Oleanane-Type Triterpenoids from the Endophytic Fungus *Pestalotiopsis clavispora* Isolated from the Chinese Mangrove Plant *Bruguiera sexangula*

by **Du-Qiang Luo***[a]), **Hui-Ying Deng**[a]), **Xiao-Long Yang**[b]), **Bao-Zhong Shi**[a]), and **Jing-Ze Zhang***[c])

[a]) College of Life Science, Key Laboratory of Medicinal Chemistry and Molecular Diagnosis of Ministry of Education, Hebei University, Baoding 071002, P. R. China (phone/fax: +86-312-5079364; e-mail: duqiangluo@163.com)

[b]) College of Pharmaceutical Science, Hebei University, Baoding 071002, P. R. China

[c]) Key Laboratory of Molecular Biology of Crop Pathogens and Insects, Ministry of Agriculture, Institute of Biotechnology, College of Agriculture & Biotechnology, Zhejiang University, Hangzhou 310029, P. R. China

Three new triterpenoid derivatives, named (15α)-15-hydroxysoyasapogenol B (**1**), (7β,15α)-7,15-dihydroxysoyasapogenol B (**2**), and (7β)-7,29-dihydroxysoyasapogenol B (**3**), were isolated from cultures of the plant endophytic fungus *Pestalotiopsis clavispora*. Their structures and relative configurations were elucidated by extensive spectroscopic analysis and X-ray crystallography.

Introduction. – Fungi of the genus *Pestalotiopsis* are known as endophytes of tropical higher plants [1][2], which are common in their distribution, and many are saprobes, while others are either pathogenic or endophytic to living plants [3]. *Pestalotiopsis clavispora*, belonging to the genus *Pestalotiopsis* (Amphisphaeriaceae), was isolated from the plant *Bruguiera sexangula* collected from Dongzhai, Hainan Province, P. R. China. Since the discovery of the anticancer agent taxol from an endophytic fungal strain of the genus *Pestalotiopsis* [4][5], the interest in bioactive compounds from this fungal genus has increased considerably. A previous chemical investigation of the genus *Pestalotiopsis* has revealed that they can produce various bioactive natural products such as ambuic acid, torreyanic acid, pestaloside, pestalotiopsins, *etc.* [6–12]. The diverse structures and activities of this genus prompted us to undertake further phytochemical investigations of *Pestalotiopsis clavispora*. As a result, the three new triterpenoid derivatives **1**–**3** (*Fig. 1*) were isolated from the culture broth of *Pestalotiopsis clavispora*. The structures of the new compounds were established by comprehensive spectroscopic analysis, X-ray-diffraction analysis, and by comparison of their NMR data with those of known related compounds. This article describes the structural characterization of these new metabolites.

Results and Discussion. – Compound **1** was assigned the molecular formula $C_{30}H_{50}O_4$, on the basis of its HR-ESI-MS ($[M+Na]^+$ at *m*/*z* 497.36136) and NMR data (*Table 1*), with six degrees of unsaturation. The ^{1}H-NMR spectrum of **1** (*Table 1*) showed seven Me *s* at δ(H) 0.88, 0.94, 1.02, 0.79, 0.86, 0.97, and 1.08, one olefinic H-atom at δ(H) 5.26 (*dd*, J = 3.4, 6.6 Hz), and five H-atoms attached to O-bearing C-atoms at δ(H) 3.19 (*dd*, J = 4.3, 9.0 Hz), 3.94 (br. *s*), 3.22 (*dd*, J = 3.2, 6.0 Hz), 3.83 (*d*,

Table 1. *NMR Data* ((D$_6$)DMSO) *of* **1**. δ in ppm, J in Hz.

	δ(H)[a]	δ(C)[b]		δ(H)[a]	δ(C)[b]
CH_2(1)	1.53–1.56 (*m*), 0.86–0.90 (*m*)	38.4	CH_2(16)	1.24 (*d*, *J* = 9.8), 1.63 (br. *s*)	39.0
CH_2(2)	1.25–1.28 (*m*), 1.31–1.35 (*m*)	27.3	C(17)		37.3
H–C(3)	3.19 (*dd*, *J* = 4.3, 9.0)	78.6	H–C(18)	1.97 (br. *s*)	45.4
C(4)		42.0	CH_2(19)	0.92 (*s*), 1.69 (*s*)	45.7
H–C(5)	0.75 (br. *s*)	55.2	C(20)		30.2
CH_2(6)	1.30–1.34 (*m*), 1.48–1.52 (*m*)	18.8	CH_2(21)	1.27 (*s*), 1.34 (*d*, *J* = 4.6)	41.1
CH_2(7)	0.73 (br. *d*, *J* = 12)	36.0	H–C(22)	3.22 (*dd*, *J* = 3.2, 6.0)	73.6
C(8)		40.4	Me(23)	1.08 (*s*)	22.8
H–C(9)	1.39 (*dd*, *J* = 7.2, 10.4)	47.4	CH_2(24)	3.83 (*d*, *J* = 8.8), 3.25 (*d*, *J* = 7.8)	63.0
C(10)		36.4	Me(25)	0.88 (*s*)	15.8
CH_2(11)	1.79–1.83 (*m*)	23.3	Me(26)	0.94 (*s*)	16.9
H–C(12)	5.26 (*dd*, *J* = 3.4, 6.6)	122.7	Me(27)	1.02 (*s*)	18.9
C(13)		145.5	Me(28)	0.79 (*s*)	21.1
C(14)		47.4	Me(29)	0.97 (*s*)	28.2
H–C(15)	3.94 (br. *s*)	65.7	Me(30)	0.86 (*s*)	32.8

[a]) Recorded at 600 MHz. [b]) Recorded at 150 MHz.

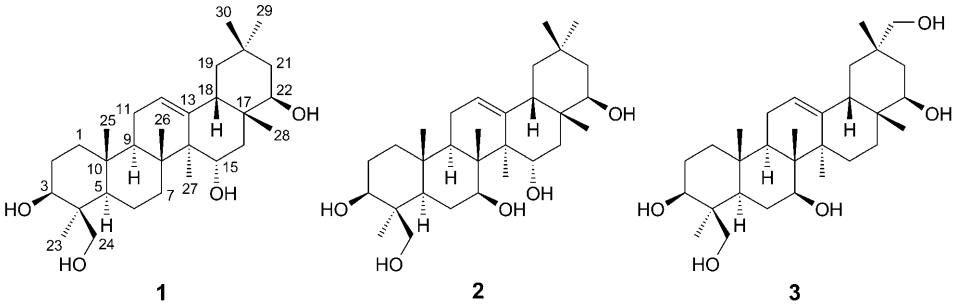

Fig. 1. *Compounds* **1**–**3**, *isolated from* Pestalotiopsis clavispora

J = 8.8 Hz), and 3.25 (*d*, J = 7.8 Hz). The ^{13}C-NMR (*Table 1*) and DEPT spectra indicated 30 C-atom signals, including seven Me, nine CH_2 (one O-bearing at δ(C) 63.0), and seven CH groups (three O-bearing at δ(C) 78.6, 65.7, and 73.6, and one olefinic at δ(C) 122.7), and seven quaternary C-atoms (one olefinic at δ(C) 145.5). A trisubstituted C=C bond deduced from the ^{13}C-NMR analysis accounted for one of the six unsaturation degrees indicating that compound **1** must be pentacyclic. Careful analysis of the ^{1}H- and ^{13}C-NMR data of **1** indicated that the structure of **1** is very similar to that of the known soyasapogenol B (=(3β,4β,22β)-olean-12-ene-3,22,24-triol) [13], except for the chemical-shift value of C(15), suggesting that **1** possesses a similar substitution pattern, *i.e.*, the CH_2(15) group of soyasapogenol B was replaced by an O-bearing CH group (δ(C) 65.7) in **1**. The presence of an OH group at C(15) of **1** was further confirmed by the ^{1}H,^{1}H-COSY and HMBC data (*Fig. 2*). The relative configuration of **1** was determined by the results of a ROESY experiment (*Fig. 3*). Slow and careful recrystallization of **1** (MeOH) furnished single crystals suitable for X-

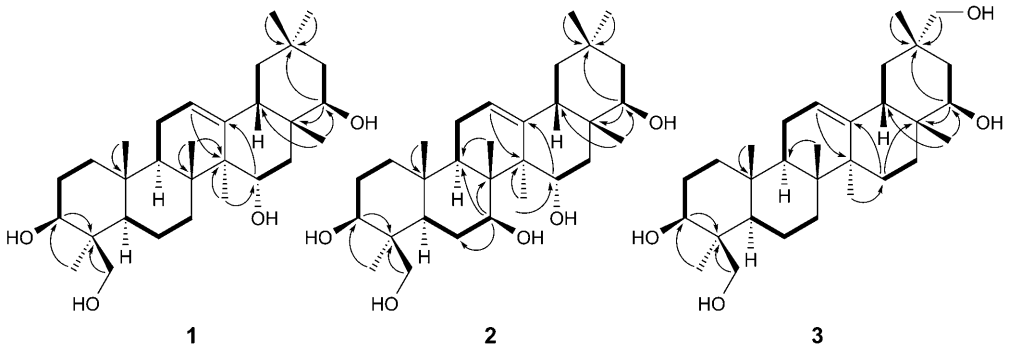

Fig. 2. *^{1}H,^{1}H-COSY* (—) *and key HMBC* (H → C) *features of* **1**–**3**

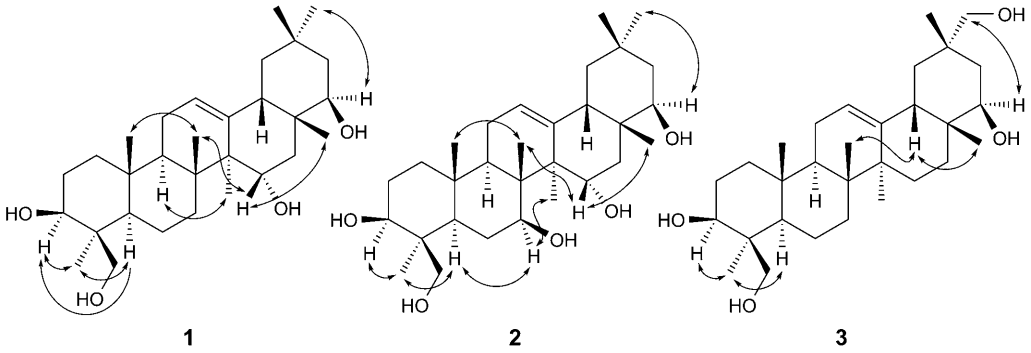

Fig. 3. *Key ROESY* (H ↔ H) *correlations of* **1**–**3**

ray analysis. Consequently, we applied single-crystal X-ray diffraction (*Fig. 4*) to determine the final structure and relative configurations of **1** as (15α)-15-hydroxy-soyasapogenol B (*Fig. 1*).

Compound **2** was obtained as a white powder. The molecular formula was deduced as $C_{30}H_{50}O_5$ from the HR-ESI-MS ($[M+Na]^+$ at *m/z* 513.35419) and ^{13}C-NMR data (*Table 2*). The ^{13}C-NMR (DEPT) spectrum revealed 30 C-atom signals, including seven Me, eight CH_2 (one O-bearing at δ(C) 65.2), and eight CH groups (four O-bearing at δ(C) 80.9, 73.2, 67.4, and 76.3 and one olefinic at δ(C) 126.1), and seven quaternary C-atoms (one olefinic at δ(C) 145.0). By careful analysis of NMR data, we found that the ^{1}H- and ^{13}C-NMR spectra of **2** were similar to those of **1**, suggesting that they possess the same skeleton. The distinct difference in the ^{13}C-NMR spectra of **2** and **1** was that the signal at δ(C) 36.0 (C(7)) of **1** was replaced by one at δ(C) 73.2 in **2**, indicating that OH C(7) of **2** is absent in **1**, and this conclusion was supported by the ^{1}H,^{1}H-COSY and HMBC data (*Fig. 2*). The same relative configuration of all chiral centers of **2** as in **1**, except for C(7), was deduced from the similar δ(C) and δ(H) and from the ROESY correlations (*Fig. 3*) found for **2**. The relative configuration of the OH group at C(7) of **2** was determined by the ROESY experiment: the NOE between

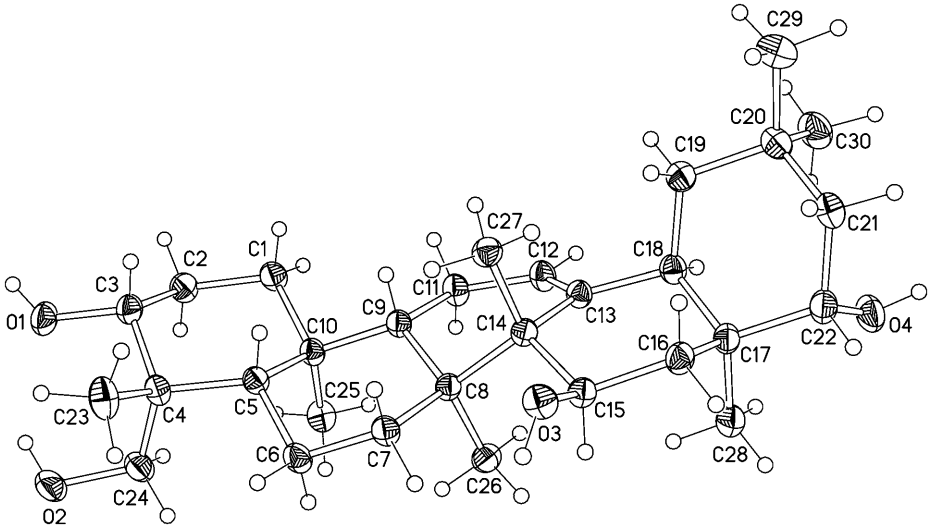

Fig. 4. *X-Ray structure of* **1** *showing the relative configuration.* Arbitrary atom numbering concerning C(29) and C(30).

Table 2. *NMR Data* (CD_3OD) *of* **2**. δ in ppm, J in Hz.

	δ(H)[a])	δ(C)[b])		δ(H)[a])	δ(C)[b])
CH_2(1)	1.71–1.74 (*m*), 0.96–1.01 (*m*)	39.7	CH_2(16)	1.78–1.81 (*m*), 1.53–1.56 (*m*)	38.8
CH_2(2)	1.78–1.81 (*m*), 1.67–1.70 (*m*)	28.4	C(17)		38.6
H–C(3)	3.33 (*dd*, *J* = 4.8, 9.0)	80.9	H–C(18)	2.11 (br. *d*)	48.3
C(4)		43.3	CH_2(19)	0.90–0.94 (*m*), 1.00–1.03 (*m*)	47.0
H–C(5)	0.75 (br. *s*)	53.9	C(20)		31.4
CH_2(6)	1.56–1.58 (*m*), 1.81–1.86 (*m*)	28.9	CH_2(21)	1.33–1.38 (*m*), 1.46–1.49 (*m*)	42.1
H–C(7)	3.81 (*dd*, *J* = 4.7, 11.2)	73.2	H–C(22)	3.39–3.41 (*m*)	76.3
C(8)		47.7	Me(23)	1.25 (*s*)	23.2
H–C(9)	1.39 (*dd*, *J* = 5.8, 9.0)	49.4	CH_2(24)	3.44 (*d*, *J* = 10.9), 4.12 (*d*, *J* = 5.9)	65.2
C(10)		38.2	Me(25)	0.99 (*s*)	16.6
CH_2(11)	1.96–1.98 (*m*), 2.06–2.09 (*m*)	24.8	Me(26)	1.01 (*s*)	10.5
H–C(12)	5.47 (*dd*, *J* = 4.2, 6.6)	126.1	Me(27)	1.16 (*s*)	18.4
C(13)		145.0	Me(28)	0.92 (*s*)	21.0
C(14)		50.6	Me(29)	1.03 (*s*)	28.9
H–C(15)	4.10 (*dd*, *J* = 5.3, 15.6)	67.4	Me(30)	0.96 (*s*)	32.6

[a]) Recorded at 600 MHz. [b]) Recorded at 150 MHz.

H_α–C(5) and H–C(7) suggested that OH–C(7) was β-oriented. Taking all data mentioned above into account, the structure of **2** was established as (7β,15α)-7,15-dihydroxysoyasapogenol B (*Fig. 1*).

Compound **3** was obtained as a white powder with the molecular formula $C_{30}H_{50}O_5$ as established by the HR-ESI-MS ($[M + Na]^+$ at m/z 513.35656), implying six degrees

of unsaturation. Detailed interpretation of the NMR (*Table 3*), ^{1}H,^{1}H-COSY, HMBC (*Fig. 2*), and ROESY data (*Fig. 3*) of **3** indicated that it possesses the same skeleton as **2**. The distinct differences between **3** and **2** were an OH group at C(15) of **2** (δ(C) 67.4 (*d*)), absent in **3** (δ(C) 28.4 (*t*)), and an OH group at C(29) of **3** (δ(C) 72.9 (*t*)), is absent in **2** (δ(C) 28.9 (*q*)). These differences were further confirmed by the key HMBCs, CH_2(15)/C(17), Me(27)/C(15), and CH_2(29)/C(20) of **3**. The same relative configuration of all chiral centers of **3** as in **2** was deduced from the similar δ(C) and δ(H), and from the ROESY correlations (*Fig. 3*) found for **3**. In light of the evidences mentioned above, the structure of **1** was finally established as (7β)-29-dihydroxysoyasapogenol B (*Fig. 1*).

Table 3. *NMR Data* (CD_3OD) *of* **3**. δ in ppm, *J* in Hz.

	δ(H)[a]	δ(C)[b]		δ(H)[a]	δ(C)[b]
CH_2(1)	1.67–1.71 (*m*), 0.94–0.98 (*m*)	39.7	CH_2(16)	1.48–1.52 (*m*), 1.25–1.29 (*m*)	30.1
CH_2(2)	1.72–1.76 (*m*), 1.63–1.67 (*m*)	25.0	C(17)		38.5
H–C(3)	3.32 (*dd*, *J* = 12.5, 4.0)	81.0	H–C(18)	2.09 (br. *d*, *J* = 13.2)	47.1
C(4)		43.2	CH_2(19)	1.87 (*dd*, *J* = 12.8, 8.8)	41.2
H–C(5)	0.91 (*dd*, *J* = 15.8, 7.0)	54.4	C(20)		37.0
CH_2(6)	1.70–1.74 (*m*), 1.28–1.31 (*m*)	30.4	CH_2(21)	1.50–1.54 (*m*), 1.26–1.28 (*m*)	36.7
CH_2(7)	3.85 (*dd*, *J* = 12.6, 4.5)	74.7	H–C(22)	3.43 (*dd*, *J* = 12.8, 3.2)	76.9
C(8)		46.5	Me(23)	1.21 (*s*)	24.8
H–C(9)	1.42 (*dd*, *J* = 11.6, 5.8)	49.8	CH_2(24)	3.32 (*d*, *J* = 6.9), 4.0 (*d*, *J* = 11.2)	65.2
C(10)		38.0	Me(25)	0.96 (*s*)	16.6
CH_2(11)	1.96 (*dd*, *J* = 11.7, 2.4),	24.3	Me(26)	0.97 (*s*)	10.4
	1.89 (*dd*, *J* = 11.0, 5.9)		Me(27)	1.20 (*s*)	23.2
H–C(12)	5.31 (br. *s*)	123.9	Me(28)	0.83 (*s*)	20.2
C(13)		144.9	CH_2(29)	3.2 (br. *s*)	72.9
C(14)		44.7	Me(30)	0.99 (*s*)	25.0
CH_2(15)	1.90–1.94 (*m*), 1.52–1.56 (*m*)	28.4			

[a]) Recorded at 600 MHz. [b]) Recorded at 150 MHz.

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Experimental Part

General. Fermentor: *VCT-500* fermentor (*Yangzhou Weikete Bioengineering Equipment Co., Ltd.*, P. R. China). Column chromatography (CC): silica gel (SiO_2; 200–300 mesh; *Yantai Zhi Fu Chemical Co., Ltd.*, P. R. China), *RP-18* (12 nm, *S-50* μm; *YMC Co., Ltd.*, Japan), TLC: silica gel GF_{254} plates (*Yantai Zhi Fu Chemical Co., Ltd*, P. R. China) and *Sephedax-LH-20* gel (25–100 μm; *GE Healthcare, Ltd.*, Sweden). M.p.: *XRC-1* micro- melting-point apparatus. Optical rotations: *Perkin-Elmer-341* spectropolarimeter. UV Spectra: *UV-210* spectrometer; λ_{max} (log ε) in nm. IR Spectra: *Perkin-Elmer-577* spectrometers; KBr pellets; $\tilde{\nu}$ in cm^{-1}. NMR Spectra: *Bruker-AM-600* spectrometer; δ in ppm rel. to Me_4Si as internal standard, *J* in Hz. FT-MS: *Bruker-Apex-ultra* 7.0 T spectrometer; in *m*/*z*.

Fungal Material. The strain of *Pestalotiopsis clavispora* was isolated from the plant *Bruguiera sexangula* collected from Dongzhai, Hainan Province, P. R. China. The isolate was identified by *J.-Z. Z.*,

and assigned the accession number L480. The fungal strain was cultured on slants of potato dextrose agar (CPDA) at 28° for 5 d. The agar plugs were used to inoculate into 1000 ml *Erlenmeyer* flasks, each containing 600 ml of media (CPDA), and the final pH of the media was adjusted to 6.5 before sterilization. Flask cultures were incubated at 28° on a rotary shaker at 150 rpm for 5 d. The seed cultures were incubated in a fermentor containing 300 l of liquid medium (0.03 g/ml soybean meal, 0.02 g/ml glucose) at 28° at 150 rpm for 4 d (ventilation: 0–12 h: 1/0.4; 12–24 h: 1/0.6; 24–36 h: 1/1; 36–48 h: 1/0.6).

Extraction and Isolation. The fermented material was concentrated and extracted with AcOEt (3 × 10 l), the org. solvent evaporated, and the crude extract (80.0 g) fractionated by CC (SiO_2, stepwise elution with petroleum ether/AcOEt/MeOH). Compound **1** (30 mg) was obtained as colorless crystals from the fraction (100 mg) eluted with petroleum ether/AcOEt 6:1. The fraction (80 mg) eluted with AcOEt/MeOH 5:1 was further purified by repeated CC (SiO_2, petroleum ether/acetone 3:1; *Sephadex LH-20*, MeOH) and prep. TLC (petroleum ether/acetone 4:1): **2** (8 mg) and **3** (15 mg).

(15α)-15-Hydroxysoyasapogenol B (*=(13β,4β,15α,22β)-Olean-12-ene-3,15,22,24-tetrol*; **1**): Colorless crystals (MeOH). M.p. 201–202°. $[\alpha]_D^{25} = +130.8$ ($c = 0.02$, MeOH). UV (MeOH): 199 (4.25). IR (KBr): 3395 (OH), 2947, 1655, 1461, 1039 (C–O–C). ^{1}H- and ^{13}C-NMR (CD_3OD): *Table 1*. HR-ESI-MS: 497.3614 ($[M+Na]^+$, $C_{30}H_{50}NaO_4^+$; calc. 497.3601).

(7β,15α)-7,15-Dihydroxysoyasapogenol B (*=(3β,4β,7β,15α,22β)-Olean-12-ene-3,7,15,22,24-pentol*; **2**): White powder. $[\alpha]_D^{20} = +45.0$ ($c = 0.02$, MeOH). UV ($CHCl_3$): 199 (4.45). IR (KBr): 3398 (OH), 2948, 1650, 1460, 1030 (C–O–C). ^{1}H- and ^{13}C-NMR (CD_3OD): *Table 2*. HR-ESI-MS: 513.3542 ($[M+Na]^+$, $C_{30}H_{50}NaO_5^+$; calc. 513.3551).

(7β)-7,29-Dihydroxysoyasapogenol B (*=(3β,4β,7β,20α,22β)-Olean-12-ene-2,7,22,24,29-pentol*; **3**). White powder. $[\alpha]_D^{20} = +95.0$ ($c = 0.01$, MeOH). UV($CHCl_3$): 199 (3.93). IR (KBr): 3396 (OH), 2930, 1650, 1456, 1029 (C–O–C). ^{1}H- and ^{13}C-NMR (CD_3OD): *Table 3*. HR-ESI-MS: 513.3566 ($[M+Na]^+$, $C_{30}H_{50}NaO_5^+$; calc. 513.3551).

X-Ray Crystallographic Analysis of **1**[1]). Upon crystallization from MeOH by the vapor-diffusion method, colorless crystals were obtained for **1**. A crystal (1.00 × 0.71 × 0.66 mm) was separated from the sample and mounted on a glass fiber, and data were collected with a *Bruker-SMART-1000-CCD* diffractometer and graphite-monochromated MoK_α radiation ($\lambda = 0.71073$ Å) at 296(2) K. Crystal data: $C_{30}H_{50}O_4$, M_r 474.36, space group orthorhombic, $P2_12_12_1$; unit cell dimensions $a = 10.081(5)$ Å, $b = 14.298(6)$ Å, $c = 10.918(5)$ Å, $V = 1551.9(12)$ Å^3, $Z = 4$, $D_{calc.} = 1.084$ Mg/m^3, $\mu = 0.071$ mm^{-1}, $F(000) = 560$. The structure was solved by direct methods with SHELXL-97 [14] and refined by full-matrix least-squares difference *Fourier* techniques. All non-H-atoms were refined with anisotropic displacement parameters, and all H-atoms were placed in idealized positions and refined as riding atoms with the relative isotropic parameters. Absorption corrections were applied with the *Siemens* area detector absorption program (SADABS) [15]. The 13515 measurements yielded 9222 independent reflections after equivalent data were averaged, and *Lorentz* and polarization corrections were applied. The final refinement gave $R_1 = 0.043$ and $wR_2 = 0.1073$ ($I > 2\sigma(I)$).

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