Pergamon

Tetrahedron 54 (1998) 6415–6426

TETRAHEDRON

A Glycosylphosphatidylinositol-Anchoring Inhibitor with an Unusual Tetracarbocyclic Sesterterpene Skeleton from the Fungus *Codinaea simplex*

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Received 23 February 1998; accepted 2 April 1998

Abstract

A novel sesterterpene, YW3699, was isolated from fermentation of the fungus *Codinaea simplex*. Based on spectroscopic evidence, including UV, IR, FAB-MS, ESI-HRMS, ^{1}H and ^{13}C NMR, DQ-COSY, HQSC and HMBC, YW3699 was shown to have an unusual tetracarbocyclic sesterterpene skeleton. Its relative stereochemistry and solution conformation were elucidated with ^{1}H-^{1}H coupling constants and detailed analysis of its ROESY spectrum, in conjunction with inspection of Dreiding stereomodels. A possible biosynthetic route for YW3699 via the mevalonate pathway is proposed and discussed. YW3699 was found to be an inhibitor of glycosylphosphatidylinositol (GPI) synthesis with a MIC of 3.5 μM.

Key Words: *biosynthesis; pharmacologically active compounds; stereochemistry; terpenes and terpenoids*

1. Introduction

Glycosylphosphatidylinositol (GPI)-anchoring represents a mechanism for attaching proteins to the cell surface that is used among all eucaryotes[1]. In our search for inhibitors of GPI anchor biosynthesis[2], we have discovered a number of sesterterpenes including the novel compound YW3699, which was isolated from the fungus *Codinaea simplex* and was found to possess an unusual tetracarbocyclic skeleton. This compound was shown to inhibit *in vitro* GPI synthesis in yeast at micromolar concentrations. We report here on the fermentation, extraction, isolation and, in particular, on the structural elucidation and stereochemical analysis of this novel tetracyclic sesterterpene based on 1D and 2D NMR spectroscopy.

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0040-4020/98/$19.00
PII: S0040-4020(98)00322-6

2. Results and Conclusions

2.1. Fermentation and isolation

Precultures of the fungus *Codinaea simplex* (F/94-493108) were grown in shake cultures for one week at 24 °C and the seed cultures inoculated into a bioreactor and fermented at 21°C for 4 days with stirring and maintaining the pH at 5. Extraction of the mycelia with MeOH/H_2O (2:1) and workup with ethyl acetate extract followed by gel filtration and chromatography on reversed-phase gave YW3699, which was finally purified by two steps of preparative HPLC.

2.2. Structure of YW3699

YW3699 was obtained in the form of a white powder. The UV absorption at 270 nm indicates the presence of a conjugation system with an α, β-unsaturated ketone. Moreover, the FT-IR spectrum contains bands at 1710 and 1655 cm^{-1}, which reveal the presence of carbonyl group and carbon-carbon double bonds. ^1H and ^{13}C NMR spectra reveal 34 carbons, 49 carbon-bound protons and 3 oxygen-bound protons in hydroxyl groups. Interpretation of the HSQC and HMBC spectra suggests the following carbon types: 8 CH_3, 7 CH_2, 8 CH, 7 C, 1 CH_2= , 1 CH= and 2 C=O.

The positive FAB-MS of YW3699 gave a molecular weight of 556 by showing quasimolecular ion at m/z 557 ($[M + H]^+$). This was confirmed by the quasimolecular ion at m/z 563 ($[M + Li]^+$) in FAB-MS with LiI. The molecular formula $C_{34}H_{52}O_6$ was determined by high resolution mass measurement (positive ESI-HRMS, found *m/z* 557.3839, calculated *m/z* 557.3842, $[M+H]^+$), in conjunction with the ^1H and ^{13}C NMR spectrum data. This requires nine degrees of unsaturation in YW3699 ($n = n_C + 1 + (n_N - n_H)/2 = 9$). Due to the presence of three carbon-carbon double bonds and two carbonyl functions, there should be four rings in the molecule.

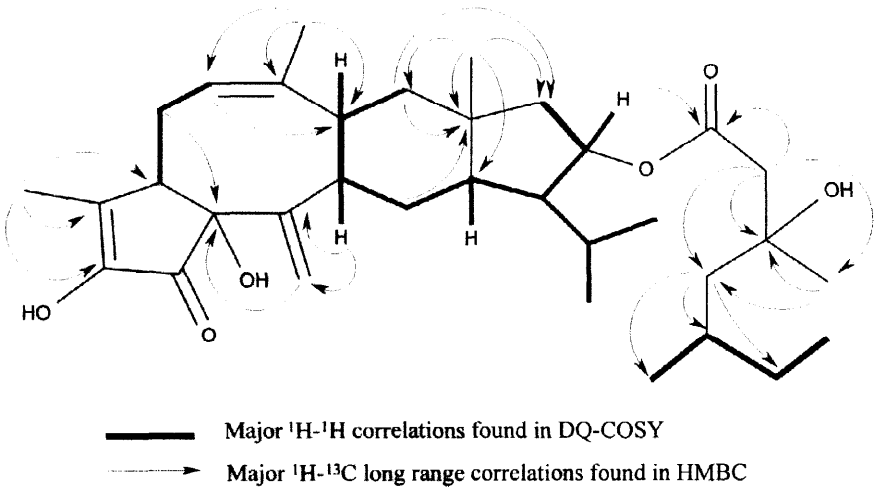

Figure 1. Major connectivity data found in DQ-COSY and HMBC spectra of YW3699.

Based on the DQ-COSY spectrum (Table 1), three fragments in YW3699 can be obtained as indicated in Figure 1. Detailed analysis of HSQC and HMBC (Table 2) suggests the connectivity among these three fragments with other parts in the molecule. As depicted in Figure 1, YW3699 is an unusual tetracarbocyclic sesterterpene esterified with a novel aliphatic acid, 3,5-dimethyl-3-hydroxylheptanoic acid ($C_9H_{18}O_3$). The two most prominent peaks of 365 (100%, $[M - C_9H_{17}O_3 - H_2O]^+$) and 383 (32%, $[M - C_9H_{17}O_3]^+$) in FAB-MS strongly support the combination of sesterterpene and this acid. The cross peak between H-C(10) and C(26) in the HMBC spectrum is a clear evidence for the esterification of HO-C(10) with the carboxylic group of the aliphatic acid.

2.3. Solution conformation and relative stereochemistry of YW3699

From the ^1H-^1H coupling constants, detailed analysis of ROESY spectrum in conjunction with studies of Dreiding models, the solution conformation and relative stereochemistry of YW3699 were elucidated and are depicted in Figure 2. The couplings and NOEs in ring C are consistent with a six-membered chair conformation whereas those in cyclopentane ring D suggest an envelope conformation. The five-membered ring A contains an α, β-unsaturated ketone and forms a regular planar pentagon. The favoured conformation for a simple cyclooctane is the boat-chair, which is in equilibrium with a few tenths of a percent of the crown conformation. However, the eight-membered ring B in YW3699 has the crown-like conformation, because of the presence of double bonds at C(15) and C(16), and at C(5) and C(20).

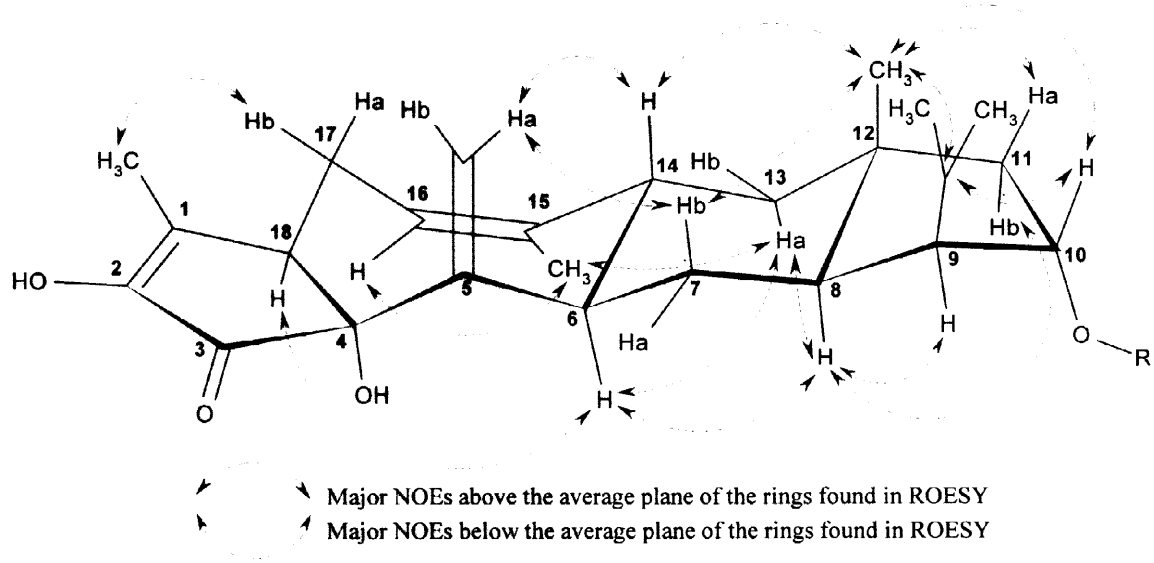

Figure 2. Solution conformation and relative stereochemistry of the tetracarbocyclic skeleton of YW3699, indicating major NOEs above and below the average plane of the rings.

The observed vicinal coupling constant of 12.5 Hz between H-C(6) and H-C(14) strongly suggests a *trans* B/C ring junction. This is also consistent with the NOEs among H-C(6), H-C(8) and Ha-C(13), and the NOEs among H-C(14), Ha-C(20) and Me(24). The NOEs among Me(24)

and Hb-C(7), H-C(14), H-C(21), and among H-C(8) and H-C(6), Ha-C(13), readily support the trans C/D ring junction and require H-C(8) and Me(24) to be *trans*-diaxial. A *cis* ring junction is also implicated between rings A and B based on the NOEs between H-C(18) and H-C(6), and between Hb-C(17) and Me(19). Coupled with inspection of Dreiding models, the axial orientation of the double bond on C(5), clearly defined with the NOE results, is additional evidence for a *cis* A/B ring junction by requiring an axial HO-C(4) in *cis*-relation to the axial H-C(18).

The axial orientation of bridgeheads protons H-C(6), H-C(8), H-C(14), H-C(18) and Me(24) are confirmed by their interactions with various axial protons or the protons on ring substituents. The major NOEs above and below the average plane of the rings obtained in ROESY spectrum are shown in Figure 2. The NOEs from geminal and vicinal protons readily observed are not indicated in the figure. Detailed analysis of the ROESY spectrum and all observed NOEs are summarised in Table 1.

Besides the NOEs, the analysis of chemical shifts of geminal protons also corroborates their configuration. Because of the C-C deshielding effect in cyclohexane, equatorial Ha-C(7) and Hb-C(13) on ring C are found to be at δ 1.98 and δ 1.86, downfield 0.55 ppm and 0.63 ppm than Hb-C(7) (δ 1.43) and Ha-C(13) (δ 1.23), respectively. The double bond between C(5) and C(20) has an axial orientation above the average plane of ring B. As expected, Hb-C(7), H-C(14) and Me(24) deshield Ha-C(20) (δ 4.96) giving rise to a signal 0.29 ppm further downfield than that for Hb-C(20) (δ 4.67). Consistently, Ha-C(17) (δ 3.06) and Ha-C(11) (δ 2.27) are deshielded markedly and their resonance signals shifted downfield by as much as 0.8 ppm and 1.08 ppm further than Hb-C(17) (δ 2.26) and Hb-C(11) (δ 1.19) respectively, due to the interaction of Ha-C(17) with Ha-C(20), Hb-C(20) and H-C(14), and the interaction of Ha-C(11) with H-C(10), H-C(21), Me(22), Me(23) and Me(24).

By means of 2D homonuclear and heteronuclear correlation in DQ-COSY and HSQC spectra, all the signals in both ^{1}H and ^{13}C NMR spectra, including those with severe overlaps in some regions, could be unambiguously assigned. The complete assignment of ^{1}H and ^{13}C NMR spectra, together with a detailed analysis of all related cross-peaks found in DQ-COSY, ROESY, HSQC and HMBC spectra, are summarised in Table 1 and Table 2, respectively. Based on careful analysis of various spectral data, the structure and relative stereochemistry of YW3699 can therefore be defined as that represented in Figure 3.

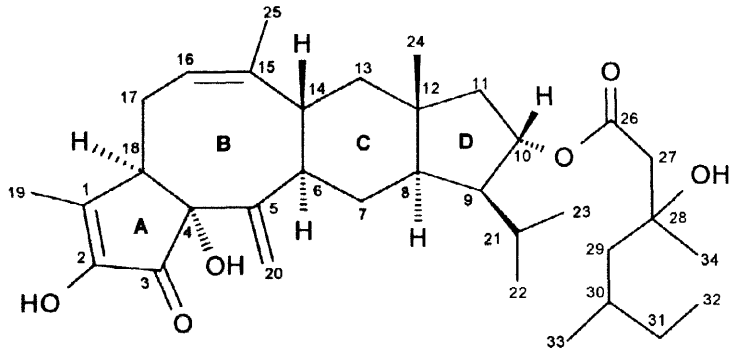

Figure 3. Structure and relative stereochemistry of YW3699 with arbitrary numbering for the skeleton.

Table 1
^{1}H NMR Data in $CDCl_3$ for YW3699

	^{1}H	DQ-COSY	ROESY
H-C(6)	2.98 (ddd, J=12.5, 12.5, 2.5)	Ha-C(7), Hb-C(7), H-C(14), Hb-C(20)	Ha-C(7), H-C(8), Ha-C(13), H-C(14), Ha-C(17), H-C(18)
Ha-C(7)	1.98 (ddd, J=12.5, 2.5, 2.5)	H-C(6), Hb-C(7), H-C(8)	H-C(6), Hb-C(7), H-C(8), H-C(9), Me(23)
Hb-C(7)	1.43 (ddd, J=12.5, 12.5, 12.5)	H-C(6), Ha-C(7), H-C(8)	Ha-C(7), H-C(8), Ha-C(20), Me(23), Me(24)
H-C(8)	2.11 (ddd, 12.5, 12.5, 2.5)	Ha-C(7), Hb-C(7), H-C(9)	H-C(6), Ha-C(7), Hb-C(7), H-C(9), Hb-C(11), Ha-C(13)
H-C(9)	1.76 (m)	H-C(8), H-C(10), H-C(21)	Ha-C(7), H-C(8), Me(22), Me(23)
H-C(10)	5.30 (ddd, J=7.5, 7.5, 3.5)	H-C(9), Ha-C(11), Hb-C(11)	Ha-C(11), H-C(21), Me(22), Me(24)
Ha-C(11)	2.27 (dd, J=12.5, 7.5)	H-C(10), Hb-C(11)	H-C(10), Hb-C(11), Ha-C(13), Hb-C(13), H-C(14), Me(24)
Hb-C(11)	1.19 (m)	H-C(10), Ha-C(11)	H-C(8), Ha-C(11)
Ha-C(13)	1.23 (dd, J=12.5, 12.5)	Hb-C(13), H-C(14)	H-C(6), H-C(8), Hb-C(13)
Hb-C(13)	1.86 (dd, J=12.5, 2.5)	Ha-C(13), H-C(14)	Ha-C(11), Ha-C(13), H-C(14), Me(24), Me(25)
H-C(14)	2.10 (dd, J=12.5, 12.5, 2.5)	H-C(6), Ha-C(13), Hb-C(13)	H-C(6), Ha-C(11), Hb-C(13), Ha-C(20), Me(24), Me(25)
H-C(16)	5.07 (dd, J=8.5, 8.5)	Ha-C(17), Hb-C(17), Me(25)	Hb-C(17), Me(19), Me(25)
Ha-C(17)	3.06 (ddd, J=16.0, 8.5, 3.5)	H-C(16), Hb-C(17), H-C(18)	H-C(6), Hb-C(17), H-C(18)
Hb-C(17)	2.26 (ddd, J=16.0, 8.5, 4.0)	H-C(16), Ha-C(17), H-C(18)	H-C(16), Ha-C(17), H-C(18), Me(19)
H-C(18)	2.91 (m)	Ha-C(17), Hb-C(17), Me(19)	H-C(6), Ha-C(17), Hb-C(17), Me(19)
Me(19)	2.02 (s)	H-C(18)	H-C(16), Hb-C(17), H-C(18)
Ha-C(20)	4.96 (s)	-	Hb-C(7), H-C(14), Hb-C(20)
Hb-C(20)	4.67 (s)	H-C(6)	Ha-C(20)
H-C(21)	1.72 (m)	H-C(9), Me(22), Me(23)	H-C(10), Me(24)
Me(22)	0.95 (d, J=6.5)	H-C(21)	H-C(9), H-C(10)
Me(23)	0.91 (d, J=6.5)	H-C(21)	Ha-C(7), Hb-C(7), H-C(9)
Me(24)	0.99 (s)	-	Hb-C(7), H-C(10), Ha-C(11), Hb-C(13), H-C(14), H-C(21), Me(25)
Me(25)	1.54 (s)	H-C(16)	Ha-C(13), Hb-C(13), H-C(14), H-C(16), Me(24)
Ha-C(27)	2.51 (d, J=15.8)	Hb-C(27)	Hb-C(27), Ha-C(29), Hb-C(29), Me(34)
Hb-C(27)	2.41 (d, J=15.8)	Ha-C(27)	Ha-C(27), Ha-C(29), Hb-C(29), Me(34)
Ha-C(29)	1.35 (dd, J=15.5, 8.0)	Hb-C(29)	Ha-C(27), Hb-C(27), Hb-C(29)
Hb-C(29)	1.55 (m)	Ha-C(29)	Ha-C(27), Hb-C(27), Ha-C(29), H-C(30), Ha-C(31), Hb-C(31), Me(33), Me(34)
H-C(30)	1.56 (m)	Hb-C(31), Me(33)	Hb-C(29), Me(33)
Ha-C(31)	1.20 (m)	Hb-C(31), Me(32)	Hb-C(29), Hb-C(31), Me(32)
Hb-C(31)	1.41 (m)	H-C(30), Ha-C(31)	Hb-C(30)
Me(32)	0.87 (dd, J=7.5, 7.5)	Ha-C(31)	Me(34)
Me(33)	0.94 (d, J=6.5)	H-C(30)	Hb-C(29), H-C(30)
Me(34)	1.25 (s)	-	Ha-C(27), Hb-C(27), Hb-C(29), Me(32)

Table 2
^{1}H and ^{13}C NMR Data in $CDCl_3$ for YW3699

	C Type	^{13}C	HSQC	HMBC
C(1)	C	144.4	-	-
C(2)	C	147.2	-	-
C(3)	C	202.3	-	-
C(4)	C	81.1	-	-
C(5)	C	152.0	-	-
H-C(6)	CH	45.7	C(6)	C(5), C(7), C(14), C(20)
Ha-C(7)	CH_2	35.5	C(7)	C(6), C(12), C(14)
Hb-C(7)			C(7)	C(6), C(8), C-(14)
H-C(8)	CH	51.2	C(8)	C(6), C(7), C(9), C(12), C(13), C(21), C(24)
H-C(9)	CH	53.3	C(9)	C(8), C(10), C(12), C(21), C(22), C(23)
H-C(10)	CH	80.1	C(10)	C(21), C(26)
Ha-C(11)	CH_2	47.4	C(11)	C(8), C(9), C(10), C(12), C(24)
Hb-C(11)			C(11)	C(10), C(12), C(13)
C(12)	C	42.7	-	-
Ha-C(13)	CH_2	44.7	C(13)	C(6), C(8), C(11), C(12), C(14), C(15), C(24)
Hb-C(13)			C(13)	C(6), C(8), C(11), C(12), C(14), C(24)
H-C(14)	CH	47.2	C(14)	C(12), C(15), H(16), C(25)
C(15)	C	143.1	-	-
H-C(16)	CH	118.5	C(16)	C(14), C(17), C(25)
Ha-C(17)	CH_2	24.5	C(17)	C(1), C(15), C(16), C(18)
Hb-C(17)			C(17)	C(4), C(15), C(16), C(18)
H-C(18)	CH	51.5	C(18)	C(16), C(17)
Me(19)	CH_3	12.0	C(19)	C(1), C(2), C(18)
Ha-C(20)	CH_2	112.5	C(20)	C(4), C(6), C(20)
Hb-C(20)			C(20)	C(4), C(6), C(20)
H-C(21)	CH	29.9	C(21)	C(9), C(22), C(23)
Me(22)	CH_3	23.8	C(22)	C(9), C(23)
Me(23)	CH_3	21.8	C(23)	C(9), C(22)
Me(24)	CH_3	20.7	C(24)	C(8), C(11), C(12), C(13)
Me(25)	CH_3	25.9	C(25)	C(14), C(15), C(16)
C(26)	C	172.9	-	-
Ha-C(27)	CH_2	45.7	C(27)	C(26), C(28), C(29), C(34)
Hb-C(27)			C(27)	C(26), C(28), C(29), C(34)
C(28)	C	71.6	-	-
Ha-C(29)	CH_2	48.2	C(29)	C(27), C(28), C(30), C(31), C(33), C(34)
Hb-C(29)			C(29)	C(27), C(28), C(30), C(31), C(33), C(34)
H-C(30)	CH	30.3	C(30)	C(28), C(29), C(31), C(33)
Ha-C(31)	CH_2	31.1	C(31)	C(30), C(33)
Hb-C(31)			C(31)	C(30), C(33)
Me(32)	CH_3	11.3	C(32)	C(30), C(31)
Me(33)	CH_3	21.4	C(33)	C(29), C(31)
Me(34)	CH_3	27.3	C(34)	C(27), C(28), C(29)

2.4. Biosynthesis

The terpenoids form a large and structurally diverse family of natural products derived from C_5 isoprene units joined in a head-to-tail fashion. The linear arrangement of isoprene units in the structure of YW3699 can be easily recognised, because no complicated rearrangement reactions have taken place. As usual in fungal terpenoids[3], the biosynthesis of sesterterpene YW3699 might be also via the mevalonate pathway.

A possible pathway for the biosynthesis of YW3699 is suggested in Figure 4. The biochemically active isoprene units, derived from acetate metabolism by way of mevalonic acid, are dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP). Combination of DMAPP and IPP via the enzyme dimethylallyl-transferase and further addition of IPP form geranylfarnesyl pyrophosphate (GFPP). A series of cyclization steps from precursor GFPP which could occur in a concerted fashion formally shown transient intermediates [A], [B] and [C] below, could lead to the unusual tetracarbocyclic sesterterpene skeleton. Further oxidation, followed by esterification with 3,5-dimethyl-3-hydroxylheptanoic acid, would then generate the complete structure of YW3699.

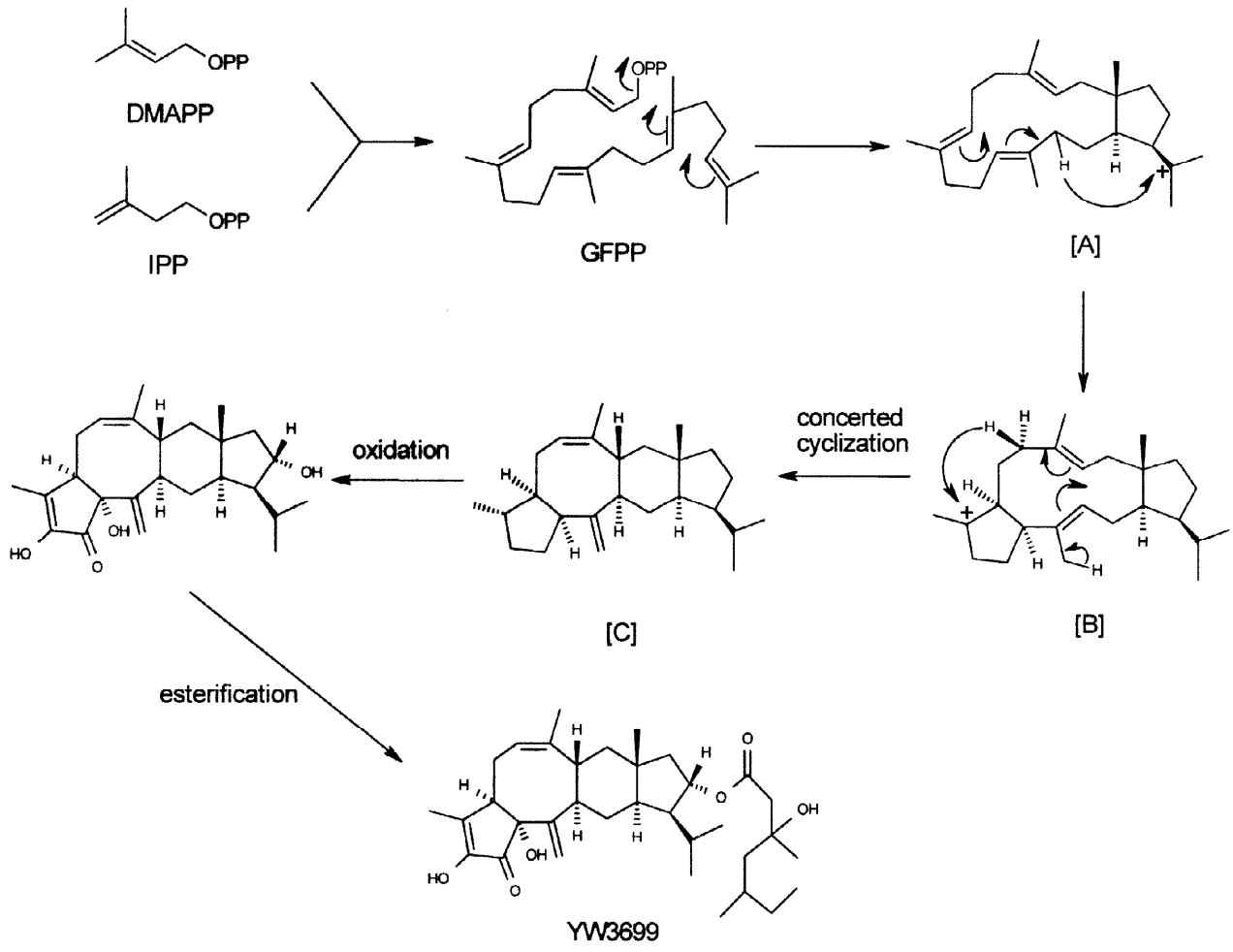

Figure 4. Hypothetical biosynthesis of YW3699 via the mevalonate pathway

2.5. Biological activity

YW3699 was tested for its inhibitory activity on glycosylphosphatidylinositol (GPI) synthesis using the *in vitro* GPI synthesis system from yeast, or by halo assay on agar plates. The minimal inhibitory concentration of YW3699 was determined as 3.5 μM[2].

3. Discussion

Sesterterpenes have been obtained from widely differing sources including terrestrial fungi, plants and insects as well as from marine sponges and nudibranchs. Sesterterpenes of marine origin represent a majority. Naturally occurring sesterterpenes were first encountered during the late 1950's, and as a consequence they are often viewed as a rare category of natural products[4]. New sesterterpenes were discovered slowly; by 1972 only 12 sesterterpenes were known which represented one acyclic and two carbocyclic frameworks[5]. Since then, there have been reports of numerous other new sesterterpenes with the total count now standing about 350 compounds, which have been summarised in a series of major reviews[4, 6-9]. Strictly speaking, a sesterterpene ought to have twenty-five contiguous carbons divisible into five isoprene units. However, there are a large number of nor- and alkylated-sesterterpenes. Their basic skeletons are very diverse and have been classified into about thirty different carbon frameworks[10].

YW3699 is a tetracarbocyclic sesterterpene. The majority of tetracarbocyclic sesterterpenes belong to the scalarane series, which are particularly common in marine sponges and are exemplified by scalarin[11]. Few tetracarbocyclic sesterterpenes containing an 8-membered ring has been identified from marine sponges, plants or insects. As secondary metabolites from microorganisms, only a couple of tetracarbocyclic sesterterpenes, *e.g.* variecolin[12], citreohydridone A[13] and aleurodiscal[14], were found around 1990. Variecolin is a hybrid of the ophiobolin and ceriferene identified from *Aspergillus variecolor*[12]. Aleurodiscal is a hydroxysesterterpene aldehyde β-D-xyloside isolated from the basidiomycetes *Aleurodiscus mirabilis*[14]. Both variecolin and aleurodiscal have novel tetracarbocyclic sesterterpene skeletons, including an eight-membered ring. The structure of aleurodiscal was determined by a single-crystal X-ray diffraction analysis because of the high complexity of the NMR spectra. It is the only known sesterterpene related to YW3699, but with different relative stereochemistry and oxygenation pattern.

Natural sesterterpene derivatives include three categories, namely degraded sesterterpenes with 22-24 contiguous carbon atoms, alkylated sesterterpenes with 26 or 27 contiguous carbon atoms, and sesterterpenes containing an additional non-terpenoid unit. In the last category, a few of sesterterpenes with an esterified aliphatic acid side chain have been identified only from marine sponges, such as foliaspongin from *Phyllospongia foliascens* collected on the Great Barrier reef, Australia[15], scalarane sesterterpenes from the Okinawan marine sponge *Strepsichordaia lendenfeldi*[16] and phyllactones from the South China Sea sponge *Phyllospongia foliascens*[17,18]. As an unusual fungal metabolite in this category, YW3699 is a

tetracarbocyclic sesterterpene connected by an ester bond with a novel branched aliphatic acid, 3,5-dimethyl-3-hydroxylheptanoic acid.

Another GPI synthesis inhibitor from the same fungus, YW3548, is biogenetically closely related to YW3699, but showed much stronger activity with an MIC of 3.4 nM[2,19]. Replacement of the six-membered δ-lactone ring in YW3548 by the five-membered α, β-unsaturated ketone ring in YW3699 causes a reduction of activity of about one thousand fold. Therefore, it seems that the lactone ring in YW3699 is essential for the very potent inhibitory activity on GPI synthesis.

4. Experimental

4.1. General methods

TLC: silica gel 60 F254 precoated plates (Merck); RP-18 W/UV254 precoated plates (Merck); HPTLC: Diol F254s precoated plates (Merck); detection at 254 nm. Open column chromatography (CC): *Sephadex LH-20* (Pharmacia). Analytical HPLC: Hewlett Packard HP 1090 system with a photodiode array UV detector and a HP Chemstation; column, *LiChrosphere* RP-18 (5 μm, 5 x 100 mm id. Merck). Preparative HPLC: Labomatic-HD-200 pump coupled with a Labomat-VS-200 gradient controller and a Labocorod-700 UV detector; column, *LiChrosorb* RP-18 (7 μm, 50 x 250 mm id. Merck). *LiChrospher* Diol (10 μm, 50 x 250 mm id. Merck). IR: FT-IR spectrometer Bruker IFS 66. ^{1}H- and ^{13}C-NMR: Bruker AMX-400 or Bruker Avance DMX-500 spectrometer at 400 or 500 and 100.62 or 125.77 MHz, resp.; in (D_6)DMSO or (D)chloroform; TMS as internal standard. MS (*m/z* (%)): VG-7044SE or Finnigan-MAT-212 spectrometer, 8 keV Xenon with nitrobenzyl-alcohol or thioglycerin as matrix operating in the FAB positive-ion mode. HR-MS: Micromass Quattro II, electrospray positive mode with capillary 3 kV, cone voltage 30 V and source temperature 70°C; flow injection (loop 50 μl) with H_2O/MeCN (50:50) + formic acid (0.05%) + ammonia (0.1%) at flow rate of 5 μl/min; scan range 487 to 627 amu in 5 s; resolution 1698 (10% peak height) and 3243 (FWHH); PEGMME 350, ions $[M+NH_4]^+$ as reference compound.

4.2. Bioassay

The inhibitory activity on glycosylphosphatidylinositol (GPI) synthesis was evaluated using an *in vitro* system, by Western blot analysis or by halo assay. Detailed procedures are described in the reference[2].

4.3. Fermentation

The active fungus strain, which produces inhibitors of GPI anchor biosynthesis, was tentatively identified as *Codinaea simplex* S. J. Hughes & Kendrick. Precultures of 1 l from 1 frozen ampoule of *Codinaea simplex* (F/94-493108) were grown in medium containing 0.1% Bacto Agar, 0.4% Yeast Extract (Gistex-X-II), 2% malt extract (Wander) in conical flasks with shaking for one week at 24 °C. The seed cultures (2.5 l) were inoculated into 50 l bioreactor

containing medium containing 0.72% Bacto Yeast extract (Difco) and 2% corn starch. The cultures were fermented at 21 °C for 4 days. The bioreactor was stirred at 150 rpm (pressure 0.5 bar, antifoam: M13), and air was introduced at a rate of 1 l/min per l of medium. The pH was maintained at 5.5 with 2N H_2SO_4 and 2 N NaOH.

4.4. Extraction

The fermentation broth (70 l) was filtered through diatomaceous earth on a Buchner funnel. The filtrate was discarded and the mycelia resuspended in approximately 2 l of methanol and 1 l deionized water. After homogenisation and agitation in Turrax mixer at top speed for 10 minutes at room temperature, the solution was again filtered and the filtrate was evaporated until the methanol was removed. The water phase was extracted three times with ethyl acetate. NaCl was added to improve the phase separation, and the combined ethyl acetate phases were washed with water, dried over sodium sulphate and evaporated to dryness, yielding 18 g of crude extract (brown oil).

4.5. Isolation

The extract was subjected to gel filtration (*Sephadex LH-20*, 5 x 94 cm id., MeOH, 40ml/fr.). All fractions were analysed with TLC (SiO_2, toluene/*iso*-propanol 95:5). Fractions 12 to 15 were combined (*ca.* 4.09 g) and chromatographed in two runs by preparative HPLC on reversed-phase (RP-18, 50 x 250 cm id., gradient elution with MeCN/H_2O 0% to 100% in 60 min., 50 ml/min.). YW3699 (32.8 mg) was obtained from one HPLC Fraction (fr. 37, 204 mg) with final purification by two steps of preparative HPLC on normal phase (Diol, 50 x 250 cm id., the first step: gradient elution with hexane/ethyl acetate 100% to 90 % in 20 min., 90% to 70% in 30 min. and 70% to 0% in 30 min.; the second step: gradient elution with toluene/ethyl acetate 100% to 70% in 40 min, 70% to 0% in 20 min., 50 ml/min.)

4.6. Sesterterpene YW3699

$C_{34}H_{52}O_6$. A white powder. TLC (SiO_2): Rf 0.48 (CH_2Cl_2/MeOH 95:5), 0.21 (*n*-hexane/*iso*-propanol 90:10), 0.26 (toluene/*iso*-propanol 95:5). HPLC (RP-18, gradient elution with MeCN/H_2O 0:100 to 100:0 in 15 min. and continued with MeCN/H_2O 100:0 for 5 min): Rt 11.9 min. UV λ_{max} (MeOH) nm: 270 (7745). UV λ_{max} (MeOH + HCl) nm: 279 (7622). UV λ_{max} (MeOH + NaOH) nm: 307 (6177). IR (KBr) cm^{-1}: 3430*m* (OH), 2964*s*, 2930*s*, 2874*m* (CH, aliphatic), 1710*s* (C=O), 1655*m* (C=C), 1463*m*, 1410*m*, 1379*m*, 1336*m*, 1195*s*(C-O), 1137*m*, 1062*w*, 1045*m*, 994*m*. ^{1}H-NMR, DQ-COSY and ROESY ($CDCl_3$): Table 1. ^{13}C-NMR, HSQC and HMBC ($CDCl_3$): Table 2. FAB-MS (pos.-ion mode) *m/z* (relative intensity %): 557 (8, $[M + H]^+$), 538 (6, $[M + H - H_2O]^+$), 383 (32, $[M - C_9H_{17}O_3]^+$), 365 (100, $[M - C_9H_{17}O_3 - H_2O]^+$), 347 (4), 339 (5), 321 (5), 307 (3), 289 (3), 281 (4), 255 (18), 241 (3), 229 (7), 201 (12). FAB-MS (+LiI, pos.-ion mode): 563 (17, $[M + Li]^+$). ES-HRMS (pos. -ion mode): observed $[M + H]^+$ 557.3839 (12 measurements with standard deviation 1.7 mmu); calculated $[M + H]^+$) 557.3842.

5. Acknowledgements

We appreciate Daniel Besnosko's technical assistance. We would also thank Hans-Ulrich Gremlich, Christian Guenat and Charles Quiquerez for recording UV, FT-IR, ESI-HRMS and FAB-MS spectra. Trevor Payne is acknowledged for the critically reading of the manuscript.

6. References

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