Carbohydrate Research 346 (2011) 1898–1902

Contents lists available at ScienceDirect

Carbohydrate Research

journal homepage: www.elsevier.com/locate/carres

A novel amino-oligosaccharide isolated from the culture of *Streptomyces* strain PW638 is a potent inhibitor of α-amylase

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ARTICLE INFO

Article history:
Received 19 April 2011
Received in revised form 31 May 2011
Accepted 6 June 2011
Available online 13 June 2011

Keywords:
α-Amylase inhibitor
Amino-oligosaccharide
SF638-1
Streptomyces
Diabetes

ABSTRACT

A novel amino-oligosaccharide, named SF638-1, was isolated from the culture filtrate of the *Streptomyces* strain PW638. Its chemical structure was determined by electrospray ionization tandem mass spectrometry (ESI-MS/MS) and two-dimensional nuclear magnetic resonance spectroscopy. The novel compound was a mixed inhibitor of human pancreatic α-amylase, with a K_i value in the same order of magnitude as that of the α-amylase inhibitor, acarbose. SF638-1 inhibited starch hydrolysis and glucose transfer in vitro, and suppressed postprandial blood glucose elevation in vivo. These results suggest that SF638-1 may be a potent antidiabetic agent.

1. Introduction

α-Amylase enzymes (EC 3.2.1.1) are highly evolutionarily conserved in nature, being present in organisms ranging from microbes to mammals. They catalyze the hydrolysis of α-(1→4)-D-glycosidic linkages of starch, glycogen, and various maltodextrins.[1] In humans, the pancreatic α-amylase (HPA)[2] is responsible for cleaving large malto-oligosaccharides to smaller oligosaccharides, which are substrates for intestinal α-glucosidases. This digestion process is important for glucose absorption from the intestine to the blood, and in fact, HPA activity has been correlated to postprandial blood glucose levels.[3–5] Indeed, inhibitors of α-amylase have been successfully used in the treatment of diseases such as diabetes and obesity where control of blood glucose level is essential.[6] Arguably, the most studied inhibitor of α-amylase is the naturally occurring and commercially available drug, acarbose,[7] which has a K_i value in the low nanomolar range.[2,8]

A great number of medicinal drugs have been derived from natural products. The richness and diversity of the secondary metabolism of *Streptomyces* have made these organisms valuable sources of antibiotics and other bioactive molecules.[9] A key research focus of our group is the search for bioactive compounds that inhibit enteric enzymes including α-glucosidase and α-amylase. We have previously isolated and purified six amino-oligosaccharides (acarviostatins I03, II03, III03, IV03,[8] II23, and II13[10]) from the culture filtrate of *Streptomyces coelicoflavus* ZG0656.

We had investigated the inhibitory effect of the GIB638 complex (extracted from *Streptomyces* sp. PW638) on α-amylase.[11] Its potent inhibitory activity encouraged us to purify compounds from this complex. Here, we describe the isolation of a novel α-amylase inhibitor designated as SF638-1. The bioactivity of the new amino-oligosaccharide was also tested using in vitro and in vivo enzymology techniques.

2. Results and discussion

The complex GIB638 (524.6 mg, half-maximal inhibitory concentration (IC_{50}) = 654.53 μg/mL) was isolated from the culture filtrate of *Streptomyces* strain PW638 as described in Section 3. It was then separated by semi-preparative high performance liquid chromatography (HPLC) to afford SF638-1. Initially, a reversed-phase HPLC was used, giving a fraction at 12.8 min with inhibitory activities against HPA. This fraction (150.6 mg, IC_{50} = 33.15 μg/mL) was further separated on a cation-exchange HPLC. A chromatographic peak with strong inhibitory activity against HPA was collected at 3.7 min. Although three other chromatographic peaks were evident at later time points, none of these contained significant inhibitory activity against HPA. After salt-removal and lyophilization, the compound, SF638-1, was obtained from the 3.7 min fraction (45 mg, IC_{50} = 5.06 μg/mL). The purity of SF638-1

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doi:10.1016/j.carres.2011.06.005

was determined by positive ion mode electrospray ionization mass spectrometry (ESI-MS) analysis. Detailed procedures are listed in Section 3.

2.1. Structural determination of SF638-1

SF638-1 was obtained as a white amorphous powder. The molecular formula was determined to be $C_{18}H_{35}NO_{16}$ based on data from combined positive high-resolution electrospray ionization mass spectrometry (HR-ESI-MS) (Anal. Found $[M+H]^+$ 522.2206, Calcd 522.2201) and nuclear magnetic resonance (NMR). The infrared (IR) spectrum of SF638-1 was characterized by broad bands at 3371 and 1023 cm^{-1}. Complete acidic hydrolysis followed by monosaccharide analysis using the 1-phenyl-3-methyl-5-pyrazolone (PMP) pre-column derivatization HPLC method revealed that SF638-1 was composed of two structural units, D-glucose and an unknown moiety. According to data describing its behavior in MS and NMR (methods described in detail below), the unknown moiety was confirmed as a special amino-containing saccharide.

The positive full-scan ESI-MS of SF638-1 showed a strong [M+H]+ signal at *m/z* 522. The ESI tandem mass spectrometry (MS/MS) spectrum from [M+H]+ is shown in Figure 1. We analyzed the structure of SF638-1 in accordance with the fragmentation patterns of this amino-oligosaccharide in the ESI-MS/MS spectrum, namely that (1) cleavage occurred on every C-1-oxygen bond; and (2) each abundant ion in ESI positive-ion mode possessed at least one nitrogen-containing moiety.[12] As a result of this analysis we concluded that the ions at *m/z* 505 ($[M-H_2O+2H]+$) and *m/z* 487 ($[M-2H_2O+2H]+$) indicated the ordinal loss of one and two water molecules from the parent ion, respectively. The daughter ion at *m/z* 343 (Y_2 and B_2) was due to the loss of one glucose unit from the reducing or non-reducing end of the original molecule. The product ion at *m/z* 325 ($[Y_2-H_2O]^+$ and $[B_2-H_2O]^+$) was consistent with the further loss of one water molecule from fragments Y_2 and B_2. These features of the MS/MS spectrum are predictive of the SF638-1 structure outlined in Figure 1.

SF638-1 was also tested using ^1H, ^{13}C, total correlation spectroscopy (TOCSY), heteronuclear single quantum correlation (HSQC), and heteronuclear multiple bond correlation (HMBC) NMR techniques (see Supplementary data). Three proton signals at δ 5.12 (overlapped), 4.56 (d, 0.6H, $J_{1,2}$ = 8.1 Hz), and 3.26 (t, 0.6H, J_{vic} = 8.1 Hz) were correlated to carbon signals at δ 92.1, 95.9, and 75.4, respectively, in the HSQC spectrum, a typical characteristic of a reducing terminal glucose unit. One methine signal at δ_C 70.3/δ_H 3.42 (overlapped) for C-4 indicated one glucose unit at the non-reducing terminus. The configuration of the glycosidic bond between ring A and B was confirmed to be α-(1→4) because a methine signal at about δ_C 78.2/δ_H 3.26 (overlapped) for A-4 indicated that its hydroxyl group was glycosylated. Furthermore, an anomeric C-1 methine on ring B at δ_C 100.4/δ_H 5.28 (m) with a ~3.0 Hz proton coupling constant suggested that its reducing hydroxyl group was glycosylated in the α-form. The connectivity between rings A and B was also established as α-(1→4) by the HMBC experiment, which revealed correlation of H-C(A4) with C(B1) and H-C(B1) with C(A4). The configuration of the bond between ring B

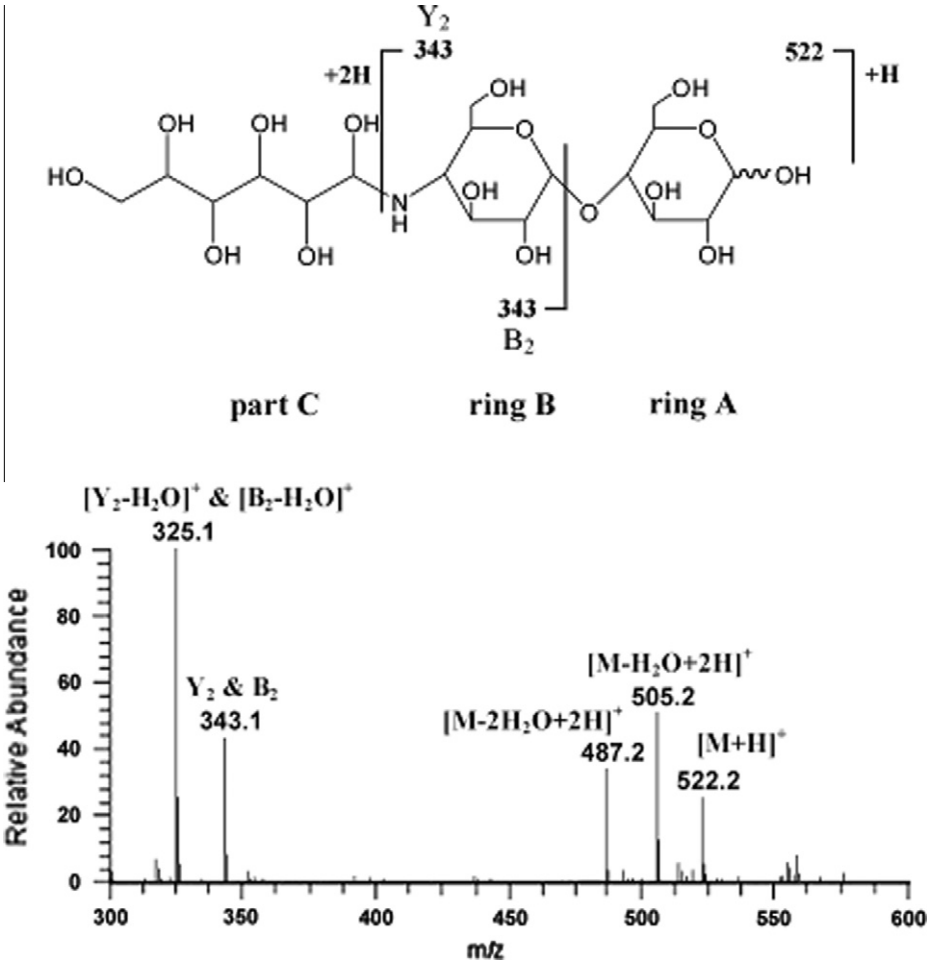

Figure 1. Positive ESI-MS/MS fragmentation and spectrum of $[M+H]^+$ of SF638-1 at *m/z* 522.

and part C was determined to be N-(1→4) because a methine signal at δ_C 69.8/δ_H 2.84 (t, J_{vic} 8.4 Hz) for B-4 went to a higher field than would be expected of a normal O-linked carbon atom. This suggests attachment to a nitrogen atom, compared with the N-pseudoglycosidic bond in the reference compound, acarviostatin I03.[8] An anomeric C-1 methine on part C at δ_C 76.4/δ_H 3.24 (overlapped) was also upfield shifted, revealing a nitrogen atom connection. Finally, the putative N-(1→4) connection between ring B and part C was also based on HMBC correlation of H-C(B4) with C(C1) and H-C(C1) with C(B4).

The complete proton and carbon signal assignments of SF638-1, aided by TOCSY, HSQC, and HMBC experiments are shown in Table 1, in comparison with reported data for trestatin,[13] butyrtatins M03 and M13,[14] acarviostatin I03,[8] and maltotriose.[15] According to the structure elucidations, SF638-1 (Fig. 1) was determined to be a novel compound.

2.2. Assay for kinetic measurement of SF638-1 inhibitory activity against α-amylase

We initially investigated the inhibitory effects of SF638-1 and acarbose on α-amylase derived from rat small intestine. As shown in Table 2, SF638-1 was a potent inhibitor of rat α-amylase with an IC_{50} (5.06 μg/mL) comparable to that of acarbose (1.54 μg/mL).

A Lineweaver–Burk plot of 1/*v* (velocity) versus 1/[S] (substrate concentration) (see Supplementary data) was constructed to determine the type of HPA inhibition induced by SF638-1. The series of lines intersect to the left of the vertical axis and above the horizontal axis, indicating that these inhibitors were mixed inhibitors in which both the enzyme–inhibitor complex (EI) and the enzyme–substrate–inhibitor complex (ESI) were formed. From the intersection of lines in Dixon plots of 1/*v* versus [I] (inhibitor concentration) (Fig. 2), the K_i values (dissociation constant of EI) of acarbose and SF638-1 against HPA were determined to be 1.47 and 1.68 μM, respectively (Table 2), suggesting that they have similar inhibitory effects.

2.3. Inhibition of starch hydrolysis and glucose transfer by SF638-1 in vitro using the everted intestinal model

The inhibition of α-amylase by SF638-1 was further tested in vitro using the rat everted intestinal sac system. Similar to acarbose, SF638-1 (3 μg/mL) significantly inhibited starch hydrolysis (P <0.01), glucose production (P <0.01), and glucose transfer to blood (P <0.01) (Table 3). These results indicated that SF638-1 could inhibit α-amylase within the intestine to reduce blood glucose level.

2.4. Suppressive effect of SF638-1 on postprandial blood glucose elevation in normal mice

We administered starch orally in normal mice to test the effects of SF638-1 on blood glucose elevation. Blood glucose level in the control group reached a maximum value at 30 min after oral starch administration (Table 4). However, compared with the control group, blood glucose levels in mice treated with either acarbose or SF638-1 were significantly (P <0.01) reduced at 30 min after starch loading. These results demonstrate that SF638-1 effectively suppresses the postprandial elevation in blood glucose level

Table 2
Effects of α-amylase inhibitors

Compounds	Rat α-amylase IC_{50}[a] (μg/mL)	Human pancreatic α-amylase Type of inhibition (K_i[b] (μM))
SF638-1	5.06 ± 0.21	Mixed(1.68 ± 0.23)
Acarbose	1.54 ± 0.15	Mixed(1.47 ± 0.14)

[a] IC_{50} is the half-maximal inhibitory concentration.
[b] K_i is the inhibition constant, defined as [E][I]/[EI].

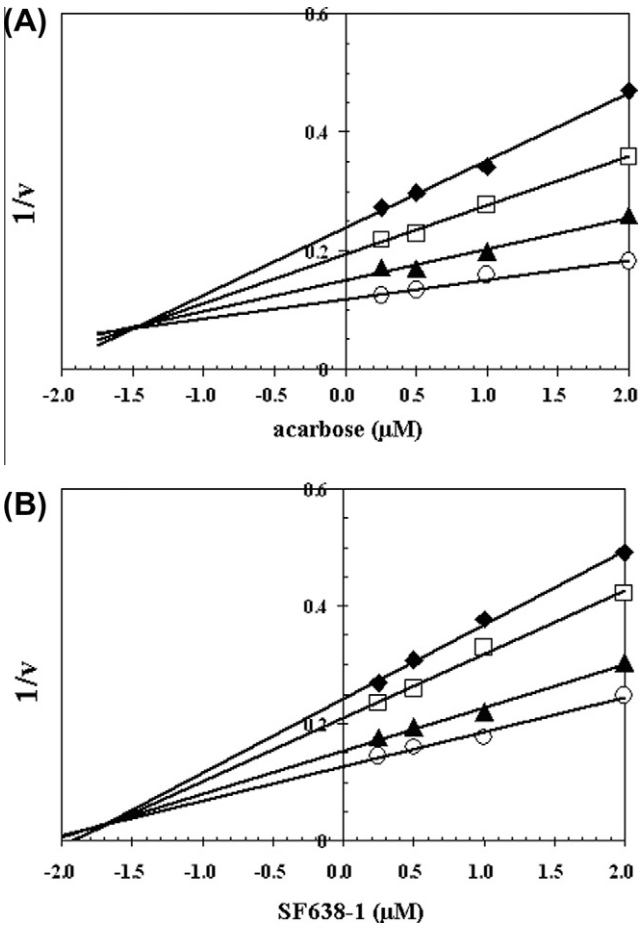

Figure 2. Dixon plots showing the kinetics of starch hydrolysis by HPA in the presence of (A) acarbose and (B) SF638-1. Initial concentrations of starch for lines in each plot are: (○) 0.5%, (▲) 0.25%, (□) 0.1%, and (♦) 0.05%; *v* is defined as μg maltose equivalent/mL/min.

Table 1
^1H and ^{13}C NMR data of SF638-1 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	92.1	5.12	A1β	95.9	4.56	B1	100.4	5.28	C1	76.4	3.24
A2α	72.1	3.56	A2β	75.4	3.26	B2	72.1	3.61	C2	73.1	3.57
A3α	74.9	3.96	A3β	77.3	3.75	B3	74.7	3.73	C3	74.3	3.66
A4α	78.2	3.26	A4β	77.6	3.65	B4	69.8	2.84	C4	70.3	3.42
A5α	71.6	3.93	A5β	69.2	3.61	B5	72.0	3.83	C5	72.0	3.83
A6α	61.1	~3.85	A6β	61.1	~3.90	B6	61.1	~3.85	C6	61.1	~3.85

[a] Assignments are supported by TOCSY and HSQC experiments.

Table 3
Inhibition of starch hydrolysis and glucose transfer by inhibitors using the in vitro everted intestinal model

Group	Dose (mg/mL)	Starch hydrolysis E/E_0 (%)	Glucose production E/E_0 (%)	Glucose transfer E/E_0 (%)
Control	—	100	100	100
Acarbose	1.5	45.6 ± 9.4**	68.8 ± 14.8**	54.8 ± 25.2**
SF638-1	0.75	75.2 ± 11.7**	87.7 ± 12.1*	79.6 ± 20.5*
	1.5	67.6 ± 5.2**	72.5 ± 7.1**	76.6 ± 18.7**
	3.0	51.3 ± 8.6**	70.4 ± 9.3**	59.4 ± 14.7**

Results are mean ± SD, $n = 5$.
* $P < 0.05$ compared with the control group.
** $P < 0.01$ compared with the control group.

Table 4
Suppressive effect of SF638-1 on postprandial blood glucose elevation in normal rats after starch loading

Group	Dose (mg/kg)	Blood glucose level (mM)			
		0 min	30 min	60 min	90 min
Control	—	4.32 ± 0.29	14.32 ± 0.91	9.24 ± 0.77	7.50 ± 0.22
Acarbose	1.5	4.17 ± 0.37	11.41 ± 1.96**	10.10 ± 1.32	7.56 ± 0.79
SF638-1	0.75	4.36 ± 0.25	12.21 ± 1.58*	8.78 ± 1.63	7.26 ± 0.36
	1.5	4.20 ± 0.17	11.63 ± 0.48**	8.53 ± 1.30	7.06 ± 0.82
	3.0	4.22 ± 0.41	11.57 ± 1.77**	9.70 ± 1.74	8.62 ± 0.93

Results are mean ± SD, $n = 8$.
* $P < 0.05$ compared with the control group.
** $P < 0.01$ compared with the control group.

induced by oral administration of starch in normal mice, and may have wider relevance to the treatment of diabetes.

3. Experimental

3.1. General methods

Optical rotation was measured on a Perkin–Elmer 241 MC polarimeter. IR spectrum was recorded on a Bio-Rad FTS 6000 spectrometer and as KBr pellets. MS was conducted on a Waters Q-TOF Premier with ESI source. NMR experiments were conducted on a Mercury 300BB instrument at ambient temperature. The D_2O solutions were used, with sodium 4,4-dimethyl-4-silapentatane-5-sulfonate (DSS), as the external standard, and NMR spectra were recorded in 5 mm tubes at 300.07 MHz for ^1H and 75.46 MHz for ^{13}C spectra. Semi-preparative HPLC was carried out on a Shimadzu series instrument.

Two-dimensional NMR spectra were recorded at 25 °C on a Bruker AVANCE 300 using a z-gradient triple resonance probe. The parameters for the phase-sensitive ^1H–^1H TOCSY spectra were as follows: spectral width, 4807.7 Hz; acquisition time, 0.213 s; relaxation delay, 1.0 s; spin-locking pulse, 60 ms; and 16 scans for each of the 160 increments. ^1H–^{13}C HSQC experiments were acquired with a spectral width of 4807.7 Hz in the F2 (^1H) dimension and 1798.5 Hz in the F1 (^{13}C) dimension; acquisition time, 0.17 s; relaxation delay, 2 s; and 32–128 scans per increment (256 increments). The ^1H–^{13}C HMBC spectra were acquired with spectral widths of 4807.7 Hz in the F2 (^1H) dimension and 18115.9 Hz in the F1 (^{13}C) dimension; acquisition time, 0.213 s; relaxation delay, 1.0 s; mixing time, 65 ms; and 32 scans per increment (256 increments).

3.2. Strain and enzymes

Streptomyces strain PW638 was isolated from soil at Fujian province, China, in 2005. Rat α-amylase was prepared from rat intestinal acetone powder (Sigma Aldrich Japan Co.). Active human pancreatic α-amylase (HPA) was derived by over-expression in a *Pichia pastoris* expression system (GS115) in our laboratory.[2]

3.3. Animals

This study was carried out using 72 Kunming mice (18–22 g, 6 weeks old) and Wistar rats (120–140 g, 4 weeks old) from the Experimental Animal Center of Academy of Military Medical Sciences (Beijing, China). Animals were maintained in a clean room at a temperature of 23–26 °C and a relative humidity of 50–60% with a 12 h light-dark cycle. Both male and female mice were used, with each mouse housed in a separate cage with food and water freely available. The experimental protocol was approved by the Animal Ethics Committee of Nankai University, in accordance with Principles of Laboratory Animal Care and Use in Research (Ministry of Health, Beijing, China).

3.4. Preparation of the GIB638 complex

The culture filtrate (about 7 L) of *Streptomyces* sp. PW638, fermented with Gause's No. 1 synthetic medium for seven days in a 10 L fermenter (Biotech-10BG2), was concentrated in vacuo to ~500 mL. This was then passed through a series number 201 × 4 macroporous resin column (300 × 40 mm, Chemical Plant of Nankai University, Tianjin, China) to partly remove pigments, followed by further filtration through a series number 001 × 7 cation exchange resin column (300 × 40 mm, Chemical Plant of Nankai University, Tianjin, China). This column was washed with water, eluted with 0.1 M ammonia, and fractions with inhibitory activities against HPA were collected. Ethanol (~9-fold volume) was added to the concentrated elution, and the supernatant was discarded after centrifugation. The pellet was lyophilized to give the GIB638 complex.

3.5. Purification of SF638-1

The GIB638 complex 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 MeCN/water (10:90) at a flow rate of 5 mL/min with UV detection at 205 nm. The fraction containing inhibitory activities against HPA was collected at 12.8 min, and this was further separated on a

Waters type S5-SCX semi-preparative HPLC at 25 °C. The mobile phase was water/ammonia/acetic acid (1000:8:8). Four chromatographic peaks were evident, and the peak at 3.7 min (containing inhibitory activities against HPA) was collected. A Sephadex G-25 column (eluted with water) was used finally to remove the salts and yielded, after lyophilization, 45 mg of the SF638-1. The purity of SF638-1 was determined by positive ion mode ESI-MS analysis. This test demonstrated a pure preparation with few contaminants.

3.6. Assay for rat α-amylase inhibition

The inhibitory effect on rat intestinal α-amylase was investigated using methods described previously.[16] Briefly, the assay mixture consisted of soluble starch substrate (200 μL, 1 mg / mL) and 100 μl of either acarbose or SF638-1. The mixture was pre-incubated for 5 min at 37 °C, and the reaction was initiated by adding 100 μL of crude enzyme solution (as α-amylase, 5 U/mL). This mixture was incubated for 30 min at 37 °C, and the reaction was terminated by heating for 5 min in a boiling water bath. Lugol's iodine solution [50 μl, containing distilled water (94%), potassium iodide (4%) and iodine (2%)] was added to the assay mixture to label any remaining substrate, and absorbance was measured at 630 nm using a microplate reader (BIO-RAD 680). The IC_{50} values were determined using GraphPad Prism 5 statistics software.

3.7. Kinetics of HPA inhibition by SF638-1

Kinetic analysis of α-amylase inhibition by the sample was performed using a modified version of the method of Geng et al.[8] The enzyme and test compounds were incubated with increasing concentrations of soluble amylose (0.05–0.25%, w/v). The substrate solution (100 μL), containing various concentrations of inhibitors (acarbose or SF638-1) were pre-incubated at 37 °C for 5 min before reactions were started by adding 50 μL of HPA enzyme solution (1 U/mL). A 50 μL sample was taken every 5 min, and the reaction was stopped by adding 50 μL of 3 M NaOH, followed by addition of 75 μL of 3,5-dinitrosalicylic acid (DNS) and boiling for 5 min prior to determination of absorbance at 490 nm using a microarray reader. Curves of product amount (in terms of glucose equivalents) versus time (in minutes) were obtained. The initial velocities (v_i) were then determined from the slope of the linear portion (within 30 min) of the curve. Kinetics of α-amylase inhibition by the sample were analyzed using Dixon plots of $1/v$ versus inhibitor concentration to determine the type of inhibition and the inhibition constant, K_i.

3.8. Effects of SF638-1 on blood glucose level in the in vitro everted intestinal sac system and the in vivo mouse model

Rat everted intestinal sac experiments were performed in vitro according to the method of Lyon et al.[17] An everted intestinal sac (4 cm in length) filled with Krebs–Henseleit buffer was suspended in a tube in buffer containing 1% starch and the required drug (acarbose or SF638-1). After incubation, the solutions from the serosal side (for transfer to blood) and the mucosal side (for reaction in intestine) were both collected to measure the concentrations of remnant starch or produced glucose. Results for each group were compared with the control and the value of E/E_0 indicates the degree of inhibition.

The effects of SF638-1 on postprandial blood glucose elevation in mice were determined as described by Geng et al.[18] Mice were subjected to an overnight fast and orally loaded simultaneously with starch (4 g/kg) and SF638-1 (various concentrations). Blood samples (20 μL) were collected from the tail vein of each mouse before and 30, 60, and 90 min after administration. The plasma was separated from the collected blood, and the concentration of glucose in this plasma was measured.

Results were expressed as mean ± SD (standard deviation). Statistical significance of differences between groups was analyzed using Dunnett's multiple comparison test based on analysis of variance test (ANOVA).

3.9. SF638-1

White amorphous powder: $[\alpha]_D^{18}$ +107 (*c* 0.1, water); UV (water): end absorption; IR ν_{max} (KBr): 3371, 2936, 1560, 1419, 1361, 1023, 576 cm^{-1}; HR-ESI-MS (pos.): *m/z* 522.2206 [M+H]+ ($C_{18}H_{35}NO_{16}$ requires 522.2201). For ESI-MS/MS (pos.), see Figure 1; for ^1H and ^{13}C NMR data, see Table 1; for ^1H, ^{13}C, TOCSY, HSQC, and HMBC spectra, see Supplementary data.

4. Conclusion

SF638-1 was produced by *Streptomyces* sp. PW638. The compound is a novel amino-oligosaccharide acting as a mixed noncompetitive inhibitor of HPA. SF638-1 inhibited HPA with a K_i value in the same order of magnitude as that of acarbose. Furthermore, SF638-1 showed a suppressive effect on starch hydrolysis and postprandial blood glucose elevation in both in vitro and in vivo tests. We conclude that SF638-1 may have potential as a novel therapeutic for the treatment of diabetes.

Acknowledgments

This work was supported by grants from the National Natural Science Foundation of China (21002052 and 30900015), the Tianjin Municipal Science and Technology Commission, China (10JCYBJC14300 and 11JCYBJC09300) and the Fundamental Research Funds for the Central Universities of China (65011161).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carres.2011.06.005.

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