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Two butylated aminooligosaccharides isolated from the culture filtrate of *Streptomyces luteogriseus*

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Abstract

Two novel aminooligosaccharides, butytatins M03 and M13 were isolated and purified from the culture filtrate of *Streptomyces luteogriseus*. Analysis by liquid chromatography coupled to electrospray ionization mass spectrometry indicated their resemblance to isovalertatin, with a four-carbon acyl group. Their structures were established by NMR as aminooligosaccharide derivatives possessing a butylated side chain.

Keywords: *Streptomyces luteogriseus*; Aminooligosaccharide; Butytatin M03; Butytatin M13; LC–ESIMS

1. Introduction

The actinomycetes microorganism *Streptomyces luteogriseus* is able to produce aminooligosaccharides.[1] This family of secondary metabolites had potent inhibitory activity against several saccharide hydrolases, making them valuable for the potential applications in medicine and agriculture.[2,3] WC670, an aminooligosaccharide-containing complex, was extracted from the culture filtrate of *S. luteogriseus*. The major components in the complex included isovalertatins M03, M13, M23, D03, and D23. The chemical structures of these isovalertatins were previously proposed,[4] and recently revised by our laboratory.[5,6] ESIMS analysis of WC670 indicated it to contain at least two more aminooligosaccharides, of the molecular weights 1039 and 1201 Da, respectively, and possessed of a butyl group side chain. This paper describes the isolation and structural elucidation of the two novel oligomers, designated as butytatins M03 (**1**) and M13 (**2**).

2. Results and discussion

The aminooligosaccharide-containing complex, WC670, extracted from the culture filtrate of *S. luteogriseus*, was dissolved in water and resolved by semipreparative reversed-phase HPLC on a Spherisorb C_8 column with the mobile phase MeCN:1.5 mmol/L aqueous ammonia with UV detection at 206 nm, affording two oligomers **1** and **2**.

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ESIMS analysis.—Solutions (10 μg/mL) of the two oligomers were prepared in the LC mobile phase, and were flow injected onto the ESI source at 5 μL/min to acquire their positive full-scan mass spectra. Each of them generated a strong $[M+H]^+$ signal at m/z 1040, and 1202, respectively, and a $[M+Na]^+$ signal at the relatively low intensity of $\sim 10\%$ at m/z 1062, and 1224, respectively. The difference of the molecular weight between **1** and **2** was 162 Da, suggesting **2** to possibly be a homologue of **1** appended by a hexose unit.

The protonated molecular ions for **1** and **2** yielded CID spectra that were useful for structural elucidation. Compared to the CID spectrum of isovalertatin M03, **1** has a similar fragmentation pattern.[5] Moreover, every fragment in **1**, including the esterified hexose moiety (ring D) displayed a lower mass-to-charge ratio by 14 mass units, but the fragment excluding ring D displayed the same m/z value as isovalertatin M03, suggesting that **1** has a four-carbon acyl group attached to ring D instead of the isovaleryl group in isovalertatin M03.

The LC–ESIMS analysis of **1** and **2** was conducted on a C_8 column with a gradient of MeCN–alkaline water as the mobile phase, and selected ion monitoring (SIM) was used. The addition of ammonia (1.5 mmol/L) increased the pH to 9. The highly polar aminooligosaccharides, in their free-base form, tend to be retained on the reversed-phase column more strongly, and do not interact with the surface silanols of the stationary phase, and tailing is avoided.[7] The retention times of **1** and **2** were 8.93 and 7.85 min, respectively, whereas those for isovalertatin M03 and M13 were 14.76 and 12.65 min, respectively,[5] which accorded with the suggestion of a four-carbon acyl side-chain in **1** and **2**. The lipophilicity of a four-carbon acylated derivative is lower than that of an isovalerylated homologue, and the former is thus retained more weakly in the column.

Structural determination of **1**.—Butytatin M03 (**1**), a white amorphous powder, gave positive reactions with silver nitrate–sodium hydroxide and with anthrone. The molecular formula was deduced as $C_{41}H_{69}NO_{29}$ by combined high-resolution positive ESIMS (Anal. Found $[M+H]^+$ 1040.4037, Calcd 1040.4034) and the NMR data. Careful inspection of the CID spectrum from $[M+H]^+$ of **1** (Fig. 1) revealed that the fragment ions at m/z 1022 and 1004 corresponded to the neutral loss of 1–2 water molecules, the ions at m/z 860 (B_5), 698 (B_4), and 536 (B_3) fitted with the neutral

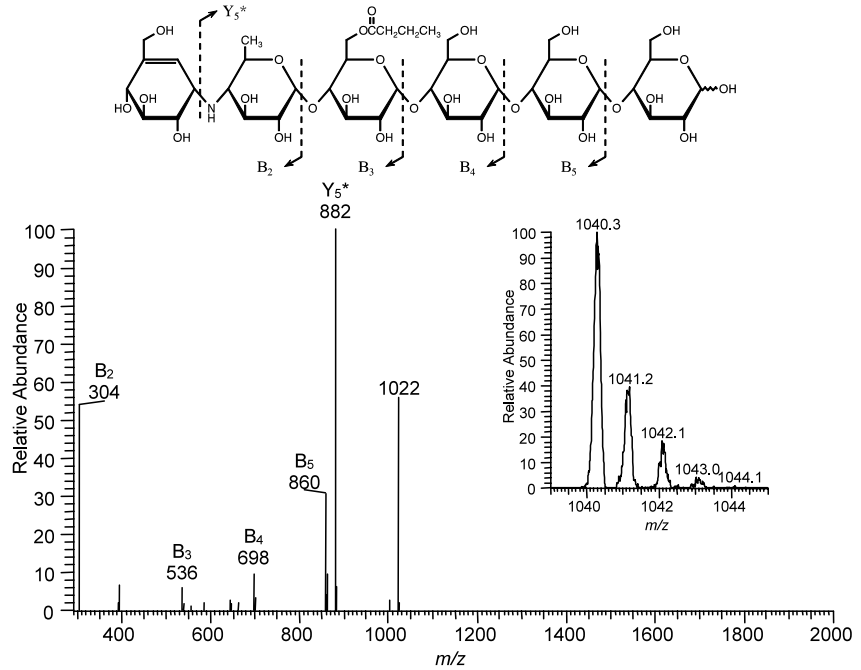

Fig. 1. CIDMS–MS fragmentation and spectrum of $[M+H]^+$ of **1** at m/z 1040. Insert: zoom scan MS of **1**.

loss of 1–3 hexose groups attached to the reducing end, and the base peak at m/z 882 (Y_5^*) was due to rupture of the cyclohexitol–nitrogen bond. The precursor ion at m/z 1040 fragmented to generate the abundant peak at m/z 304 (B_2) corresponding to cleavage of the quinovosidic bonds. The absence of a $[M+H]^+ - 162$ fragment ion, added by the diagnostic ion at m/z 304, suggested that no hexose group was linked to the nonreducing end. These features of the CID spectrum allowed the structure outlined in Fig. 1 to be proposed, but the types of hexose units and the four-carbon acyl group, the linkage positions, and their details remained undetermined.

The total correlation spectroscopy (TOCSY) spectrum of **1** revealed the presence of two spin–spin coupling systems including protons resonated at δ 5.25 (d, $J_{1,2}$ 3.5 Hz), 2.43 (t, J_{vic} 9.0 Hz), 1.28 (d, 3 H, $J_{5,6}$ 6.0 Hz), and others at δ 3.50 (t, $J_{1,2}$ 5.0 Hz), 4.01 (d, $J_{3,4}$ 6.5 Hz), 4.09 (d, J_{gem} 14.0 Hz), 4.20 (d, J_{gem} 13.5 Hz), and 5.87 (d, $J_{1,7}$ 3.0 Hz). These proton signals correlated with resonances for carbon atoms at δ 101.7 (d), 66.2 (d), 18.5 (q), and δ 57.1 (d), 72.4 (d), 62.8 (t), 125.0 (d), respectively, in the heteronuclear multiple quantum correlation (HMQC) spectrum. This indicated the presence of a unit of acarviosine in the structure.[8]

Three proton signals at δ 5.20 (d, 0.3 H, $J_{1,2}$ 3.5 Hz), 4.63 (d, 0.7 H, $J_{1,2}$ 8.0 Hz), and 3.25 (t, 0.7 H, J_{vic} 8.5 Hz), which correlated to carbon signals at δ 93.1 (d), 97.0 (d), and 75.2 (d), respectively, in the HMQC spectrum, are typical characteristics for a reducing terminal glucose unit.[9,10] The chemical shifts for three anomeric protons of rings B–D ranged near δ 5.38 (3 H, overlapped), displaying couplings of $\sim$3.0 Hz. These data, in conjunction with biogenetic characteristics of the aminooligosaccharides from the culture of *S. luteogriseus*,[5,6] indicated the hexose in rings A–D to be D-glucopyranose. It is well known that the NMR signals for the methine group at C-4 in a glucose unit always resonate at δ_H 3.42 and δ_C 70.4.[11] In the HMQC spectrum of **1**, four C-4 methines signals of four glucose units appeared near δ_H 3.60–3.70 (overlapped) and δ_C 78.0–78.3 (d), while no correlation near δ_H 3.42/δ_C 70.4 was found, suggesting that all of the four C-4 hydroxyl groups of four glucose units was glycosylated. Thus, the configuration of the four glycosidic bonds in **1** was assigned as α-(1→4).[11]

The ^{1}H NMR signals, comprising a spin–spin coupling system in the TOCSY spectrum, at δ 0.91 (t, 3 H, J_{vic} 7.5 Hz), 1.67 (h, 2 H, J_{vic} 7.5 Hz), 2.42 (t, 2 H, J_{vic} 7.5 Hz), and the corresponding ^{13}C signals at δ 14.1 (q), 19.1 (t), 36.8 (t), as well as a carbonyl carbon atom, resonating at δ 177.9 (s), in the HMQC spectrum, confirmed the existence of a butyl group. The NMR signals for the methylene group at position 6 in the glucose unit always appeared at δ_H 3.90/3.78 and δ_C 61.4.[11] Both the 1 H signals at δ 4.43 (dd, J_{gem} 14.0 Hz) and at δ 4.20 (dd, J_{gem} 13.5 Hz) had correlations with a methylene carbon atom resonating at δ 64.6 (t), in the HMQC spectrum, suggesting that the 6-hydroxyl group of a glucose unit was esterified with the butyl group. The esterification caused downfield shifts for the methylene group, $\sim$0.53 ppm for the proton resonance and $\sim$3.2 ppm for the carbon resonance. Thus, the chemical structure of **1** was established as shown in Fig. 1. Complete assignment of the proton and carbon shifts, aided by distortionless enhancement by polarization transfer (DEPT), TOCSY, and HMQC experiments and the comparison with reported data of acarbose and isovalertatin M03,[8] is shown in Table 1.

Structural determination of **2**.—Butytatin M13 (**2**), obtained as white amorphous powder, was assigned the molecular formula of $C_{47}H_{79}NO_{34}$ by high-resolution positive ESIMS (Anal. Found $[M+H]^+$ 1202.4567, Calcd 1202.4562) and NMR data. The color reactions (with silver nitrate–sodium hydroxide and with anthrone), spectroscopic characteristics, and liquid-chromatographic behavior closely resembled those of **1**, suggesting **2** to be an analogue having an appended glucose unit.

The CID spectrum of its protonated molecular ion at m/z 1202 exhibited major fragment ions at m/z 1184, 1166, 1040, 1022, 882, 864, 860, 698, and 466. Among these fragments, that at m/z 1184 corresponded to the neutral loss of a water molecules, the ions at m/z 1022

Table 1
^{1}H and ^{13}C NMR data of **1** in D_2O [a]

Position	δ_H	δ_C	Position	δ_H	δ_C	Position	δ_H	δ_C
A-1α	5.20 (0.3 H)	93.1	B-1,C-1	5.37–5.39	100.6–100.8	E-1	5.25	101.7
A-2α	3.53	72.5	B-2,C-2	3.56–3.62	72.4–72.7	E-2	3.59	72.1
A-3α	3.90–3.95	74.3–74.5	B-3,C-3	3.90–3.95	74.3–74.5	E-3	3.54	74.2
A-4α	3.62	79.1	B-4,C-4	3.59–3.65	78.0–78.3	E-4	2.43	66.2
A-5α	3.88	71.1	B-5,C-5	3.74–3.78	72.3–72.5	E-5	3.66	71.0
A-6α	3.70–3.78	61.6–61.9	B-6,C-6	3.70–3.78	61.6–61.9	E-6	1.28	18.5
A-1β	4.63 (0.7 H)	97.0	D-1	5.37–5.39	100.6–100.8	F-1	3.50	57.1
A-2β	3.25 (0.7 H)	75.2	D-2	3.56–3.62	72.4–72.7	F-2	3.58	73.9
A-3β	3.75	77.4	D-3	3.90–3.95	74.3–74.5	F-3	3.69	74.2
A-4β	3.59–3.65	78.0–78.3	D-4	3.59–3.65	78.0–78.3	F-4	4.01	72.7
A-5β	3.57	75.7	D-5	4.01	70.1	F-5		140.2
A-6β	3.70–3.78	61.6–61.9	D-6	4.20/4.43	64.6	F-6	4.09/4.20	62.8
Butyl group						F-7	5.87	125.0
CH_3	0.91	14.1	CH_2	1.62	19.1			
CH_2	2.42	36.8	C=O		177.9			

[a] Assignments are supported by DEPT, TOCSY, and HMQC experiments.

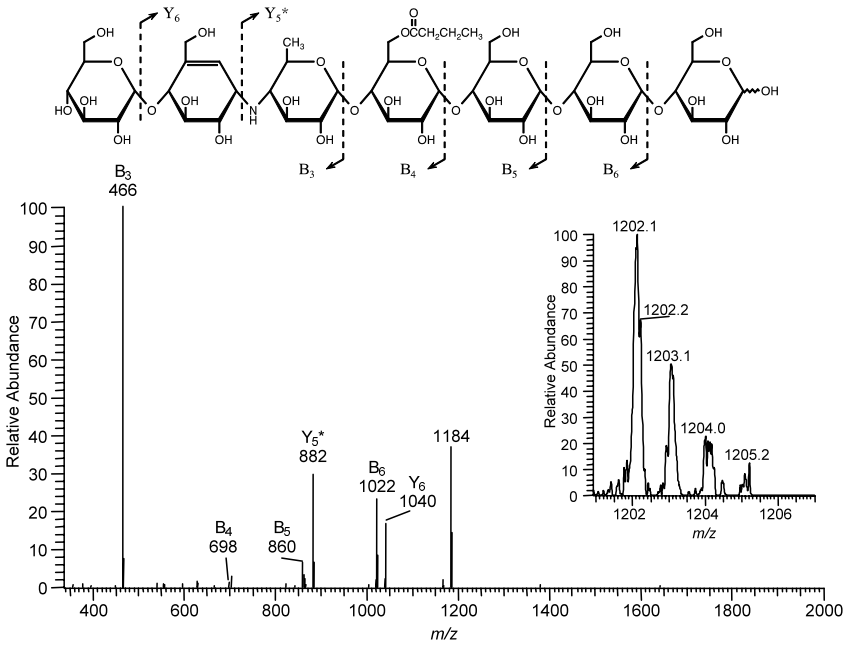

Fig. 2. CIDMS–MS fragmentation and spectrum of $[M+H]^+$ of **2** at m/z 1202. Insert: zoom scan MS of **2**.

(B_6), 860 (B_5), and 698 (B_4) indicated the neutral loss of 1–3 glucose molecules attached on the 'right side', and the abundant peak at m/z 882 (Y_5^*) resulted from the cleavage of the cyclohexitol–nitrogen bond. The product ion at m/z 1040 (Y_6) revealed the presence of a nonreducing terminal glucose unit, and the abundant peak at m/z 466 (B_3), from dissociation of the quinovodic bond, confirmed that the glucose group was attached on the 'left side' to the acarviosine moiety (Fig. 2).

Because the yield of **2** was low, we recorded only its ^{1}H NMR spectrum. The ^{1}H NMR spectrum of **2** resembled much that of **1**, showing the characteristic signals for a butyl group at δ 0.91 (t, 3 H, J_{vic} 7.3 Hz), 1.62 (h, 2 H, J_{vic} 7.5 Hz), 2.41 (d, 2 H, J_{vic} 7.5 Hz), for an acarviosine moiety at δ 5.25 (d, $J_{1,2}$ 3.5 Hz), 2.43 (t, J_{vic} 8.5 Hz), 1.28 (d, 3 H, $J_{5,6}$ 6.5 Hz), 3.50 (t, J_{vic} 5.0 Hz), 4.12 (d, J_{gem} 13.5 Hz), 4.18 (m), 5.95 (d, $J_{1,7}$ 4.5 Hz), for an esterified hydroxyl group at C-6 of a glucose

unit at δ 4.43 (dd, J_{gem} 10.5 Hz), 4.20 (m), as well as the typical signals at δ 5.20 (d, 0.4 H, $J_{1,2}$ 3.5 Hz), 4.63 (d, 0.6 H, $J_{1,2}$ 8.0 Hz), 3.25 (t, 0.6 H, J_{vic} 8.0 Hz) for a reducing glucose terminus. The configuration of the five glycosidic bonds in **2** were assigned as α because of the diagnostic anomeric proton signals at δ 5.37 having coupling constants of $\sim$3.0 Hz. Slight differences were found in signals for protons at positions F-4–F-7 from δ 4.01, 4.09, 4.20, 5.87 in **1** to δ 4.12 (d, $J_{3,4}$ 7.5 Hz), 4.12 (d, J_{gem} 13.8 Hz), 4.18 (d, J_{gem} 13.8 Hz), 5.95 (d, $J_{1,7}$ 4.5 Hz) in **2**. These alterations resembled the differences between isovalertatins M03 and M13, suggesting a glycosylated hydroxyl group attached to position F-4 in **2** instead of a free hydroxyl group in **1**.[8] Other differences included two additional signals at δ 3.40 (t, J_{vic} 9.5 Hz), indicating a free hydroxyl group at position G-4,[11] and at δ 5.35 (d, $J_{1,2}$ 4.0 Hz) for the anomeric proton of G-1.[12] All of the foregoing evidence confirmed that the appended glucose unit was linked to position F-4 through an *O*-α-glycosidic bond. Thus, the chemical structure of **2** was established as shown in Fig. 2.

3. Experimental

General.—HPLC was carried out on a Hewlett–Packard series 1100 instrument. The analytical LC experiment coupled to MS detection was taken on a Spherisorb C_8 column (250 × 4.6 mm i.d., 5 μm, Elite Co., Dalian, China) with 3:17 (v/v) MeCN–1.5 mmol/L aq NH_3 as mobile phase at 0.5 mL/min. MS analysis was run on a Finnigan LCQ ion-trap mass spectrometer equipped with an electrospray ionization source. The positive-ion mode was employed, and the spray voltage was set at 4.5 kV. The capillary voltage was fixed at 5.0 V, and its temperature maintained at 180 °C. The HPLC fluid was nebulized using N_2 as both the sheath gas, at a flow rate of 1.2 L/min, and the auxiliary gas at a flow rate of 0.15 L/min. CID experiments were conducted using helium as the collision gas, and the relative collision energy was set at 28%. HRESIMS data were acquired on Autospec–Ultima ETOF mass spectrometer with PEG1450 as the internal standard. NMR measurements were conducted on a Varian Inova-500 instrument at 25 °C. The D_2O solutions (0.7 mol/L, pH $\sim$ 7) were used with sodium 4,4-dimethyl-4-silapentanoate (DSS) as the external standard, and the NMR spectra were recorded in 5-mm tubes at 500.13 MHz for ^1H and 125.76 MHz for ^{13}C. Optical rotations were measured on a Perkin–Elmer 241MC polarimeter. IR spectra were taken on a Bruker IFS 55 FT-IR spectrometer and recorded in KBr pellets.

Microorganism.—*S. luteogriseus* strain 670, collected in soil near Kunming, China, in 1988, was identified by the Institute of Microbiology, Academia Sinica. A reference specimen (CCGMC 0331) is deposited in the Center for Collection of General Microbiological Cultures, Institute of Microbiology, Academia Sinica.

Preparation of WC670 complex.—The culture (60 L) of *S. luteogriseus* was acidified to pH 3.0 with oxalic acid and filtered. The filtrate (50 L) was adjusted to pH 8.0 and passed through a column of No. 312 macroporous resin. After being washed with water followed by 10% aq Me_2CO, the column was eluted with 20% aq Me_2CO, and the eluate was concentrated in vacuo. About a twofold volume of MeOH and eightfold Me_2CO was added to the concentrated aqueous solution, and the filtrate was discarded. The residue (13.5 g) was redissolved in water and applied to a low-pressure reversed-phase C_{18} column, which was eluted successively with water, 1:9 water–MeOH and 1:4 water–MeOH. The fraction of 20% aq MeOH was lyophilized to gave the complex, WC670 (6.6 g).

Purification of butytatins.—The amino-oligosaccharide-containing complex, WC670 (1.0 g), was dissolved in water and filtered through a 0.45 μm membrane and applied to a semi-preparative reversed-phase HPLC using a stainless-steel column packed with 10 μm Spherisorb C_8 at 18 °C, using the isocratic mobile phase of 3:22 (v/v) MeCN–1.5/mmol L aq NH_3 at a flow rate of 3.0 mL/min with 206 nm as the detection wavelength, to give a combined fraction from the 7.7–10.2 min eluate. This eluate was further separated on the same column at 34 °C with 8.5:91.5 (v/v)

MeCN–1.5 mmol/L aq NH_3 as mobile phase, to give two fractions from the 21.5- and 24.8-min chromatographic peaks. These residues were finally purified by the same LC system at 36 °C with 7.5:92.5 (v/v) MeCN–1.5 mmol/L aq NH_3 as the isocratic mobile phase to afford **2** (10.5 mg) from the 25.5-min peak, and **1** (24.2 mg) from the 29.0-min peak. The purity of these oligomers was detected by positive-ion mode ESIMS analysis.

Butytatin M03 (**1**).—White amorphous powder. The retention time on the analytical LC–ESIMS system at 18 °C was 8.93 min; $[\alpha]_D^{18}$ + 156.8° (*c* 0.1, water); UV (water): end absorption; IR ν_{max} (KBr): 3388, 2928, 1720, 1655, 1382, 1025, 577 cm^{-1}; HRESIMS (pos.): *m/z* 1040.4037 $[M+H]^+$ ($C_{42}H_{72}NO_{29}$ requires 1040.4034); ESI–CIDMS of *m/z* 1040 (pos.): *m/z* (rel. int.) 1022(55.7), 1004(2.5), 882(100), 864(9.5), 860(30.4), 698(9.5), 536(5.8), 304(53.8); For ^1H and ^{13}C NMR data, see Table 1.

Butytatin M13 (**2**).—White amorphous powder. The retention time on the analytical LC–ESIMS system at 18 °C was 7.85 min; $[\alpha]_D^{18}$ + 158.2° (c 0.1, water); UV (water): end absorption; IR ν_{max} (KBr): 3386, 2930, 1718, 1655, 1384, 1028, 581 cm^{-1}; HRESIMS (pos.): *m/z* 1202.4567 $[M+H]^+$ ($C_{42}H_{72}NO_{29}$ requires 1202.4562); ESI–CIDMS of *m/z* 1202 (pos.): *m/z* (rel. int.) 1184(37.0), 1166(1.8), 1040(16.6), 1022(23.1), 1004(0.5), 882(29.6), 864(2.1), 860(6.6), 698(1.3), 466(100); ^1H NMR (500 MHz, D_2O): δ 0.91 (t, 3 H, J_{vic} 7.3 Hz, $COCH_2CH_2CH_3$), 1.28 (d, 3 H, $J_{E5,E6}$ 6.5 Hz, E-6), 1.62 (h, 2 H, J_{vic} 7.5 Hz, $COCH_2CH_2CH_3$), 2.41 (d, 2 H, J_{vic} 7.5 Hz, $COCH_2CH_2CH_3$), 2.43 (t, 1 H, J_{vic} 8.5 Hz, E-4), 3.25 (t, 0.6 H, J_{vic} 8.0 Hz, A-2β), 3.40 (t, 1 H, J_{vic} 9.5 Hz, G-4), 3.50 (t, 1 H, J_{vic} 5.0 Hz, F-1), 3.54–3.97(overlapping), 4.01 (m, 1 H, D-5), 4.12 (d, 1 H, J_{gem} 13.5 Hz, F-6a), 4.18 (m, 1 H, F-6b), 4.20 (m, 1 H, D-6a), 4.43 (dd, 1 H, J_{gem} 10.5 Hz, D-6b), 4.63 (d, 0.6 H, $J_{A\text{-}1\beta,A\text{-}2\beta}$ 8.0 Hz, A-1β), δ 5.20 (d, 0.4 H, $J_{A\text{-}1\alpha,A\text{-}2\alpha}$ 3.5 Hz, A-1α), 5.25 (d, 1 H, $J_{E\text{-}1,E\text{-}2}$ 3.5 Hz, E-1), 5.35 (d, 1 H, $J_{G\text{-}1,G\text{-}2}$ 4.0 Hz, G-1), 5.36–5.38 (overlapping, 3 H, B-1–D-1), 5.95 (d, 1 H, $J_{F\text{-}1,F\text{-}7}$ 4.5 Hz, F-7).

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