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Four acarviosin-containing oligosaccharides identified from *Streptomyces coelicoflavus* ZG0656 are potent inhibitors of α-amylase

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Abstract—Four aminooligosaccharides were isolated and purified from the culture filtrate of *Streptomyces coelicoflavus* ZG0656. Their chemical structures were determined by electrospray ionization tandem mass spectrometry (ESI-MS/MS) and two-dimensional nuclear magnetic resonance (NMR) spectroscopy. The names acarviostatins I03, II03, III03, and IV03 were given to the oligomers due to their acarviosin core structures. Acarviostatins III03 and IV03, which contain three and four acarviosin–glucose moieties, respectively, were identified as novel compounds. The four acarviostatins were all mixed noncompetitive inhibitors of porcine pancreatic α-amylase (PPA). The inhibition constants (K_i) for acarviostatins III03 and IV03 were 0.008 and 0.033 μM, respectively. Acarviostatin III03 is the most effective α-amylase inhibitor known to date, with a K_i value 260 times more potent than acarbose.

Keywords: *Streptomyces coelicoflavus*; Aminooligosaccharides; Acarviosin; Acarviostatin III03; Acarviostatin IV03

1. Introduction

α-Amylase (EC 3.2.1.1) is an endo-acting enzyme that catalyzes the hydrolysis of α-(1→4)-D-glycosidic linkages of starch, amylose, amylopectin, glycogen, and various maltodextrins. α-Amylases are produced by a variety of organisms, including bacteria, fungi, plants, and animals. α-Amylase inhibitors have many medical applications, such as controlling blood glucose and serum insulin levels, and starch loading tests in animals and humans.[1]

In the course of our screening program to find new α-amylase inhibitors, we previously discovered a complex, AIB656, in the culture filtrate of a strain of *Streptomyces coelicoflavus* to which the name *S. coelicoflavus* ZG0656 was given. The complex was composed of aminooligosaccharide derivatives, showing remarkable inhibitory activity against porcine pancreatic α-amylase (PPA).[2] Two novel compounds, acarviostatin II13 and II23, were separated from the complex by our groups and have been reported previously.[3] This paper reports some additional components in the complex.

Acarviosin[4] is composed of a cyclohexitol unit (a hydroxymethylconduritol residue) and a 4-amino-4,6-dideoxy-D-glucopyranose unit (a 4-amino-4-deoxy-D-quinovopyranose residue). Acarbose was the first reported acarviosin-containing α-amylase inhibitor.[5] Because of the acarviosin moiety with one nitrogen atom in, it binds to α-amylase tighter than other α-amylase inhibitors. As a result, acarbose is 1–3 orders of magnitude more potent than other known α-amylase inhibitors.[1] Several acarviosin-containing α-amylase inhibitors have been found in recent years, including natural product inhibitors from microbes, such as trestatins,[6] isovalertatins,[7] butytatins,[8] and acarviostatins[3] as

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well as artificial acarbose analogs transformed by special enzymes, such as 4^{IV}-α-maltohexaosyl acarbose (G_6-Aca) and 4^{IV}-α-maltododecaosyl acarbose (G_{12}-Aca).[1] These acarbose analogs containing more glucose residues were found to display increased inhibitory potencies in common.

Mass spectrometry (MS) is becoming the method of choice for the analysis of oligosaccharides. Studies applying various soft ionization modes, such as fast-atom bombardment (FAB) and matrix-assisted laser desorption/ionization (MALDI), for the structural elucidation of oligosaccharides have suggested the utility of MS in the determination of monosaccharide compositions and their sequence, linkage, and branching features. Development of the electrospray ionization ion (ESI) trap mode has increased remarkably the sensitivity of the method and has decreased dramatically the amount of test sample required. Development of the MS/MS technique enables one to get the product ion spectrum from a parent ion and to obtain considerable structural information about the corresponding components. Furthermore, aminooligosaccharide derivatives show high sensitivity by positive ESI-MS analysis because amine residues readily form protonated molecules or fragments.[9] Therefore, the chemical structures of the oligomers obtained in our studies were determined mainly by ESI-MS and MS/MS techniques. Acidic hydrolysis and 2D nuclear magnetic resonance (NMR) experiments were also used to complete the structure elucidations.

This paper describes the isolation, structural elucidation, and inhibitory activities towards α-amylase of some acarviosin-containing oligosaccharides identified from *S. coelicoflavus* ZG0656.

2. Experimental

2.1. General methods

Optical rotations were measured on a Perkin–Elmer 241MC polarimeter. Infrared (IR) spectra were recorded on a Bruker IFS 55 FTIR spectrometer and as KBr pellets. Nuclear magnetic resonance (NMR) experiments were conducted on a Bruker UltraShield-300 instrument at 25 °C. The D_2O solutions were used, with sodium 4,4-dimethyl-4-silapentane-5-sulfonate (DSS) as the external standard, and the NMR spectra were recorded in 5 mm tubes at 300.07 MHz for ^1H and 75.45 MHz for ^{13}C spectra. Semi-preparative high performance liquid chromatography (HPLC) was carried out on a Shimadzu series instrument.

2.2. Mass spectrometry

Mass spectrometric detection was conducted on a Thermofinnigan LCQ Advantage mass spectrometer equipped with an ESI source and a mass range up to m/z 2000. 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 was maintained at 220 °C. The solvent was nebulized using N_2 as both the sheath gas and the auxiliary gas at a flow rate of 0.80 L/min and 0.08 L/min, respectively. Multistage MS experiments were performed using helium as the collision gas, and the relative collision energy was set at 35% from the $[M+H]^+$ or $[M+2H]^{2+}$ ions of the different molecules studied. Electrospray ionization-Fourier transformed mass spectrometry (ESI-FTMS) data were acquired on an IonSpec QFT mass spectrometer with PEG1450 as the internal standard. The conditions were identical to those above.

2.3. Microorganism

S. coelicoflavus strain ZG0656, which was collected from soil at the Nankai University campus, Tianjin, China, in 2005 was identified by the Department of Microbiology, Nankai University. A voucher specimen (CGMCC 2097) was deposited in China General Microbiological Culture Collection Center, Institute of Microbiology, Academia Sinica.

2.4. Preparation of the AIB656 complex

The culture (5 L) of *S. coelicoflavus* ZG0656 was filtered and concentrated in vacuo. About a onefold volume of EtOH was added to the concentrated aqueous solution, and the residue was discarded after centrifugation. The supernatant was concentrated to 200 mL in vacuo, and passed through a D301R macroporous resin column (300 × 40 mm) to partly remove pigments, followed by a column of X-5 macroporous resin (400 × 35 mm, washed with water, eluted with 40% aqueous EtOH), and a column of 001 × 7 cation-exchange resin (200 × 20 mm, washed with water, eluted with 1 M aqueous NH_3). A Sephadex G-25 column (eluted with water) was used at the end to obtain the 500–2000 Da fractions, which were lyophilized to give the AIB656 complex (1.3 g).[3]

2.5. Purification of acarviostatins

The aminooligosaccharide-containing complex, AIB656 (1.3 g), was dissolved in water, filtered through a 0.45-μm membrane, and separated by semi-preparative reversed-phase HPLC using a stainless-steel column filled with Kromasil C_{18} (250 × 10 mm, i.d., 10 μm) at 25 °C. The mobile phase was 7:93 (v/v) MeCN–1.5 mM aqueous NH_3 at a flow rate of 3.0 mL/min with UV detection at 210 nm. Four fractions were collected with peaks at 5.9, 10.9, 19.2, and 36.0 min. These fractions were further purified on the same column at

25 °C with MeCN–1.5 mM aqueous NH_3 as the mobile phase. Using 6:94 (v/v) MeCN–aqueous NH_3, the first fraction gave **1** (58.8 mg) at 7.1 min, and the second fraction gave **2** (43.4 mg) at 14.3 min; meanwhile, using 8:92 (v/v) MeCN–aqueous NH_3, the third fraction gave **3** (19.2 mg) at 9.7 min, and the fourth fraction gave **4** (10.0 mg) at 13.9 min. The purities of these oligomers were determined by positive-ion mode ESI-MS analysis.

2.6. Kinetics of PPA inhibition by acarviostatins

Soluble amylose (0.05–0.5% w/v) in 25 mM imidazolium–HCl buffer (pH 6.5) containing 1.0 mM $CaCl_2$ was used as the substrate solution for the PPA inhibition studies. The substrate solution (450 μL), containing various concentrations of inhibitors (acarbose, acarviostatins I03, II03, III03, and IV03) was preincubated at 37 °C for 10 min. The reactions were started by adding 50 μL of PPA (Sigma, USA) enzyme solution (5 U/mL). One unit (U) of α-amylase was defined as the hydrolysis of 1 μmol of α-(1→4)-glycosidic linkages per minute. A 50 μL sample was taken every 5 min, and the reaction was stopped by adding 50 μL of 3 M NaOH. The reducing value was measured by adding 75 μL of 3,5-dinitrosalicylic acid (DNS) and boiling for 5 min to acquire absorbance at 490 nm, using maltose as a standard. The curves of product amount, in terms of μg maltose equivalents, versus time in minutes were obtained. The initial velocities (v) were then determined from the slope of the linear portion of the curve. Dixon plots of $1/v$ versus inhibitor concentration for four concentrations of soluble amylose were used to determine the type of inhibition and the inhibition constants, K_i and K_i'.[1,3]

2.7. Acarviostatin I03 (1)

White amorphous powder; $[\alpha]_D^{18}$ +159 (c 0.1, water); UV (water): end absorption; IR ν_{max} (KBr): 3385, 2927, 1638, 1382, 1029, 580 cm^{-1}; ESI-FTMS (pos.): m/z 970.3609 $[M+H]^+$ ($C_{37}H_{63}NO_{28}$ requires 970.3610); for ESI-MS/MS (pos.), see Figure 1; for ^1H and ^{13}C NMR data, see Table 1.

2.8. Acarviostatin II03 (2)

White amorphous powder; $[\alpha]_D^{18}$ +165 (c 0.1, water); UV (water): end absorption; IR ν_{max} (KBr): 3385, 2926, 1660, 1384, 1041, 579 cm^{-1}; ESI-FTMS (pos.): m/z 1435.5438 $[M+H]^+$ ($C_{56}H_{94}N_2O_{40}$ requires 1435.5456); for ESI-MS/MS (pos.), see Figure 2; for ^1H and ^{13}C NMR data, see Table 2.

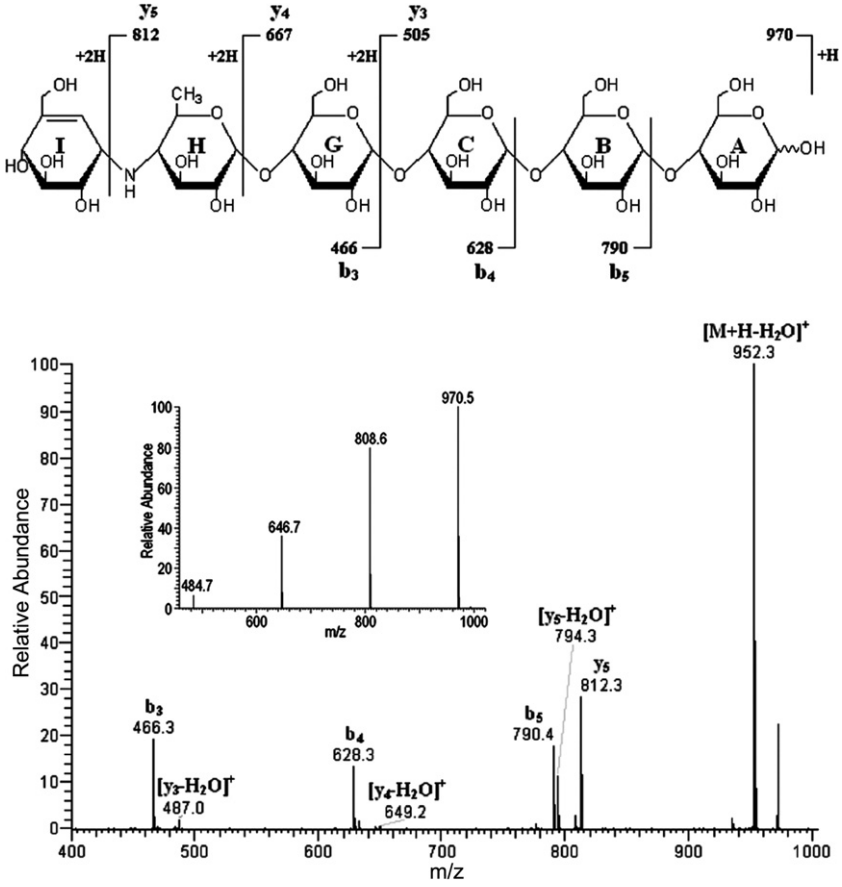


Figure 1. Positive ESI-MS/MS fragmentation and spectrum of $[M+H]^+$ of **1** at m/z 970 (inset: full scan MS of **1**).

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

Position	δ_C	δ_H	Position	δ_C	δ_H	Position	δ_C	δ_H
A1α	93.0	5.16	B1,C1,G1	~100.7	~5.33	H1	101.0	5.24
A2α	72.6	3.50	B2,C2,G2	~72.6	~3.52	H2	70.7	3.68
A3α	74.4	4.01	B3,C3,G3	~74.4	~3.86	H3	73.8	3.52
A4α	79.3	3.56	B4,C4,G4	~78.0	~3.57	H4	66.0	2.40
A5α	71.0	3.91	B5,C5,G5	~72.2	~3.76	H5	70.7	3.72
A6α	61.5	~3.69	B6,C6,G6	~61.5	~3.76	H6	18.4	1.27
A1β	96.8	4.58				I1	57.1	3.46
A2β	75.1	3.22				I2	74.0	3.60
A3β	77.3	3.63				I3	73.8	3.66
A4β	77.8	3.58				I4	72.2	3.96
A5β	75.6	3.50				I5	140.1	—
A6β	61.5	~3.70				I6	62.6	4.07/4.15
						I7	124.8	5.82

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

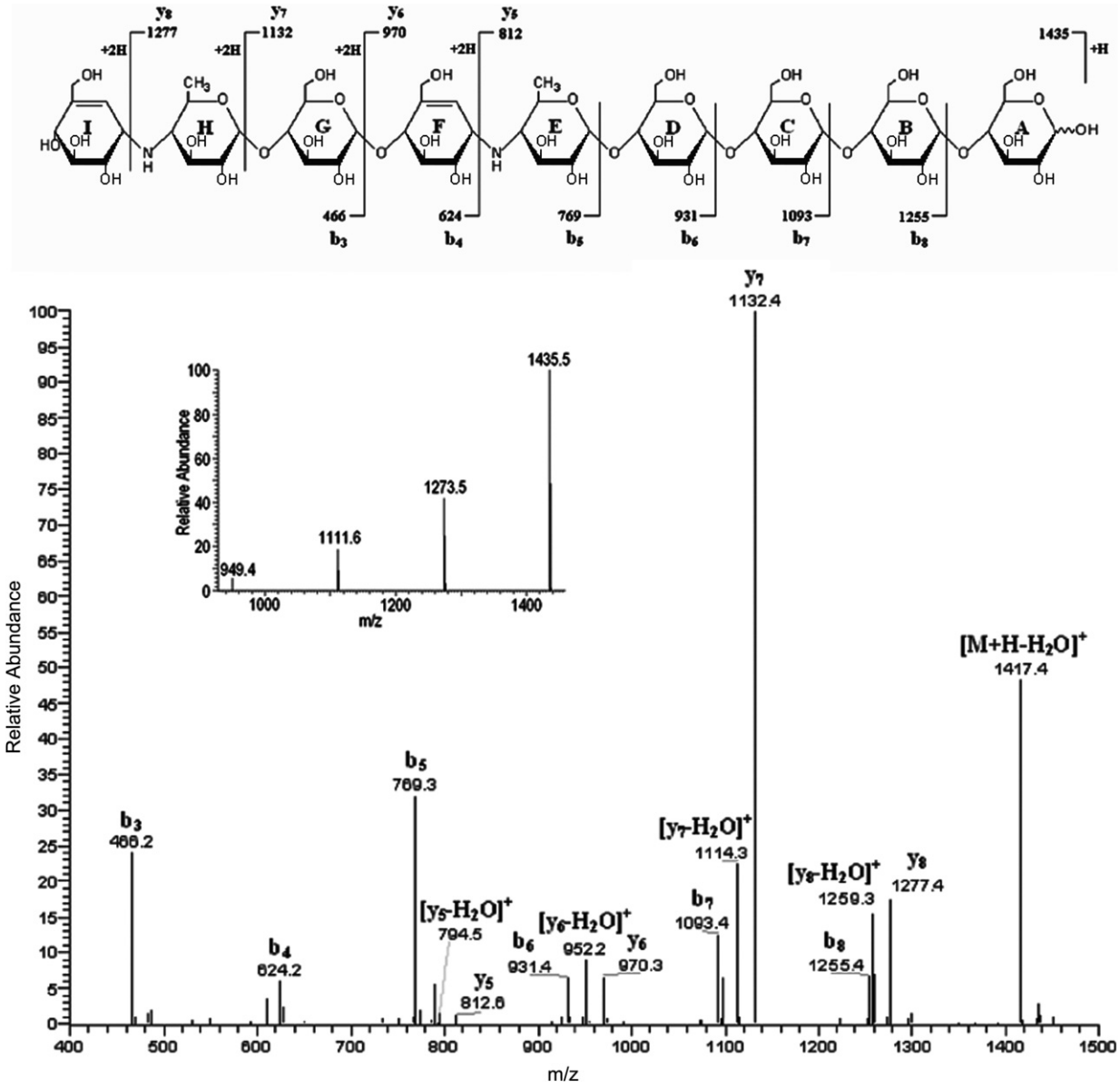


Figure 2. Positive ESI-MS/MS fragmentation and spectrum of $[M+H]^+$ of **2** at *m/z* 1435 (inset: full scan MS of **2**).

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

Position	δ_C	δ_H	Position	δ_C	δ_H	Position	δ_C	δ_H	Position	δ_C	δ_H
A1α	93.1	5.16	B1,C1,D1	~100.8	~5.33	E1	101.1	5.26	H1	101.1	5.26
A2α	72.5	3.49	B2,C2,D2	~72.5	~3.52	E2	70.6	3.67	H2	70.6	3.67
A3α	74.4	4.00	B3,C3,D3	~74.4	~3.89	E3	73.7	3.52	H3	73.7	3.52
A4α	79.3	3.55	B4,C4,D4	~77.9	~3.55	E4	65.2	2.40	H4	66.1	2.40
A5α	71.0	3.91	B5,C5,D5	~72.2	~3.75	E5	70.4	3.73	H5	70.4	3.73
A6α	61.5	~3.68	B6,C6,D6	~61.5	~3.75	E6	18.4	1.27	H6	18.4	1.27
A1β	97.1	4.58	G1	98.5	5.30	F1	56.1	3.45	I1	57.0	3.45
A2β	75.1	3.20	G2	72.5	3.72	F2	70.6	3.67	I2	74.0	3.60
A3β	77.3	3.63	G3	74.4	3.86	F3	71.8	4.07	I3	73.7	3.65
A4β	77.9	3.56	G4	77.9	3.55	F4	77.1	4.16	I4	72.2	3.95
A5β	75.6	3.50	G5	72.2	3.83	F5	137.5	—	I5	140.1	—
A6β	61.5	~3.71	G6	61.5	~3.80	F6	63.1	4.07/4.12	I6	62.6	4.05/4.12
						F7	127.4	5.90	I7	124.8	5.82

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

2.9. Acarviostatin III03 (3)

White amorphous powder; $[\alpha]_D^{18}$ +168 (*c* 0.1, water); UV (water): end absorption; IR ν_{max} (KBr): 3416, 2927, 1660, 1406, 1044, 571 cm^{-1}; ESI-FTMS (pos.): *m/z* 950.8675 $[M+2H]^{2+}$ ($C_{75}H_{125}N_3O_{52}$ requires 950.8687); for ESI-MS/MS (pos.), see Figure 3; for ^{1}H and ^{13}C NMR data, see Table 3.

2.10. Acarviostatin IV03 (4)

White amorphous powder; $[\alpha]_D^{18}$ +153 (*c* 0.1, water); UV (water): end absorption; IR ν_{max} (KBr): 3405, 2926, 1660, 1383, 1039, 578 cm^{-1}; ESI-FTMS (pos.): *m/z* 1182.9544 $[M+2H]^{2+}$ ($C_{94}H_{156}N_4O_{64}$ requires 1182.9570); for ESI-MS/MS (pos.), see Figure 4; for ^{1}H and ^{13}C NMR data, see Table 3.

3. Results and discussion

The complex, AIB656, was isolated from the culture filtrate of *S. coelicoflavus* ZG0656. It was then separated by semi-preparative HPLC to afford four aminooligosaccharide derivatives **1**–**4**. Complete acidic hydrolysis followed by monosaccharide analysis with the

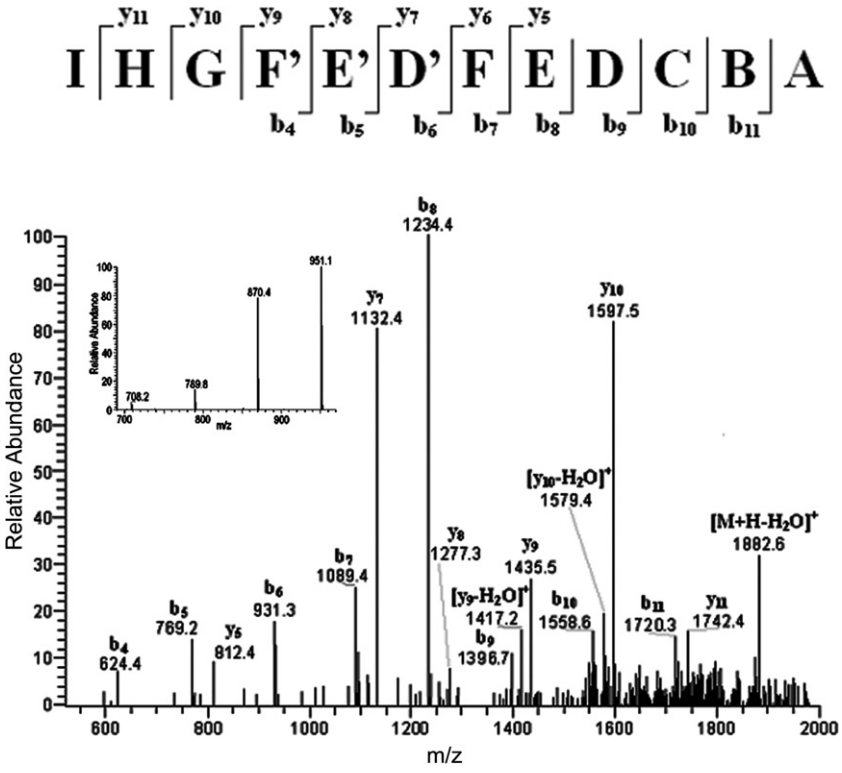


Figure 3. Positive ESI-MS/MS fragmentation and spectrum of $[M+2H]^{2+}$ of **3** at *m/z* 951 (inset: full scan MS of **3**).

Table 3. ^{1}H and ^{13}C NMR data of **3** and **4** in D_2O[a]

Position	3		4		Position	3		4	
	δ_C	δ_H	δ_C	δ_H		δ_C	δ_H	δ_C	δ_H
A1α	93.0	5.16	92.9	5.17	B1,C1,D1	~100.9	~5.34	~100.9	~5.34
A2α	72.6	3.50	72.6	3.51	B2,C2,D2	~72.6	~3.54	~72.6	~3.54
A3α	74.6	4.02	74.6	4.02	B3,C3,D3	~74.4	~3.90	~74.4	~3.91
A4α	79.3	3.56	79.4	3.56	B4,C4,D4	~77.9	~3.57	~77.9	~3.58
A5α	71.0	3.92	71.1	3.92	B5,C5,D5	~72.2	~3.77	~72.2	~3.77
A6α	61.5	~3.70	61.5	~3.70	B6,C6,D6	~61.5	~3.77	~61.5	~3.77
A1β	96.8	4.59	96.9	4.60	D′1[b],G1	98.5	5.31	98.5	5.32
A2β	75.1	3.21	75.1	3.21	D′2,G2	72.6	3.73	72.6	3.73
A3β	77.3	3.64	77.3	3.64	D′3,G3	74.4	3.87	74.4	3.88
A4β	77.9	3.57	77.9	3.58	D′4,G4	77.9	3.57	77.9	3.58
A5β	75.6	3.51	75.6	3.52	D′5,G5	72.2	3.85	72.2	3.85
A6β	61.5	~3.72	61.5	~3.72	D′6,G6	61.5	~3.81	61.5	~3.80
E1[c]	100.9	5.27	100.9	5.28	H1	100.9	5.27	100.9	5.28
E2	70.6	3.69	70.7	3.69	H2	70.6	3.69	70.7	3.69
E3	73.6	3.53	73.6	3.53	H3	73.6	3.53	73.6	3.53
E4	65.2	2.40	65.2	2.41	H4	66.1	2.40	66.1	2.41
E5	70.5	3.74	70.5	3.75	H5	70.5	3.74	70.5	3.75
E6	18.4	1.26	18.4	1.27	H6	18.4	1.26	18.4	1.27
F1[d]	56.1	3.47	56.1	3.47	I1	57.1	3.47	57.1	3.47
F2	70.6	3.69	70.7	3.69	I2	74.0	3.60	74.0	3.61
F3	71.8	4.09	71.8	4.09	I3	73.7	3.66	73.7	3.67
F4	77.0	4.16	77.0	4.16	I4	72.2	3.97	72.2	3.97
F5	137.5	—	137.5	—	I5	140.0	—	140.1	—
F6	63.1	4.09/4.12	63.1	4.09/4.13	I6	62.6	4.07/4.12	62.6	4.07/4.13
F7	127.4	5.90	127.4	5.91	I7	124.8	5.82	124.9	5.82

[a] Assignments are supported by TOCSY, HMQC, and HMBC experiments.
[b] Assignments of D″ in **4** are identical to those of D′.
[c] Assignments of E′ and E″ are identical to those of E.
[d] Assignments of F′ and F″ are identical to those of F.

1-phenyl-3-methyl-5-pyrazolone (PMP) pre-column derivatization HPLC method[10] revealed that **1**–**4** were composed of two structural units, D-glucose and acarviosin, when compared to acarbose. Acarviosin cannot be further hydrolyzed under these acidic conditions.[11]

The structural elucidations (in detail below) display that **1**–**4** have a core, pseudotrisaccharide, formed by an acarviosin unit and a D-glucopyranose group through an α-(1→4) quinovosidic bond. Aminooligosaccharides are therefore named acarviostatins followed by a Roman numeral and two numbers,[3] i.e. acarviostatins I03, II03, III03, and IV03. *Acarvios* originates from the acarviosin core; the Roman numeral I, II, III, or IV represents one, two, three, or four pseudotrisaccharide residues, respectively; the middle digit represents the number of glucose units at the non-reducing end, and the last digit represents the number of glucose units at the reducing end.

The formation of b_i and y_j fragment ions is characteristic of glycosidic bond dissociation of protonated oligosaccharides. However, acarviostatin molecules contain cyclohexitol, a pseudosaccharide unit. The cyclohexitol–nitrogen bond is defined as a pseudoglycosidic bond, and the cleavage of the pseudoglycosidic bond also yields b_i and y_j fragment ions.[3]

3.1. Structural determination of 1

Acarviostatin I03 (**1**) was a white amorphous powder. The molecular formula was deduced as $C_{37}H_{63}NO_{28}$ by combined positive ESI-FTMS (Anal. Found $[M+H]^+$ 970.3609, Calcd 970.3610) and NMR data. The IR spectrum of **1** displayed absorption at 1638 cm^{-1} for the C=C double bond and broad bands at 3385 and 1029 cm^{-1} for the oligosaccharide.

The positive full-scan ESI-MS of **1** showed a strong $[M+H]^+$ signal at *m*/*z* 970 (Fig. 1). The signals at *m*/*z* 808, 646, and 484 were due to the loss of one to three glucose units from the molecule. The ESI-MS/MS spectrum from $[M+H]^+$ is shown in Figure 1. The daughter ion at *m*/*z* 952 corresponded to the neutral loss of one water molecule. The product ions at *m*/*z* 790 (b_5), 628 (b_4), and 466 (b_3) agreed with the ordinal loss of one to three glucose units attached to the reducing end. The fragment ion at *m*/*z* 812 (y_5) resulted from the loss of a cyclohexitol group attached to the non-reducing end. The ion at *m*/*z* 649 ($[y_4-H_2O]^+$) indicated the loss of an acarviosin moiety and a water molecule. The ion at *m*/*z* 487 ($[y_3-H_2O]^+$) was due to the loss of a pseudotrisaccharide residue and a water molecule. Generally, the cleavages occurred on every C-1-oxygen bond and every abundant fragment possessed a nitrogen-contain-

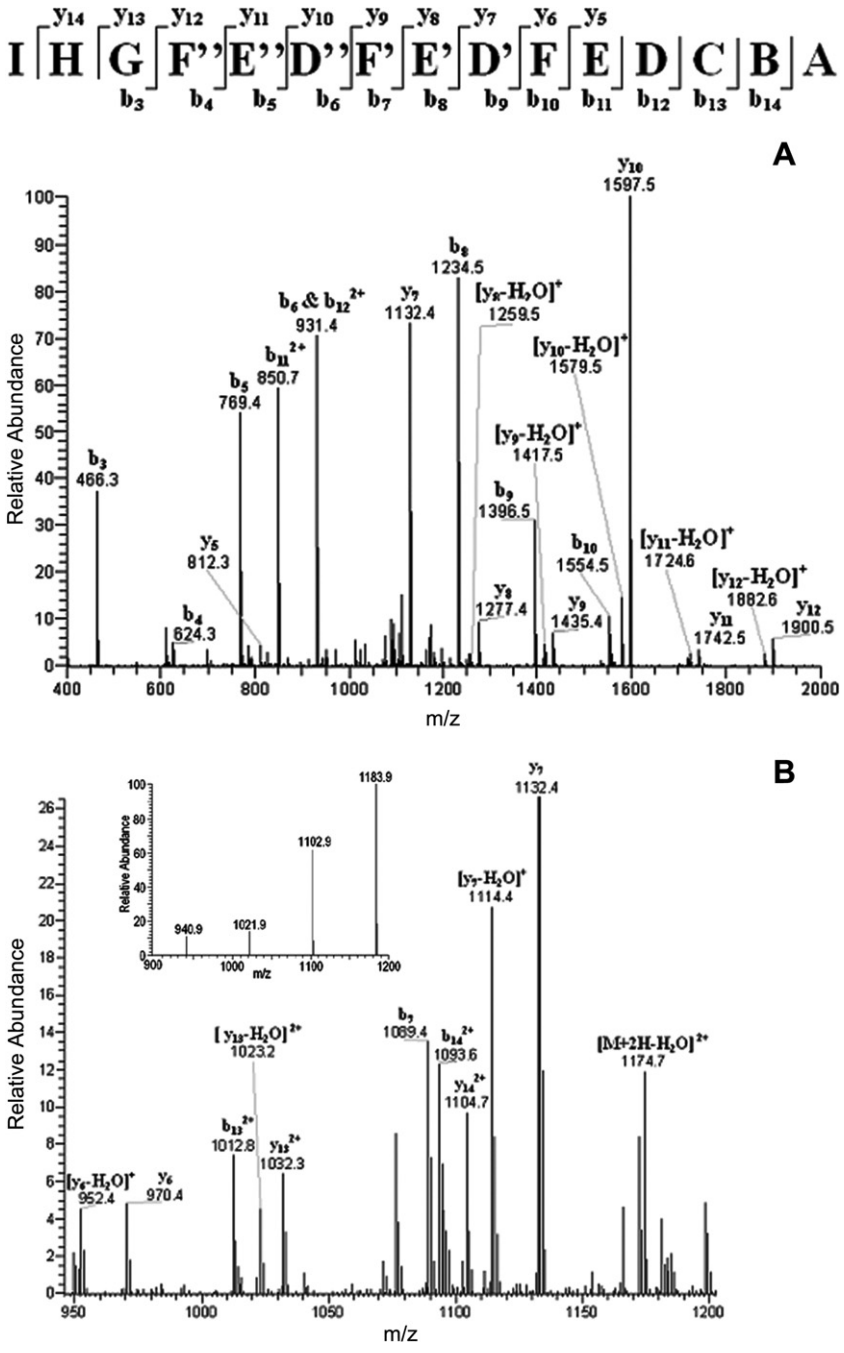
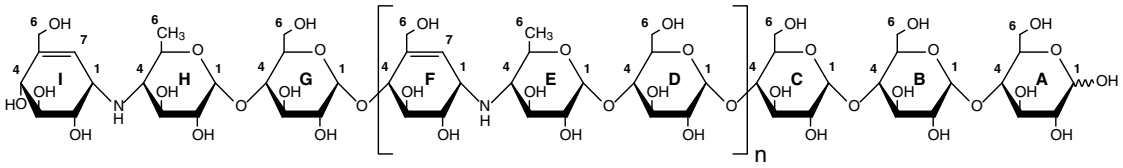


Figure 4. Positive ESI-MS/MS fragmentation and spectrum of $[M+2H]^{2+}$ of **4** at *m*/*z* 1183 (A: full scan spectrum; B: amplified spectrum from *m*/*z* 950 to *m*/*z* 1200; inset: full scan MS of **4**).

ing group. This characteristic could differentiate a monosaccharide unit on the 'left side' from one on the 'right side'. The ions y_j could further form a water-loss fragment due to the existence of a hydroxyl group at the reducing terminus, whereas b_i could not because of the absence of a reducing hydroxyl group. These features of the MS/MS spectrum indicated the structure of **1** outlined in Figure 1 and Scheme 1.

The ^{1}H, ^{13}C, total correlation spectroscopy (TOCSY), and heteronuclear multiple quantum correlation (HMQC) NMR spectra of **1** showed the presence of one terminal unit of acarviosin in the non-reducing end,[6–8] including δ_C 101.0/ δ_H 5.24 (d, $J_{1,2} = 3.3$ Hz), δ_C 66.0/δ_H 2.40 (t, $J_{vic} = 9.6$ Hz), δ_C 18.4/δ_H 1.27 (d, 3H, $J_{5,6} = 6.3$ Hz), δ_C 57.1/δ_H 3.46 (t, $J_{vic} = 5.1$ Hz), δ_C 72.2/δ_H 3.96 (d, $J_{3,4} = 7.2$ Hz), δ_C 62.6/δ_H 4.07 (m)/ 4.15 (d, $J_{gem} = 14.1$ Hz), δ_C 124.8/δ_H 5.82 (d, $J_{1,7} =$ 5.1 Hz), and δ_C 140.1. The NMR data confirmed that there was no glucose unit attached to the non-reducing end of **1**. The HMQC spectrum of **1** showed typical



Acarviostatin I03 (**1**): n=0

Acarviostatin II03 (**2**): n=1

Acarviostatin III03 (**3**): n=2

Acarviostatin IV03 (**4**): n=3

Scheme 1.

characteristic signals for the reducing terminal glucose unit[3,7,8] at δ_C 93.0/δ_H 5.16 (t, 0.4H, $J_{1,2} = 3.6$ Hz), δ_C 96.8/δ_H 4.58 (d, 0.6H, $J_{1,2} = 7.8$ Hz), and δ_C 75.1/δ_H 3.22 (t, 0.6H, $J_{vic} = 8.1$ Hz). It is well known that the NMR signals for the methine group at C-4 in a glucose unit normally resonate at δ_C 70.4/δ_H 3.42.[3,7,8] In the HMQC spectrum of **1**, four C-4 methine signals of four glucose units appeared at about δ_C 77.8–79.3/δ_H 3.56–3.58 (overlapped), while no correlation at about δ_C 70.4/δ_H 3.42 was found, revealing that all four C-4 hydroxyl groups of the four glucose units were glycosylated. As the chemical shifts for three anomeric C-1 methines on rings B, C, and G occurred at δ_C 100.7–101.0/δ_H 5.33 (m, 3H), and the coupling constants of the protons were ~3.0 Hz, the configuration of the glycosidic bonds was determined to be α-(1→4).[3,7,8]

The complete proton and carbon signal assignments of **1**, aided by TOCSY, HMQC, and heteronuclear multiple bond correlation (HMBC) experiments, and comparisons with reported data for compound 375372-11-5,[12] trestatin A,[6] isovalertatin M03,[7] and butytatin M03[8] are shown in Table 1. The structure of acarviostatin I03 (**1**) was identical to that of compound 375372-11-5.[12]

3.2. Structural determination of 2

Acarviostatin II03 (**2**), obtained as a white amorphous powder, was assigned the molecular formula of $C_{56}H_{94}N_2O_{40}$ by positive ESI-FTMS (Anal. Found $[M+H]^+$ 1435.5438, Calcd 1435.5456) and NMR data. The spectroscopic characteristics and liquid chromatographic behavior of **2** closely resembled those of **1**, suggesting **2** to be an analog with a repeating moiety appended.

The full-scan ESI-MS of **2** showed an abundant $[M+H]^+$ signal at *m*/*z* 1435 (Fig. 2). The signals at *m*/*z* 1273, 1111, and 949 were attributed to the loss of one to three glucose units from the molecule. Careful inspection of the ESI-MS/MS spectrum from $[M+H]^+$ (Fig. 2) revealed that the fragmentation pattern was quite similar to that of **1**. The fragment ion at *m*/*z* 1417 corresponded to the neutral loss of one water molecule. The product ions at *m*/*z* 1255 (b_8), 1093 (b_7), and 931 (b_6) matched with the ordinal loss of one to three glucose units attached to the reducing end. The most abundant daughter ions at *m*/*z* 1132 (y_7) and 769 (b_5) were produced by the cleavages of the bond between the quinovopyranose and glucose units, which indicated the relatively weaker intensity compared to the other ordinary glycosidic bonds in the molecule. The fragment ions at *m*/*z* 1277 (y_8), 812 (y_5), and 624 (b_4) resulted from the dissociations of the pseudoglycosidic bond within the acarviosin moiety. The ions at *m*/*z* 970 (y_6) and 466 (b_3) were due to the cleavage of the glycosidic bond between two pseudotrisaccharide residues. The ions y_j could also further form a water-loss fragment. These features of the MS/MS spectrum allowed the structure of **2** outlined in Figure 2 and Scheme 1 to be proposed.

The ^1H, ^{13}C, TOCSY, and HMQC NMR spectra of **2** appeared very similar to those of **1**, showing the characteristic signals for a terminal acarviosin moiety at δ_C 101.1/δ_H 5.26 (d, $J_{1,2} = 2.7$ Hz), δ_C 66.1/δ_H 2.40 (t, $J_{vic} = 9.6$ Hz), δ_C 18.4/δ_H 1.27 (d, 3H, $J_{5,6} = 6.0$ Hz), δ_C 57.0/δ_H 3.45 (t, $J_{vic} = 3.6$ Hz), δ_C 72.2/δ_H 3.95 (d, $J_{3,4} = 6.9$ Hz), δ_C 62.6/δ_H 4.05 (m)/4.12 (m), δ_C 124.8/δ_H 5.82 (d, $J_{1,7} = 3.9$ Hz), and δ_C 140.1. However, NMR spectra of **2** also agreed with the presence of one inner unit of acarviosin[3,6,7] at δ_C 65.2/δ_H 2.40 (t, $J_{vic} = 9.6$ Hz), δ_C 56.1/δ_H 3.45 (t, $J_{vic} = 3.6$ Hz), δ_C 77.1/δ_H 4.16 (d, $J_{3,4} = 5.4$ Hz), δ_C 63.1/δ_H 4.07 (m)/4.12 (m), δ_C 127.4/δ_H 5.90 (d, $J_{1,7} = 3.3$ Hz), and δ_C 137.5. The data confirmed two acarviosin moieties in the structure of **2**. The NMR spectra of **2** also showed the signals for the reducing glucose terminus at δ_C 93.1/δ_H 5.16 (t, 0.4H, $J_{1,2} = 3.6$ Hz), δ_C 97.1/δ_H 4.58 (d, 0.6H, $J_{1,2} = 7.8$ Hz), and δ_C 75.1/δ_H 3.20 (t, 0.6H, $J_{vic} = 8.1$ Hz). The configurations of the glycosidic bonds in **2** were also determined to be α-(1→4) because of the diagnostic anomeric C-1 methine signals at δ_C 100.8/δ_H 5.26–5.34 (m, 6H, ~3.0 Hz), and C-4 signals at about δ_C 77.9/δ_H 3.55 (overlapped).

The complete proton and carbon signal assignments of **2**, aided by TOCSY, HMQC, and HMBC experi-

ments, and comparisons with reported data for trestatin B,[6] isovalertatin M13,[7] butytatin M13,[8] and acarviostatin II23[3] are shown in Table 2. There were several revisions in the assignments compared to the reported data of compound 375372-10-4,[12] although the structure of acarviostatin II03 (**2**) was identical to that of the known oligomer.

3.3. Structural determination of 3

Acarviostatin III03 (**3**) was obtained as a white amorphous powder. Its molecular formula was established as $C_{75}H_{125}N_3O_{52}$ via positive ESI-FTMS (Anal. Found $[M+2H]^{2+}$ 950.8675, Calcd 950.8687) and NMR data. The spectroscopic characteristics and liquid chromatographic behavior of **3** closely resembled those of **1**, suggesting **3** to be an analog with two additional acarviosin–glucose moieties.

The full-scan ESI-MS of **3** showed a strong $[M+2H]^{2+}$ signal at *m/z* 951 (Fig. 3). The signals at *m/z* 870, 789, and 708 resulted from the loss of one to three glucose units from the molecule. The ESI-MS/MS spectrum from $[M+2H]^{2+}$ (Fig. 3) showed that the fragmentation pattern was quite similar to that of **1** and **2**. The daughter ion at *m/z* 1882 indicated the loss of one water molecule. The product ions at *m/z* 1720 (b_{11}), 1558 (b_{10}), and 1396 (b_9) agreed with the ordinal loss of one to three glucose units attached to the reducing end. The abundant daughter ions at *m/z* 1597 (y_{10}), 1132 (y_7), 1234 (b_8), and 769 (b_5) were produced by the cleavages of the quinovosidic bond. The fragment ions at *m/z* 1742 (y_{11}), 1277 (y_8), 812 (y_5), 1089 (b_7), and 624 (b_4) resulted from the dissociations of the pseudoglycosidic bond. The ions at *m/z* 1435 (y_9) and 931 (b_6) were due to the cleavages of the glycosidic bond between pseudotrisaccharide residues. Some water-loss fragments produced by the ions y_j were still found. The fragmentation pathway indicated the structure of **3** outlined in Figure 3 and Scheme 1.

The ^1H, ^{13}C, TOCSY, and HMQC NMR spectra of **3** appeared almost the same as those of **2** besides the relative abundance of different signals. They showed the diagnostic signals for a terminal acarviosin moiety at δ_C 100.9/δ_H 5.27 (m), δ_C 66.1/δ_H 2.40 (t, $J_{vic} = 9.3$ Hz), δ_C 18.4/δ_H 1.26 (d, 3H, $J_{5,6} = 6.0$ Hz), δ_C 57.1/δ_H 3.47 (t, $J_{vic} = 3.3$ Hz), δ_C 72.2/δ_H 3.97 (d, $J_{3,4} = 6.9$ Hz), δ_C 62.6/δ_H 4.07 (m)/4.12 (m), δ_C 124.8/δ_H 5.82 (d, $J_{1,7} = 5.1$ Hz), and δ_C 140.0; for two inner acarviosin moieties at δ_C 65.2/δ_H 2.40 (t, 2H, $J_{vic} = 9.3$ Hz), δ_C 56.1/δ_H 3.47 (t, 2H, $J_{vic} = 3.3$ Hz), δ_C 77.0/δ_H 4.16 (d, 2H, $J_{3,4} = 5.7$ Hz), δ_C 63.1/δ_H 4.09 (m)/4.12 (m), δ_C 127.4/δ_H 5.90 (d, 2H, $J_{1,7} = 3.6$ Hz), and δ_C 137.5; and for the reducing glucose terminus at δ_C 93.0/δ_H 5.16 (t, 0.4H, $J_{1,2} = 3.6$ Hz), δ_C 96.8/δ_H 4.59 (d, 0.6H, $J_{1,2} = 8.1$ Hz), and δ_C 75.1/δ_H 3.21 (t, 0.6H, $J_{vic} = 8.1$ Hz). The configurations of the glycosidic bonds in **3** were also determined to be α-(1→4) because of the characteristic anomeric C-1 methine signals at δ_C 100.9/δ_H 5.27–5.34 (m, 8H, ~3.0 Hz), and C-4 signals at about δ_C 77.9/δ_H 3.57 (overlapped).

The complete proton and carbon signal assignments of **3**, aided by TOCSY, HMQC, and HMBC experiments, and comparisons with data for **2**, trestatin C,[6] isovalertatins,[7] butytatins,[8] and acarviostatins[3] are shown in Table 3. Although **3** had the same molecular formula as the known compound trestatin C, the spectral characteristics of **3** determined that it contained a reducing glucose terminus and lacked α-(1→1) glycosidic bonds, which differed from trestatin C. Therefore, **3** is a novel compound and was named acarviostatin III03.

3.4. Structural determination of 4

Acarviostatin IV03 (**4**), obtained as a white amorphous powder, was assigned the molecular formula of $C_{94}H_{156}N_4O_{64}$ by positive ESI-FTMS (Anal. Found $[M+2H]^{2+}$ 1182.9544, Calcd 1182.9570) and NMR data. The spectroscopic characteristics and liquid chromatographic behavior of **4** closely resembled those of **1**, suggesting **4** to be an analog with three appended acarviosin–glucose moieties.

The $[M+2H]^{2+}$ ion at *m/z* 1183 of **4** was found in the full-scan ESI-MS (Fig. 4). The signals at *m/z* 1102, 1021, and 940 corresponded to the loss of one to three glucose units from the molecule. The ESI-MS/MS spectrum from $[M+2H]^{2+}$ displayed a quite similar fragmentation pathway compared to that of **3**. The doubly protonated ion at *m/z* 1174 indicated the loss of one water molecule. The ordinal loss of one to three glucose units attached to the reducing end produced doubly protonated ions at *m/z* 1093 (b_{14}), 1012 (b_{13}), and 931 (b_{12}). Similar to **2** and **3**, the quinovosidic bond cleavages yielded doubly protonated daughter ions at *m/z* 1032 (y_{13}) and 850 (b_{11}), and abundant single protonated fragment ions at *m/z* 1597 (y_{10}), 1132 (y_7), 1234 (b_8), and 769 (b_5). The dissociations of the pseudoglycosidic bond resulted in doubly protonated ion at *m/z* 1104 (y_{14}) and single protonated ions at *m/z* 1742 (y_{11}), 1277 (y_8), 812 (y_5), 1554 (b_{10}), 1089 (b_7), and 624 (b_4). The cleavages of the glycosidic bond between pseudotrisaccharide residues led to the ions at *m/z* 1900 (y_{12}), 1435 (y_9), 970 (y_6), 1396 (b_9), 931 (b_6), and 466 (b_3). A number of water-loss fragments produced by the ions y_j were easily observed. In particular, the fragments with relatively high molecular weights, such as y_{13}, y_{14}, and b_{11} to b_{14}, formed doubly protonated ions, whereas the fragments with relatively low molecular weights formed single protonated ions. The cleavage pathway suggested the structure of **4** outlined in Figure 4 and Scheme 1.

The ^1H, ^{13}C, TOCSY, and HMQC NMR spectra of **4** appeared almost the same as those of **3** besides the relative abundance of different signals. They also showed

the characteristic signals for a terminal acarviosin moiety at δ_C 100.9/δ_H 5.28 (d, $J_{1,2} = 3.0$ Hz), δ_C 66.1/$\delta_H = 2.41$ (t, $J_{vic} = 9.6$ Hz), δ_C 18.4/δ_H 1.27 (d, 3H, $J_{5,6} = 6.0$ Hz), δ_C 57.1/δ_H 3.47 (m), δ_C 72.2/δ_H 3.97 (d, $J_{3,4} = 6.9$ Hz), δ_C 62.6/δ_H 4.07 (m)/4.13 (m), δ_C 124.9/δ_H 5.82 (d, $J_{1,7} = 5.1$ Hz), and δ_C 140.1; for three inner acarviosin moieties at δ_C 65.2/δ_H 2.41 (t, 3H, $J_{vic} =$ 9.6 Hz), δ_C 56.1/δ_H 3.47 (m), δ_C 77.0/δ_H 4.16 (d, 3H, $J_{3,4} = 5.4$ Hz), δ_C 63.1/δ_H 4.09 (m)/4.13 (m), δ_C 127.4/δ_H 5.91 (d, 3H, $J_{1,7} = 3.9$ Hz), and δ_C 137.5; for the reducing glucose terminus at δ_C 92.9/δ_H 5.17 (t, 0.4H, $J_{1,2} = 3.3$ Hz), δ_C 96.9/δ_H 4.60 (d, 0.6H, $J_{1,2} =$ 8.1 Hz), and δ_C 75.1/δ_H 3.21 (t, 0.6H, $J_{vic} = 8.1$ Hz). The configurations of the glycosidic bonds in **4** were also determined to be α-(1→4) because of the diagnostic anomeric C-1 methine signals at δ_C 100.9/δ_H 5.28–5.34 (m, 10H, ~3.0 Hz), and C-4 signals at about δ_C 77.9/δ_H 3.58 (overlapped).

The complete proton and carbon signal assignments of **4**, aided by TOCSY, HMQC, and HMBC experiments, and comparisons with data for **2** and **3**, trestatins,[6] isovalertatins,[7] butytatins,[8] and acarviostatins[3] are shown in Table 3. The spectral characteristics of **4** determined that the oligomer contained four acarviosin–glucose moieties and a glucose at the reducing terminus. Thus, **4** was named acarviostatin IV03. This is the first report of a compound containing four acarviosin moieties.

3.5. ESI-MS/MS fragmentation pattern of acarviostatins

All four acarviostatins showed similar ESI-MS/MS fragmentation patterns as follows: (1) Every glycosidic bond could dissociate easily, including the pseudo-glycosidic bond within the acarviosin moiety. The quinovosidic bond cleavage happened much more readily than that of an ordinary glucosidic bond. As a result, the daughter ions due to the quinovosidic bond cleavages always displayed the most abundant signals. (2) Almost all of the cognizable fragment ions originated from single bond cleavages. The product ions derived from more than one bond cleavage were rarely observed. (3) Each abundant ion in ESI positive-ion mode possessed at least one nitrogen-containing moiety. Because secondary amine residues exhibit fairly strong basicity, ESI positive-ion detections are usually enhanced by the presence of amine-containing molecules or fragments. (4) The fragments with relatively high molecular weights were likely to form doubly protonated ions. However, those with relatively low molecular weights normally formed single protonated ions. Although each secondary amine residue could be protonated in theory, it was difficult to discover multi-protonated ions from fragments containing more than two amine residues. (5) Signals due to the loss of a water molecule from fragments containing reducing hydroxyl groups, such as the precursor ion and y_j ions, were easily found; whereas ions without reducing hydroxyl groups, such as b_i ions, normally could not form product ions with the loss of water.

3.6. Kinetics of PPA inhibition by acarviostatins

The Lineweaver–Burk plots of $1/v$ versus $1/[S]$ (data not shown) and Dixon plots of $1/v$ versus [I] (Fig. 5) were made to determine the type of inhibition each of the four acarviostatins had against PPA. All four acarviostatins were mixed noncompetitive inhibitors in which both

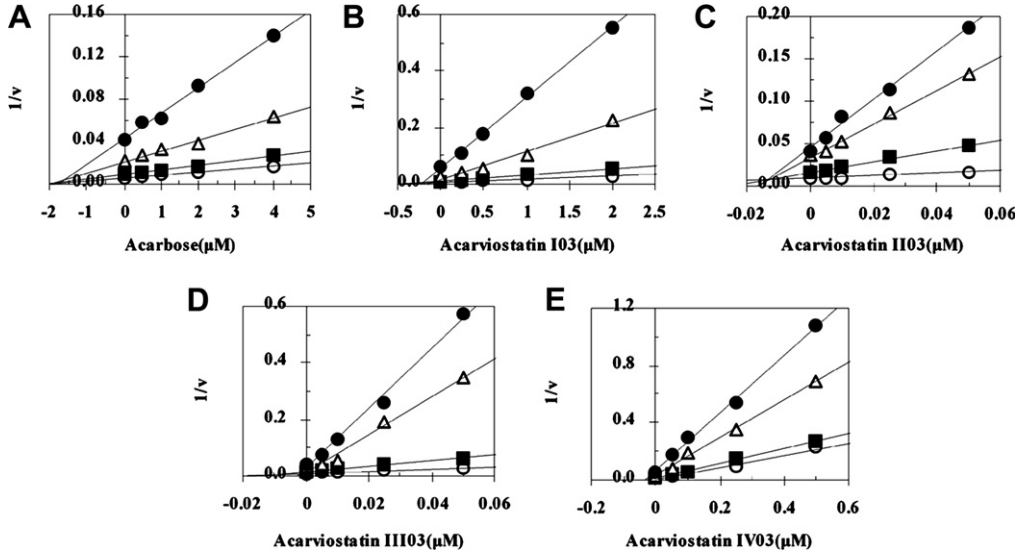


Figure 5. Dixon plots showing the velocity of amylose hydrolysis by PPA in the presence of (A) acarbose, (B) acarviostatin I03, (C) acarviostatin II03, (D) acarviostatin III03, and (E) acarviostatin IV03. Initial concentrations of amylose for lines in each plot are: (○) 0.5%, (■) 0.25%, (△) 0.1%, and (●) 0.05%; v is defined as μg maltose equivalent/mL/min.

Table 4. Inhibition constants of acarbose and acarviostatins for PPA

Inhibitor	Inhibition type	K_i[a] (μM)	K_i'[b] (μM)	Inhibition potency[c]
Acarbose	Mixed	2.077 ± 0.463	2.066 ± 0.681	1
Acarviostatin I03	Mixed	0.346 ± 0.141	8.057 ± 1.778	6
Acarviostatin II03	Mixed	0.013 ± 0.002	0.018 ± 0.003	160
Acarviostatin III03	Mixed	0.008 ± 0.002	0.018 ± 0.007	260
Acarviostatin IV03	Mixed	0.033 ± 0.009	0.011 ± 0.004	63

[a] K_i is the inhibition constant, defined as [E][I]/[EI].
[b] K_i' is the inhibition constant, defined as [ES][I]/[ESI].
[c] Inhibition potency was obtained by dividing the K_i of acarbose by the K_i of acarviostatins.

the enzyme–inhibitor complex (EI) and the enzyme–substrate–inhibitor complex (ESI) were formed. The kinetic parameters, V_m, K_m, K_i, and K_i' were determined from the Michaelis–Menten equation for mixed noncompetitive inhibition, using linear regression analysis[1,3]

$$v = V_m[S]/(K_m(1 + ([I]/K_i)) + [S](1 + ([I]/K_i')))$$

where v_i is the initial velocity, [S] and [I] are the concentrations of substrate and inhibitor, respectively, V_m is the maximum velocity, K_m is the Michaelis–Menten constant, K_i is the dissociation constant of EI, and K_i' is the dissociation constant of ESI. The inhibition constants, K_i and K_i', for each compound are given in Table 4. The relative inhibition potencies of the acarviostatins, compared to acarbose, were calculated from the ratio of K_i.

Acarviostatin III03, which contained two more acarviosin–glucose moieties at the non-reducing terminus than acarbose, was the most potent inhibitor of PPA compared to all of the known compounds,[1] with a K_i value of 0.008 μM, 260 times better than acarbose. That is to say, acarviostatin III03 is the most effective α-amylase inhibitor observed to date. Acarviostatin I03 inhibited PPA six times better than acarbose due to its two additional glucose moieties at the reducing terminus. Acarviostatins II03 and IV03 were also potent PPA inhibitors with K_i values 160 and 63 times stronger than acarbose due to one and three additional acarviosin–glucose moieties at the non-reducing terminus, respectively. These results show that a molecule with three repeating acarviosin–glucose moieties binds PPA more tightly so as to inhibit it most effectively.

4. Conclusions

Acarviostatins I03, II03, III03, and IV03 are produced by *S. coelicoflavus* ZG0656. They are all acarviosin-containing oligosaccharides as mixed noncompetitive inhibitors of PPA. Acarviostatins III03 and IV03, which contain three and four acarviosin–glucose moieties, respectively, are both novel compounds. Acarviostatin III03 is the most effective α-amylase inhibitor known to date, with a K_i value of 0.008 μM, 260 times more potent than acarbose.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carres.2008.01.020.

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