Conformation and Relative Configuration of a Very Potent Glycosylphosphatidylinositol-Anchoring Inhibitor with an Unusual Tricarbocyclic Sesterterpenoid δ-Lactone Skeleton from the Fungus *Paecilomyces inflatus*

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In the course of our screening for glycosylphosphatidylinositol (GPI) inhibitors, we found a fungal strain, *Paecilomyces inflatus*, which inhibited GPI anchoring in yeast. Bioassay-guided fractionation with gel filtration, MPLC on normal phase and prep. HPLC on reversed-phase yielded a minor secondary metabolite, the substituted hexadecahydroindeno[5′,6′:4,5]cycloocta[1,2-*c*]pyranyl heptanoate **1**, that inhibited GPI synthesis *in vitro* by yeast microsomes with a *MIC* of 3.4 nM. Ester **1** specifically inhibited GPI synthesis in eukaryotic, including mammalian cells, but had no significant activity in protozoa. Based on spectroscopic evidence, including UV, FT-IR, FAB-MS, ESI-HR-MS, ^{1}H-NMR, ^{13}C-NMR, DQ-COSY, ROESY, HSQC, and HMBC data, the metabolite **1** was shown to have an unusual tricarbocyclic sesterterpenoid δ-lactone skeleton. Its solution conformation and relative configuration was elucidated with ^{1}H,^{1}H coupling constants and detailed analysis of its ROESY data, coupled with inspection of *Dreiding* models. In the preliminary investigations on structure-activity relationships, the three derivatives **2**–**4** of ester **1** were prepared by acetylation, catalytic hydrogenation, and intramolecular alkene addition of a hydroxy group, respectively. The test results revealed that the modifications of substituents at the δ-lactone ring or of the C=C bonds of **1** caused a loss of activity by a factor of *ca*. 500 to 5000. Therefore, it seems that the δ-lactone ring and the C=C bonds in **1** are essential for the potent GPI-inhibitory activity.

Introduction. – Several cell-surface proteins which serve as receptors or co-receptors for ligands that modulate signal transduction are anchored to the outer surface of the cell membrane by glycosylphosphatidylinositol (GPI) moieties. GPI Anchoring represents a mechanism for attaching proteins to the cell surface that is used among all eucaryotes [1]. Inhibitor compounds in the pathways of GPI biosynthesis could, therefore, be potential leads in drug discovery.

Inhibitors have also been used to study the GPI-anchor biosynthesis. 2-Fluoro-2-deoxyglucose and amphomycin are known inhibitors of dolichol-P-mannose synthase, and they block the mannosylation of GPI intermediates [2]. The amino-sugar mannosamine appears to block the addition of the third mannose to the intermediate structure in mammalian cells [3]. (Phenylmethyl)sulfonyl fluoride was shown to inhibit GPI anchoring in African trypanosomes [4] and specifically blocks the inositol acylation process that converts glycolipid precursor A into precursor C, but is not active in mammalian cells [5]. Diisopropyl fluorophosphate also affects the inositol deacylation step in trypanosomes [6]. However, all these inhibitors do not seem to be specific for GPI anchoring.

We have developed a screen based on the maturation of the major yeast GPI-anchored protein Gas1p [7]. In our screening for natural products which inhibit GPI anchoring in yeast and block Gas1p transport to the Golgi, an extract with potent inhibitory activity was identified from the fungus *Paecilomyces inflatus*. To identify the bioactive secondary metabolite that is responsible for the inhibitory activity of the fungal extract, fractionation and separation of the active extract were guided by the growth-inhibitory activity on a yeast lawn and by testing Gas1p maturation by Western-blot analysis.

Results. – Precultures of the fungus *Paecilomyces inflatus* (F/92-802010) were grown in shake cultures for one week at 21° and the seed cultures inoculated into a bioreactor and fermented at 21° for 6 days with stirring. The fermentation broth was extracted with AcOEt. The extract was subjected to gel filtration on *Sephadex LH-20*, followed by MPLC on silica gel. As a minor secondary metabolite and a very potent GPI-anchoring inhibitor, the metabolite **1** was finally purified by prep. HPLC on reversed-phase *RP-18*. The whole fractionation and purification procedures were guided by the growth-inhibitory activity on a yeast lawn and by testing Gas1p maturation by Western-blot analysis.

The UV spectrum of **1** showed only an end absorption. The FT-IR spectrum contained bands at 1734 and 1705 cm^{-1} which suggest the presence of a δ-lactone and a carbonyl group. ^{1}H- and ^{13}C-NMR spectra revealed 34 C-atoms, 51 C-bound protons and 3 O-bound protons in hydroxy groups. Interpretation of the HSQC and HMBC spectra suggests the following C-atom types: 8 Me, 7 CH_2, 10 CH, 5 C, 1 CH_2=, 1 CH=, and 2 C=O groups. The positive-ion-mode FAB-MS of **1** indicated a molecular mass of 574 by showing quasimolecular ions at *m/z* 597 ($[M+Na]^+$) and 575 ($[M+H]^+$). This was confirmed by the quasimolecular ion at *m/z* 581 ($[M+Li]^+$) in FAB-MS with LiI. The molecular formula $C_{34}H_{54}O_7$ was determined by a high-resolution mass measurement (ESI-HR-MS (pos.): 575.3939 ($[M+H]^+$; calc. 575.3948)), in conjunction with the ^{1}H- and ^{13}C-NMR data. This requires eight degrees of unsaturation in **1** ($n=n_C+1+(n_N-n_H)/2=8$). Due to the presence of two C=C bonds and two C=O functions, there should be four rings in the molecule.

By means of 2D homonuclear and heteronuclear correlation using DQ-COSY and HSQC, 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 data, together with a detailed analysis of all related cross-peaks found in DQ-COSY, ROESY, HSQC, and HMBC, are summarized in *Tables 1* and *2*. Based on careful analysis of various spectral data, the structure and relative configuration of metabolite **1** could be established as that shown in *Fig. 1*.

The cross-peaks in DQ-COSY (*Table 1*) and HSQC and HMBC (*Table 2*) suggested that **1** is an unusual tricarbocyclic sesterterpenoid δ-lactone esterified with an aliphatic acid, 3-hydroxy-3,5-dimethylheptanoic acid ($C_9H_{18}O_3$) (*Fig. 1*). The two most prominent peaks at *m/z* 383 (100%, $[M-C_9H_{17}O_3-H_2O]^+$) and 401 (92%, $[M-C_9H_{17}O_3]^+$) in the FAB-MS strongly support the combination of the tricarbocyclic sesterterpenoid δ-lactone and this aliphatic acid.

From the ^{1}H,^{1}H coupling constants, detailed analysis of ROESY data in conjunction with studies of *Dreiding* models, the solution conformation and relative configuration of **1** were elucidated (see *Fig. 2*). The couplings and NOEs in 6-ring C are consistent

Table 1. *^{1}H-NMR Data* ($CDCl_3$) *of Metabolite* **1**[1]). δ in ppm, J in Hz.

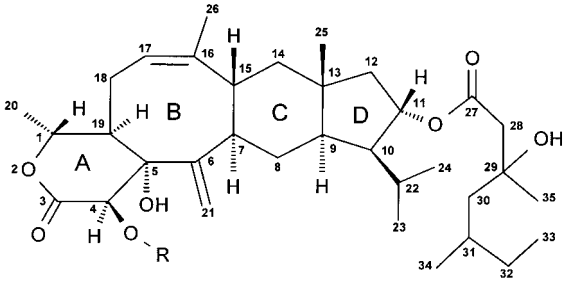
	δ(H)	DQ-COSY	ROESY
H−C(1)	4.39 (*dddd*, J = 14, 7, 7, 7)	H−C(19), Me(20)	H−C(17), Me(20), H_b−C(21)
H−C(4)	4.24 (*s*)	OH−C(4)	OH−C(4), OH−C(5), H−C(19), H_b−C(21)
OH−C(4)	3.07 (br. *s*)	H−C(4)	H−C(4), H_b−C(21)
OH−C(5)	2.78 (*s*)	–	H−C(4), H−C(7), H_a−C(8)
H−C(7)	3.10 (*ddd*, J = 12.5, 12.5, 3.0)	H_a−C(8), H_b−C(8), H−C(15), H_b−C(21)	OH−C(5), H_a−C(8), H−C(9), H_a−C(14)
H_a−C(8)	1.83 (*ddd*, J = 12.5, 3.0, 3.0)	H−C(7), H_b−C(8), H−C(9)	H−C(7), H_b−C(8), H−C(9), Me(23)
H_b−C(8)	1.38 (*ddd*, J = 12.5, 12.5, 12.5)	H−C(7), H_a−C(8), H−C(9)	H_a−C(8), H−C(9), H−C(15), Me(23), Me(25)
H−C(9)	2.04 (*ddd*, J = 12.5, 12.5, 3.0)	H_a−C(8), H_b−C(8), H−C(10)	H−C(7), H_a−C(8), H_b−C(8), H−C(10), H_b−C(12), H_a−C(14)
H−C(10)	1.78 (*m*)	H−C(9), H−C(11)	H−C(9), Me(23), Me(24)
H−C(11)	5.31 (*ddd*, J = 7.5, 7.5, 3.5)	H−C(10), H_a−C(12), H_b−C(12)	H−C(10), H_a−C(12), H−C(22), Me(23), Me(24), Me(25)
H_a−C(12)	2.28 (*dd*, J = 12.0, 7.5)	H−C(11), H_b−C(12)	H−C(11), H_b−C(12), Me(25)
H_b−C(12)	1.17 (*m*)	H−C(11), H_a−C(12)	H−C(9), H_a−C(12), H_a−C(14)
H_a−C(14)	1.09 (*dd*, J = 12.5, 12.5)	H_b−C(14), H−C(15)	H−C(7), H−C(9), H_b−C(12), H_b−C(14)
H_b−C(14)	1.98 (*dd*, J = 12.5, 2.5)	H_a−C(14), H−C(15)	H_a−C(14), H−C(15), Me(25)
H−C(15)	2.21 (*ddd*, J = 12.5, 12.5, 2.5)	H−C(7), H_a−C(14), H_b−C(14)	H_b−C(8), H_b−C(14), H_a−C(21), Me(25), Me(26)
H−C(17)	5.06 (*dd*, J = 8.5, 8.5)	H_a−C(18), H_b−C(18), Me(26)	H−C(1), H_a−C(18), H_b−C(18), Me(20), Me(26)
H_a−C(18)	3.14 (*ddd*, J = 16.0, 9.5, 3.5)	H−C(17), H_b−C(18), H−C(19)	H−C(17), H_b−C(18)
H_b−C(18)	1.97 (*ddd*, J = 16.0, 8.5, 5.0)	H−C(17), H_a−C(18), H−C(19)	H−C(17), H_a−C(18), Me(20)
H−C(19)	2.06 (*m*)	H−C(1), H_b−C(18)	H−C(4), Me(20)
Me(20)	1.48 (*d*, J = 7.0)	H−C(1)	H−C(1), H−C(17), H_b−C(18), H−C(19), H_a−C(21)
H_a−C(21)	5.16 (*d*, J = 1.0)	H_b−C(21)	H−C(15), Me(20), H_b−C(21), Me(25)
H_b−C(21)	4.72 (*d*, J = 1.0)	H−C(7), H_a−C(21)	H−C(1), H−C(4), OH−C(4), H_a−C(21)
H−C(22)	1.72 (*m*)	Me(23), Me(24)	H−C(11), Me(23), Me(24), Me(25)
Me(23)	0.95 (*d*, J = 6.3)	H−C(22)	H_a−C(8), H−C(10), H−C(11), H−C(22)
Me(24)	0.91 (*d*, J = 6.3)	H−C(22)	H−C(10), H−C(11), H−C(22)
Me(25)	0.99 (*s*)	–	H−C(11), H_a−C(12), H_b−C(14), H−C(15), H_a−C(21), H−C(22)
Me(26)	1.65 (*s*)	H−C(17), H_a−C(18)	H−C(15), H−C(17)
H_a−C(28)	2.51 (*d*, J = 15.8)	H_b−C(28), Me(35)	H_b−C(28), OH−C(29), H_a−C(30), H_b−C(30), Me(34), Me(35)
H_b−C(28)	2.40 (*d*, J = 15.8)	H_a−C(28)	H_a−C(28), OH−C(29), H_a−C(30), Me(34), Me(35)
OH−C(29)	3.63 (*s*)	Me(35)	H_a−C(28), H_b−C(28), Me(35)
H_a−C(30)	1.35 (*dd*, J = 15.5, 7.5)	H_b−C(30)	H_a−C(28), H_b−C(28), Me(35)
H_b−C(30)	1.55 (*m*)	H_a−C(30)	H_a−C(28), Me(35)
H−C(31)	1.56 (*m*)	H_a−C(32), H_b−C(32), Me(34)	Me(33), Me(34)
H_a−C(32)	1.20 (*m*)	H−C(31), H_b−C(32), Me(33)	H_b−C(32), Me(33), Me(34)
H_b−C(32)	1.40 (*m*)	H−C(31), H_a−C(32), Me(33)	H_a−C(32), Me(33), Me(34)
Me(33)	0.88 (*dd*, J = 7.4, 7.4)	H_a−C(32), H_b−C(32)	H−C(31), H_a−C(32), H_b−C(33)
Me(34)	0.95 (*d*, J = 7.4)	H−C(31)	H_a−C(28), H_b−C(28), H−C(31), H_a−C(32), H_b−C(32)
Me(35)	1.25 (*s*)	H_a−C(28), OH−C(29)	H_a−C(28), H_b−C(28), OH−C(29), H_a−C(30), H_b−C(30)

Table 2. *^{1}H- and ^{13}C-NMR Data* ($CDCl_3$) *of Metabolite* **1**[1]). δ in ppm.

	δ(C)	HSQC	HMBC
C(1)	77.6	C(1)	
C(3)	173.1		
H–C(4)	74.5	C(4)	
OH–C(5)	76.4		
C(6)	150.5		
H–C(7)	43.4	C(7)	C(5), C(7), C(14), C(20)
H_a–C(8)	35.0	C(8)	C(6), C(12), C(14)
H_b–C(8)		C(8)	C(6), C(8), C(14)
H–C(9)	50.6	C(9)	C(6), C(7), C(9), C(12), C(13), C(21), C(24)
H–C(10)	53.3	C(10)	C(8), C(10), C(12), C(21), C(22), C(23)
H–C(11)	79.9	C(11)	C(21), C(26)
H_a–C(12)	47.3	C(12)	C(8), C(9), C(10), C(12), C(24)
H_b–C(12)		C(12)	C(10), C(12), C(13)
C(13)	42.3		
H_a–C(14)	45.8	C(14)	C(6), C(8), C(12), C(14), C(15), C(24)
H_b–C(14)		C(14)	
H–C(15)	45.8	C(15)	C(12), C(15), H(16), C(25)
C(16)	143.1		
H–C(17)	116.7	C/17)	C(14), C(17), C(25)
H_a–C(18)	21.6	C(18)	C(1), C(15), C(16), C(18)
H_b–C(18)		C(18)	C(4), C(15), C(16), C(18)
H–C(19)	49.4	C(19)	C(16), C(17)
Me(20)	19.5	C(20)	C(1), C(2), C(18)
H_a–C(21)	111.0	C(21)	C(4), C(6), C(20)
H_b–C(21)		C(21)	C(4), C(6), C(20)
H–C(22)	29.7	C(22)	C(9), C(22), C(23)
Me(23)	24.0	C(23)	C(9), C(23)
Me(24)	21.8	C(24)	C(9), C(22)
Me(25)	20.5	C(25)	C(8), C(11), C(12), C(13)
Me(26)	26.4	C(26)	C(14), C(15), C(16)
C(27)	172.9		
H_a–C(28)	45.6	C(28)	C(26), C(28), C(29), C(34)
H_b–C(28)		C(28)	C(26), C(28), C(29), C(34)
OH–C(29)	71.6		
H_a–C(30)	48.2	C(30)	C(27), C(28), C(30), C(31), C(33), C(34)
H_b–C(30)		C(30)	C(27), C(28), C(30), C(31), C(33), C(34)
H–C(31)	30.3	C(31)	C(28), C(29), C(31), C(33)
H_a–C(32)	31.1	C(32)	C(30), C(33)
H_b–C(32)		C(32)	C(30), C(33)
Me(33)	11.4	C(33)	C(30), C(31)
Me(34)	21.4	C(34)	C(29), C(31)
Me(35)	27.3	C(35)	

with a chair conformation, whereas those in 5-ring D suggest an envelope conformation. The 6-ring A contains a δ-lactone and forms a regular chair conformation. 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 8-ring B in **1** has the crown-like conformation because of the presence of double bonds C(16)=C(17), and C(6)=C(21)[1]).

[1]) Arbitrary numbering (see *Fig. 1*). For systematic names, see *Exper. Part.*



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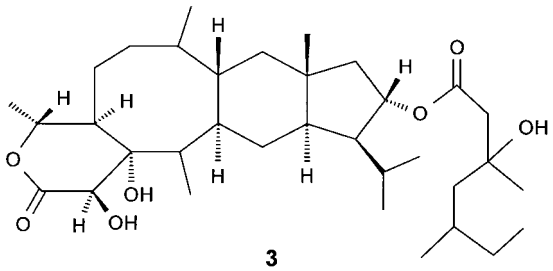

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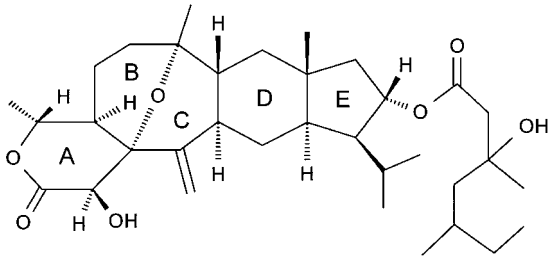

4

Fig. 1. *Structure and relative configuration of metabolite* **1** *and its derivatives* **2**–**4**[1])

The observed vicinal coupling constant of 12.5 Hz between H–C(7) and H–C(15) of **1** strongly suggests a *trans* B/C ring junction. This is also consistent with the NOEs H–C(7)/OH–C(5), H–C(9), and H_a–C(14), and the NOEs H–C(15)/H_b–C(8), H_a–C(21), and Me(25). The NOEs Me(25)/H_b–H(8), H–C(11), H_a–C(12), H–C(15), and H–C(22) and H–C(9)/H–C(10) and H_b–C(12) readily support the *trans* C/D ring junction and require H–C(9) and Me(25) to be *trans*-diaxial. A *cis* ring junction is also implicated between rings A and B according to the NOEs H–C(19)/H–C(4) and Me(20) and OH–C(5)/H–C(4) and H–C(7).

The axial orientation of the bridgehead groups and protons OH–C(5), H–C(7), H–C(9), H–C(15), H–C(19), and Me(25) are confirmed by their interactions with various axial protons or the protons of ring substituents. Detailed analysis of the ROESY data and all observed NOEs are summarized 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 H_a–C(8) and H_b–C(14) on ring C are found to be at δ 1.83 and 1.98, *i.e.*, shifted downfield by 0.45 and 0.89 ppm, respectively, with respect to H_b–C(8)

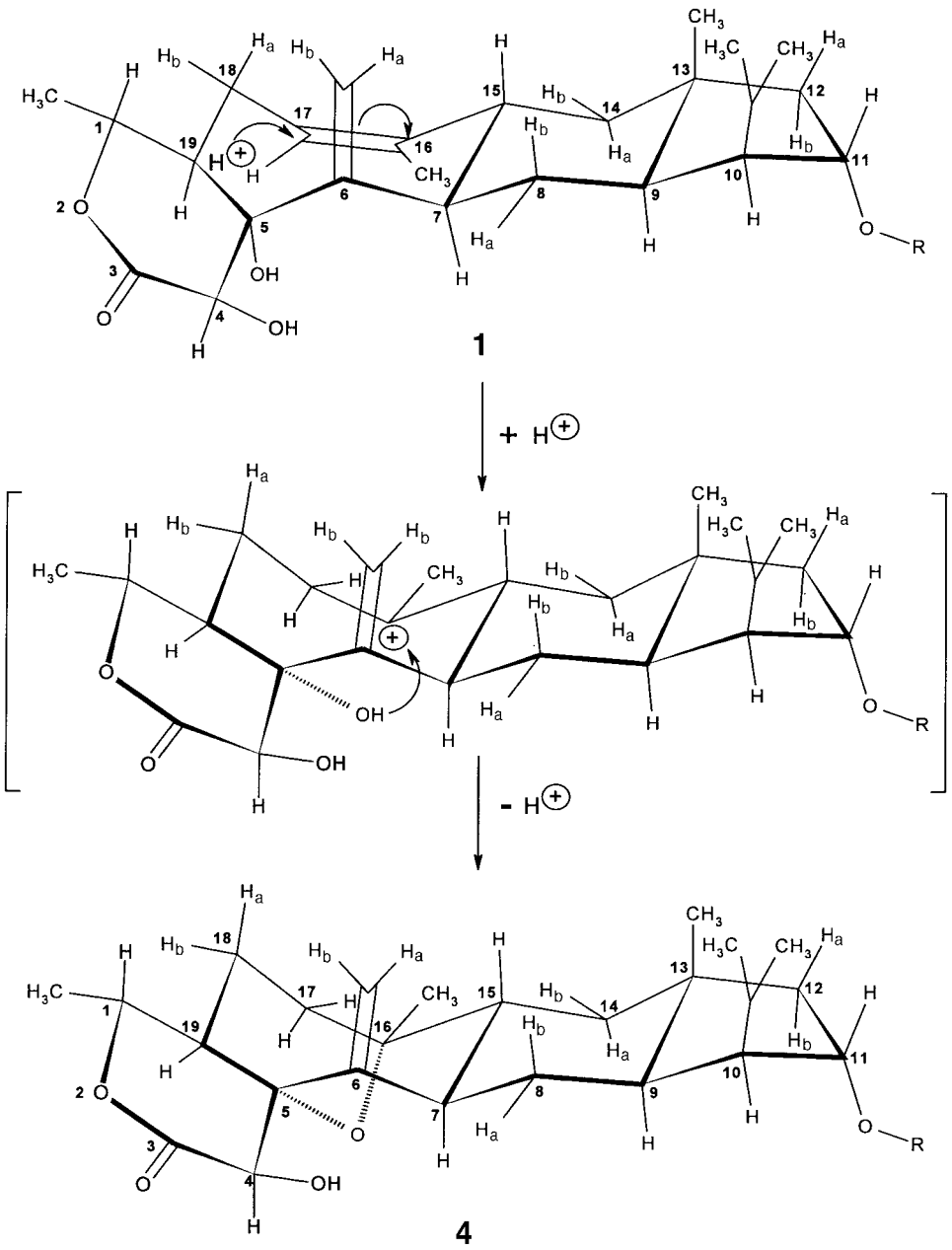

Fig. 2. *Intramolecular alkene addition of the hydroxy group of* **1** *yielding* **4** *and the conformations of* **1** *and* **4** ($R = C_9H_{17}O_2$)[1])

(δ 1.38) and H_a–C(14) (δ 1.09). The C(6)=C(21) bond has a pseudo-axial orientation above the average plane of ring B. As expected, H_b–C(8), H–C(15), and Me(25) deshield H_a–C(21) (δ 5.16) which appears 0.44 ppm further downfield than H_b–C(21) (δ 4.72). Consistently, H_a–C(18) (δ 3.14) and H_a–C(12) (δ 2.28) are

Table 3. *^{1}H- and ^{13}C-NMR Data* ($CDCl_3$) *of Acetyl Derivative* **2** *and OH-Addition Compound* **4**[1]). δ in ppm, J in Hz.

	2		**4**	
	δ(H)	δ(C)[a]	δ(H)	δ(C)[b])
H−C(1)	4.36 (*dddd*, *J* = 12.0, 6.0, 6.0, 6.0)	76.7	4.43 (*dddd*, *J* = 12.0, 6.0, 6.0, 6.0)	78.3
C(3)				172.9
H−C(4)	5.45 (*s*)	74.6	4.18 (*d*, *J* = 1.5)	76.0
OH−C(4)			3.07 (*d*, *J* = 1.5)	
OH−C(5)	2.69 (*s*)			77.6
C(6)				149.2
H−C(7)	3.05 (*ddd*, *J* = 12.5, 12.5, 3.0)	43.1	2.11 (*ddd*, *J* = 12.5, 12.5, 3.0)	41.4
H_a−C(8)	1.76 (*ddd*, *J* = 12.5, 3.0, 3.0)	35.4	2.12 (*ddd*, *J* = 12.5, 3.0, 3.0)	26.2
H_b−C(8)	1.38 (*ddd*, *J* = 12.5, 12.5, 12.5)		1.37 (*ddd*, *J* = 12.5, 12.5, 12.5)	
H−C(9)	2.02 (*ddd*, *J* = 12.5, 12.5, 3.0)	50.7	1.99 (*ddd*, *J* = 12.5, 12.5, 3.0)	48.4
H−C(10)	1.76 (*m*)	53.4	1.82 (*m*)	53.0
H−C(11)	5.32 (*ddd*, *J* = 7.5, 7.5, 2.5)	80.0	5.30 (*ddd*, *J* = 7.5, 7.5, 3.5)	80.0
H_a−C(12)	2.28 (*dd*, *J* = 12.0, 7.5)	47.5	2.26 (*dd*, *J* = 12.0, 7.5)	47.7
H_b−C(12)	1.17 (*m*)		1.19 (*m*)	
H_a−C(14)	1.08 (*dd*, *J* = 12.5, 12.5)	45.9	1.33 (*dd*, *J* = 12.5, 12.5)	42.5
H_b−C(14)	1.98 (*dd*, *J* = 12.5, 2.5)		1.53 (*dd*, *J* = 12.5, 2.5)	
H−C(15)	2.22 (*ddd*, *J* = 12.5, 12.5, 2.5)	45.7	1.48 (*ddd*, *J* = 12.5, 12.5, 2.5)	38.1
C(16)				79.5
H−C(17)	5.07 (*dd*, *J* = 8.5, 8.5)	116.9	1.52 (*m*)	38.0
H_a−C(18)	3.15 (*dd*, *J* = 16.0, 8.5)	21.7	1.53 (*m*)	18.9
H_b−C(18)	1.97 (*ddd*, *J* = 16.0, 8.5, 5.0)		1.53 (*m*)	
H−C(19)	2.07 (*dd*, *J* = 12.0, 8.5)	50.6	1.80 (*m*)	47.2
Me(20)	1.48 (*d*, *J* = 7.0)	19.6	1.48 (*d*, *J* = 7.0)	19.7
H_a−C(21)	5.19 (*d*, *J* = 1.0)	112.1	5.13 (br. *s*)	107.1
H_b−C(21)	4.88 (*d*, *J* = 1.0)		4.68 (br. *s*)	
H−C(22)	1.72 (*m*)	29.8	1.75 (*m*)	29.7
Me(23)	0.95 (*d*, *J* = 6.3)	24.0	1.03 (*d*, *J* = 6.3)	24.1
Me(24)	0.91 (*d*, *J* = 6.3)	22.0	0.92 (*d*, *J* = 6.3)	21.9
Me(25)	0.99 (*s*)	20.6	0.84 (*s*)	20.1
Me(26)	1.65 (*s*)	26.5	1.19 (*s*)	25.7
C(27)				172.9
H_a−C(28)	2.51 (*d*, *J* = 15.8)	45.9	2.52 (*d*, *J* = 15.8)	45.8
H_b−C(28)	2.41 (*d*, *J* = 15.8)		2.42 (*d*, *J* = 15.8)	
OH−C(29)	3.58 (*s*)		3.62 (*s*)	71.4
H_a−C(30)	1.35 (*dd*, *J* = 15.5, 7.5)	48.5	1.35 (*dd*, *J* = 15.5, 7.5)	48.4
H_b−C(30)	1.55 (*m*)		1.55 (*m*)	
H−C(31)	1.56 (*m*)	30.4	1.56 (*m*)	30.4
H_a−C(32)	1.20 (*m*)	31.2	1.21 (*m*)	31.1
H_b−C(32)	1.40 (*m*)		1.41 (*m*)	
Me(33)	0.88 (*dd*, *J* = 7.4, 7.4)	11.4	0.88 (*dd*, *J* = 7.4, 7.4)	11.4
Me(34)	0.95 (*d*, *J* = 7.4)	21.5	0.96 (*d*, *J* = 7.4)	21.5
Me(35)	1.25 (*s*)	27.5	1.26 (*s*)	27.5
AcO−C(4)	2.22 (*s*)	20.8		

[a]) Extracted from the HSQC spectrum. [b]) Extracted from the HSQC and HMBC spectra.

deshielded markedly and their resonance signals shifted downfield by as much as 1.17 and 1.11 ppm, respectively, as compared to those of H_b−C(18) (δ 1.97) and H_b−C(12) (δ 1.17), due to the interaction of H_a−C(18) with H−C(1), H_a−C(21), H_b−C(21), and H−C(15), and the interaction of H_a−C(12) with H−C(11), H−C(22), Me(23), Me(24), and Me(25).

In an attempt to explore some preliminary structure-activity relationships, the three derivatives **2**–**4** of metabolite **1** were prepared by acetylation, catalytic hydrogenation, and intramolecular alkene addition of a hydroxy group, respectively. The activity of all the three derivatives were also tested using the *in vitro* system.

The acetylated derivative **2** was formed under quite mild conditions. Its FAB-MS established a molecular mass increased by 42 with respect to **1**, indicating the acetylation of only 1 OH group. Due to the steric hindrance of both tertiary OH groups of **1**, *i.e.* OH–C(5) and OH–C(29), only the secondary one, OH–C(4), could be easily acetylated. This was confirmed by the ^{1}H- and ^{13}C-NMR data (**1**: H–C(4) at δ 4.24, C(4) at δ 74.5; **2**: H–C(4) at δ 5.45, C(4) at δ 74.6; see *Tables 1–3*).

After catalytic hydrogenation of **1**, the FAB-MS of the product **3** established a molecular mass of 578, *i.e.*, 4 amu higher than that of **1**, suggesting hydrogenation of both C=C bonds of **1**. The lack of signals for C=C bonds in the ^{1}H-NMR spectrum of **3** also supported these changes.

The intramolecular cyclization of the OH group at C(5) onto the C(16)=C(17) bond of **1** afforded an unusual addition product **4**. An unchanged molecular mass of **4** revealed that neither the lactone ring nor the ester bond were hydrolysed under the acidic conditions. The structure of **4** was established by spectroscopic means.

In comparison with the ^{1}H-NMR spectrum of **1**, the disappearance of the H–C(17) signal at δ 5.06 and the shift of Me(26) from δ 1.65 to 1.19 in **4** suggested saturation of the C(16)=C(17) bond. The lack of the OH–C(5) signal at δ 2.78 was an indication that this OH group was involved in the addition of the C=C bond. Because the unsaturation degree was unchanged, there must be five rings present in **4**, one more than in the parent compound **1**. In the HMBC spectrum of **4**, a cross-peak between Me(26) (δ 1.19) and the new quaternary C(16) (δ 79.5) provided strong evidence that an O-bridge was formed between C(5) and C(16). These changes were also confirmed by upfield shifts for related protons, *e.g.* the signals of H–C(15) and H–C(19) were shifted from δ 2.21 and 2.06 in **1** to δ 1.48 and 1.80 in **4**, resp.

The intramolecular alkene addition of OH–C(5) of **1** to C(16)=C(17) involves protonation at C(17) and a conformation which favours concerted and high-yield addition of OH–C(5) to the transient carbonium ion at C(16) to give **4**. As described in the conformation analysis (see above), the eight-membered ring B of **1** has a crown-like conformation, which is less stable than a boat-chair conformation for a simple cyclooctane. After the intramolecular alkene addition yielding **4**, the eight-membered ring adopts the favoured boat-chair conformation in which the newly formed ring B and ring C have a chair and a boat conformation, respectively (see *Fig. 2*). Due to steric crowding, the ester bond was not hydrolysed under the mild acidic conditions.

The bioassay-guided fractionation and isolation yielded the very potent GPI-anchoring inhibitor **1**. As determined by *in vitro* GPI synthesis in yeast [7], the minimal inhibitory concentration (*MIC*) of **1** is 3.4 nM. All the three derivatives **2**–**4** were also tested using the *in vitro* system for GPI synthesis using yeast microsomes. The *MIC* of **2**, **3**, and **4** were 3.2, 1.7, and 17 μM, respectively. These test results reveal that the modifications of substituents at the lactone ring or of C=C bonds of **1** caused a loss of activity by a factor of *ca*. 500 to 5000. Therefore, it seems that the substituted δ-lactone ring and the C=C bonds in **1** are essential for the very potent GPI-inhibitory activity.

3. Discussion. – The sesterterpenoids are the least common family of terpenoids although they have been isolated from a wide range of sources, such as terrestrial fungi,

plants, insects, marine sponges, and nudibranchs. Their basic skeletons are very diverse and have been classified into about thirty different C-frameworks [8]. Metabolite **1** is a tricarbocyclic sesterterpenoid. The majority of tricarbocyclic sesterterpenoids belong to the ophiobolane type, which has a C_5-C_8-C_5 tricarbocyclic skeleton and are represented by a series of ophiobolins [9]. Other known types of tricarbocyclic sesterterpenoids are exemplified by conulosins from the Australian sponge *Latrunculia convulosa* (C_6-C_6-C_6 skeleton) [10], cerorubenols from *Ceroplastes rubens* (C_6-C_7-C_7 skeleton) [11], gascardic acid from *Gascardia madagascariensis* (C_5-C_6-C_7 skeleton) [12], stellatic acid from *Aspergillus stellatus* (C_5-C_6-C_{11} skeleton) [13], floridenol from *Ceroplastes floridensis* (C_6-C_6-C_{11} skeleton) [14], and variculanol from *Aspergillus variecolor* (C_5-C_{12}-C_{15} skeleton) [15]. It is noteworthy that **1** represents a tricarbocyclic sesterterpenoid type with an unusual C_8-C_6-C_5 skeleton.

The formation of a γ-lactone or α,β-unsaturated γ-lactone moiety is particularly common in acyclic sesterterpenoids, and has also been observed in some monocyclic, bicyclic, tricyclic, and tetracyclic sesterterpenoids [16][17]. However, few sesterterpenoid δ-lactones have been reported. Although soulameolide is a C_{25} compound with an α,β-unsaturated δ-lactone, as a triterpenoid degradation product, it has been excluded from sesterterpenoids [18]. Therefore, the δ-lactone moiety in **1** is another unusual structural feature for the sesterterpenoids.

After we had finished our work on the metabolite **1** [7], we read a Japanese patent [19] in which a series of antifungal substances BE-49385 were described. One of the analogues, BE-49385A, had the same molecular formula as **1** and, the tricarbocyclic δ-lactone skeleton was depicted as its enantiomer. Nevertheless, only 2D structures were reported in the patent for the whole BE-49385 analogues without any assignment of the spectroscopic data, and their relative configuration and conformation are still unknown. The full-structure on-line literature with *Sci-Finder*® linked to the *Chemical Abstracts* databases shows that the tricarbocyclic sesterterpenoid δ-lactone **1** and all the three derivatives **2**–**4** are novel compounds.

As a very potent GPI inhibitor, compound **1** is just a minor secondary metabolite identified from the fungus *Paecilomyces inflatus*. After preliminary optimization of fermentation conditions, its yield is still only *ca*. 0.04 mg/l in the fermentation broth or 44 μg/g in the crude broth extract. Later on, another fungus, *Codinaea simplex*, was found to produce the same secondary metabolite **1** [7]. The isolation from *Codinaea simplex* gave a much higher yield, around 4.2 mg/l of **1** in the fermentation broth. Therefore, *Codinaea simplex* was used to produce large amounts of **1** for the derivatization and further biochemical evaluations. As a by-product, tetracarbocyclic sesterterpene YW3699 was also isolated from the fungus *Codinaea simplex*, which is biogenetically closely related to **1** but showed much weaker GPI-inhibitory activity with an *MIC* of 3.5 μM [7][20].

There are examples where lactone analogues of sugars have been found to be inhibitors of glycosyltransferases as they have a planar geometry like the oxonium intermediates involved in the reaction. However, it is difficult to imagine that **1** mimics the transition state of the oxonium ion from mannose as the relative configuration at the OH-substituted atoms C(4) and C(5) would be incorrect and the function of the terpene backbone would be difficult to explain unless it interacted with an accessory site on the enzyme surface not involved in substrate binding. The possibility that it

binds to the enzyme in place of Man_2-GlcN-acylPI would be more attractive, but again, a clear structural relationship is not obvious.

Further investigations have shown that **1** specifically blocks the addition of the third mannose to the intermediate structure Man_2-GlcN-acylPI and perhaps also prevents addition of an aminoethyl phosphate to the first mannose [21]. Consistent with the block in GPI synthesis, **1** prevents the incorporation of [^{3}H]*myo*-inositol into proteins, and the transport of GPI-anchored proteins to the Golgi, and is toxic. Compound **1** inhibits the same step of GPI synthesis in mammalian cells but has no significant activity in protozoa [7]. Therefore, despite the conserved core structure, the GPI biosynthetic machinery may be different enough between mammalian and protozoa to represent a target for anti-protozoan chemotherapy.

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Experimental Part

General. TLC: silica gel *60 F_{254}* precoated plates (*Merck*); *RP-18-W/UV254* precoated plates (*Merck*). Open column chromatography (CC): *Sephadex LH-20* (*Pharmacia*). MPLC: silica gel 40–63 μm (*Merck*). Anal. HPLC: *Waters-Alliance-2690* separation module with *Millennium-2020*-networked system or *Hewlett-Packard-HP-1090* system with a *HP-Chemstation*, coupled with photodiode array UV detectors; *LiChrosphere-RP-18* column (5 μm, 5 × 100 mm i.d.; *Merck*); t_R in min. Prep. 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 × 250 mm i.d.; *Merck*). IR Spectra: FT-IR spectrometer *Bruker IFS 66*; $\tilde{\nu}$ in cm^{-1}. NMR Spectra: for ^{1}H, DQ-COSY, and ROESY, *Bruker-AMX-400*, *Bruker-Avance-DMX-500*, or *Bruker-DRX-600* spectrometer at 400, 500, or 600 MHz, resp.; for ^{13}C, HSQC, and HMBC, *Bruker-Avance-DMX-500* spectrometer at 125.77 MHz; in (D_6)DMSO or $CDCl_3$; $SiMe_4$ as internal standard. MS (*m*/*z* (%)): *VG-7044SE* or *Finnigan-MAT-212* spectrometer, 8 keV Xenon with 3-nitrobenzyl alcohol or thioglycerol as matrix operating in the FAB positive-ion mode. HR-MS: *Micromass Quattro II*, electrospray positive mode with capillary 3.5 kV, cone voltage 30 V, and source temp. 70°; flow injection (loop 50 μl) with H_2O/MeCN 50 : 50, formic acid (0.05%), and ammonia (0.1%), at a flow rate of 5 μl/min; scan range 517–657 amu in 5 s; resolution 1938 (10% peak height) and 3682 (FWHH); *PEG 600*, ions $[M + NH_4]^+$ as reference compound.

Bioassay. The inhibitory activity on glycosylphosphatidylinositol (GPI) synthesis was evaluated using an *in vitro* GPI synthesis system with yeast microsomes, by Western-blot analysis or by halo assay. Detailed procedures are described in [7].

Fermentation. The fungal strain was tentatively identified as *Paecilomyces inflatus* (BURNSIDE) CARMICHAEL. Precultures from one frozen ampoule of *Paecilomyces inflatus* (F/92-802010) were grown in 200 ml of medium composed of *Bacto* agar (0.1%), yeast extract (*Gistex-X-II*; 0.4%), and malt extract (*Wander*; 2%) in deionized H_2O (pH adjusted to 5.5 before sterilization at 121° for 25 min) in conical flasks (500 ml). The inoculated flasks were incubated on a rotary shaker (200 rpm) at 21° for 7 days. After a further culture in 1 l of the same medium in 2 l of conical flasks for another week, the seed cultures were inoculated into a 50-l bioreactor containing the same medium. The cultures were fermented at 21° for 7 days. The bioreactor content 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 cultures were transferred into a 500-l bioreactor containing the production medium composed of *Bacto* agar (0.1%), $CaCl_2 \cdot 6\ H_2O$ (0.002%), K_2HPO_4 (0.01%), $MgSO_4 \cdot 7\ H_2O$ (0.005%), BBL yeast extract (0.1%), and peptone (*Pharmatone*; 2%) in deionized H_2O (pH adjusted to 6.5 with KOH and H_2SO_4 before sterilization at 121° for 25 min). The cultures were fermented at 21° for 6 days. The bioreactor content was stirred at 100 rpm (pressure 0.5 bar, antifoam *M13*), and air was introduced at a rate of 1 l/min per l of medium.

Extraction. The fermentation broth (500 l) and AcOEt (500 l) were homogenized with a *Dispax* reactor for 2 h and agitated for 3 h at r.t. The org. phase was separated from extracted broth with a *Westfalia* separator. The extraction was repeated once with the same procedure and afforded a total of 470 g of AcOEt extract.

Isolation. The extract (470 g) was subjected to gel filtration (10 kg of *Sephadex LH-20*, MeOH). *Fr. 8* (6.2 g) from the *Sephadex* column was further chromatographed by MPLC (500 g SiO_2, gradient hexane/i-PrOH/AcOH 99:1:0.5 →80:20:0.5 in 80 min) *Fr. 32–35* of the MPLC column were combined (218 mg) and purified again by prep. HPLC (*RP-18*, 50 × 250 cm i.d., gradient MeCN/H_2O 50:50 →100:0 in 80 min, 40 ml/min), yielding 20 mg of **1** from *Fr. 51* of the HPLC column.

3-Hydroxy-3,5-dimethylheptanoic Acid rel-*(1*R,*4*R,*4a*R,*7a*R,*8a*R,*10*S,*11*S,*11a*R,*12a*R,*13a*R*)-1,2,4,4a,5,7a,8,8a,9,10,11,11a,12,12a,13,13a-Hexadecahydro-1,13a-dihydroxy-4,7-dimethyl-13-methylene-11-(1-methylethyl)-2-oxoindeno[5′,6′:4,5]cycloocta[1,2-c]pyran-10-yl Ester* (**1**). White powder. TLC (SiO_2): R_f 0.24 (hexane/i-PrOH/AcOH 90:10:0.1), 0.32 (toluene/i-PrOH 95:5). HPLC (*RP-18*, gradient MeCN/H_2O 0:100 →100:0 in 15 min, then MeCN/H_2O 100:0 for 5 min): t_R 12.9. HPLC (*RP-18*, gradient MeCN/H_2O 60:40 →100:0 in 10 min, then MeCN/H_2O 100:0 for 5 min): t_R 6.49. UV (MeOH): end absorption. IR (KBr): 3462*m* (OH), 2962*s*, 2932*s*, 2874*m* (aliph. CH), 1734*s* (C=O, δ-lactone), 1705*s* (C=O), 1464*m*, 1381*m*, 1337*m*, 1277*m*, 1198*s*, 1123*s*, 1086*w*, 1045*w*, 979*w*. ^{1}H-NMR, DQ-COSY, ROESY ($CDCl_3$): *Table 1*. ^{13}C-NMR, HSQC, HMBC ($CDCl_3$): *Table 2*. FAB-MS (pos.): 597 (47, $[M+Na]^+$), 575 (67, $[M+H]^+$, $C_{34}H_{55}O_7^+$), 557 (8, $[M+H-H_2O]^+$), 401 (92, $[M-C_9H_{17}O_3]^+$), 383 (100, $[M-C_9H_{17}O_3-H_2O]^+$), 365 (29, $[M-C_9H_{17}O_3-2H_2O]^+$), 355 (23). FAB-MS (+LiI, pos.): 597 (20, $[M+Na]^+$), 581 (100, $[M+Li]^+$). ES-HR-MS (pos.): $[M+H]^+$ 575.3939 (11 measurements with standard deviation 1.7 mmu, $[M+H]^+$; calc. 557.3842; $\Delta = -0.9$ mmu (−1.6 ppm)).

3-Hydroxy-3,5-dimethylheptanoic Acid rel-*(1*R,*4*R,*4a*R,*7a*R,*8a*R,*10S*,*11*S,*11a*R,*12a*R,*13a*R*)-1-(Acetyloxy)-1,2,4,4a,5,7a,8,8a,9,10,11,11a,12,12a,13,13a-hexadecahydro-13a-hydroxy-4,7-dimethyl-13-methylene-11-(1-methylethyl)-2-oxoindeno[5′,6′:4,5]cycloocta[1,2-c]pyran-10-yl Ester* (**2**). A mixture of **1** (14.5 mg) and dry pyridine (0.5 ml) was treated at r.t. for 15 h with freshly distilled Ac_2O (0.5 ml), then poured into ice-water, and extracted 3 times with AcOEt. The AcOEt phase was washed 3 times with H_2O and evaporated: 11 mg of **2**. White powder. TLC (SiO_2, toluene/i-PrOH 95:5): R_f 0.27. ^{1}H-NMR ($CDCl_3$), ^{13}C-NMR (HSQC, $CDCl_3$): *Table 3*. FAB-MS (pos.): 617 (12, $[M+H]^+$, $C_{36}H_{57}O_8^+$), 599 (5, $[M+H-H_2O]^+$), 576 (6), 443 (26), 425 (100, $[M-C_9H_{17}O_3-H_2O]^+$), 401 (15), 383 (64), 365 (58). FAB-MS (+LiI, pos.): 623 (100, $[M+Li]^+$).

3-Hydroxy-3,5-dimethylheptanoic Acid rel-*(1*R,*4*R,*4a*R,*7a*R,*8a*R,*10*S,*11*S,*11a*R,*12a*R,*13a*R*)-1,2,4,4a,5,6,7,7a,8,8a,9,10,11,11a,12,12a,13,13a-Octadecahydro-1,13a-dihydroxy-4,7,13-trimethyl-11-(1-methylethyl)-2-oxoindeno[5′,6′:4,5]cycloocta[1,2-c]pyran-10-yl Ester* (**3**). A soln. of **1** (14.4 mg) in EtOH (3 ml) was stirred under H_2 with 10% Pd/C for 7 h. The catalyst was filtered off over talc and washed with EtOH and AcOEt, the filtrate evaporated, and the residue purified by prep. HPLC (*RP-18*, MeCN/H_2O 60:40, isocratic): **3** (7 mg). White powder. TLC (SiO_2, toluene/i-PrOH 95:5): R_f 0.27. FAB-MS (pos.): 601 (6, $[M+Na]^+$), 579 (12, $[M+H]^+$, $C_{34}H_{59}O_7^+$), 405 (100, $[M-C_9H_{17}O_3]^+$), 387 (65). FAB-MS (+LiI, pos.): 578 (100, $[M+Li]^+$).

3-Hydroxy-3,5-dimethylheptanoic Acid rel-*(1*R,*4*R,*4a*R,*7*S,*7a*R,*8a*R,*10*S,*11*S,*11a*,R,*12a*R,*13a*R*)-1,2,4a,5,6,7,7a,8,8a,9,10,11,11a,12,12a,13-Hexadecahydro-1-hydroxy-4,7-dimethyl-13-methylene-11-(1-methylethyl)-2-oxo-4*H-*7,13a-epoxyindeno[5′,6′:4,5]cycloocta[1,2-c]pyran-10-yl Ester* (**4**). A soln. of **1** (15.2 mg) in EtOH (5 ml) and 1N HCl (5 ml) were refluxed. The reaction was checked by TLC after 30 and 75 min, then stopped after 105 min. EtOH was evaporated, the aq. phase extracted 3 times with AcOEt, the AcOEt phase washed 4 times with H_2O and evaporated, and the residue purified by prep. HPLC (*RP-18*, MeCN/H_2O 65:35, isocratic): **4** (9.5 mg). White powder. TLC (SiO_2, toluene/i-PrOH 95:5): R_f 0.51. ^{1}H-NMR ($CDCl_3$), ^{13}C-NMR (HSQC, $CDCl_3$): *Table 3*. FAB-MS (pos.): 575 (10, $[M+H]^+$, $C_{34}H_{55}O_7^+$), 460 (8), 412 (4), 401 (100, $[M-C_9H_{17}O_3]^+$), 383 (5). FAB-MS (+LiI, pos.): 581 (100, $[M+Li]^+$).

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