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Antifungal nortriterpene and triterpene glycosides from the sea cucumber *Apostichopus japonicus* Selenka

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ABSTRACT

A nortriterpene glycoside, 26-nor-25-oxo-holotoxin A_1 (**1**), four triterpene glycosides, including both holostane and non-holostane types analogues, holotoxins D–G (**2**–**5**), together with three known triterpene glycosides, holotoxins A_1 and B (**6**, **7**), and cladoloside B (**8**), were isolated from the warty sea cucumber *Apostichopus japonicus* Selenka, a traditional tonic with high economic value in China. The structures of the new compounds were elucidated by a combination of detailed spectroscopic analysis and chemical methods. This is the first report of a nortriterpene glycoside isolated from sea cucumbers. These compounds showed potent antifungal activities in the *in vitro* biotest. A preliminary structure–activity analysis suggests that the 18(20) lactone group and the Δ^{25} terminal double bond may increase the activity. The component of the carbohydrate chain seems play an important role whereas the double bond transformation from $\Delta^{9(11)}$ to Δ^{7} in the aglycone moiety contributes little to the bioactivity.

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1. Introduction

Sea cucumbers belonging to the family Stichopodidae (order Aspidochirotida, class Holothurioidea) are popular in China, usually served as a culinary delicacy and traditional tonic (Cui et al., 2007). Among the members of this family, *Apostichopus japonicus* Selenka (also identified as *Stichopus japonicus* Selenka) is regarded as the best edible species with the highest economic value (Liao, 1997). In the *Supplement to Compendium of Materia Medica* by Xue-Min Zhao (1719–1805) in Qin dynasty, *S. japonicus* Selenka was specifically described as a good remedy similar to that of ginseng for "yin deficiency" of kidney, ischaemia, dysentery and ulcers. Currently, it is one of the most important cultured aquatic species in China and many other Asian countries.

Chemical investigation of *S. japonicus* Selenka can be traced back to 1969 when its glycosidic components were reported to have antifungal activity (Shimada, 1969). However, the sensitive techniques at that time were insufficient for separation and characterisation of the active molecules. Subsequent research on the species led to the isolation and structural elucidation of four triterpene glycosides, namely holotoxins A, A_1, B, and B_1. These molecules possess the same holostane type of aglycone, characterised by functionalities of a 9(11) double bond, a 25(26) terminal double bond, and a 16-ketone group. The structure of the holotoxin C remained unknown due to the minor quantity isolated (Isao et al., 1978; Kitagawa, Sugawara, & Yosioka, 1976; Maltsev, Stonik, Kalinovsky, & Elyakov, 1984). More recently, holotoxins A_1 and B_1 were repeatedly isolated from the title animals collected from the Yellow Sea of China (Xue et al., 2010). This investigation also resulted in another known glycoside, cladoloside B, once obtained from the sea cucumber *Cladolabes* sp (Avilov & Stonik, 1988).

During our ongoing screening for biologically active constituents from sea cucumbers (Han, Xu, Tang, Yi, & Gong, 2010; Liu et al., 2007; Yuan et al., 2008, 2009; Zhang, Yi, & Tang, 2006; Zou et al., 2005), we recently investigated the cold water sea cucumber *A. japonicus*, collected off the Dalian coast, Bohai Sea of China, which led to the isolation and structural elucidation of a nortriterpene glycoside, 26-nor-25-oxo-holotoxin A_1 (**1**), four new triterpene glycosides, including two holostane type glycosides, holotoxin D and E (**2**, **3**), and two uncommon non-holostane type glycosides, holotoxin F (**4**) and G (**5**), together with three known triterpene analogues, holotoxins A_1 (**6**) (Maltsev et al., 1984; Xue et al., 2010) and B (**7**) (Isao et al., 1978; Kitagawa et al., 1976), and cladoloside B (**8**) (Avilov & Stonik, 1988; Xue et al., 2010). We report herein the isolation, structure elucidation, and bioactivity of these compounds.

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2. Material and methods

2.1. General

Preparative HPLC was carried out using an Agilent 1100 liquid chromatograph, refractive index detector, Zorbax 300 SB-C18 column (25 cm × 9.4 mm i.d.) and a YMC-Pack C_8 column (1.0 × 25 cm i.d.). Column chromatography used silica gel (200–300 mesh, 10–40 mm; Yantai, PR China), ODS (40–63 mm; Merck) and Sephadex LH-20 (Pharmacia). TLC applied the precoated silica-gel GF_{254} plate (10–40 mm; Yantai). Melting point measurements were made using an XT5-XMT apparatus. Optical rotations were measured using a Perkin-Elmer 341 polarimeter. NMR spectra were obtained from Varian Inova-400 and Inova-500 spectrometers with standard pulse sequences. ESI-MS and HR-ESI-MS were collected using a Micromass Quattro mass spectrometer. GC/MS was carried out using a Finnigan Voyager apparatus with DB-5 column (30 m × 0.25 mm i.d., 0.25 μm).

2.2. Animal material

Specimens of *A. japonicus* Selenka were collected off the Dalian coast, Bohai Sea of China, in October 2006, and authenticated by Prof. Yulin Liao (Institute of Oceanology, Chinese Academy of Science, PR China). A voucher specimen (No.AJ-2006-10) was deposited at the Research Center for Marine Drugs, School of Pharmacy, Second Military Medical University, Shanghai.

2.3. Extraction and isolation

The sea cucumbers were freeze-dried (3.5 kg), pulverised and refluxed with 60% EtOH (3 L × 6, 3 h), and then evaporated under reduced pressure. The EtOH extract was submitted to a DA-101 resin column eluting with H_2O and 70% EtOH. The 70% EtOH extract was resuspended in H_2O and partitioned with *n*-BuOH 5 times. The *n*-BuOH extract (105.3 g) was subjected to column chromatography on Si gel eluting with $CHCl_3$/MeOH/H_2O (10:1:0.1, 9:1:0.1, 8:2:0.2, 7:3:0.5, 6:4:0.8) gradient to give 6 fractions (Fr. A–F). Fractions C–E were further purified by reverse-phase silica MPLC eluting with an aq. CH_3OH (45–85%) gradient to give 14 fractions (Fr. H_1–H_6 from Fraction C, Fr. H_7–H_{11} from Fraction D, Fr. H_{11}–H_{14} from Fraction E). Fraction H_9–H_{14} was gel-filtered on Sephadex LH-20 (MeOH/H_2O, 1:1) followed by semipreparative HPLC (Zorbax 300 SB-C18 and YMC-Pack C8) to yield glycoside **1** (3.6 mg, t_R 29.3 min, C18, 73% aq. MeOH, 1.0 mL/min) from fraction H_8, **2** (11.7 mg, t_R 32.2 min, C18, 75% aq. MeOH, 1.0 mL/min) from fraction H_{10}, **3** (15.5 mg, t_R 35.8 min, C18, 75% aq. MeOH, 1.0 mL/min) from fraction H_{11}, **7** (6.2 mg, t_R 12.8 min, C18, 80% aq. MeOH, 1.5 mL/min) from fraction H_{12}, **6** (250 mg, t_R 32.2 min, C18, 85% aq. MeOH, 1.0 mL/min) and **8** (30 mg, t_R 33.4 min, C18, 85% aq. MeOH, 1.0 mL/min) from fraction H_{13}, **4** (7.0 mg, t_R 26.0 min, YMC-Pack C8, 73% aq. MeOH, 1.0 mL/min) and **5** (4.4 mg, t_R 27.8 min, C8, 73% aq. MeOH, 1.0 mL/min) from fraction H_{14}.

2.4. Spectral measurements

26-Nor-25-oxo-holotoxin A_1 (**1**), colourless amorphous powder; $[\alpha]_D^{20}$ − 28 (*c* 0.30, pyridine); ^{1}H-NMR chemical shifts of the aglycone moiety (400 MHz in C_5D_5N): δ_H 1.53(1H, m, H-1β), 1.95 (1H, m, H-1α), 2.03 (1H, m, H-2β), 2.17 (1H, m, H-2α), 3.33 (1H, m, H-3), 1.00 (1H, m, H-5), 1.76 (2H, m, H-6), 1.30 (1H, m, H-7β), 1.62 (1H, m, H-7α), 3.33 (1H, m, H-8), 5.71 (1H, m, H-11), 2.55 (2H, m, H-12), 2.27 (1H, d *J* = 16.0 Hz, H-15β), 2.44 (1H, d *J* = 16.0 Hz, H-15α), 2.84 (1H, s, H-17), 1.49 (3H, s, H-19), 1.45 (3H, s, H-21), 1.72 (1H, m, H-22β), 1.87 (1H, m, H-22α), 1.80 (1H, m, H-23β), 1.95 (1H, m, H-23α), 2.40 (2H, m, H-24), 2.10 (3H, s, H-27), 1.20 (3H, s, H-30), 1.37 (3H, s, H-31), 0.97 (3H, s, H-32); ^{13}C-NMR chemical shifts of the aglycone moiety (100 MHz in C_5D_5N): δ_C 36.5 (CH_2, C-1), 27.3 (CH_2, C-2), 89.0 (CH, C-3), 40.1 (C, C-4), 53.1 (CH, C-5), 21.3 (CH_2, C-6), 28.7 (CH_2, C-7), 38.9 (CH, C-8), 151.5 (C, C-9), 39.9 (C, C-10), 111.2 (CH, C-11), 32.2 (CH_2, C-12), 55.9 (C, C-13), 42.2 (C, C-14), 52.1 (CH_2, C-15), 213.6 (C, C-16), 61.5 (CH, C-17), 176.2 (C, C-18), 22.3 (CH_3, C-19), 83.3 (C, C-20), 26.8 (CH_3, C-21), 38.2 (CH_2, C-22), 19.0 (CH_2, C-23), 43.5 (CH_2, C-24), 207.7 (C, C-25), 29.7 (CH_3, C-27), 16.8 (CH_3, C-30), 28.2 (CH_3, C-31), 20.8 (CH_3, C-32); ^{1}H- and ^{13}C-NMR chemical shifts of the sugar moiety see holotoxins A_1 (**6**) (Maltsev et al., 1984; Xue et al., 2010). ESI-MS (positive ion mode) *m*/*z*: 1417 $[M + Na]^+$; ESI-MS (negative ion mode) *m*/*z*: 1393 $[M–H]^+$; HRESI-MS (positive ion mode) *m*/*z*: 1417.6257 $[M + Na]^+$ (calcd. $C_{65}H_{102}O_{32}Na$, 1417.6252); ESI-MS-MS (negative ion mode) *m*/*z*: 1393 $[M–H]^+$ (33), 1349 $[M–H–CO_2]^+$ (100), 1173 $[M–H–CO_2–MeGlc]^+$ (33), 1041 $[M–H–CO_2–MeGlc–Xyl]^+$ (30), 865 $[M–H–CO_2–MeGlc–Xyl–MeGlc]^+$ (28), 703 $[M–H–CO_2–MeGlc–Xyl–MeGlc–Glc]^+$ (68), 557 $[M–H–CO_2–MeGlc–Xyl–MeGlc–Qui]^+$ (12).

Holotoxin D (**2**): colourless amorphous powder; $[\alpha]_D^{23}$ − 49 (*c* 0.56, pyridine); ^{1}H-NMR chemical shifts of the sugar moiety (400 MHz in C_5D_5N): Xyl^1 (1→C-3), δ_H 4.84 (1H, d, *J* = 7.2 Hz, H-1), 4.20 (1H, m, H-2), 4.26 (1H, m, H-3), 4.33 (1H, m, H-4), 3.94 (1H, m, H-5β), 4.26 (1H, m, H-5α); Glc^2 (1→2Xyl^1), δ_H 5.32 (1H, d, *J* = 7.0 Hz, H-1), 3.93 (1H, m, H-2), 4.24 (1H, m, H-3), 4.46 (1H, m, H-4), 3.99 (1H, m, H-5), 4.53 (1H, m, H-6β), 4.67 (1H, m, H-6α); Xyl^3 (1→4Glc^2), δ_H 5.19 (1H, d, *J* = 7.3 Hz, H-1), 4.07(1H, m, H-2), 4.28 (1H, m, H-3), 4.29 (1H, m, H-4), 3.68 (1H, m, H-5β), 4.22 (1H, m, H-5α); $MeGlc^4$ (1→3Xyl^3), δ_H 5.34 (1H, d, *J* = 7.9 Hz, H-1), 4.07 (1H, m, H-2), 3.79 (1H, m, H-3), 4.18 (1H, m, H-4), 4.05 (1H, m, H-5), 4.31 (1H, m, H-6β), 4.55 (1H, m, H-6α), 3.94 (3H, s, OMe); Glc^5 (1→4Xyl^1) δ_H 5.18 (1H, d, *J* = 7.4 Hz, H-1), 4.07 (1H, m, H-2), 4.09 (1H, m, H-3), 4.16 (1H, m, H-4), 4.02 (1H, m, H-5), 4.31 (1H, m, H-6β), 4.55 (1H, m, H-6α); $MeGlc^6$ (1→3Glc^5), δ_H 5.31 (1H, d, *J* = 7.7 Hz, H-1), 4.07 (1H, m, H-2), 3.79 (1H, m, H-3), 4.18 (1H, m, H-4), 4.05 (1H, m, H-5), 4.31 (1H, m, H-6β), 4.55 (1H, m, H-6α), 3.94 (3H, s, OMe); ^{13}C-NMR chemical shifts of the sugar moiety (100 MHz in C_5D_5N): Xyl^1 (1→C-3), δ_C 105.4 (CH, C-1), 83.2 (CH, C-2), 76.8 (CH, C-3), 77.7 (CH, C-4), 64.1 (CH_2, C-5); Glc^2 (1→2Xyl^1), δ_C 105.6 (CH, C-1), 76.9 (CH, C-2), 75.8 (CH, C-3), 80.5 (CH, C-4), 78.5 (CH, C-5), 61.4 (CH_2, C-6); Xyl^3 (1→4Glc^2), δ_C 105.1 (CH, C-1), 73.8 (CH, C-2), 87.8 (CH, C-3), 69.3 (CH, C-4), 66.7 (CH_2, C-6); $MeGlc^4$ (1→3Xyl^3), δ_C 105.9 (CH, C-1), 75.3 (CH, C-2), 88.2 (CH, C-3), 70.7 (CH, C-4), 78.5 (CH, C-5), 62.4 (CH_2, C-6), 60.9 (CH_3, OMe); Glc^5 (1→4Xyl^1) δ_C 103.0 (CH, C-1), 73.3 (CH, C-2), 88.1 (CH, C-3), 69.9 (CH, C-4), 78.5 (CH, C-5), 62.2 (CH_2, C-6); $MeGlc^6$ (1→3Glc^5), δ_C 105.9 (CH, C-1), 75.3 (CH, C-2), 88.2 (CH, C-3), 70.7 (CH, C-4), 78.5 (CH, C-5), 62.4 (CH_2, C-6), 60.9 (CH_3, OMe); ^{1}H- and ^{13}C-NMR chemical shifts of the aglycone moiety see holotoxins A_1 (**6**) (Maltsev et al., 1984; Xue et al., 2010). ESI-MS (positive-ion mode) *m*/*z* 1431 $[M + Na]^+$; ESI-MS (negative-ion mode) *m*/*z* 1407 $[M–H]^+$. HR-ESI-MS (positive-ion mode) *m*/*z* 1431.6406 $[M + Na]^+$ (calcd. for $C_{66}H_{104}O_{32}Na$, 1431.6408). ESI-MS/MS (negative-ion mode) *m*/*z* 1407 $[M–H]^+$ (95), 1363 $[M–H–CO_2]^+$ (89), 1187 $[M–H–CO_2–MeGlc]^+$ (38), 1099 $[M–H–MeGlc–Xyl]^+$ (65), 1055 $[M–H–CO_2–MeGlc–Xyl]^+$ (51), 923 $[M–H–MeGlc–Xyl–MeGlc]^+$ (22), 879 $[M–H–CO_2–MeGlc–Xyl–MeGlc]^+$ (35), 761 $[M–H–MeGlc–Xyl–MeGlc–Glc]^+$ (42), 717 $[M–H–CO_2–MeGlc–Xyl–MeGlc–Glc]^+$ (55), 599 $[M–H–MeGlc–Xyl–MeGlc–Glc–Glc]^+$ (9), 555 $[M–H–CO_2–MeGlc–Xyl–MeGlc–Glc–Glc]^+$ (22), 423 $[M–H–CO_2–MeGlc–Xyl–MeGlc–Glc–Glc–Xyl]^+$ (27).

Holotoxin E (**3**): colourless amorphous powder; $[\alpha]_D^{23}$ − 49 (*c* 0.56, pyridine); ^{1}H-NMR chemical shifts of the sugar moiety (400 MHz in C_5D_5N): Xyl^1 (1→C-3), δ_H 4.83 (1H, d, *J* = 7.0 Hz, H-1), 4.12 (1H, m, H-2), 4.30 (1H, m, H-3), 4.34 (1H, m, H-4),

3.68 (1H, m, H-5β), 4.28 (1H, m, H-5α); Qui^2 (1→2Xyl^1), δ_H 5.23 (1H, d, J = 7.4 Hz, H-1), 4.12 (1H, m, H-2), 4.16 (1H, m, H-3), 3.73 (1H, m, H-4), 3.85 (1H, m, H-5), 1.86 (2H, d, J = 5.9 Hz); Xyl^3 (1→4Qui^2), δ_H 4.94 (1H, d, J = 7.5 Hz, H-1), 4.07 (1H, m, H-2), 4.19 (1H, m, H-3), 4.11 (1H, m, H-4), 3.70 (1H, m, H-5β), 4.48 (1H, m, H-5α); MeGlc^4 (1→3Xyl^3), δ_H 5.37 (1H, d, J = 7.8 Hz, H-1), 4.16 (1H, m, H-2), 3.79 (1H, m, H-3), 4.21 (1H, m, H-4), 4.03 (1H, m, H-5), 4.34 (1H, m, H-6β), 4.62 (1H, m, H-6α), 3.94 (3H, s, OMe); Glc^5 (1→4Xyl^1) δ_H 5.08 (1H, d, J = 7.8 Hz, H-1), 4.08 (1H, m, H-2), 4.19 (1H, m, H-3), 4.48 (1H, m, H-4), 4.03 (1H, m, H-5), 4.30 (1H, m, H-6β), 4.53 (1H, m, H-6α); Glc^6 (1→3Glc^5), δ_H 5.33 (1H, d, J = 8.0 Hz, H-1), 4.03 (1H, m, H-2), 4.06 (1H, m, H-3), 4.27 (1H, m, H-4), 4.17 (1H, m, H-5), 4.30 (1H, m, H-6β), 4.51 m(1H, m, H-6α); ^{13}C-NMR chemical shifts of the sugar moiety (100 MHz in C_5D_5N): Xyl^1 (1→C-3), δ_C 105.5 (CH, C-1), 83.7 (CH, C-2), 76.0 (CH, C-3), 77.7 (CH, C-4), 64.3 (CH_2, C-5); Qui^2 (1→2Xyl^1), δ_C 105.8 (CH, C-1), 76.7 (CH, C-2), 75.7 (CH, C-3), 86.1 (CH, C-4), 72.0 (CH, C-5), 18.2 (CH_2, C-6); Xyl^3 (1→4Qui^2), δ_C 105.4 (CH, C-1), 73.7 (CH, C-2), 87.7 (CH, C-3), 69.3 (CH, C-4), 66.7 (CH_2, C-6); MeGlc^4 (1→3Xyl^3), δ_C 105.7 (CH, C-1), 75.7 (CH, C-2), 88.2 (CH, C-3), 70.8 (CH, C-4), 78.5 (CH, C-5), 62.7 (CH_2, C-6), 60.9 (CH_3, OMe); Glc^5 (1→4Xyl^1) δ_C 103.1 (CH, C-1), 73.4 (CH, C-2), 88.4 (CH, C-3), 69.9 (CH, C-4), 78.4 (CH, C-5), 62.4 (CH_2, C-6); Glc^6 (1→3Glc^5), δ_C 106.0 (CH, C-1), 78.5 (CH, C-2), 75.3 (CH, C-3), 71.8 (CH, C-4), 78.8 (CH, C-5), 62.3 (CH_2, C-6); ^{1}H- and ^{13}C-NMR chemical shifts of the aglycone moiety see liouvillosides A1 and A2 (Antonov et al., 2008), and neothyonidioside and mollisosides B1 (Moraes et al., 2005). ESI-MS (positive-ion mode) m/z 1401 [M + Na]$^+$; ESI-MS (negative-ion mode) m/z 1377 [M–H]$^+$. HR-ESI-MS (positive-ion mode) m/z 1401.6313 [M + Na]$^+$ (calcd. for $C_{66}H_{104}O_{32}Na$, 1401.6303). ESI-MS-MS (negative-ion mode) m/z 1377 [M–H]$^+$ (94), 1333 [M–H–CO_2]$^+$ (100), 1157 [M–H–CO_2–MeGlc]$^+$ (11), 1069 [M–H–MeGlc–Xyl]$^+$ (26), 1025 [M–H–CO_2–MeGlc–Xyl]$^+$ (22), 863 [M–H–CO_2–MeGlc–Xyl–Glc]$^+$ (31), 701[M–H–CO_2–MeGlc–Xyl–Glc–Glc]$^+$ (38), 555 [M–H–CO_2–MeGlc–Xyl–Glc–Glc–Qui]$^+$ (8), 423 [M–H–CO_2–MeGlc–Xyl–Glc–Glc–Qui–Xyl]$^+$ (24).

Holotoxin F (**4**): colourless amorphous powder; $[\alpha]_D^{20}$ – 102 (c 0.15, pyridine); ^{1}H-NMR chemical shifts of the aglycone moiety (500 MHz in C_5D_5N): δ_H 1.48 (1H, m, H-1β), 1.83 (1H, m, H-1α), 2.02 (1H, m, H-2β), 2.28 (1H, m, H-2α), 3.30 (1H, dd, J = 3.9, 11.7 Hz, H-3), 1.00 (1H, m, H-5), 1.54 (1H, m, H-6β), 1.78 (1H, m, H-6α), 1.61 (2H, m, H-7), 2.39 (1H, m, H-8), 5.45 (1H, m, H-11), 2.23 (1H, m, H-12β), 2.49 (1H, m, H-12α), 2.19 (1H, d J = 17.7 Hz, H-15β), 2.41 (1H, d J = 17.7 Hz, H-15α), 2.97 (1H, s, H-17), 1.20 (3H, s, H-18), 1.18 (3H, s, H-19), 1.57 (3H, s, H-21), 2.01 (1H, m, H-22), 1.81 (1H, m, H-23β), 1.92 (1H, m, H-23α), 2.19 (2H, m, H-24), 4.92 (2H, d, J = 16.1 Hz, H-26), 1.81 (3H, s, H-27), 1.20 (3H, s, H-30), 1.37 (3H, s, H-31), 1.00 (3H, s, H-32); ^{13}C-NMR chemical shifts of the aglycone moiety (125 MHz in C_5D_5N): δ_C 36.4 (CH_2, C-1), 27.3 (CH_2, C-2), 88.9 (CH, C-3), 40.2 (C, C-4), 53.1 (CH, C-5), 21.4 (CH_2, C-6), 28.6 (CH_2, C-7), 40.8 (CH, C-8), 149.7 (C, C-9), 39.8 (C, C-10), 115.4 (CH, C-11), 36.8 (CH_2, C-12), 44.2 (C, C-13), 41.3 (C, C-14), 49.8 (CH_2, C-15), 221.0 (C, C-16), 64.7 (CH, C-17), 17.2 (C, C-18), 22.6 (CH_3, C-19), 74.5 (C, C-20), 26.6 (CH_3, C-21), 41.9 (CH_2, C-22), 22.6 (CH_2, C-23), 38.8 (CH_2, C-24), 146.5 (C, C-25), 110.6 (CH_2, C-26), 22.2 (CH_3, C-27), 16.9 (CH_3, C-30), 28.3 (CH_3, C-31), 19.0 (CH_3, C-32); ^{1}H- and ^{13}C-NMR chemical shifts of the aglycone moiety see cladoloside B (**8**) (Avilov & Stonik, 1988; Xue et al., 2010). ESI-MS (positive ion mode) m/z: 1227 [M + Na]$^+$; ESI-MS (negative ion mode) m/z: 1203 [M–H]$^+$; HRESI-MS (positive ion mode) m/z: 1227.6147 [M + Na]$^+$ (calcd. $C_{59}H_{96}O_{25}Na$, 1227.6138); ESI-MS-MS (negative ion mode) m/z: 1203 [M–H]$^+$ (20), 1185 [M–H–H_2O]$^+$ (36), 1009 [M–H–H_2O–MeGlc]$^+$ (23), 895 [M–H–MeGlc–Xyl]$^+$ (100), 877 [M–H–H_2O–MeGlc–Xyl]$^+$ (68), 769 [M–H–MeGlc–Xyl–Glc]$^+$ (65), 715 [M–H–H_2O–MeGlc–Xyl–Glc]$^+$ (47), 455 [M–H–MeGlc–Xyl–Glc–Qui–Xyl]$^+$ (38).

Holotoxin G (**5**): colourless amorphous powder; $[\alpha]_D^{20}$ – 92 (c 0.15, pyridine); ^{1}H-NMR chemical shifts of the sugar moiety (500 MHz in C_5D_5N): Xyl^1 (1→C-3), δ_H 4.82 (1H, d, J = 7.3 Hz, H-1), 4.11 (1H, m, H-2), 4.28 (1H, m, H-3), 4.39 (1H, m, H-4), 3.76 (1H, m, H-5β), 4.48 (1H, m, H-5α); Qui^2 (1→2Xyl^1), δ_H 5.25 (1H, d, J = 7.6 Hz, H-1), 4.19 (1H, m, H-2), 4.18 (1H, m, H-3), 3.73 (1H, m, H-4), 3.87 (1H, m, H-5), 1.83 (2H, d, J = 5.8 Hz); Xyl^3 (1→4Qui^2), δ_H 4.95 (1H, d, J = 7.6 Hz, H-1), 4.08 (1H, m, H-2), 4.18 (1H, m, H-3), 4.12 (1H, m, H-4), 3.71 (1H, m, H-5β), 4.29 (1H, m, H-5α); Glc^4 (1→3Xyl^3), δ_H 5.41 (1H, d, J = 7.9 Hz, H-1), 4.19 (1H, m, H-2), 4.29 (1H, m, H-3), 4.27 (1H, m, H-4), 4.07 (1H, m, H-5), 4.41 (1H, m, H-6β), 4.61 (1H, m, H-6α); Glc^5 (1→4Xyl^1) δ_H 5.08 (1H, overlap, H-1), 4.10 (1H, m, H-2), 4.29 (1H, m, H-3), 4.27 (1H, m, H-4), 4.07 (1H, m, H-5), 4.41 (1H, m, H-6β), 4.61 (1H, m, H-6α); ^{13}C-NMR chemical shifts of the sugar moiety (125 MHz in C_5D_5N): Xyl^1 (1→C-3), δ_C 105.5 (CH, C-1), 83.6 (CH, C-2), 76.1 (CH, C-3), 77.5 (CH, C-4), 64.4 (CH_2, C-5); Qui^2 (1→2Xyl^1), δ_C 105.8 (CH, C-1), 76.7 (CH, C-2), 75.7 (CH, C-3), 86.2 (CH, C-4), 72.0 (CH, C-5), 18.3 (CH_2, C-6); Xyl^3 (1→4Qui^2), δ_C 105.4 (CH, C-1), 73.7 (CH, C-2), 87.9 (CH, C-3), 69.3 (CH, C-4), 66.7 (CH_2, C-6); Glc^4 (1→3Xyl^3), δ_C 105.8 (CH, C-1), 75.7 (CH, C-2), 78.4 (CH, C-3), 71.8 (CH, C-4), 79.0 (CH, C-5), 62.7 (CH_2, C-6); Glc^5 (1→4Xyl^1) δ_C 103.6 (CH, C-1), 74.5 (CH, C-2), 78.4 (CH, C-3), 71.8 (CH, C-4), 78.8 (CH, C-5), 62.7 (CH_2, C-6); ^{1}H- and ^{13}C-NMR chemical shifts of the aglycone moiety see cladoloside B (**8**) (Avilov & Stonik, 1988; Xue et al., 2010). ESI-MS (positive ion mode) m/z: 1213 [M + Na]$^+$; ESI-MS (negative ion mode) m/z: 1189 [M–H]$^+$; HRESI-MS (positive ion mode) m/z: 1213.5988 [M + Na]$^+$ (calcd. $C_{58}H_{94}O_{25}Na$, 1213.5982); ESI-MS-MS (negative ion mode) m/z: 1189 [M–H]$^+$ (16), 1171 [M–H_2O]$^+$ (100), 1009 [M–H_2O–Glc]$^+$ (15), 877 [M–H_2O–Glc–Xyl]$^+$ (23), 715 [M–H_2O–Glc–Xyl–Glc]$^+$ (7).

2.5. Acid hydrolysis of *2*, *3* and *4*

Each glycoside (3 mg) was heated in an ampoule with 5 mL of aqueous 2 M CF_3COOH at 120 °C for 2 h. The aglycone was extracted with dichloromethane, and the aqueous residue was evaporated *in vacuo*. To this dry residue was added 1 mL of pyridine and 2 mg of NH_2OH·HCl, and the mixtures were heated at 90 °C for 1 h. After the reaction mixtures were cooled, 1.5 mL of Ac_2O were added and the mixtures were heated at 90 °C for 1 h. The reaction mixtures were evaporated *in vacuo*, and the resulting aldononitrile peracetates as reference were analysed by GC–MS using the corresponding authentic D-xylose, D-quinovose, D-glucose and 3-*O*-methyl-D-glucose treated by the same procedure. The oligosaccharides of the glycosides were determined as D-xylose, D-glucose and 3-*O*-methyl-D-glycose in a ratio of 1:1:1 for **2**, D-xylose, D-quinovose, D-glucose and 3-*O*-methyl-D-glucose in a ratio of 2:1:2:1 for **3**, and D-xylose, D-glucose and 3-*O*-methyl-D-glucose in a ratio of 2:1:1:1 for **4**, respectively.

2.6. Fungus strains and positive controls

The fungus strains *Candida albicans* SC5314 and *Cryptococcus neoformans* BLS108 were provided by the Changzheng hospital, The Second Military Medical University (SMMU), PR China, and *Candida tropicalis*, *Trichophyton rubrum* 0501124, *Microsporum gypseum* 31388 and *Aspergillus fumigatus* 0504656 were provided by the Changhai hospital, SMMU, PR China. Positive controls Itraconazole (ICZ), Terbinafine (TRB), Ketoconazole (KCZ), Amphotericin B (AMB), Voriconazole (VCZ) and Fluconazole (FCZ) were supplied by the Department of Pharmacology, School of Pharmacy, Second Military Medical University, Shanghai.

2.7. Bioassays

The antifungal activities of the compounds **1**–**8** were tested against six strains. The data of the antifungal activities were evaluated by measuring optical density (*OD*) at 630 nm using an automatic microplate reader (Zhang et al., 2006). The MIC_{80} was defined as the first well with an approximate 80% reduction in growth compared to the growth of the drug-free-well. The data represented the means of three independent experiments in which each compound concentration was tested in three replicate wells. Itraconazole (ICZ), Terbinafine (TRB), Ketoconazole (KCZ), Amphotericin B (AMB), Voriconazole (VCZ) and Fluconazole (FCZ) were used as positive controls.

3. Results and discussion

3.1. Chemical investigation

26-Nor-25-oxo-holotoxin A_1 (**1**) was obtained as a minor component. The molecular formula was established as $C_{65}H_{102}O_{32}$ by HR-ESI-MS from the pseudo-molecular ion at *m*/*z* 1417.6257 $[M + Na]^+$ (calcd. for $C_{65}H_{102}O_{32}Na$, 1417.6252). ^1H and ^{13}C NMR spectra of **1** showed presence of a trisubstituted double bond (δ_C 151.5, C and 111.2, CH; δ_H 5.71, m), two keto groups (δ_C 213.6 and 207.7), one lactone carbonyl group (δ_C 176.2), and an oligosaccharide chain composed of six sugar units. Analysis of the NMR spectra of **1** revealed a great similarity to those of the co-occurring glycoside holotoxin A_1 (**6**), except that the 25(26) terminal double bond (δ_C 145.5, C and 110.5, CH_2; δ_H 4.76, 2H) (Maltsev et al., 1984; Xue et al., 2010) in **6** was replaced by a keto group (δ_C 207.7) in **1**. The keto group was assigned at C-25 due to the obvious HMBC correlations from H_3-27 to both C-24 and C-25. This assignment was in agreement with the observation of marked downfield shift values of NMR signals for C-24 (δ_C 43.5 and δ_H 2.40 in **1**, δ_C 38.1, δ_H 1.97 in **6**) and C-27 (δ_C 29.7 and δ_H 2.10 in **1**, δ_C 22.5, δ_H 1.67 in **6**). The substructure of the aglycone part of **1** was then elucidated as holosta-26-nor-9(11)-en-16,25-dione-3β-ol.

The NMR signals of the carbohydrate chain of **1** were identical to those of glycosides in holotoxin A_1 (**6**) and stichloroside C_1 (Kitagawa et al., 1981), having a nonlinear hexasaccharide carbohydrate chain constructed of one unit of both D-quinovose and D-glucose, and two units of both D-xylose and 3-*O*-methyl-D-glucose, respectively. The presence of six monosaccharide units in the carbohydrate chain of glycoside **1** was deduced from the ^{13}C and ^1H NMR spectra, showing six anomeric protons and corresponding carbons (δ_H 4.82, 4.93, 5.06, 5.23, 5.33, 5.37; δ_C 103.0, 105.4 × 2, 105.6, 105.8 × 2). The interglycosidic linkages in the hexasaccharide chain of **1** and its connectivity to the aglycone were proven by HMBC experiments the same as those in **6**, and were confirmed by the analysis of the Q-TOF MS/MS spectra (section 2.4). Complete assignments were achieved by using HSQC, HMBC, ^1H-^1H COSY, TOCSY and NOESY experiments (Fig. 1). Finally, the structure of **1** was established as 3β-*O*-{2-*O*-[3-*O*-methyl-β-D-glucopyranosyl-(1→3)-β-D-xylopyranosyl-(1→4)-β-D-quinovopyranosyl]-4-*O*-[3-*O*-methyl-β-D-glucopyranosyl,-(1→3)-β-D-glucopyranosyl]-β-D-xylopranosyl}-holosta-26-nor-9(11)-en-16,25-dione, namely 26-nor-25-oxo-holotoxin A_1.

Holotoxin D (**2**) was isolated as a colourless amorphous powder. It had a molecular formula of $C_{66}H_{104}O_{32}$ on the basis of HR-ESI-MS analysis (positive ion mode, *m*/*z*: 1431.6406 $[M + Na]^+$, calcd. for $C_{66}H_{104}O_{32}Na$, 1431.6408). ^1H and ^{13}C NMR data for the aglycone of **2** were identical with those of holotoxin A_1 (**6**). Their difference was recognised in the sugar moieties.

The sugar moiety of **2** also closely resembled that of **6**, containing six monosaccharides. However, the D-quinovose in **6** was replaced by a D-glucose in **2** due to the 1:1:1 ratio of D-xylose, D-glucose and 3-*O*-methyl-D-glycose as determined by an acidic hydrolysis experiment. The interglycosidic linkages and their connectivity to the aglycone in **2** were proven in the same way as those in **6** except for the replacement of the D-quinovose by a D-glucose. The above conclusion was supported by the HMBC correlations between H-1 of Xyl^1 and C-3 of the aglycone, C-2 of Xyl^1 and H-1 of Glc^2, C-4 of Glc^2 and H-1 of Xyl^3, C-3 of Xyl^2 and H-1 of $MeGlc^4$, C-4 of Xyl^1 and H-1 of Glc^5, C-3 of Glc^5 and H-1 of $MeGlc^6$. The Q-TOF-MS/MS experiment showed further proof by peaks for fragments of $[M–H–CO_2–MeGlc]^+$, $[M–H–MeGlc–Xyl]^+$, $[M–H–CO_2–MeGlc–Xyl]^+$, $[M–H–MeGlc–Xyl–MeGlc]^+$, $[M–H–CO_2–MeGlc–Xyl–MeGlc]^+$, $[M–H–MeGlc–Xyl–MeGlc–Glc]^+$, $[M–H–CO_2–MeGlc–Xyl–MeGlc–Glc]^+$, $[M–H–MeGlc–Xyl–MeGlc–Glc–Glc]^+$, and $[M–H–CO_2–MeGlc–Xyl–MeGlc–Glc–Glc–Xyl]^+$ (section 2.4). The above evidence indicated the structure of holotoxin D (**2**) to be 3β-*O*-{2-*O*-[3-*O*-methyl-β-D-glucopyranosyl-(1→3)-β-D-xylopyranosyl-(1→4)-β-D-glucopyranosyl]-4-*O*-[3-*O*-β-D-glucopyranosyl-(1→3)-β-D-glucopyranosyl]-β-D-xylopranosyl}-holosta-9(11),25(26)-dien-16-one.

Holotoxin E (**3**), colourless amorphous powder; the molecular formula was established as $C_{65}H_{102}O_{31}$ by HR-ESI-MS at *m*/*z*

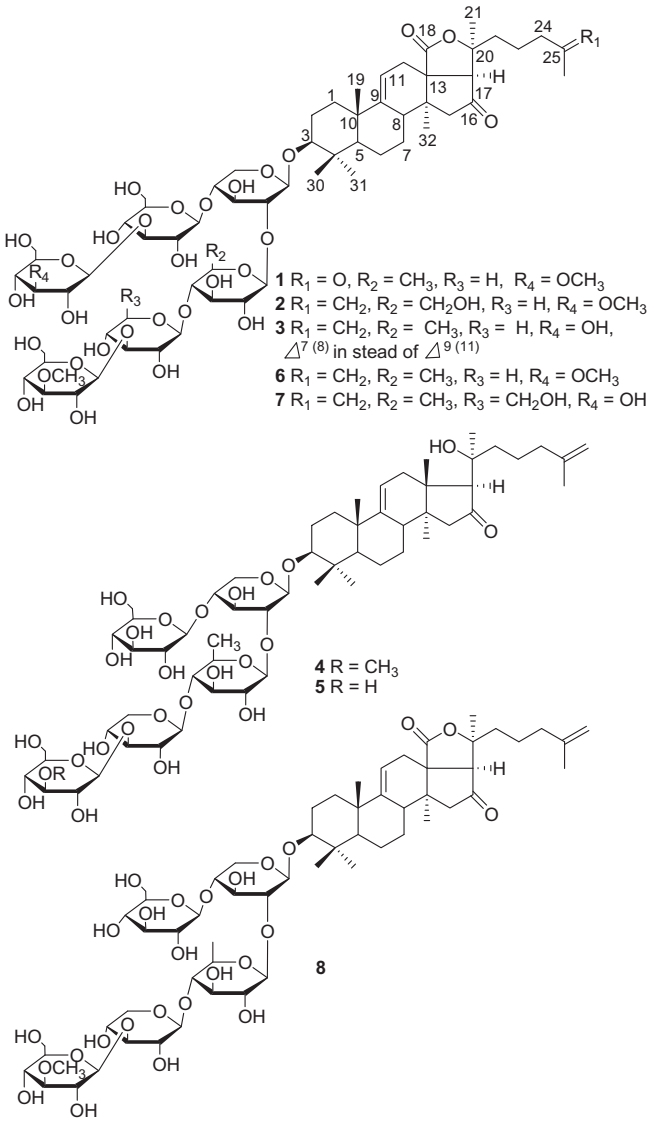

Fig. 1. Structures of compounds **1**–**8**.

Table 1
Antifungal activities of glycosides **1–8** (MIC_{80}, μM).

Compounds	*Candida albicans* SC5314	*Cryptococcus neoformans* BLS108	*Candida tropicalis*	*Trichophyton rubrum* 0501124	*Microsporum gypseum* 31388	*Aspergillus fumigatus* 0504656
1	>45.91	>45.91	>45.91	45.91	5.73	11.48
2	6.64	6.64	13.29	13.29	6.64	13.29
3	13.45	6.72	13.45	13.45	6.72	26.89
4	5.58	2.84	5.68	5.68	1.42	5.68
5	5.81	2.90	5.81	2.90	1.45	11.61
6	11.49	1.44	1.44	1.44	0.18	5.75
7	11.36	2.84	5.68	11.36	0.71	11.36
8	3.28	3.28	1.64	0.41	0.82	3.28
ICZ[a]	0.09	0.18	0.18	0.18	0.09	2.83
TRB[a]	27.45	1.72	13.73	0.86	0.43	0.86
KCZ[a]	0.1	0.12	0.24	0.12	<0.24	1.88
AMB[a]	17.31	34.63	–	34.63	2.16	34.63
VCZ[a]	0.04	0.04	–	0.18	0.36	0.72
FCZ[a]	1.63	3.26	1.63	13.05	3.26	Inactive

[a] Positive antifungal activity control.

1401.6313 $[M + Na]^+$ (calcd. for $C_{66}H_{104}O_{32}Na$, 1401.6303). The aglycone of **3** differed from that of **6** by containing a 7(8)-double bond (δ_C 122.1, CH; 144.2, C) (Antonov et al., 2008; Moraes et al., 2005; Silchenko et al., 2008) other than a 9(11)-double bond, which was confirmed by HMBC correlations from H_3-32 (δ_H 1.25) to C-8 (δ_C 144.2). The MeGlc^6 in oligosaccharide chain of **6** was replaced by a D-glucose in **3** with other saccharides and the interglycosidic linkages remained intact. The component of oligosaccharide (D-xylose:D-quinovose:D-glucose:3-*O*-methyl-D-glucose, 2:1:2:1) was determined on the basis of an acid hydrolysis and GC–MS analysis. The sugar sequence and its connectivity to the aglycone were deduced from cross-peaks between H-1 of Xyl^1 and C-3 of the aglycone, C-2 of Xyl^1 and H-1 of Qui^2, C-4 of Qui^2 and H-1 of Xyl^3, C-3 of Xyl^2 and H-1 of MeGlc^4, C-4 of Xyl^1 and H-1 of Glc^5, C-3 of Glc^5 and H-1 of Glc^6 in HMBC spectrum, and further confirmed by peaks in Q-TOF-MS/MS as shown in section 2.4. The structure of holotoxin E (**3**) was thus determined as 3*β*-*O*-{2-*O*-[3-*O*-methyl-*β*-D-glucopyranosyl-(1→3)-*β*-D-xylopyranosyl-(1→4)-*β*-D-quinovopyranosyl]-4-*O*-[*β*-D-glucopyranosyl-(1→3)-*β*-D-glucopyranosyl]-*β*-D-xylopranosyl}-holosta-7(8), 25(26)-dien-16-one.

Holotoxin F (**4**) was obtained as a colourless amorphous powder. The HRESI-MS of **4** exhibited a pseudo-molecular ion peak at *m*/*z* 1227.6147 $[M + Na]^+$ (calcd. for $C_{59}H_{96}O_{25}Na$, 1227.6138), allowing the determination of the molecular formula as $C_{59}H_{96}O_{25}$. Comparison of the NMR spectral data of **4** with those of **8** showed great similarity. The 18(20) lactone group (δ_C 176.0) in **8** (Avilov & Stonik, 1988; Xue et al., 2010), however, was replaced by a methyl group (δ_C 17.2, δ_H 1.20) in **4**. Furthermore, the apparent upfield shifted ^{13}C NMR resonance of C-20 (δ_C 74.5 in **4**, 83.1 in **8**) supported the presence of an 18-methyl instead of an 18(20) lactone group in the structure, suggesting a non-holostane triterpenoid skeleton. This was also confirmed by the reported ^{13}C NMR data of C-20 (δ_C 74.1) in DS-penaustrosides B, a non-holostane glycoside isolated from sea cucumber *Pentacta australis* (Miyamoto, Togawa, Higuchi, Komori, & Sasaki, 1992). The *S* configuration at C-20 was proposed by comparing the resonances of H_3-21 (δ_H 1.57, δ_C 26.6) in **4** with that in DS-penaustrosides B (δ_H 1.54, δ_C 26.2) (Miyamoto et al., 1992). Therefore, the aglycone moiety of **4** was tentatively determined as (20*S*)-lanosta-9(11),25(26)-dien-16-one-3*β*-ol.

The monosaccharide units in **4** were identical to those of **8**, having D-xylose, D-quinovose, 3-*O*-methyl-D-glucose and D-glucose in a 2:1:1:1 ratio, based on an acidic hydrolysis and the following GC–MS experiments. The interglycosidic linkages in the pentasaccharide chain of **4** and its bonding to the aglycone were proven in the same manner as those in **8** by HMBC, NOESY and Q-TOF-MS/MS experiments. All these data indicated that the structure of holotoxin F (**4**) has to be assigned as (20*S*)-3*β*-*O*-{2-*O*-[3-*O*-methyl-*β*-D-glucopyranosyl-(1→3)-*β*-D-xylopyranosyl-(1→4)-*β*-D-quinovopyranosyl]-4-*O*-[*β*-D-glucopyranosyl]-*β*-D-xylopranosyl}-lanosta-9(11),25(26)-dien-16-one.

Holotoxin G (**5**) was isolated as a colourless amorphous powder. The molecular formula of **5** was determined as $C_{58}H_{94}O_{25}$ by HRES-I-MS at *m*/*z*: 1213.5988 $[M + Na]^+$ (calcd. for $C_{58}H_{94}O_{25}Na$, 1213.5982), and thus possessing 14 mass units more than **4**. A general inspection of the ^{13}C and ^1H NMR spectra revealed a non-holostane triterpene glycoside of compound **5**, showing almost identical signals with those of **4**. In fact, the absence of an oxygenated methyl (δ_H 3.95, δ_C 61.1) as compared to **4** was the only difference recognised in the spectroscopic data of **5**. These facts suggested that a D-glucose instead of a 3-*O*-methyl-D-glucose was included in the sugar chain of **5**. The conclusion was confirmed by the Q-TOF-MS/MS fragment peaks. Therefore, compound **5** was identified as (20*S*)-3*β*-*O*-{2-*O*-[*β*-D-glucopyranosyl-(1→3)-*β*-D-xylopyranosyl-(1→4)-*β*-D-quinovopyranosyl]-4-*O*-[*β*-D-glucopyranosyl]-*β*-D-xylopranosyl}-lanosta-9(11),25(26)-dien-16-one.

3.2. Antifungal activity

Triterpene glycosides **1–8** were tested *in vitro* for antifungal activity against six strains: *C. albicans* SC5314, *C. neoformans* BLS108, *C. tropicalis*, *T. rubrum* 0501124, *M. gypseum* 31388 and *A. fumigatus* 0504656. Itraconazole (ICZ), Terbinafine (TRB), Ketoconazole (KCZ), Amphotericin B (AMB), Voriconazole (VCZ) and Fluconazole (FCZ) were used as positive controls. Glycosides **1**, **4** and **5** exhibited selective antifungal activities against *C. albicans*, *C. neoformans* and *M. gypseum* while **2**, **3**, **6–8** had significant growth inhibitory activities against six strains, with MIC_{80} values of 0.18–26.89 μM (Table 1). These facts suggest that the 18(20) lactone group and the Δ^{25} terminal double bond may increase the activity. The component of the carbohydrate chain seems play an important role whereas the position of trisubstituted double bond in aglycone moiety (Δ^7 or $\Delta^{9(11)}$) contributes little to the bioactivity.

4. Conclusions

Chemical investigation of the sea cucumber *A. japonicus* Selenka, a traditional tonic in China, led to the isolation of a nortriterpene glycoside with a 26-nor holostane skeleton, two rare non-holostane type glycosides and two holostane-type triterpene

glycosides, together with three known triterpene glycosides, demonstrating an example of chemical diversity. *In vitro* assays on the compounds displayed potent antifungal activity, and resulted in a preliminary analysis on the structure–activity. The interesting discovery may encourage further investigations on the complex metabolites, the antifungal activity, and the structure–activity relationship.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.foodchem.2011.10.080.

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