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cis-Fused caryophyllenes from liquid cultures of *Hebeloma longicaudum*

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Abstract

Three new caryophyllenes, hebelophyllenes A–C were isolated from the ectomycorrhizal fungus *Hebeloma longicaudum*. Their structures were determined by modern spectroscopic methods.

Keywords: *Hebeloma longicaudum*; Ectomycorrhizal fungi; Sesquiterpenes; Caryophyllenes; Hebelophyllenes; Circular dichroism; Absolute configuration

1. Introduction

The fungus *Hebeloma longicaudum* (Pers.:Fr.) Kummer was isolated from a fruiting body associated with Norway spruce growing in Sweden. It is an ectomycorrhizal fungus which produces abundant ectomycorrhizae associated with pines and spruces and occurs in diverse forest habitats throughout the world. It has great potential for large-scale nursery inoculation of conifer seedlings for superior growth and survival in the field after outplanting (Boyle, Robertson, & Salonius, 1987; Boyle & Hellenbrand, 1991). This paper describes some of the metabolites produced when the fungus is grown in liquid cultures.

2. Results and discussion

The fungus *H. longicaudum* was grown in potato dextrose liquid shake culture and was harvested after 3 weeks. The ethyl acetate extract of the filtered broth was subjected to flash column chromatography on silica gel and the crude fractions recrystallized to afford several crystalline products. In this study we report the structural elucidation of three new sesquiterpenes, hebelophyllenes A (**1**), B (**2**) and C (**4**).

The molecular formula of hebelophyllene A was determined to be $C_{15}H_{22}O_3$ from HRMS, indicating five degrees of unsaturation. As two double bonds and one carbonyl group are present, as indicated by the ^1H and ^{13}C NMR spectral data (Tables 1 and 2), hebelophyllene A must be bicyclic. The IR bands at 3428 and 3281 cm^{-1}, and the presence of 20 signals in the ^1H NMR spectrum in CD_3OD and signals at 71.0 and 77.5 ppm in the ^{13}C NMR spectrum (Table 2) lead to the conclusion that the remaining two oxygen atoms constitute two hydroxyl groups. The NMR spectral data also show that hebelophyllene A has two aliphatic and one olefinic methyl groups (singlets at 1.19 and 1.26 ppm and a broad singlet at 1.47 ppm, respectively). The ^{13}C chemical shift of the olefinic methyl group (17.7 ppm) is indicative of an *E*-double bond. At this point all spectral data were suggestive of a caryophyllane sesquiterpene skeleton. The complete assignment of all ^1H and ^{13}C NMR chemical shifts achieved on the basis of ^1H–^1H COSY, HMQC and HMBC, established structure **1** for hebelophyllene A.

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Table 1
^{1}H NMR data of sesquiterpenes **1**, **2** and **4** (δ, multiplicity, J in Hz)

Assignment[a]	**1**[b]	**2**[c]	**4**[c]
H-1	2.82 (br.t, 11.0)	2.80 (t, 11.0)	2.72 (ddd, 11.4, 10.5, 0.8)
H-2	3.72 (dd, 11.0, 2.5)	3.74 (dd, 11.0, 2.0)	4.35 (d, 11.4)
H-3	4.13 (d, 2.5)	4.01 (br.s)	
H-4			3.12 (qdd, 6.7, 4.0, 0.6)
H-5	6.08 (tquint, 8.5, 1.0)	5.58 (ddquint, 12.8, 5.2, 1.3)	4.82 (dt, 10.4, 4.0)
H-6 α	3.10 (dd, 19.0, 8.5)	2.51 (dddd, 11.7, 7.3, 5.2, 1.7)	2.52 (dd, 13.5, 10.4)
H-6 β	3.21 (ddq, 19.0, 8.0, 1.6)	2.29 (ddd, 12.8, 11.7, 9.1)	3.25 (ddd, 13.5, 4.0, 0.6)
H-7		3.95 (br.t, 8.2)	
H-9	3.04 (ddd, 11.0, 10.5, 5.7)	2.74 (dddd, 11.0, 10.4, 6.6, 1.1)	3.49 (dtt, 7.0, 10.0, 1.5)
H-10α	2.13 (dd, 12.6, 10.5)	2.12 (dd, 12.0, 10.4)	1.94 (ddd, 12.5, 9.3, 0.8)
H-10β	1.73 (ddd, 12.6, 5.7, 0.9)	1.60 (dd, 12.0, 6.6)	2.01 (br.dd, 12.5, 7.0)
H-12	1.26 (s)	1.24 (s)	1.21 (s)
H-13	1.19 (s)	1.16 (s)	1.23 (s)
H-14	1.48 (br.s)	1.56 (d, 1.3)	1.09 (d, 6.7)
H-15a	5.53 (s)	5.18 (d, 1.0)	5.83 (d, 1.5)
H-15b	6.01 (s)	5.33 (d, 1.0)	6.00 (d, 1.5)

[a] The assignments are based on coupling constants and ^{1}H–^{1}H COSY spectra.
[b] $CDCl_3$, 600 MHz.
[c] CD_3OD, 600 MHz.

The location of the hydroxyl groups at C-2 and C-3, the presence of a $CH_2C(CH_3)_2$ fragment as part of a four membered ring adjacent to an α,β-unsaturated ketone and the presence of a double bond (C-4 to C-5) adjacent to C-3 follows from the key HMBC correlations shown in Table 2. The cross peaks from C-7 and C-4 to H-6 establish the position of the second methylene group at C-6 of the nine-membered ring. The constitution of hebelophyllene A is closely related to that of naematolin, a *cis*-caryophyllene bearing an additional acetoxy group at C-6 (Backens, Steffan, Steglich, Zechlin, & Anke, 1984; Tsuboyama, Sakuray, Tsuboyama, & Doi, 1986). The coupling constants $J_{\text{H-1, H-2}}$ and $J_{\text{H-2, H-3}}$ of **1** and naematolin are almost identical, suggesting analogous stereochemistry. Conclusive support for the relative stereochemistry of **1** came from the TROESY (transverse rotating-frame Overhauser effect spectroscopy (Hwang & Shaka, 1993)) (Fig. 1), which not only provides direct evidence for *cis*-fusion of the rings (cross peaks between H-1 and H-9, as well as between Ha-15 and H-2), but also confirms the *cis* position of the hydroxyl groups at C-2

Table 2
^{13}C NMR assignments and HMBC data of sesquiterpenes **1**, **2** and **4**

No.[a]	**1**[b]	**2**[c]	**4**[d]
C-1	46.6 (3, 10 β, 12, 13)	48.0 (3, 9, 12, 13)	49.2 (2,9,10 α, 10 β, 12, 13)
C-2	71.0 (1, 3)	71.1 (1, 3)	72.2 (1)
C-3	77.5 (5, 14)	78.9 (5, 14)	214.1 (1, 4, 14)
C-4	139.2 (3,6 α, 6 β, 14)	135.7 (3, 6 α, 6 β, 14)	52.1 (3, 6 α, 6 β, 14)
C-5	118.1 (3, 6 α, 6 β, 14)	119.8 (3, 6 α, 6 β, 14)	68.9 (4, 6 α, 6 β, 14)
C-6	39.5 (5)	35.7 (7)	46.4 (4)
C-7	205.0 (6 α, 6 β, 9, 15a, 15b)	77.7 (6 α, 6 β, 9, 15a, 15b)	205.7 (6 α, 6 β, 15a, 15b)
C-8	154.9 (1, 9, 10 α, 10 β)	157.9 (6 α, 7, 9, 10 α, 10 β, 15a, 15b)	152.2 (1, 6 α, 9, 10 α, 10 β, 15a, 15b)
C-9	33.8 (1, 10 α, 10 β, 15a, 15b)	38.4 (1, 7, 10 α, 10 β, 5a, 15b)	31.1 (1, 10 α, 10 β, 15a, 15b)
C-10	41.8 (9, 12, 13)	43.5 (12, 13)	35.0 (9, 12, 13)
C-11	34.6 (1, 9, 10 α, 10 β, 12, 13)	34.1 (1, 10 α, 10 β, 12, 13)	35.1 (1, 10 α, 10 β, 12, 13)
C-12	24.0 (1, 10 α, 10 β, 13)	24.6 (1, 10 α, 10 β, 13)	25.6 (1, 10 α, 10 β, 13)
C-13	33.6 (1, 10 α, 10 β, 12)	33.0 (1, 10 α, 10 β, 12)	32.6 (1, 10 α, 10 β, 12)
C-14	17.7 (5)	18.1 (5)	9.0 (4)
C-15	122.9 (9)	109.7 (7, 9)	121.6 (9)

[a] The multiplicities were verified with APT and HMQC spectra.
[b] In $CDCl_3$, 50 MHz.
[c] In $CDCl_3$, 100 MHz.
[d] In CD_3OD, 50 MHz.

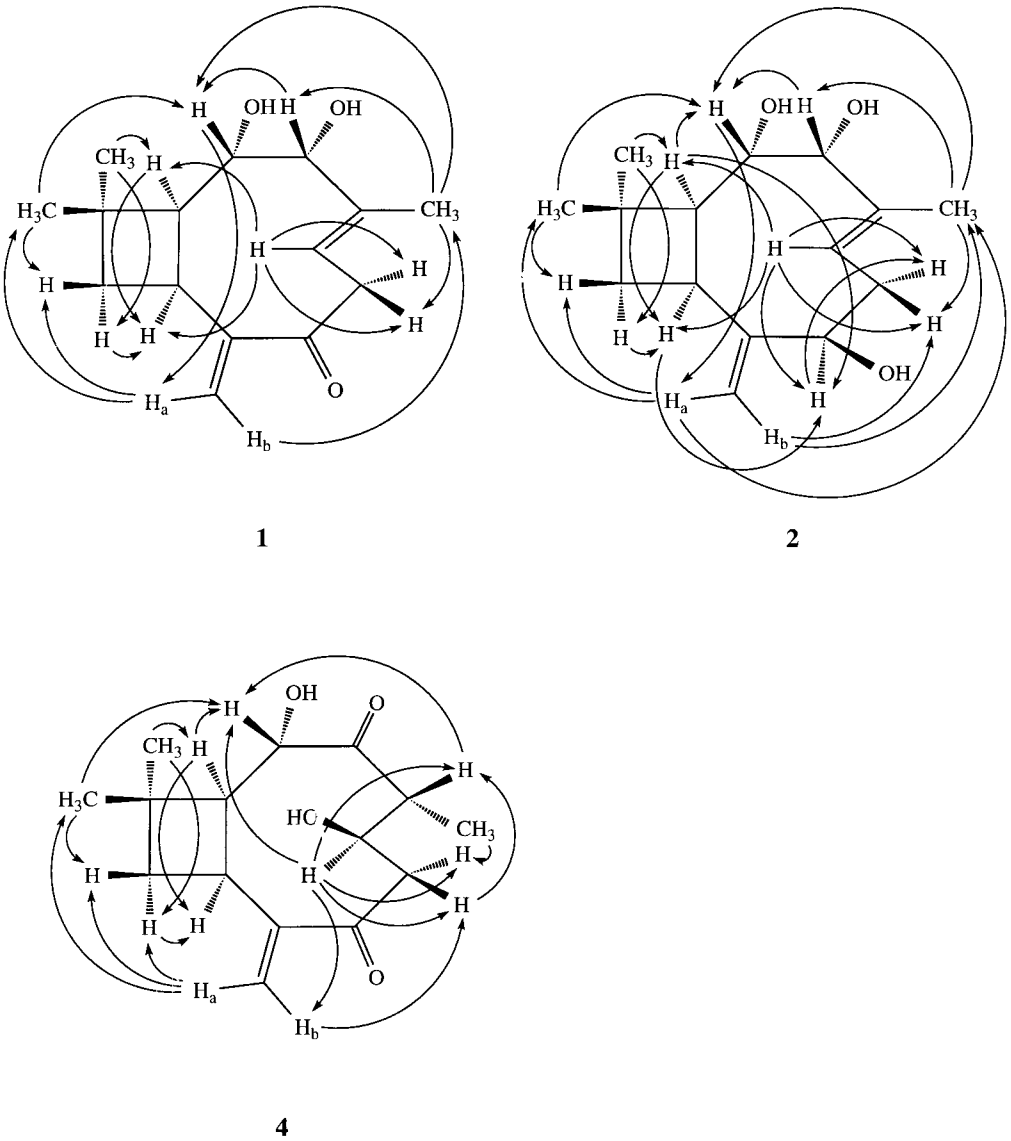
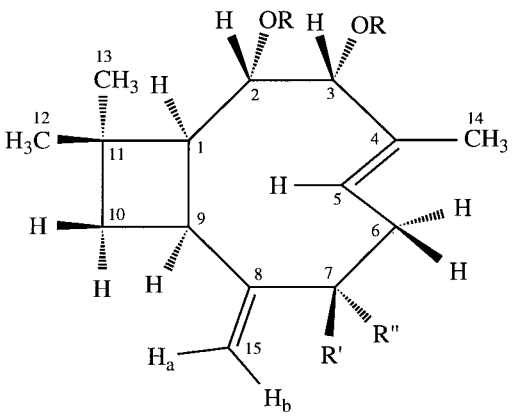


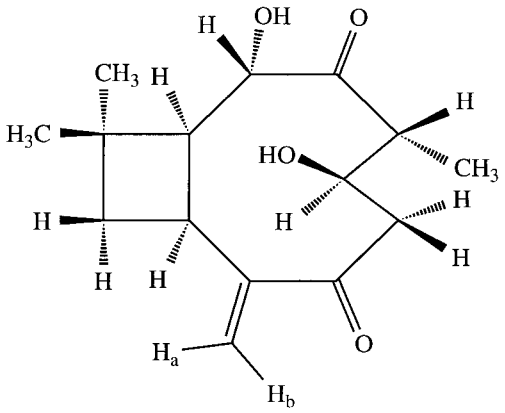
Fig. 1. Pertinent TROESY correlations for compounds **1**, **2** and **4**; all geminal and 1,3-correlations in the four-membered ring are omitted.

and C-3 and shows that the C-4–C-5 double bond is twisted in such a way as to bring H-5 close to H-1 and H-9, and H-14 close to H-2 and H-3.

In order to determine the absolute configuration of hebelophyllene A (**1**), the di(*p*-nitrobenzoate) derivative **3** was prepared. The CD spectrum of **3** exhibits split Cotton effects with the first sign negative ($\Delta\varepsilon_{267}$ −19.52), which is in agreement with a negative chirality between the *p*-nitrobenzoate chromophores observed in structure **3** (Harada & Nakanishi, 1983). Thus, the absolute configuration of **1** is 1*S*,2*S*,3*R*,9*S*, the same as that of naematolin (Bohlman & Zdero, 1978; Tsuboyama et al., 1986).

Hebelophyllene B has the molecular formula $C_{15}H_{24}O_3$ as established by HRMS and indicates four unsaturations. The lack of a carbonyl group and the presence of two double bonds as indicated by the NMR spectra leads to a bicyclic structure. The NMR data of hebelophyllene B are very similar to those of **1**. Unlike **1**, however, which has a C-7 carbonyl group, hebelophyllene B has a CH(OH) group at C-7 (triplet at 3.95 ppm in the ^{1}H NMR and doublet at 77.7 ppm in the ^{13}C NMR spectra). Consequently, the signals of H-6 (Table 1) and especially of C-15 (Table 2) of hebelophyllene B are shifted upfield as compared to those of **1** and the geminal coupling $J_{\text{H-6}\alpha,\text{H-6}\beta}$ is significantly reduced, from 19.0 Hz for **1** to 11.7 Hz for hebelophyllene B. The TROESY correlations (Fig. 1) show most of the cross peaks observed for hebelophyllene A (**1**). The correlations between H-7 and H-1 and H-9 determine the stereochemistry at C-7 as shown and confirm the structure **2** for hebelophyllene B.





1 R = H, R'+ R" = O

2 R = R"=H, R' = OH

3 R = p-$O_2NC_6H_4CO$, R' + R" = O

4

The molecular formula of hebelophyllene C, $C_{15}H_{22}O_4$, was determined by CIMS, since the HRMS spectrum produced only $[M–H_2O]^+$ ions. Its NMR signals are similar to those of **1**, except for the presence of an additional, nonconjugated carbonyl group (singlet at 214.1 ppm in the ^{13}C NMR spectrum) and for the lack of a C-4 to C-5 double bond (H-14 shows a doublet, $J=6.7$ Hz, in the ^1H NMR spectrum). Cross peaks in the HMBC spectrum from the new carbonyl signal to H-1, H-2 and H-14 determine its position as C-3, while the two hydroxyl groups are at C-2 and C-5 as indicated by the key correlations from C-2 to H-1 and H-9, and from C-5 to H-6 and H-14 in the HMBC spectrum. All of the above information confirms structure **4** for hebelophyllene C.

Although many caryophyllenes have been isolated from plants and fungi, only three (Doi et al., 1986; Tsuboyama et al., 1986; Frank et al., 1999) have the *cis* ring juncture. Thus, hebelophyllene A (**1**), hebelophyllene B (**2**) and hebelophyllene C (**4**) are new representatives of the small group of *cis*-fused caryophyllene sesquiterpenes.

3. Experimental

3.1. General

Melting points are uncorrected. The HREIMS spectra were recorded on a AEI-50 mass spectrometer and CIMS spectra were recorded on a VG70/70 mass spectrometer. ^1H NMR and TROESY spectra were recorded on a Varian Inova 600 and ^1H–^1H COSY, HMQC and HMBC spectra on a Varian Inova 300 spectrometer. ^{13}C NMR and APT spectra were recorded on a Bruker AM-200 or AM-400 spectrometer. Chemical shifts are reported in ppm downfield of TMS and carbon multiplicities were measured with the APT and HMQC sequences. UV spectra were recorded on a Hewlett Packard Diode Array UV–VIS spectrometer (ICP) with a cell path of 1 mm and IR spectra on a Nicolet 7199 FTIR spectrometer in solution or under microscope (Uscope). The optical rotation was measured on a Perkin Elmer 241 polarimeter with a cell path of 10 cm and CD spectra were measured on a Jasco ORD-CD spectrometer with a cell path of 1 mm. Precoated TLC plates, SIL G-25, 20 × 20 cm, 0.25 mm layer (Macherey–Nagel) were used for preparative TLC.

3.2. Fungal growth and isolation of metabolites

H. longicaudum (strain 16) was isolated from a fruiting body associated with Norway Spruce. A voucher specimen is deposited at the Northern Forestry Centre, Edmonton, Canada, as NOF 2298. The fungus was grown in 5 l of liquid shake culture using 2.5% solution of Potato Dextrose Broth (DIFCO). After 21 days of shaking at room temperature, the cultures were filtered and the broth was concentrated in vacuo to 1 l and extracted exhaustively with ethyl acetate to yield 1.06 g of crude extract. The latter was subjected to column chromatography on silica gel 60 (230–400 mesh, Merck) with hexane–EtOAc (gradient, 25–100% EtOAc). From hexane–EtOAc (3:2) fractions A, B and C were obtained. Each fraction was concentrated in

vacuo to ca. 1 ml and kept overnight at RT. The crystalline material was filtered and washed with hexane–EtOAc (4:1) to provide sesquiterpenes **1** (13.3 mg), **2** (9.1 mg) and **4** (3.9 mg).

Hebelophyllene A (**1**) was obtained as colorless needles, m.p. 110.0–111.0°C; $[\alpha]_D$ −304.2° (MeOH, *c* 0.23); UV (MeOH) λ_{max} (ε) 203 nm (5410), 234 nm (2270), 332 nm (79); CD: $\Delta\varepsilon_{214}$ +5.58, $\Delta\varepsilon_{253}$ +1.31 $\Delta\varepsilon_{325}$ −3.20 (MeOH, *c* 0.046); IR ($CHCl_3$): 3428 (OH), 3281 (OH), 2932, 2866, 1690 (⟩C=O), 1602 (⟩C=C⟨), 1447, 1398, 1364, 1322, 1233, 1200, 1176, 1096, 1067, 1038, 1022, 1001, 975, 937, 921, 882, 841, 815, 755, 654, 630, 546, 517 cm^{-1}; ^1H NMR spectrum (Table 1); ^{13}C NMR spectrum (Table 2); HREIMS *m*/*z* (rel. int.): 250.1566 $[M]^+$ (0.63%, calc. for $C_{15}H_{22}O_3$: 250.1569), 235 $[M–C_4H_8]^{+8}$, 176(26), 166(40), 148(18), 133(12), 111(25), 109(22), 105(14), 85(100), 53(55).

Hebelophyllene B (**2**) was obtained as colorless needles, m.p. 146.0–147.0°C; $[\alpha]_D$ −152.2° (MeOH, *c* 0.23); CD: $\Delta\varepsilon_{210}$ −11.87 (MeOH, *c* 0.028); IR (Uscope): 3398 (OH), 3275 (OH), 3077, 2976, 2958, 2931, 2872, 2427, 1667 (⟩C=O), 1642, 1455, 1439, 1387, 1367, 1291, 1263, 1226, 1209, 1163, 1105, 1064, 1042, 997, 923, 897, 876, 823, 813, 711, 660, 639, 585 cm^{-1}; ^1H NMR (Table 1); ^{13}C NMR (Table 2); HREIMS *m*/*z* (rel. int.): 252.1730 $[M]^+$ (0.1%, calc. for $C_{15}H_{24}O_3$: 252.1725), 234 $[M–H_2O]^+$ (2), 219 $[M–H_2O–CH_3]^+$ (2), 216 $[M–2H_2O]^+$ (2), 196 $[M–C_4H_8]^+$, 150(36), 108(21), 85(100), 56(11), 55(42).

Hebelophyllene C (**4**) was obtained as colorless needles, m.p. 140.0–141.0°C; $[\alpha]_D$ +39.4° (MeOH, *c* 0.35); UV (MeOH) λ_{max} (ε) 238 nm (2670), 325 nm (72); CD: $\Delta\varepsilon_{233}$ −2.02, $\Delta\varepsilon_{332}$ −1.47 (MeOH, *c* 0.055); IR (Uscope): 3393 (OH), 2954, 2867, 1706 (⟩C=O), 1680 (sh, ⟩C=O), 1620 (⟩C=C⟨), 1452, 1408, 1367, 1269, 1201, 1129, 1049, 1004, 977, 941, 731, 696 cm^{-1}; ^1H NMR (Table 1); ^{13}C NMR (Table 2); CIMS (NH_3) *m*/*z* (rel.int.): 284 $[M+NH_4]^+$ (22), 267 $[M+H]^+$ (3), 266 $[M]^+$ (4); HREIMS *m*/*z* (rel. int.): 248.1401 $[M–H_2O]^+$ (1%, calc. for $C_{15}H_{20}O_3$: 248.1412), 210 $[M–C_4H_8]^+$, (9), 192(11), 182(37), 164(26), 136(14), 124(38), 109(20), 85(100), 53(34).

3.3. Preparation of diester 3

A solution of **1** (1.8 mg, 0.0072 mmol), triethylamine (5 μl, 0.036 mmol), *p*-dimethylaminopyridine (1 mg) and *p*-nitrobenzoyl chloride (4.7 mg, 0.027 mmol) in 1 ml of dry methylene chloride was kept at RT for 2 days. The reaction mixture was diluted with 30 ml of ether and washed with 15 ml of 5% Na_2CO_3 solution and then with ice water. The organic layer was separated and dried over $MgSO_4$. The solvent was removed under vacuum and the residue (3.7 mg) was subjected to preparative TLC with benzene–acetone 9:1. The UV active zone (254 nm, R_f 0.54) was extracted with methylene chloride to give 2.5 mg of **3** as a colorless solid, m.p. 96.0–97.0°C; $[\alpha]_D$ −2.61° ($CHCl_3$, *c* 0.15); UV (EtOH) λ_{max} (ε) 256 nm (26750); CD: $\Delta\varepsilon_{236}$ +6.23, $\Delta\varepsilon_{267}$ −19.52, $\Delta\varepsilon_{320}$ −4.15 (EtOH, *c* 0.020); IR ($CHCl_3$): 2960, 2926, 2856, 1729 (⟩C=O), 1690 (⟩C=O), 1608 (⟩C=C⟨), 1528, 1347, 1279, 1102, 873, 757, 720 cm^{-1}; ^1H NMR (360 MHz, $CDCl_3$): δ 1.07 (3H, s, H-12), 1.31 (3H, s, H-13), 1.78 (3H, br.*s*, H-14), 1.89 (1H, dd, *J* = 12.9, 5.5 Hz, H-10α), 2.33 (1H, dd, *J* = 12.9, 10.2 Hz, H-10β), 3.19 (1H, dd, *J* = 19.0, 8.6 Hz, H-6 α), 3.31 (1H, dd, *J* = 19.0, 8.2 Hz, H-6 β), 3.32 (1H, ddd, *J* = 11.0, 10.2, 5.5 Hz, H-9), 3.39 (1H, br.t, *J* = 11.3 Hz, H-1), 5.37 (1H, dd, *J* = 11.7, 2.2 Hz, H-2), 5.61 (1H, d, *J* = 2.2 Hz, H-3), 5.69 (1H, s, H-15a), 6.13 (1H, tquint, *J* = 8.5, 1.2 Hz, H-5), 6.20 (1H, s, H-15b), 8.10 (2H, d, *J* = 9.0 Hz), 8.25 (4H, t, *J* = 9.0 Hz), 8.37 (2H, d, *J* = 9.0 Hz). HREIMS *m*/*z* (rel. int.): 548.1803 $[M]^+$ (0.1%, calc. for $C_{29}H_{28}N_2O_9$: 548.1795).

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