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Structural revision of isovalertatins M03, M13, and M23 isolated from the culture of *Streptomyces luteogriseus*

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Abstract

Three aminooligosaccharides, isovalertatins M03 (**1**), M13 (**2**), and M23 (**3**) were isolated and purified from the culture filtrate of *Streptomyces luteogriseus*. Their physicochemical properties, liquid chromatographic behavior, and spectroscopic data were in full accordance with the reported compounds [Xu, Q.; Wang, Q.; Lu, D. CN Patent 1100756, 1995; *Chem. Abstr.* **1995**, *123*, 110278n], but their structures were reinvestigated and revised by spectroscopic methods, including ESI multistage mass spectrometry and 2-dimensional NMR techniques.

Keywords: *Streptomyces luteogriseus*; Structural revision; Aminooligosaccharide; Isovalertatin M03; Isovalertatin M13; Isovalertatin M23; ESIMS^n

1. Introduction

In a screening program searching for bioactive oligosaccharide derivatives, we discovered a novel complex, WC670, from the culture of *Streptomyces luteogriseus* strain 670. The complex showed remarkable inhibitory activity against several saccharide hydrolases.[1] Three oligomers were isolated and purified from the complex, and their molecular weights were determined as 1053, 1215, and 1377 Da by ESIMS analysis. Their physicochemical properties, liquid chromatographic behavior (direct comparisons with authentic samples), and spectroscopic data were in full accordance with the reported *Yiwutadings* F, D, and C, respectively,[2,3] suggesting that the soil microorganism, *S. luteogriseus*, could also produce aminooligosaccharide-family secondary metabolites. However, when verifying their chemical structures, we found several inconsistencies in the corresponding ESI ion-trap multistage mass spectra. This phenomenon encouraged us to reinvestigate their structures, and resulted in the structural revision of these bioactive compounds. The names for the three oligomers are re-designated isovalertatins M03, M13, and M23, respectively.

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2. Results and discussion

The aminooligosaccharide-containing complex, WC670, was isolated from the culture filtrate of *S. luteogriseus*. The complex was then dissolved in water and resolved by semi-preparative reversed-phase HPLC on a Spherisorb C_8 column with a mobile phase of MeCN–1.5 mmol/L aqueous ammonia and UV detection at 206 nm, which gave the three oligomers **1**–**3**.

Structural reinvestigation of compound **1**.— Isovalertatin M03 (**1**), a white amorphous powder, gave positive reactions with silver nitrate–sodium hydroxide and with anthrone. The molecular formula was deduced as $C_{42}H_{71}NO_{29}$ by combined high-resolution positive ESIMS (Anal. Found $[M+H]^+$ 1054.4182, Calcd 1054.4190) and NMR data. The retention time for **1** on an LC–ESIMS system, incorporating a Spherisorb C_8 column (250 × 4.6 mm, i.d., 5 μm) at 18 °C, with the mobile phase of MeCN–1.5 mmol/L aqueous ammonia (0.5 mL/min), and MS detection in the selected ion-monitoring mode, was 14.76 min. The IR spectrum of **1** displayed absorption at 1719 cm^{-1} for the ester carbonyl group, 1653 cm^{-1} for the C=C double bond, and broad bands at 3389 and 1025 cm^{-1} for the oligosaccharide. A collision-induced-dissociation (CID) mass spectrum (MS^2) of its protonated molecular ion at m/z 1054 showed main fragment ions at m/z 1036, 896, 878, 874, 712, 554, 550, and 304 (Fig. 1). However, many daughter ions, including the most abundant ion at m/z 896, could not be correlated with the reported structure of **1** (Scheme 1).[3] A further triple-stage MS (MS^3) experiment to the precursor ions at m/z 896 and 874 gave corresponding fragment-ions at m/z 878, 716, 554, and at m/z 716, 712, 550, 304, respectively. Nearly every abundant fragment ion in

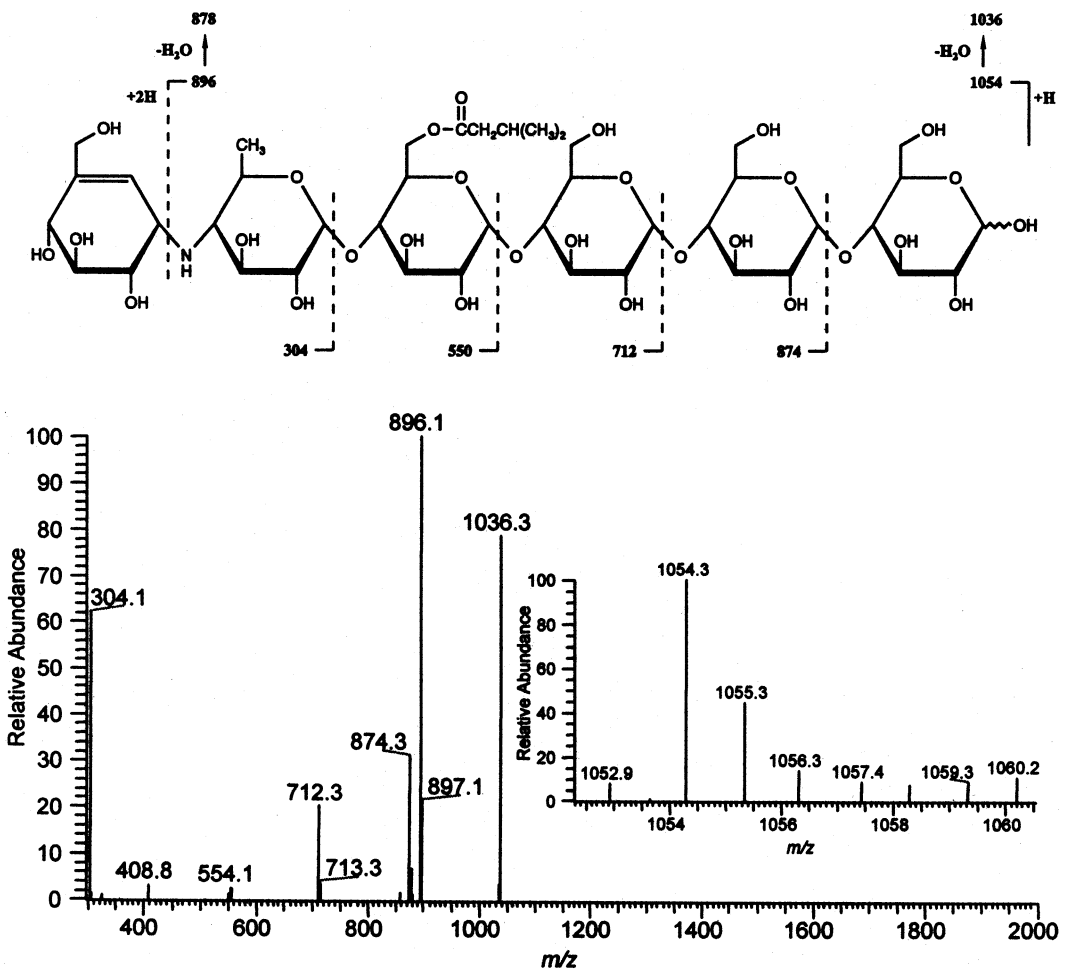
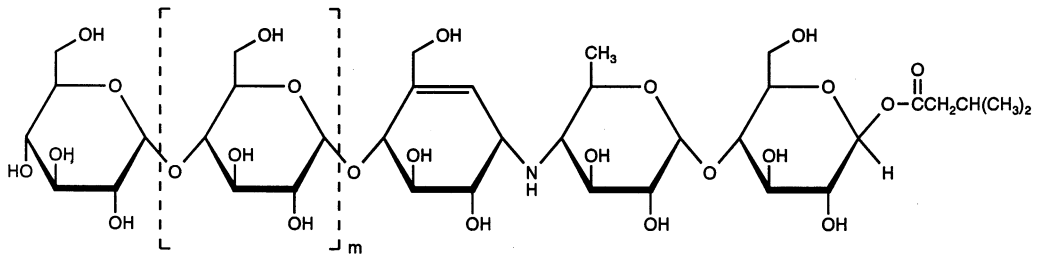


Fig. 1. Positive ESI CID mass spectrum of $[M+H]^+$ of **1** at m/z 1054. Insert: full scan MS of **1**.



1: m = 2 **2**: m = 3 **3**: m = 4

Scheme 1.

the ESI positive-ion mode should possess at least one site with significant basicity for a basic nitrogen-containing compound, and most of the abundant fragment-ions should originate from single-bond glycosidic cleavages.[4] The foregoing feature of MS^n enabled proposal of the structure outlined in Fig. 1. However, the linkage positions and the configuration of the isovaleryl group, a moiety of acarviosine,[5] and four glucose units, remained undetermined.

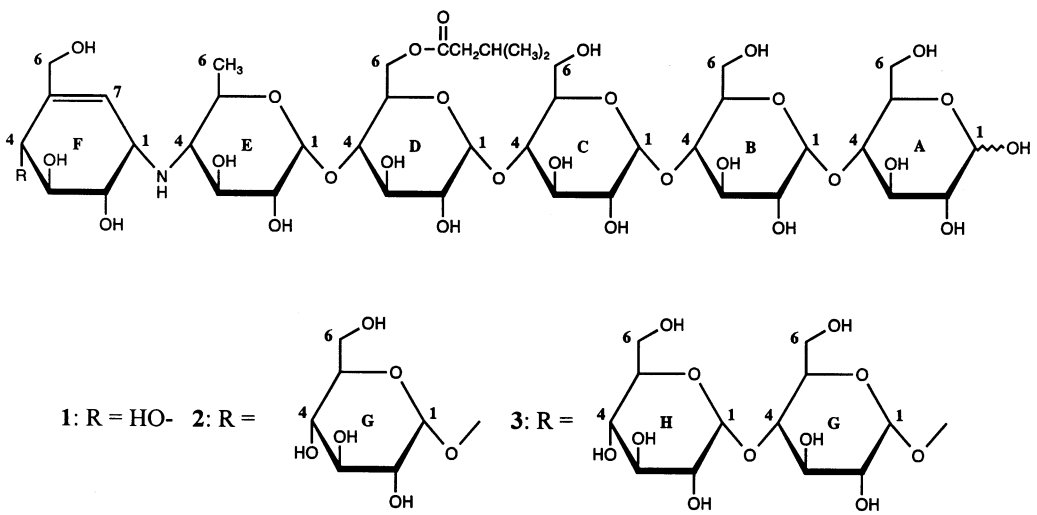
Acid hydrolysis experiments had proved that **1**–**3** were composed of D-glucose, acarviosine, and isovaleric acid.[3] The ^{1}H NMR signals, comprising a spin–spin coupling system in the TOCSY spectrum, at δ 0.94 (d), 2.05 (m), 2.32 (d), and the relevant ^{13}C signals at δ 22.6, 26.4, 43.7 as well as a carbonyl carbon atom resonating at δ 177.1, confirmed the existence of an isovaleryl group. The TOCSY spectrum of **1** also revealed the presence of two spin–spin coupling systems, including protons located at δ 5.25 (d, $J_{1,2}$ 3.3 Hz), 2.44 (t, J_{vic} 9.3 Hz), 1.29 (d, $J_{5,6}$ 6.2 Hz), and protons resonating at δ 3.51 (t, $J_{1,2}$ 4.7 Hz), 4.03 (d, $J_{3,4}$ 7.2 Hz), 4.09 (d, J_{gem} 13.8 Hz), 4.20 (d, J_{gem} 13.8 Hz), and 5.88 (d, $J_{1,7}$ 3.0 Hz). These proton signals correlated with resonances for carbon atoms at δ 101.6, 65.9, 18.4, and at δ 56.7, 72.5, 62.6, 124.8, respectively, in the HMQC spectrum. These data accorded with the presence of a unit of acarviosine in the structure.[6]

The NMR signals for the methylene group at position 6 in a glucose unit normally appear at δ_H 3.90/3.78 and δ_C 61.4.[7] Two 1 H signals at δ 4.46 (dd, J_{gem} 11.2 Hz) and 4.20 (dd, J_{gem} 11.2, J_{vic} 7.4 Hz) showed correlations with a methylene carbon atom resonated at δ 64.4, in the HMQC spectrum, suggesting that the hydroxyl group at position 6 of a glucose unit was esterified with the isovaleryl group. The esterification caused downfield shifts for the methylene protons of $\sim$0.57 ppm and $\sim$3.0 ppm for the carbon signal. In addition, an HMBC experiment further confirmed the esterified position, as the carbonyl carbon atom showed correlations with two protons at position D6.

Three proton signals at δ 5.21 (d, 0.4 H, $J_{1,2}$ 3.8 Hz), 4.63 (d, 0.6 H, $J_{1,2}$ 7.9 Hz), and 3.25 (t, 0.6 H, J_{vic} 8.4 Hz), which correlated to carbon signals at δ 92.9, 96.6, and 75.1, respectively, in the HMQC spectrum, were typical characteristics of the reducing terminal glucose unit.[8,9] It is well known that the NMR signals for the methine group at position 4 in a glucose unit always resonate at δ_H 3.42 and δ_C 70.4.[7] In the HMQC spectrum of **1**, four methines at position 4 of four glucose units appeared at about δ_H 3.62–3.66 (overlapped) and δ_C 78.1–78.4, while no correlation at about δ_H 3.42/δ_C 70.4 was found, revealing that all of the four hydroxyl groups at position 4 of four glucose units were glycosylated. As the chemical shifts for three anomeric protons on rings B–D occurred at δ 5.38 (3 H, overlapped), and the coupling constants were $\sim$3.0 Hz, the configuration of the glycosidic bonds was determined to be α-(1$\rightarrow$4).[7] The chemical structure of **1** was thus revised as shown in Scheme 2. The complete assignment of the proton and carbon shifts, aided by DEPT, TOCSY, HMQC, and HMBC experiments and by the comparison with reported data of acarbose,[8] are shown in Table 1.

Structural reinvestigation of compound **2**.— Isovalertatin M13 (**2**), obtained as a white amorphous powder, was ascribed the molecular formula of $C_{48}H_{81}NO_{34}$ via high-resolution positive ESIMS (Anal. Found $[M+H]^+$ 1216.4723, Calcd 1216.4718) and NMR data. The retention time for **2** on the same LC–ESIMS system was 12.65 min. The color reactions, spectroscopic characteristics, and liquid chromatographic behavior closely resembled that of **1**, suggesting **2** to be an analogue having a glucose unit appended. The ESIMS[2] spectrum of its protonated molecular ion at m/z 1216 exhibited main fragment-ions at m/z 1198, 1054, 1036, 896, 878, 874, 712, and 466. The fragmentation pathways shown in Fig. 1 indicate that the appending glucose unit is attached to the non-reducing end of compound **1**.

The ^{1}H and ^{13}C NMR spectra of **2** appeared much like those of **1**, showing the characteristic signals for an isovaleryl group at δ_H 0.94 (d, J_{vic} 6.5 Hz), 2.05 (m), 2.32 (d, J_{vic} 7.0 Hz), and δ_C 22.3, 26.1, 43.5, 176.9; for an acarviosine moiety at δ_H 5.26 (d, $J_{1,2}$ 3.0 Hz), 2.45 (t, J_{vic} 9.5 Hz), 1.29 (d, $J_{5,6}$ 6.0 Hz), 3.52 (t, J_{vic} 5.0 Hz), 4.12 (m), 4.19 (m), 5.95 (d, $J_{1,7}$ 3.0 Hz), and δ_C 101.3, 64.9, 18.1, 55.7, 62.7, 126.6; for an esterified hydroxyl group at position 6 of a glucose unit at δ_H 4.45 (dd, J_{gem}



Scheme 2.

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
A1α	5.21(0.4 H)	92.9	B1,C1	5.37–5.39	100.5–100.7	E1	5.25	101.6
A2α	3.56	72.3	B2,C2	3.56–3.64	72.4–72.6	E2	3.64	71.6
A3α	3.95–3.97	74.0–74.3	B3,C3	3.95–3.97	74.0–74.3	E3	3.56	74.0
A4α	3.63	79.1	B4,C4	3.62–3.66	78.1–78.4	E4	2.44	65.9
A5α	3.92	71.0	B5,C5	3.73–3.77	72.3–72.4	E5	3.71	70.7
A6α	3.75–3.77	61.6	B6,C6	3.75–3.77	61.4–61.8	E6	1.29	18.4
A1β	4.63(0.6 H)	96.6	D1	5.37–5.39	100.5–100.7	F1	3.51	56.7
A2β	3.25(0.6 H)	75.1	D2	3.56–3.64	72.4–72.6	F2	3.62	73.8
A3β	3.74	77.2	D3	3.95–3.97	74.0–74.3	F3	3.73	74.0
A4β	3.58	78.1–78.4	D4	3.62–3.66	78.1–78.4	F4	4.03	72.5
A5β	3.57	75.6	D5	4.01	70.1	F5		140.1
A6β	3.75–3.77	61.6	D6	4.20–4.46	64.4	F6	4.09–4.20	62.6
Isovaleryl group						F7	5.88	124.8
CH_3	0.94	22.6	CH_2	2.32	43.7			
CH	2.05	26.4	C=O		177.1			

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

Table 2
^{1}H and ^{13}C NMR data of **2** and **3** in D_2O [a]

Position	**2**		**3**		Position	**2**		**3**	
	δ_H	δ_C	δ_H	δ_C		δ_H	δ_C	δ_H	δ_C
A1α	5.20(0.4 H)	92.6	5.20(0.4 H)	92.6	E4	2.45	64.9	2.43	64.9
A4α	3.59	78.7	3.60	78.6	E6	1.29	18.1	1.28	18.1
A5α	3.93	70.6	3.92	70.6	F1	3.52	55.7	3.51	55.7
A1β	4.63(0.6 H)	96.5	4.63(0.6 H)	96.5	F4	4.20	76.6	4.20	76.9
A2β	3.25(0.6 H)	74.7	3.25(0.6 H)	74.7	F5		137.4		137.2
A3β	3.73	76.9	3.74	76.9	F6	4.12–4.19	62.7	4.13–4.19	62.7
A5β	3.55	75.2	3.59	75.2	F7	5.95	126.6	5.94	126.9
B1–D1	5.37–5.39	100.1–100.3	5.37–5.38	100.1–100.4	G1	5.36	98.3	5.37–5.38	100.1–100.4
D5	4.02	69.6	4.02	69.6	G4	3.40	70.2	nd [b]	nd
D6	4.19–4.45	64.0	4.19–4.45	64.0	H1			5.35	98.2
E1	5.26	101.3	5.25	101.2	H4			3.39	70.0
Isovaleryl group									
CH_3	0.94	22.3	0.93	22.3	CH_2	2.32	43.5	2.32	43.5
CH	2.05	26.1	2.05	26.1	C=O		176.9		176.8

[a] Assignments are aided by comparisons with NMR data of **1**, acarbose, and maltriose, and supported by the HMQC experiments.
[b] Not distinguished.

10.5 Hz), 4.19 (m), and δ_C 64.0, as well as the typical signals at δ_H 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} 8.8 Hz), and δ_C 92.6, 96.5, 74.7 for the reducing glucose terminus. The configurations of the five glycosidic bonds in **2** are also determined to be α because of the diagnostic anomeric proton signals at δ 5.38 with coupling constants of $\sim$3.0 Hz. Slight differences were found in signals representing the carbon atoms and protons at position F4–F7, from δ_H 4.02, 4.09, 4.20, 5.88 in **1** to δ_H 4.20 (d, $J_{3,4}$ 7.5 Hz), 4.12 (d, J_{gem} 13.8 Hz), 4.19 (d, J_{gem} 13.8 Hz), 5.95 (d, $J_{1,7}$ 3.0 Hz) in **2**, and from δ_C 72.5, 140.1, 62.6, 124.8, to δ_C 76.6, 137.4, 62.7, 126.6, in the HMQC spectrum. These alterations suggested a glycosylated hydroxyl group attached to position F4 in **2** instead of a free hydroxyl group in **1**.[6] Another difference occurred in a new correlation between signals at δ_H 3.40 (t, J_{vic} 9.5 Hz) and δ_C 70.2, in the HMQC spectrum of **2**, indicating the presence of a free hydroxyl group at position G4.[7] All of this evidence confirmed that the appending unit of glucose was linked to position F4 through an *O*-α-glycosidic bond. The chemical structure of **2** is thus revised as shown in Scheme 2. Assignment of distinctive proton and carbon signals, aided by HMQC experiments and comparisons with reported data for acarbose[8] and maltotriose,[10] are shown in Table 2.

Structural reinvestigation of compound **3**.—Isovalertatin M23 (**3**) was obtained as a white amorphous powder. Its molecular formula was established as $C_{54}H_{91}NO_{39}$ by combined high-resolution positive ESIMS (Anal. Found $[M+H]^+$ 1378.5288, Calcd 1378.5246) and NMR data. The retention time for **3** on the same LC–ESIMS system was 11.26 min. The color reactions, spectroscopic characteristics, and liquid chromatographic behavior closely resembled that of **1**, suggesting **3** to be an analogue bearing two additional glucose units. The ESIMS^2 spectrum of its protonated molecular ion at m/z 1378 showed main fragment-ions at m/z 1360, 1216, 1198, 1054, 1036, 896, 878, 874, and 628. Based on the fragmentation pathways shown in Fig. 1, the appended two glucose units are attached to the non-reducing end of compound **1**.

The ^{1}H and ^{13}C NMR spectra of **3** appeared almost the same as those of **2**. They also showed the diagnostic signals for an isovaleryl group at δ_H 0.93 (d, J_{vic} 6.5 Hz), 2.05 (m), 2.32 (d, J_{vic} 6.0 Hz), and δ_C 22.3, 26.1, 43.5, 176.8,

for an acarviosine moiety at δ_H 5.25 (d, $J_{1,2}$ 3.0 Hz), 2.43 (t, J_{vic} 9.5 Hz), 1.28 (d, $J_{5,6}$ 5.5 Hz), 3.51 (t, J_{vic} 4.5 Hz), 4.12 (m), 4.19 (m), 5.94 (d, $J_{1,7}$ 2.5 Hz), and δ_C 101.2, 64.9, 18.1, 55.7, 62.7, 126.9; for an esterified hydroxyl group at position 6 of a glucose unit at δ_H 4.45 (dd, J_{gem} 10.0 Hz), 4.19 (m), and δ_C 64.0, as well as the typical signals at δ_H 5.20 (d, 0.4 H, $J_{1,2}$ 3.0 Hz), 4.63 (d, 0.6 H, $J_{1,2}$ 8.5 Hz), 3.25 (t, 0.6 H, J_{vic} 9.2 Hz), and δ_C 92.6, 96.5, 74.7 for the reducing glucose terminus. The configuration of the six glycosidic bonds was also determined as α because of the characteristic anomeric proton signals resonating at δ 5.37 with coupling constants of $\sim$3.0 Hz. The NMR signals for the carbon atoms and protons at position F4–F7 were also located at, δ_H 4.20 (d, $J_{3,4}$ 7.5 Hz), 4.13 (d, J_{gem} 13.5 Hz), 4.19 (d, J_{gem} 13.5 Hz), 5.98 (d, $J_{1,7}$ 3.0 Hz), and δ_C 76.9, 137.2, 62.7, 126.9. The HMQC spectrum revealed a glycosylated hydroxyl group attached to position F4 in **3**. There was only one proton, located at δ_H 3.39 (t, J_{vic} 9.5 Hz) that correlated with one carbon atom resonating at δ_C 70.0 in the HMQC spectrum of **3**, indicating the existence of a free hydroxyl group at position H4, which further hinted that the free hydroxyl group at position G4 in **2** is replaced by a glycosylated hydroxyl group in **3**. All of this evidence confirmed that an appending unit of maltose was linked to position F4 through an *O*-α-glycosidic bond. The chemical structure of **3** was thus revised as shown in Scheme 2. Assignment of distinctive proton and carbon signals, aided by the HMQC experiment and the comparisons with reported data of acarbose[8] and maltotriose,[10] are shown in Table 2.

3. Experimental

General.—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. NMR experiments were conducted on Varian Inova-500 and Bruker ARX-500 instruments at 25 °C. The D_2O solutions (0.7 mol/L, pH $\sim$ 7) were used with sodium 4,4-dimethyl-4-silapentane-5-sulfonate (DSS), 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. ESI multistage MS experiments were run on a Finnigan LCQ ion-trap mass spectrometer, and HRESIMS data were acquired on Autospec-Ultima ETOF mass spectrometer with PEG1450 or PEG2000 as internal standards. Semi-preparative HPLC was carried out on a Hewlett–Packard series 1100 instrument with a Spherisorb C_8 column (300 × 8.0 mm i.d., 10 μm). Authentic samples of *Yiwutadings* F, D, and C were supplied by Novel Medicine Research Development Center, North China Pharmacy Group.

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

Preparation of the 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 concentrated in vacuo. About a twofold volume of MeOH and eightfold Me_2CO were added to the concd aq solution, and the filtrate was discarded. The residue (13.5 g) was redissolved in water and subjected 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 combined fraction eluted by 20% aq MeOH was lyophilized to give the WC670 complex (6.6 g).

Purification of isovalertatins.—The amino-oligosaccharide-containing complex, WC670 (500 mg), was dissolved in water and filtered through a 0.45-μm membrane and separated by semi-preparative reversed-phase HPLC, using a stainless-steel column filled with 10 μm Spherisorb C_8 at 18 °C. The mobile phase was (12:88, v/v) MeCN–1.5 mmol/L aq ammonia at a flow rate of 3.0 mL/min

with UV detection at 206 nm, which gave three combined fractions from the 15.8, 17.9, and 21.7-min peaks. These fractions were further purified on the same column at 25 °C with (9:91, v/v) MeCN–1.5 mmol/L aq ammonia as the mobile phase to give **3** (46.9 mg) for the 24.6-min peak, **2** (24.5 mg) for the 27.0-min peak, and **1** (22.4 mg) for the 33.0-min peak, respectively. The purities of these oligomers were verified by positive-ion mode ESIMS analysis.

Isovalertatin M03 (**1**).—White amorphous powder; $[\alpha]_D^{18}$ +157.9° (*c* 0.1, water); UV (water): end absorption; IR ν_{max} (KBr): 3389, 2929, 1719, 1653, 1384, 1025, 579 cm^{-1}; HRESIMS (pos.): *m*/*z* 1054.4182 $[M+H]^+$ ($C_{42}H_{72}NO_{29}$ requires 1054.4190); $ESIMS^2$ (pos.): *m*/*z* (rel. int.) 1036(78.5), 896(100), 878(6.5), 874(31.0), 716(3.3), 712(20.1), 554(2.5), 550(1.5), 409(2.8), 304(62.0). For ^1H and ^{13}C NMR data, see Table 1.

Isovalertatin M13 (**2**).—White amorphous powder; $[\alpha]_D^{18}$ +157.1° (*c* 0.1, water); UV (water): end absorption; IR ν_{max} (KBr): 3387, 2930, 1719, 1655, 1384, 1027, 580 cm^{-1}; HRESIMS (pos.): *m*/*z* 1216.4723 $[M+H]^+$ ($C_{48}H_{82}NO_{34}$ requires 1216.4718); $ESIMS^2$ (pos.): *m*/*z* (rel. int.) 1216(3.7), 1198(21.4), 1054(12.4), 1036(29.1), 1018(0.9), 896(37.3), 878(1.3), 874(8.1), 856(0.5), 772(1.6), 716(0.3), 712(2.7), 628(1.7), 466(100), 408(3.4). For ^1H and ^{13}C NMR data, see Table 2.

Isovalertatin M23 (**3**).—White amorphous powder; $[\alpha]_D^{18}$ +160.0° (*c* 0.1, water); UV (water): end absorption; IR ν_{max} (KBr): 3387, 2929, 1723, 1640, 1384, 1027, 578 cm^{-1}; HRESIMS (pos.): *m*/*z* 1378.5288 $[M+H]^+$ ($C_{54}H_{92}NO_{39}$ requires 1378.5246); $ESIMS^2$ (pos.): *m*/*z* (rel. int.) 1378(2.5), 1360(22.6), 1342(1.2), 1216(11.8), 1198(26.2), 1054(14.9), 1036(19.9), 1018(1.0), 896(36.2), 878(1.0), 874(0.9), 628(100), 610(1.2), 554(0.6), 466(4.5), 408(1.4). For ^1H and ^{13}C NMR data, see Table 2.

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