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ANTIBACTERIAL PHLOROGLUCINOLS AND FLAVONOIDS FROM *HYPERICUM BRASILIENSE*

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Key Word Index—*Hypericum brasiliense*; Guttiferae; phloroglucinols; hyperbrasilol A; antibacterial activity; *Bacillus subtilis*; flavonoids.

Abstract—Three known phloroglucinols (japonicine A, uliginosin A and isouliginosin B) and a new phloroglucinol (hyperbrasilol A) have been isolated from a petrol extract of the leaves and flowers of *Hypericum brasiliense*. Their structures were established by spectroscopic methods (UV, DCI-MS, ^{1}H and ^{13}C NMR, including SINEPT, HMBC, HSQC, DQFCOSY experiments). The substitution pattern of hyperbrasilol A was confirmed by X-ray crystallography. All four phloroglucinols were antibacterial against *Bacillus subtilis* in a TLC bioautographic assay. The flavonoids, kaempferol, luteolin, quercetin, quercitrin, isoquercitrin, hyperoside and guaijaverin, were isolated from a methanol extract of the same organs.

INTRODUCTION

Plants of the Guttiferae contain xanthones and other constituents with very diverse biological activities [1]. Recently, the genus *Hypericum* has been receiving attention for the putative anti-retroviral effects of hypericin from *H. perforatum* [2]. Different species of *Hypericum* are active against Gram-positive bacteria *in vitro* and it was shown that phloroglucinol and filicinic acid derivatives were responsible for these effects [3].

Extracts of *Hypericum brasiliense* from the south-east of Brazil have been found to inhibit monoamine oxidases (MAO), enzymes which are important in the regulation of some physiological amines and are thought to contribute to the management of depression. Three xanthones isolated from the stems and roots of *H. brasiliense* were found to be inhibitors of MAO [4]. The xanthones and a new γ-pyrone derivative exhibited antifungal activity. Continuing the search for the bioactive compounds from *H. brasiliense*, the isolation of four phloroglucinol derivatives and of flavonoids is reported here.

RESULTS AND DISCUSSION

Leaves and flowers of *Hypericum brasiliense* were extracted successively with petrol, dichloromethane and methanol. The petrol extract showed activity against the Gram-positive bacterium, *Bacillus subtilis*, in a bioautographic TLC assay [5]. After evaporation to dryness, this extract was treated with acetone to obtain an acetone-soluble fraction and an insoluble fatty residue. Fractionation of the bioactive acetone-soluble fraction by a combination of silica gel column chromatography, gel filtration on Sephadex LH-20, centrifugal partition chromatography (CPC) and recrystallization provided compounds **1**–**4**, sitosterol and stigmasterol. Separation of the methanol extract by repeated chromatography over Sephadex LH-20 afforded the flavonoid aglycones, kaempferol, luteolin and quercetin, together with the glycosides, isoquercitrin, hyperoside, guaijaverin and quercitrin (see Experimental).

The yellow derivatives **2**–**4** appeared as red-coloured spots on silica gel TLC plates when sprayed with Godin's reagent [6], while **1** remained colourless. The DCI mass spectrum of compound **1** gave a $[M + H]^+$ at *m/z* 461, thus indicating a M_r of 460. The NMR data revealed a dimeric structure consisting of two acyl filicinic acid moieties linked by a methylene bridge. Compound **1** proved to be identical with japonicine A (= albaspidin iBiB) which has already been found in *H. japonicum* [7] and *Dryopteris subtriangularis* [8]. Compounds **2**–**4** contained both filicinic acid and phloroglucinol moieties. The DCI mass spectrum of **2** exhibited a $[M + H]^+$ at *m/z* 501 and the compound was identified from its NMR and mass spectral data as uliginosin A, a metabolite previously isolated from *H. uliginosum* [9]. The M_r of compound **3** was deduced to be 498 since the DCI mass

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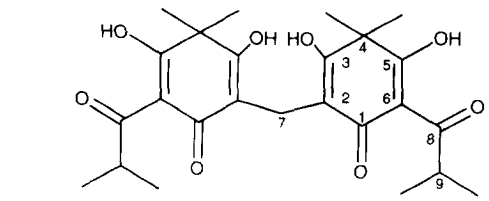

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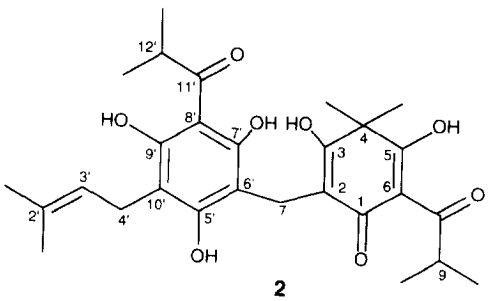

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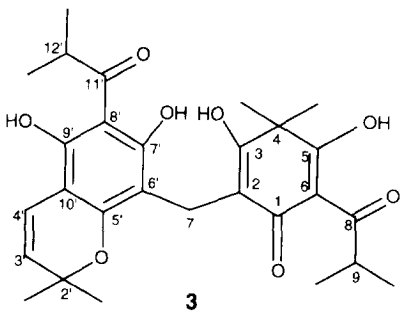

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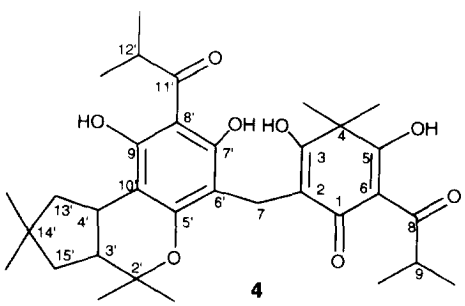

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Table 1. ^{13}C NMR spectral data of compounds **1**–**4** (multiplicities in parentheses)*

C	1	2†	3	4‡
1	199.8 (*s*)	198.9 (*s*)	199.1 (*s*)	200.1 (*s*)
2	107.1 (*s*)	106.5 (*s*)[a]	106.9 (*s*)[a]	107.9 (*s*)
3	173.3 (*s*)	182.0 (*s*)	170.9 (*s*)	172.0 (*s*)
4	44.5 (*s*)	51.9 (*s*)§	44.1 (*s*)	44.8 (*s*)
4-Me	24.3 (*q*)	24.6 (*q*)	24.5 (*q*)	24.9 (*q*)
	25.4 (*q*)	24.6 (*q*)	25.0 (*q*)	25.1 (*q*)
5	187.4 (*s*)	198.9 (*s*)	187.2 (*s*)	188.2 (*s*)
6	110.8 (*s*)	107.5 (*s*)[a]	111.3 (*s*)	112.2 (*s*)
7	18.2 (*t*)	18.0 (*t*)	17.0 (*t*)	18.0 (*t*)
8	210.6 (*s*)	209.9 (*s*)	211.9 (*s*)	211.6 (*s*)
9	36.6 (*d*)	32.2 (*d*)	36.8 (*d*)	37.4 (*d*)
9-Me	18.7 (*q*)	19.2 (*q*)	18.9 (*q*)[b]	19.6 (*q*)
	19.3 (*q*)	19.2 (*q*)	18.9 (*q*)[b]	19.7 (*q*)
2′		129.1 (*s*)	80.5 (*s*)	84.3 (*s*)
2′-Me		17.5 (*q*)	27.7 (*q*)	19.9 (*q*)
		25.3 (*q*)	27.7 (*q*)	28.2 (*q*)
3′		123.6 (*d*)	124.1 (*d*)	51.6 (*d*)
4′		21.4 (*t*)	117.1 (*d*)	36.5 (*d*)
5′		157.4 (*s*)[b]	154.6 (*s*)[c]	157.2 (*s*)
6′		103.7 (*s*)[a]	104.9 (*s*)[a]	105.6 (*s*)[a]
7′		160.8 (*s*)[b]	160.1 (*s*)[c]	158.7 (*s*)
8′		102.7 (*s*)[a]	105.7 (*s*)[a]	105.8 (*s*)[a]
9′		161.3 (*s*)[b]	160.4 (*s*)[c]	164.3 (*s*)
10′		104.6 (*s*)[a]	102.1 (*s*)[a]	106.4 (*s*)
11′		209.9 (*s*)	210.9 (*s*)	212.2 (*s*)
12′		38.0 (*d*)	39.5 (*d*)	40.0 (*d*)
12′-Me		19.2 (*q*)	19.2 (*q*)[b]	19.1 (*q*)
		19.2 (*q*)	19.2 (*q*)[b]	19.1 (*q*)
13′				47.3 (*t*)
14′				37.8 (*s*)
14′-Me				32.2 (*q*)
				32.5 (*q*)
15′				41.9 (*t*)

*Spectra recorded at 50 MHz in $CDCl_3$ (**1** and **3**), DMSO-d_6 (**2**) or acetone-d_6 (**4**).

†Only two signals observed in the region δ 190–210. It is assumed that the signal at 198.9 can be attributed to C-1 and C-5, while the signal at δ 209.9 can be attributed to C-8 and C-11′. All four resonances were observed in the $CDCl_3$ spectrum at δ 211.3, 210.8, 199.3 and 171.6.

‡Assignments supported by HMBC and HSQC data.

§Tentative assignment (low intensity); signal observed at δ 44.2 in $CDCl_3$.

[a–c]Values with same superscripts in each column are interchangeable.

spectrum gave a $[M + H]^+$ at *m/z* 499. This difference of 2 mu when compared with **2** was the result of cyclisation of the prenyl chain on C-10′ to build a 2,2-dimethyl chromene unit. Based on NMR data, in particular the OH-7′ (δ 11.69) and OH-9′ (δ 14.14) resonances, **3** was identified as isouliginosin B, a compound which has been obtained as a by-product in the synthesis of uliginosin B [10]. The reliability of the hydroxyl ^{1}H NMR signals for the determination of the orientation of the cyclisation has been recently confirmed by SINEPT experiments on drummondin C and isodrummondin C [11]. Isouliginosin B has not been previously isolated from a natural source. Since the complete ^{13}C NMR data of **1**–**3** have not been reported, they are listed in Table 1. Compound **4** had a M_r of 568, apparent from a quasimolecular ion at *m/z* 569 ($[M + H]^+$) in the DCI-mass spectrum. The molecular formula was deduced to be $C_{33}H_{44}O_8$. The ^{1}H NMR spectrum run at 500 MHz gave hydroxyl resonances at δ 9.42, 11.46, 14.07 and 18.80 which strongly suggested the same cyclization pattern as in **3**. The occurrence of two *iso*butyryl chains was inferred from multiplets in the ^{1}H-NMR spectrum at δ 4.16 and 4.08 coupling with doublets (J = 7) at δ 1.17 and 1.15, respectively. Six quaternary methyl groups were further detected as five singlets at δ 1.59, 1.52 (2×), 1.31, 1.16 and 1.07. Finally, a spin system due to two CH_2 and two CH of a cycle could be fully resolved by the use of pulsed-field-gradient DQFCOSY [12] and HSQC [13] experi-

ments. The ^{13}C NMR data confirmed that **4** contained a filicinic acid moiety identical to that found in **1**–**3**. In particular, the characteristic chemical shift of C-4 (δ 44.8) demonstrated the presence of a *gem*-dimethyl group attached to this carbon. A pulsed-field-gradient HMBC experiment [14] enabled, in conjunction with the DQFCOSY and HSQC spectra, complete characterization of the cyclopentane ring fused to the pyran heterocycle. HMBC correlations within the dimethylchromene moiety are shown in Fig. 1. ^{13}C and ^{1}H NMR spectral data of **4** are given in Tables 1 and 2, respectively. The structure of **4** was definitively confirmed by a single crystal X-ray analysis. Suitable crystals were grown from methanol. The two halves of the molecule, folded about atom C-7, are reasonably planar probably due to the formation of relatively strong intra-molecular hydrogen bonds (Fig. 2). It was not possible to determine the absolute configuration of the molecule; the relative configuration is illustrated by the perspective view of the molecule given in Fig. 2. Compound **4**, which has been named hyperbrasilol A, is a new phloroglucinol derivative.

The isolated compounds **1**–**4** were inhibitory to *B. subtilis* in a bioautography assay [5] (Table 3). The most active, **3**, was antibacterial at 0.16 μg, while the reference substance, ampicillin, inhibited growth of the Gram-positive bacterium at 0.01 μg. Apolar extracts of *Hypericum* species have already been shown to possess antibiotic activity [15]. The phloroglucinols, uliginosins A and B, from *Hypericum uliginosum* were active against *Staphylococcus aureus* and antifungal against *Trichophyton mentagrophytes* [16]. Drummondins A–F and isodrummondin D from *H. drummondii* were inhibitory to *S. aureus*, *B. subtilis* and *Mycobacterium smegmatis*, with activities comparable to or greater than streptomycin. Since these analogues have, in addition, shown cytotoxic activity in cultured P-388, KB and human cancer cell lines (breast, colon, lung, melanoma) [17, 18] it will be of interest to test **1**–**4** for possible cytotoxic effects.

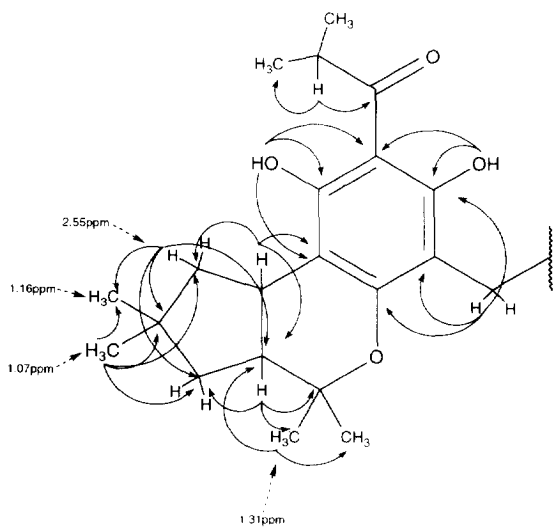

Fig. 1. Long-range heteronuclear correlations within the phloroglucinol moiety of compound **4** in a HMBC experiment. Correlations from overlapping signals have not been included.

EXPERIMENTAL

General. Mps: uncorr. ^{1}H and ^{13}C NMR: 25° at 200 and 50 MHz, respectively. ^{1}H, pulsed-field-gradient DQFCOSY, HSQC and HMBC NMR spectra of **4** were recorded at $-10°$ at 500 MHz. Distinction of carbon multiplicities used DEPT sequences. ^{1}H chemical shifts relative to TMS; ^{13}C chemical shifts referenced to solvent peak. TLC: silica gel precoated Al sheets and RP-18 HPTLC glass plates (Merck). CPC: Pharma-Tech CCC-1000, 2.6 mm i.d. Teflon coils, capacity 660 ml (Pharma-Tech, Baltimore, MD, U.S.A.). DCIMS: positive ion mode, NH_3 as reactant gas, EIMS: 70 eV.

Plant material. Hypericum brasiliense Choisy was collected in Nova Friburgo, Brazil, in March 1991. A herbarium specimen (number RFA 23.257) is deposited at the Botany Department, Institute of Biology (Federal University of Rio de Janeiro, Brazil).

Extraction and isolation. Ground, air-dried leaves and flowers (1.31 kg) were extracted successively at room temp. with petrol, CH_2Cl_2 and MeOH. After evapn to dryness, the petrol extract was treated with Me_2CO to afford an Me_2CO-sol. fr. (32 g) and an insol. fatty residue (11.8 g). A portion (17.5 g) of the Me_2CO-sol. fr. was separated by gel filtration over Sephadex LH-20 with $CHCl_3$–MeOH (1:1) to give 3 frs (A–C). Fr. B, which exhibited antibacterial properties, was further fractionated by CC on silica gel with $CHCl_3$–MeOH mixts of increasing polarity ($CHCl_3$–MeOH 1:0 → 4:1) to yield 7 frs (I–VII). Fr. III (792 mg) afforded **4** (4.5 mg) after CPC with hexane–MeCN–MeOH (8:5:2, upper phase as mobile phase) followed by CC on silica gel with hexane–EtOAc–MeOH–H_2O (10:5:5:1, upper phase). Repeated recrystallizations of fr. IV (2.1 g) gave **3** (135 mg) and subsequently **1** (5 mg). Fr. VII was separated using two CPC steps with hexane–EtOAc–MeOH–H_2O (10:5:5:1, upper phase as mobile phase) and hexane–MeCN–MeOH (40:25:10, upper phase as mobile phase), respectively. Final purification over Sephadex LH-20 with $CHCl_3$–MeOH (1:1) afforded **2** (38 mg). In addition, recrystallization of fr. VI gave a mixt. (105 mg) of stigmasterol and sitosterol.

The MeOH extract (30.0 g) was subjected to gel filtration over Sephadex LH-20 with MeOH to give 10 frs (I–X). Further chromatography over Sephadex LH-20 of fr. II with MeOH afforded isoquercitrin (115 mg), guaijaverin (24 mg) and quercitrin (5 mg). Hyperoside (513 mg), luteolin (36 mg), kaempferol (5 mg) and quercetin (315 mg) were obtained by the same procedure from frs III, V, VII and IX, respectively. Flavonoids were identified by comparing their spectroscopic (UV, DCI-MS, ^{1}H and ^{13}C NMR) and chromatographic (TLC and HPLC) properties with those of authentic samples. Identities of sugars were confirmed after acidic hydrolyses of the glycosides by comparison with ref. samples.

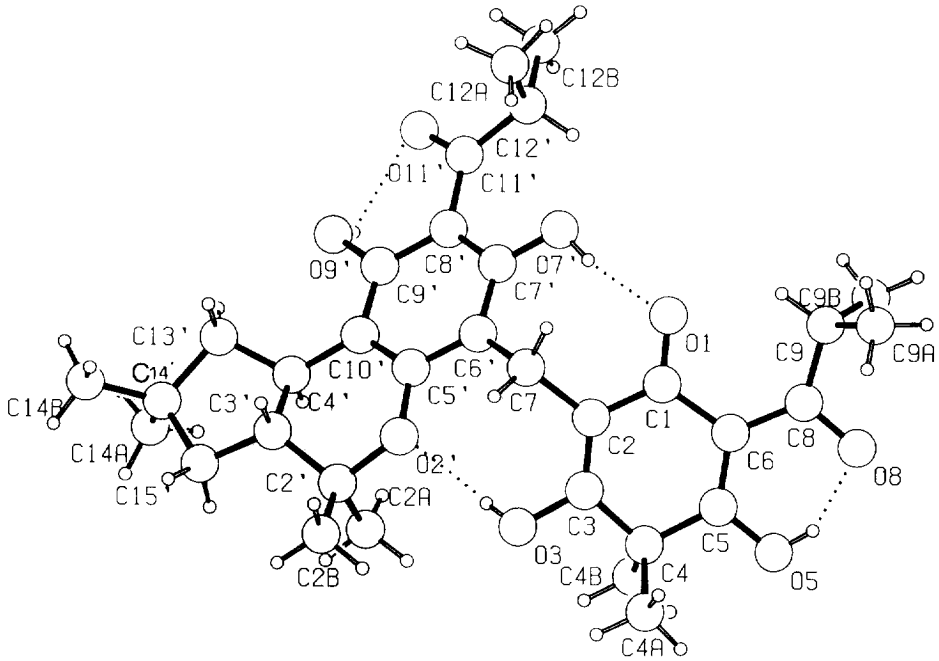

Fig. 2. Perspective view of compound **4**.

Table 2. ^{1}H NMR spectral data of compounds **2**–**4** (*J* values in Hz)*

H	2	3	4†
4-Me	1.45 (*br s*)	1.50 (*s*)	1.52 (*s*)
	1.51 (*br s*)		
7	3.53 (*br s*)	3.52 (*br m*)	3.58 (*d J* = 17)
			3.48 (*d J* = 17)
9	4.19 (*sep J* = 7)	4.19 (*sep J* = 7)	4.16 (*m*)
9-Me	1.17 (*d J* = 7)[a]	1.17 (*d J* = 7)	1.17 (*d J* = 7)[a]
2′-Me	1.79 (*s*)	1.55 (*s*)	1.31 (*s*)
	1.85 (*s*)		1.59 (*s*)
3′	5.22 (*pseudo t J* = 7)	5.46 (*d J* = 10)	1.92 (*m*)
4′	3.45 (*d J* = 7)	6.71 (*d J* = 10)	2.66 (*ddd J* = 13, 13, 6)
12′	3.92 (*sep J* = 7)	4.05 (*sep J* = 7)	4.08 (*m*)
12′-Me	1.18 (*d J* = 7)[a]	1.17 (*d J* = 7)	1.15 (*d J* = 7)[a]
13′			1.24 (*m*)
			2.55 (*dd J* = 13, 6)
14′-Me			1.07 (*s*)
			1.16 (*s*)
15′			1.22 (*m*)
			1.58 (*m*)
3-OH	10.10 (*br s*)	9.05 (*s*)	9.42 (*s*)
5-OH	18.78 (*br s*)	18.80 (*s*)	18.80 (*s*)
5′-OH	11.50 (*br s*)		
7′-OH	16.18 (*br s*)	11.69 (*s*)	11.46 (*s*)
9′-OH	6.40 (*br s*)	14.14 (*s*)	14.07 (*s*)

* Spectra recorded at 200 MHz in $CDCl_3$ (**2** and **3**) or 500 MHz in acetone-d_6 (**4**).
† Attributions supported by DQFCOSY, HSQC and HMBC data.
[a] These attributions can be reversed.

Japonicine A (*albaspidin iBiB*, **1**). TLC (silica gel, $CHCl_3$): R_f 0.70. HPTLC (RP-18, MeCN): R_f 0.17. UV λ^{MeOH} nm (log ε): 341 (4.26), 221 (4.42). ^{13}C NMR: see Table 1. EI-MS *m/z* (rel. int.): 460 ($[M]^+$, 100), 445 (36), 417 (16), 235 (17), 209 (45), 182 (45), 165 (13), 69 (33). DCI-MS *m/z*: 461 ($[M + H]^+$), 239, 172, 136, 105.

Uliginosin A (**2**). TLC (silica gel, $CHCl_3$–MeOH, 7:3): R_f 0.45. HPTLC (RP-18, MeCN): R_f 0.55. UV

Table 3. Antibacterial activities of phloroglucinols from *Hypericum brasiliense* against *Bacillus subtilis*

Sample	Activity* (μg)
Petrol extract	2
Dichloromethane extract	> 100
Methanol extract	> 100
1	0.5
2	0.2
3	0.16
4	0.32
Ampicillin	0.01
Chloramphenicol	0.001

*Minimum amount required to inhibit growth of *B. subtilis* in TLC bioassay.

λ^{hexane} nm: 290, 230. ^{13}C and ^{1}H NMR: see Tables 1 and 2. EI-MS *m/z* (rel. int.): 500 (M^+, 70), 457 (28), 445 (12), 401 (5), 277 (18), 264 (49), 221 (100), 209 (33), 165 (50), 153 (11), 121 (7), 69 (18). DCI-MS *m/z*: 501 ($[M + H]^+$).

Isouliginosin B (**3**). Crystals from MeCN, mp 152–154°. $[\alpha]_D$: − 34° ($CHCl_3$, *c* 0.025). TLC (silica gel, $CHCl_3$): R_f 0.48. HPTLC (RP-18, MeCN): R_f 0.16. UV λ^{MeOH} nm (log ε): 355 (4.15), 315 (4.18), 277 (4.41), 224 (4.33). ^{13}C and ^{1}H NMR: see Tables 1 and 2. EI-MS *m/z* (rel. int.): 498 ($[M]^+$, 81), 483 (34), 455 (14), 275 (23), 262 (40), 247 (100), 219 (33). DCI-MS *m/z*. 499 ($[M + H]^+$), 263.

Hyperbrasilol A (**4**). Crystals from MeOH, mp 151–152°. $[\alpha]_D$: + 23° ($CHCl_3$, *c* 0.025). TLC (silica gel, $CHCl_3$): R_f 0.59. HPTLC (RP-18, MeCN): R_f 0.12. UV λ^{MeOH} nm (log ε): 355 (3.96), 306 (4.10), 224 (*sh.*, 4.12), 209 (4.18). IR ν^{KBr} cm^{-1}: 3180 (*br*), 2915, 1640, 1620, 1610, 1580, 1430. ^{13}C and ^{1}H NMR: see Tables 1 and 2. EI-MS *m/z* (rel. int.): 568 ($[M]^+$, 0.4), 332 (19), 289 (100), 209 (47), 193 (47), 179 (67), 165 (67), 150 (67), 111 (54), 95 (47), 71 (41), 69 (98). DCI-MS *m/z*: 569 ($[M + H]^+$), 461, 333, 180.

Antibacterial testing. Tests were carried out against *Bacillus subtilis* ATCC 6633 using bioautography [5] on silica gel glass-backed plates.

Crystallographic data for compound **4**. $C_{33}H_{44}O_8 \cdot 0.3CH_3OH$, formula weight 578.3, orthorhombic, $P2_12_12_1$, $a = 6.035(1)$, $b = 21.475(4)$, $c = 25.840(4)$ Å, $V = 3348.9(10)$ Å^3, $Z = 4$, $D_x = 1.147$ g cm^{-3}, $\lambda = 0.71073$ Å, $\mu = 0.081$ mm^{-1}, F(OOO) = 1246. 2559 unique reflections, 2541 reflections for refinement, 403 variables, 0 = restraints, $R1 = 0.094$, $wR2 = 0.206$ (for 1165 reflections with $I > 2\sigma(I)$); $R1 = 0.228$, $wR2 = 0.364$ (all data). Max shift/sigma ratio 0.004, residual density (e/Å^3) max. 0.29, min. − 0.22. Intensity data were collected at room temp. on a Stoe AED2 4-circle diffractometer using MoK_α graphite monochromated radiation and ω/θ scans out to 45° in 2θ. The structures were solved by Direct Methods using the programme SHELXS-90 [19] and refined using the programme SHELXL-93 [20]. Neutral complex-atom scattering factors are from ref. [21]. The H-atoms were included in calculated positions. The hydroxyl H-atoms, HO-3, HO-5, HO-7′ and HO-9′, were located using the HFIX 147 command. The non-hydrogen atoms were refined anisotropically. The refinement method was full-matrix least-squares on F^2. The crystal did not diffract significantly beyond 35° in 2θ and there are very few reflections per parameter. Parts of the molecule, the *iso*propyl groups, for example, undergo considerable thermal motion. A region of disordered solvent, $0.3CH_3OH$, was located in a final difference map and the SWAT command was included in the refinement; the *g* variable refined to a value of 2.609.

Considering the poor quality of the crystal, the bond distances and angles are normal within experimental error. There are four intra-molecular hydrogen bonds involving hydroxyls O-3, O-5, O-7′ and O-9′. There are no short intermolecular (< 3.2 Å) contacts between non H-atoms in the crystal. Atomic parameters and complete tables of bond distances and angles have been deposited at the Cambridge Crystallographic Data Centre (Cambridge, UK). The numbering scheme used is illustrated in the PLUTON [22] plot, see Fig. 2. Further details may been obtained from the authors.

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