odor release. The lack of this structural feature in diastereoisomers **6–8** prevents the corresponding molecules from a distinct receptor event.

We are indebted to Mr. W. Thommen and Mr. R. Brauchli for the NMR measurements. We also wish to thank Mrs. B. Mayer-Frei for her valuable technical assistance.

Experimental Part

General. If not stated otherwise, org. extracts were washed to neutral reaction with aq. soln. of H_2SO_4 and/or NaHCO_3 and NaCl , dried (MgSO_4), and evaporated. TLC: silica-gel plates. Medium-pressure chromatography (MPLC): silica gel 60 (particle size 0.040–0.063 mm, Merck) or prepacked Lobar[®] columns (Merck). Low-pressure chromatography (LPLC): silica gel 60 (particle size < 0.230 mm, Merck). Anal. GC: fused silica capillary columns (Supelcowax[®] 10, 60 m $\times$ 0.23 mm; DB WAX, 15 m $\times$ 0.53 mm; CP-Sil 5-CB, 10 m $\times$ 0.25 mm). Prep. GC: 5% SP-1100 (polyethylene glycol) on Chromosorb G, AW-DMCS, 80–100 mesh, 2.5 m $\times$ 3 mm; 10% Carbowax 20M (polyethylene glycol) on Chromosorb W, 80–100 mesh, 3 m $\times$ 4 mm; 4% SOMB (methyl silicone) on Chromosorb G, AW-DMCS, 80–100 mesh, 2.5 m $\times$ 3 mm. M.p.: not corrected. $^1\text{H-NMR}$ (360 MHz) and $^{13}\text{C-NMR}$ (90.5 MHz): Bruker-WH-360 instrument; in CDCl_3 solns. with TMS ($= 0.00$ ppm) as internal standard; J in Hz. MS: Finnigan-MAT quadrupole instrument coupled with a GC; electron energy *ca.* 70 eV; fragment ions m/z in % of the most abundant peak.

³⁾ The threshold values were determined by the method of Guadagni and coworkers [17]. We thank Mr. A. Fuerrer, Firmenich SA, for the measurements.

Crystallographic Data for Compounds 5 and 14b. Cell parameters and reflection intensities were measured at r.t. on a Philips PW1100 diffractometer with graphite-monochromated MoK α radiation. A summary of crystal data, intensity measurements, and structure refinements is given in Table 2. The structures were solved by direct methods (MULTAN-80) [18] and refined by least-square analysis with the XRAY-76 [19] program. Crystallographic data have been deposited with the Cambridge Crystallographic Data Center, University Chemical Laboratory, Lensfield Road, Cambridge CB2 1EW, England.

1. **Compound 7.** 1,1, (±)-13,14,15,16-Tetranor-5 β ,9 β -labdane-8 β ,12-diol (= 2-(1,2,3,4,4a β ,5,6,7,8,8a-Decahydro-2 β -hydroxy-2,5,5,8a β -tetramethyl-1 α -naphthyl)ethanol; 12). A soln. of 11^b (4.3 g, 17.2 mmol) in anhyd. Et₂O (50 ml) was added dropwise to a stirred suspension of LiAlH₄ (400 mg, 10.5 mmol) in a few ml of Et₂O. The mixture was refluxed for 2 h, then cooled and hydrolyzed by addition of H₂O (0.4 ml), 15% aq. NaOH soln. (0.4 ml), and H₂O (1.2 ml). The precipitate was filtered and the filtrate evaporated: 3.5 g (80.7%) of 12. An anal. sample was recrystallized several times from petroleum ether (80–100°)/EtOH. M.p. 145–146°. ¹H-NMR: 0.89 (s, 3 H); 1.10 (s, 3 H); 1.16 (s, 3 H); 1.24 (s, 3 H–C(20)); 3.47 (m, 1 H–C(12)); 3.82 (m, 1 H–C(12)). ¹³C-NMR: Table 1. MS: 254 (0, M⁺), 239 (2), 236 (2), 221 (10), 195 (7), 177 (7), 151 (41), 137 (17), 123 (31), 109 (100), 95 (77), 81 (68), 69 (93), 55 (51), 43 (52).

1,2, (±)-5 β ,8 α ,9 β -Ambrox (= 1,2,3a,4,5,5a β ,6,7,8,9,9a,9b β -Dodecahydro-3a,6,6,9a β -tetramethylnaphtho[2,1-b]furan; 7). Diol 12 (1.0 g, 3.9 mmol) was dissolved in anhyd. pyridine (5 ml) and treated slowly with ten drops of POCl₃. After 1 h (TLC: no 12 left), the mixture was diluted with Et₂O and hydrolyzed cautiously with ice. Usual workup and bulb-to-bulb distillation of the crude product at 150°/0.2 Torr afforded pure 7 (700 mg, 76%). ¹H-NMR: 0.89 (s, 3 H); 1.14 (s, 3 H); 1.155 (s, 3 H); 1.18 (s, 3 H); 3.81 (ddd, J = 8.6, 1 H–C(12)); 3.88 (ddd, J = 8.6, 8.6, 3.6, 1 H–C(12)). ¹³C-NMR: Table 1. MS: 236 (1, M⁺), 221 (67), 203 (3), 177 (5), 137 (39), 121 (13), 109 (21), 97 (100), 81 (36), 69 (35), 55 (31), 43 (37).

2. **Compound 6.** 2,1, (±)-5 β ,8 α -Sclareotide (= 3a,4,5,5a β ,6,7,8,9,9a,9b β -Dodecahydro-3a,6,6,9a β -tetramethylnaphtho[2,1-b]furan-2(1H)-one; 13). Lactone 11 (3.2 g, ca. 90%, 11.5 mmol) was dissolved in HCOOH (50 ml) and conc. H₂SO₄ (5 ml) and stirred under Ar at 90° for 5 h. The mixture was poured onto ice and the org. material extracted with several portions of Et₂O. The combined extracts were treated in the usual way to give 3.0 g of crude material which consisted of 13 (37%) and 11 (8%) besides several undefined side products (Supelcowax[®] 10). One recrystallization from petroleum ether (80–100°) raised the purity of 13 to 92% (980 mg, 31%). An anal. sample was recrystallized several times from the same solvent. M.p. 96–96.5° [4] (non-revised structure): 99–100°. ¹H-NMR: 0.93 (s, 3 H); 1.085 (s, 3 H); 1.105 (s, 3 H); 1.525 (s, CH₃(20)); 2.475 (dd, J = 18, 7, 1 H–C(11)); 2.62 (dd, J = 18, 7, 1 H–C(11)). ¹³C-NMR: Table 1. MS: 250 (2, M⁺), 235 (33), 207 (12), 190 (8), 175 (9), 167 (22), 136 (71), 121 (77), 109 (79), 95 (62), 81 (100), 69 (75), 55 (73), 43 (90).

2,2, (±)-13,14,15,16-Tetranor-5 β -labdane-8 β ,12-diol (= 2-(1,2,3,4,4a β ,5,6,7,8,8a-Decahydro-2 β -hydroxy-2,5,5,8a β -tetramethyl-1 β -naphthyl)ethanol; 14a). Reduction of 13 (1.0 g, 4 mmol) with LiAlH₄ (200 mg, 5.3 mmol) in anhyd. Et₂O (100 ml) was carried out as described above: 1.0 g (98%) of 14a. An anal. sample was recrystallized from petroleum ether (80–100°). M.p. 138.5–139°. ¹H-NMR^c: 0.91 (s, CH₃(19)); 1.00 (s, CH₃(18)); 1.06 (s, CH₃(17)); 1.16 (s, CH₃(20)); 3.60 (dd, J = 7, 2 H–C(12)). ¹³C-NMR: Table 1. MS: 254 (1, M⁺), 236 (3), 221 (56), 195 (6), 177 (12), 165 (6), 151 (22), 137 (36), 123 (27), 109 (100), 95 (70), 81 (76), 69 (82), 55 (69), 43 (66).

2,3, (±)-5 β ,8 α -Ambrox (= 1,2,3a,4,5,5a β ,6,7,8,9,9a,9b β -Dodecahydro-3a,6,6,9a β -tetramethylnaphtho[2,1-b]furan; 6). Diol 14a (1.0 g, 3.9 mmol) was converted to 6 as described above. The crude product was bulb-to-bulb distilled at 150°/0.1 Torr to give 0.8 g (87%) of pure 6 as an oil. ¹H-NMR: 0.89 (s, 3 H); 1.075 (s, 3 H); 1.095 (s, 3 H); 1.305 (s, CH₃(20)); 3.75 (m, 2 H–C(12)). ¹³C-NMR: Table 1. MS: 236 (1, M⁺), 221 (67), 203 (2), 177 (7), 137 (41), 121 (18), 109 (32), 97 (100), 81 (44), 69 (40), 55 (41), 43 (48).

3. **Compound 5.** 3,1, (±)-3 β -Hydroxy-13,14,15,16-tetranor-5 β -labdan-12-yl Acetate (= 2-(1,2,3,4,4a β ,5,6,7,8,8a-Decahydro-2 β -hydroxy-2,5,5,8a β -tetramethyl-1 β -naphthyl)ethyl Acetate; 14b). Diol 14a (20 mg, 0.78 mmol) was acetylated in a mixture of Ac₂O (0.1 ml) and pyridine (1.0 ml) at r.t. overnight. Upon evaporation and recrystallization from pentane, pure 14b was obtained as prisms. M.p. 78–79°. ¹H-NMR: 0.94 (s, 3 H); 1.03 (s, 3 H); 1.07 (s, 3 H); 1.20 (s, CH₃(20)); 2.02 (s, CH₃CO); 4.06 (m, 2 H–C(12)). ¹³C-NMR: Table 1. MS: 296 (0, M⁺), 281 (< 1), 236 (1), 221 (5), 203 (3), 195 (7), 177 (5), 165 (8), 151 (56), 131 (21), 123 (22), 109 (78), 95 (100), 81 (60), 69 (80), 55 (49), 43 (93). Crystal data: see Table 2.

^b) Lactone 11 was prepared from dihydro- α -ionone 9 according to [20]; m.p. 103–104° [4]: 103–104°.

^c) The assignments were obtained from a ¹H, ¹³C-correlated 2D-NMR spectrum.

Table 2. Crystal Data, Intensity Measurement, and Structure Refinement for 5 β -Ambrox (5) and Monoacetate 14b

	5	14b
Formula	C ₁₆ H ₂₆ O	C ₁₈ H ₃₂ O ₂
Molecular weight	236.4	296.5
Crystal system	orthorhombic	triclinic
Space group	<i>P</i> 2 ₁ 2 ₁ 2 ₁	<i>P</i> $\bar{1}$
Crystal size [mm]	0.22 $\times$ 0.25 $\times$ 0.28	0.20 $\times$ 0.25 $\times$ 0.38
<i>a</i> [Å]	8.185(2)	7.5499(13)
<i>b</i> [Å]	10.436(3)	7.5831(9)
<i>c</i> [Å]	16.875(5)	16.105(12)
α [°]	90.0	78.83(5)
β [°]	90.0	79.74(6)
γ [°]	90.0	75.54(2)
<i>V</i> [Å ³]	1441.4(7)	867.7(7)
<i>Z</i>	4	2
<i>F</i> _{calc}	328	328
<i>D</i> _c [g·cm ⁻³]	1.09	1.14
μ [mm ⁻¹]	0.061	0.070
$\sin(\theta/\lambda)_{\max}$ [Å ⁻¹]	0.60	0.51
No. measured reflections	1499	1864
No. observed reflections	943	1472
Criterion for observed	$ F_o > 4\sigma(F_o)$	$ F_o > 4\sigma(F_o)$
Refinement (on <i>F</i>)	full-matrix	full-matrix
No. of parameters	154	190
Weighting scheme	$w = 1$	$w = 1$
Max. and average Δ/σ	0.0002, 0.0001	0.014, 0.004
Max. and min. $\Delta\rho$ [e·Å ⁻³]	0.35, -0.35	0.25, -0.41
<i>R</i> (= wR) [%]	7.2	6.5

3.2. ($\pm$)-13,14,15,16-Tetranor-5 β -labda-7-en-12-yl and -8(20)-en-12-yl Acetate (= 2-(1,4,4a β ,5,6,7,8,8a-Octahydro-2,5,5,8a-tetramethyl-1 β -naphthyl)ethyl Acetate and 2-(1,2,3,4,4a β ,5,6,7,8,8a-Decahydro-5,5,8a-trimethyl-2-methylidene-1 β -naphthyl)ethyl Acetate, resp.; 17 and 18, resp.). The procedure was adapted from [8]. To a soln. of 14a (4.97 g, 16.8 mmol) in benzene (17 ml) was added TiNCO (2.8 ml, 18.4 mmol). After stirring at r.t. for 2 h, the solvent was evaporated. The intermediate urethane was heated at 160° under stirring, until CO₂ evolution had ceased (15 min). The crude olefin mixture was purified by MPLC (Lobar* column, size C; hexane/AcOEt 8:2): thick oil (4.19 g, 89%) which contained 87% of 17 and 12% of 18. On SP-1000 and SOMB GC columns, 17 preceded 18. Anal. samples were collected by prep. GC. Data of 17. ¹H-NMR: 0.81 (s, 3 H); 0.84 (s, 3 H); 0.87 (s, 3 H); 1.69 (br. s, CH₃(20)); 2.06 (s, CH₃CO); 4.02 (m, 1 H-C(12)); 4.26 (m, 1 H-C(12)); 5.35 (d, *J* = 4.3, 1 H-C(7)). MS: 278 (< 1, *M*⁺), 218 (3), 203 (1), 190 (1), 124 (7), 109 (61), 94 (100), 79 (44), 43 (17).

Data of 18. ¹H-NMR: 0.89 (s, 3 H); 1.03 (s, 3 H); 1.06 (s, 3 H); 2.03 (s, CH₃CO); 3.86 (m, 1 H-C(12)); 4.02 (m, 1 H-C(12)); 4.52 (s, 1 H-C(20)); 4.74 (s, 1 H-C(20)). MS: 278 (< 1, *M*⁺), 218 (8), 203 (11), 190 (2), 162 (4), 137 (66), 123 (33), 109 (37), 95 (70), 81 (100), 69 (61), 43 (60).

3.3. ($\pm$)-7 β ,8- and 8,20-Epoxy-13,14,15,16-tetranor-5 β -labda-12-yl Acetate (= 2-(2 β ,3 β -Epoxy-1,2,3,4,4a β ,5,6,7,8,8a-decahydro-5,5,8a-trimethyl-1 β -naphthyl)ethyl Acetate and 2-(3,4,4a β ,5,6,7,8,8a-Octahydro-5,5,8a-trimethylspiro[naphthalene-2(1H),2'-oxiran]-1 β -yl)ethyl Acetate, resp.; 19 and 20 resp.). To a slightly cooled soln. of 17/18 (4.19 g, 15.1 mmol) in anh. CH₂Cl₂ (165 ml) was added 3-chloroperbenzoic acid (Aldrich; ca. 85%, 3.41 g, 16.8 mmol) in small portions. After stirring at r.t. for 5 h (TLC: no 17/18 left), the soln. was washed with 10% aq. NaHSO₃ and NaHCO₃ solns., dried (MgSO₄), and evaporated: 4.62 g (100%) of crude product as a thick oil. GC/MS (Supelcowax*10): 3 isomers, ca. 87:6:8. The major isomer was isolated in pure form by prep. GC (SP-1000). The two minor isomers were coeluted under these conditions.

Major isomer 19. ¹H-NMR: 0.87 (s, 3 H); 0.89 (s, 3 H); 0.93 (s, 3 H); 1.31 (s, CH₃(20)); 2.06 (s, CH₃CO); 2.94 (d, *J* = 7.2, H-C(7)); 4.13 (m, 1 H-C(12)); 4.26 (m, 1 H-C(12)). MS: 294 (< 1, *M*⁺), 279 (3), 234 (3), 219 (10), 161 (9), 149 (9), 135 (12), 124 (24), 109 (64), 95 (52), 81 (47), 69 (56), 55 (42), 43 (100).

Minor Isomers 20. $^1\text{H-NMR}$: 2.53, 2.58 (*qm*, $\text{H-C}(20)$); 4.04 (*m*, $\text{H-C}(12)$). MS (isomer 1): 294 (0, M^{++}), 279 (2), 234 (4), 219 (11), 180 (5), 156 (11), 136 (22), 121 (31), 109 (75), 95 (63), 81 (72), 69 (96), 55 (77), 43 (100). MS (isomer 2): 294 (0, M^{++}), 279 (2), 234 (3), 219 (4), 180 (5), 156 (12), 136 (22), 123 (29), 109 (74), 95 (72), 81 (75), 69 (89), 55 (68), 43 (100).

3.4. $(\pm)$ -13,14,15,16-Tetranor-5 β -labdane-8 α ,12-diol ($=$ 2-(1,2,3,4,4a β ,5,6,7,8,8a-Decahydro-2a-hydroxy-2,5,5,8a-tetramethyl-1 β -naphthyl)ethanol; **15**). A suspension of anhyd. AlCl_3 (733 mg, 5.5 mmol) and LiAlH_4 (690 mg, 18.1 mmol) in anhyd. Et_2O (70 ml) was prepared at -10° under Ar. A soln. of 4.60 g (ca. 15 mmol) of **19/20** in 60 ml of anhyd. Et_2O was added dropwise. The mixture was stirred at -5 to 0° for 1 h, then at r.t. for 1 h. The reaction was quenched with MeOH (3.6 ml) and the mixture poured into cold sat. aq. NH_4Cl soln. The org. material was extracted into Et_2O and treated in the usual way. Separation of the crude product (4.20 g) into the pure components was achieved by MPLC (Lobar[®] column, size C; hexane/AcOEt 8:2). $(\pm)$ -7 β ,8-Epoxy-13,14,15,16-tetranor-5 β -labdane-12-ol ($=$ 2-(2 β ,3 β -epoxy-1,2,3,4,4a β ,5,6,7,8,8a-decahydro-5,5,8a-trimethyl-1 β -naphthyl)ethanol; **21**; 3.26 g, 86%) was eluted first. An anal. sample was recrystallized from hexane. M.p. 66–66.5°. $^1\text{H-NMR}$: 0.88 (*s*, 3 H); 0.89 (*s*, 3 H); 0.94 (*s*, 3 H); 1.31 (*s*, $\text{CH}_3(20)$); 2.94 (*d*, $J = 6.5$, 1 H-C(7)); 3.70 (*m*, 1 H-C(12)); 3.83 (*m*, 1 H-C(12)). MS: 252 (1, M^{++}), 237 (19), 221 (10), 207 (2), 193 (2), 135 (15), 123 (40), 109 (100), 97 (77), 81 (70), 69 (78), 55 (59), 43 (79).

The next fractions contained 200 mg (5%) of **14a**. Diol **15** was eluted last (240 mg, 6%). It was recrystallized from AcOEt/hexane. M.p. 136–136.5°. $^1\text{H-NMR}$: 0.885 (*s*, $\text{CH}_3(17)$); 0.95 (*s*, $\text{CH}_3(19)$); 1.06 (*m*, $\text{H-C}(5)$); 1.13 (*s*, $\text{CH}_3(18)$); 1.27 (*s*, $\text{CH}_3(20)$); 3.47 (*m*, 1 H-C(12)); 3.79 (*m*, 1 H-C(12)). $^{13}\text{C-NMR}$: Table 1. MS: 254 (0, M^{++}), 239 (1), 221 (3), 195 (7), 177 (11), 165 (7), 151 (15), 139 (11), 123 (22), 109 (70), 95 (77), 83 (74), 69 (10), 55 (52), 43 (50).

3.5. $(\pm)$ -5 β -Ambrox ($=$ 1,2,3a,4,5,5a β ,6,7,8,9,9a,9b α -Dodecahydro-3a β ,6,6,9a β -tetramethylnaphtho[2,1-*b*]-furan; **5**). A soln. of **15** (125 mg, 0.49 mmol) in anhyd. pyridine (1.5 ml) was cooled to 0° , and TsCl (210 mg, 1.1 mmol) was added. After stirring at 0° for 30 min, the mixture was abandoned in the refrigerator overnight. (TLC: no **15** left). The mixture was diluted with ice/ H_2O , and extracted with several portions of Et_2O . The combined extracts were treated in the usual way: 120 mg of crude **5**. It was purified by MPLC (Lobar[®] column, size B; hexane/ Et_2O 4:1): 100 mg (86%) of pure **5** which was bulb-to-bulb distilled at $100^\circ/0.05$ Torr and crystallized from hexane. M.p. 52–53°. $^1\text{H-NMR}$: 0.92 (*s*, $\text{CH}_3(17)$); 0.96 (*s*, $\text{CH}_3(19)$); 1.06 (*s*, $\text{CH}_3(18)$); 1.12 (*m*, $\text{H-C}(5)$); 1.14 (*s*, $\text{CH}_3(20)$); 1.94 (*dd*, $J = 6.5$, 13.0, $\text{H-C}(9)$); 3.80 (*ddd*, $J = 7.9$, 1 H-C(12)); 3.86 (*ddd*, $J = 3.5$, 7.9, 7.9, 1 H-C(12)). $^{13}\text{C-NMR}$: Table 1. MS: 236 (6, M^{++}), 221 (57), 203 (3), 177 (5), 151 (7), 137 (46), 124 (22), 109 (27), 97 (100), 81 (41), 69 (33), 55 (32), 43 (40). Crystal data: see Table 2.

4. Acid-Catalyzed Isomerization of 5. A soln. of 1 mg of **5** in 50 μl of nitromethane was heated in the presence of one crystal of TsOH at 60° for 1 h. A few mg of solid Na_2CO_3 were added, and the supernatant was analyzed by capillary GC (Supelcomax[®] II). Compound **5** had disappeared and besides a new unknown (67%), 27% of a product was observed, the retention time of which was identical with the one of 5 β ,8 α -ambrox (**6**). Isomer **6** was eluted immediately after **5**.

5. Correlation of Epoxide 21 with 14b. The procedure is adapted from [21]. To a soln. of LDA (5 mmol) in anhyd. Et_2O (63 ml) was added dropwise and at 0° a soln. of **21** (504 mg, 2 mmol) in 4 ml of Et_2O . The mixture was allowed to warm to r.t. and stirred overnight. It was poured onto ice and extracted with Et_2O . The crude product was acetylated with Ac₂O/pyridine 1:2 (3 ml) overnight. Excess reagent was removed by repeated evaporation in the presence of toluene. Thus, 560 mg of material were obtained which still contained much unreacted epoxide in the form of **19** as shown by TLC. The mixture was separated by MPLC (Lobar[®] column, size B; hexane/ EtOAc 3:1): 420 mg (71%) of **19** and 120 mg (20%) of the more polar $(\pm)$ -8 β -hydroxy-13,14,15,16-tetranor-5 β -labd-6-en-12-yl acetate ($=$ 2-(1,2,4a β ,5,6,7,8,8a-octahydro-2 β -hydroxy-2,5,5,8a-tetramethyl-1 β -naphthyl)ethyl acetate; **22**). After two recrystallizations from hexane, the m.p. was 64–65°. $^1\text{H-NMR}$: 0.82 (*s*, 3 H); 0.94 (*s*, 3 H); 0.96 (*s*, 3 H); 1.22 (*s*, $\text{CH}_3(20)$); 2.06 (*s*, CH_3O); 4.13 (*m*, 2 H-C(12)); 5.69 (*d*, $J = 9.7$, $\text{H-C}(7)$); 5.80 (*dd*, $J = 5.8$, 9.7, $\text{H-C}(6)$). MS: 294 (0, M^{++}), 279 (2), 234 (2), 219 (30), 133 (55), 123 (30), 107 (35), 95 (41), 84 (72), 69 (69), 55 (36), 43 (100).

A soln. of **22** (120 mg, 0.47 mmol) in EtOH (2 ml) was hydrogenated in the presence of PtO_2 (10 mg) until H_2 uptake had ceased. The catalyst was filtered off and the filtrate concentrated to give **14b**. M.p., $^1\text{H-NMR}$ and chromatographic data, identical with those of starting **14b**.

6. Compound 8. **6.1. Methyl 1,2,3,4,4a β ,5,6,7,8,8a-Decahydro-5,5,8a-trimethyl-2-oxo-1 β -naphthalenecarboxylate (24).** To an efficiently stirred soln. of **23** (4.7 g, 18.6 mmol; prepared from **9** according to [22]) in nitromethane (320 ml) was added dropwise stannic chloride (4 ml, 34.2 mmol) at r.t. under Ar. After 45 min (TLC: no **23** left after 15 min), the mixture was worked up in the usual way. The crude product was bulb-to-bulb distilled at $190^\circ/0.1$

Torr: 3.5 g of material containing 39% of **24** and of 26% of the corresponding known 4aa,8aβ-isomer [23] (*DB WAX*, *cis*-fused **24** preceded *trans*-fused isomer). Pure **24** was isolated by repetitive LPLC on silica gel (cyclohexane/Et₂O 95:5). ¹H-NMR: 1.04 (s, 3 H); 1.16 (s, 3 H); 1.18 (s, 3 H); 3.70 (s, CH₃O); 3.87 (s, H-C(1)). MS: 252 (2, *M*⁺), 234 (9), 219 (12), 205 (33), 136 (100), 69 (98), 55 (83), 41 (73).

6.2. *3,4,4aβ,5,6,7,8,8a-Octahydro-5,5,8aβ-trimethylnaphthalen-2(1H)-one (25)*. An anal. sample of **24** was treated with NaOH in aq. MeOH at reflux temperature for 3 h. The soln. was acidified and worked up with Et₂O. Bulb-to-bulb distillation gave **25**. GC and NMR: identical with the ones of the authentic sample [11].

6.3. *Methyl 1,2,3,4,4aβ,5,6,7,8,8a-Decahydro-5,5,8aβ-trimethyl-2-oxo-1-(prop-2'-enyl)naphthalene-1-carboxylate (26)*. Procedure according to [10]: A soln. of **24** (1.6 g, 6.3 mmol) in 15 ml of DMF was treated with NaH (6.3 mmol) and stirred at r.t. overnight. The mixture was cooled to 0° and allyl bromide (0.7 ml, 8.1 mmol) added dropwise. Stirring was continued at r.t. overnight. The mixture was poured onto ice and extracted with Et₂O. Evaporation left 1.1 g of enol ether of **24** which was heated at reflux temperature in 20 ml of xylene during 30 h. The soln. was evaporated and the crude **26** (18:57 epimeric mixture; *CP-Sil 5-CB*) was separated into the two components by LPLC (cyclohexane/EtOH 95:5).

Minor Epimer: less polar, 0.2 g. ¹H-NMR: 0.89 (s, 3 H); 1.18 (s, 3 H); 1.46 (s, 3 H); 3.61 (s, CH₃O); 4.93 (d, *J* = 10.8, 1 H); 5.00 (d, *J* = 16.2, 1 H); 5.75 (m, 1 H). ¹³C-NMR: 208.0 (C(2)); 171.3 (C=O); 136.4 (C(2')); 116.5 (C(3')); 69.1 (C(1)); 51.3 (CH₃O); 47.9 (C(8a)); 39.5 (C(8)); 34.5 (C(5)); 33.4 (CH₂-C(5)); 33.3 (C(3)); 32.9 (C(6)); 31.2 (CH₂-C(5)); 27.1 (C(1)); 26.3 (C(4)); 24.2 (CH₂-C(8a)); 18.6 (C(7)). MS: 292 (2, *M*⁺), 259 (11), 233 (5), 169 (38), 141 (48), 137 (81), 123 (100), 114 (36), 109 (80), 95 (60), 81 (90), 67 (57), 55 (71), 41 (100).

Major Epimer: more polar 0.6 g. ¹H-NMR: 0.95 (s, 3 H); 1.11 (s, 3 H); 1.12 (s, 3 H); 3.71 (s, CH₃O); 4.97 (d, *J* = 10.8, 1 H); 4.05 (d, *J* = 16.2, 1 H); 5.61 (m, 1 H). ¹³C-NMR: 207.7 (C(2)); 170.8 (C=O); 134.1 (C(2')); 117.4 (C(3')); 51.4 (CH₃O); 48.0 (C(4a)); 44.5 (C(1)); 38.7 (C(8)); 35.5 (C(6)); 34.5 (C(8a)); 33.4 (C(3)); 33.3 (C(5)); 31.3 (CH₂-C(5)); 28.7 (C(1)); 25.0 (CH₂-C(8a)); 23.5 (C(4)); 18.6 (C(7)). MS: 292 (2, *M*⁺), 260 (3), 233 (3), 169 (43), 136 (80), 123 (100), 114 (32), 109 (100), 95 (95), 81 (91), 69 (74), 55 (80), 41 (98).

6.4. *3,4,4a,5,6,7,8,8a-Octahydro-5,5,8aβ-trimethyl-1-(prop-2'-enyl)naphthalen-2(1H)-one (27)*. A stirred mixture of **26** (major epimer; 500 mg, 1.71 mmol), CaCl₂·2H₂O (1 g), and DMSO (10 ml) was heated at reflux for 2 h. The mixture was then poured into H₂O and extracted with Et₂O. The crude product was bulb-to-bulb distilled at 140°/0.09 Torr: 400 mg (100%) of **27** (major epimer). ¹H-NMR: 0.82 (s, 3 H); 1.02 (s, 3 H); 1.21 (s, 3 H); 4.92 (d, *J* = 10.8, 1 H); 5.00 (d, *J* = 16.2, 1 H); 5.83 (m, 1 H). ¹³C-NMR: 213.8 (C(2)); 138.7 (C(2')); 115.0 (C(1)); 50.0 (C(4a)); 43.0 (C(8a)); 42.3 (C(6)); 39.9 (C(3)); 37.5 (C(8)); 34.8 (C(5)); 34.4 (C(1)); 33.9 (CH₂-C(5)); 26.3 (CH₂-C(8a)); 26.3 (CH₂-C(5)); 22.9 (C(4)); 18.5 (C(7)). MS: 234 (6, *M*⁺), 219 (3), 201 (2), 109 (22), 96 (100), 69 (24), 55 (23), 41 (26).

6.5. When the epimeric mixture **26** (ratio 18:57; 3.7 g, 12.6 mmol) was demethoxycarbonylated under the same conditions, a 18:65 C(1)-epimeric mixture **27** (*CP-Sil 5-CB*) was obtained. The minor diastereoisomer was not isolated in pure form.

6.6. *(±)-5β,9β-Ambrox (= 1,2,3a,4,5,5aβ,6,7,8,9,9a,9bβ-Dodecahydro-3aβ,6,6,9aβ-tetramethylnaphthol-2,1-bis-furan; 8)*. The mixture **27** (18:65; 1.0 g, 4.2 mmol) in Et₂O (10 ml) was added to the Grignard reagent prepared from Mg (1.0 g) and MeI (2 ml) in Et₂O (20 ml) and heated at reflux for 30 min. It was decomposed with aq. NH₄Cl soln. and extracted with Et₂O. The residue (1.1 g; 2 alcohols in the ratio of 18:65; *CP-Sil 5-CB*) was dissolved in AcOEt (20 ml) and ozonized at -70°. Upon addition of NaBH₄ (1.0 g), the mixture was allowed to warm to r.t. overnight. It was hydrolyzed (H₂O) and worked up with Et₂O, giving **28/14a** (1.1 g) in a ratio of ca. 18:65. The major isomer was identical with **14a** obtained above (by TLC and GC). The diol mixture was converted to **8/6** (18:65) by the method described above (POCl₃/pyridine). Separation was achieved by repetitive MPLC (*Lobar*[®] column; cyclohexane/Et₂O 98:2). The major isomer was identical with **6** (*Supelcowax*[®] 10) and eluted well after **8**. Compound **8** remained a thick oil. ¹H-NMR: 0.915 (s, 3 H); 1.055 (s, 3 H); 1.08 (s, 3 H); 1.10 (s, 3 H); 3.71 (m, 2 H-C(12)). ¹³C-NMR: *Table 1*. MS: 236 (0, *M*⁺), 221 (100), 203 (3), 137 (47), 97 (75), 81 (33), 69 (32), 55 (33), 41 (41).

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