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Two Selective Novel Triterpene Glycosides from Sea Cucumber, *Telenata Ananas*: Inhibitors of Chemokine Receptor-5

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Abstract—The aqueous methanolic extract of a sea cucumber was found to contain two triterpene glycosides **1** and **2**. The structures of **1** and **2** were established based on high-resolution NMR studies. Compounds **1** and **2** exhibited inhibitory activity (K_i) of 30 and 5 μM, respectively, in a chemokine receptor subtype 5 (CCR5) assay. Both compounds did not show any significant inhibition in a CXCR2 assay at 50 μM, suggesting their selectivity for the CCR5 receptor.

Chemokine receptors comprise a large family of seven transmembrane domain G-protein coupled receptors that are differentially expressed in diverse cell types.[1] They are defined by their ability to signal upon binding to one or more members of the chemokine superfamily of chemotactic cytokines. In addition to their role in regulating cell trafficking, several chemokine receptors have been identified to act as cofactors for HIV-1 attachment and entry into target cells. Among the receptors shown to act as HIV-1 co-factors, CCR5[2] and CXCR4 are considered the most clinically relevant since all HIV-1 isolates can utilize one or both of these receptors to gain entry into cells. Recently, much attention has been focused on targeting these receptors for antiviral therapy. The CCR5 receptor has been particularly attractive since it is the most commonly used receptor by HIV-1[2] strains and is thought to be important in viral transmission. Furthermore, in vivo evidence from both animal knockout models and humans suggests that functional CCR5 is not essential for immune competence and that blockade of these receptors by a specific antagonist will not severely affect normal immune function. Several small molecule antagonists of CCR5 are being developed for HIV therapy, one of which, SCH-C,[3] is currently in clinical trials.

As part of our continuing efforts to discover new leads for our CCR5 antagonist program, we have utilized a CCR5 binding assay screen to our natural product library. Using this assay, we screened aqueous methanolic extracts of several marine animals. A methanolic extract of a sea cucumber identified to be *Telenata ananas* sp. displayed distinct activity in the CCR5 assay. Bioassay guided fractionation of this extract led to the isolation of two triterpene glycosides **1** and **2**.

The sea cucumbers (2 kg) were collected from Andaman and Nicobar Islands (India) by Scuba diving, were preserved in MeOH and transported to the lab. They were identified as *T. ananas*. After 2 weeks, the solution was decanted, filtered, and concentrated under vacuum to a semi-solid form. This was resuspended in between water and saturated *n*-butanol and partitioned. The organic layer was dried to afford 385 mg of the extract and 370 mg of this solid was chromatographed through a CHP-20 (0.5 × 15 inches) column eluting with a water and methanol gradient. The elution of active compounds was monitored by their activity in the CCR5 assay. The active fractions were collected and dried to yield 48.7 mg of enriched complex. Separation of the active compounds was achieved by reverse-phase preparative HPLC on a Phenomenex Luna C-18 silica column (21.2 × 250 mm), eluting with a mixture of acetonitrile and water (62:38 v/v). Acetonitrile was removed from the active peak eluates, and the aqueous solution was freeze-dried. Fourteen

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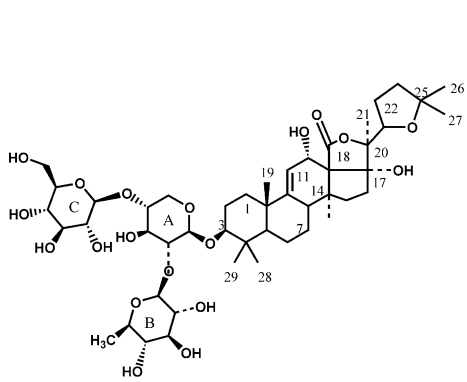

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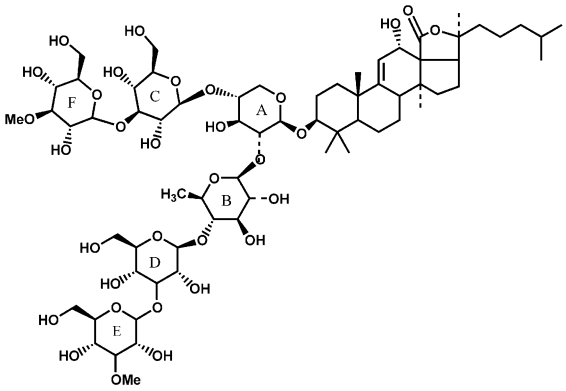

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milligrams of enriched complex yielded 3.8 and 0.7 mg of **1** and **2**, respectively.

Compound **1** showed a sodiated ion m/z 965 $(M+Na)^+$ in the FAB MS suggesting a molecular weight 942 daltons. The molecular formula of **1** was established as $C_{47}H_{74}O_{19}$ by HRMS (high-resolution MS).[4] The UV spectrum (MeOH) showed end absorption and the IR spectrum in KBr showed peaks at 3306, 2960, 1715, 1636, and 1540 cm^{-1}, suggesting the presence of an ester functionality. ^1H and ^{13}C NMR chemical shifts of **1** and ^{13}C NMR chemical shifts of **2** are listed in Table 1. The ^1H NMR of 1 indicated the presence of eight methyls, several methylenes and the presence three sugars. The ^{13}C NMR also showed 47 carbon signals in agreement with the number of carbons revealed by HRMS. APT ^{13}C NMR identified them as one >C=O, two olefinic (one =CH–, one =C<), three O–CH–O, 14 >CH–O, three >C–O, four >C<, two –CH–, two O–CH_2, eight >CH_2 and eight –CH_3. Seven methyls were tertiary and the eighth was a secondary methyl as revealed by ^1H NMR. This information led us to believe the compound to be a triterpene glycoside.

Table 1. ^1H chemical shifts for **1** and ^{13}C NMR chemical shifts for **1** and **2**

C no.	^1H	Sugar	C no.	^1H	Sugar	C no.	1	2	3^{10}	4^7	Sugar	C no.	1	2
1	1.86 (m), 1.47 (m)	Sugar A	1′	4.74 (d, 7.5 Hz)		1	36.7	36.8	35.5	36.4	Sugar B	1^2	106.6	106.0
2	1.97 (m), 2.16 (m)		2′	4.10 (dd, J=7.5, 9 Hz)		2	27.4	27.5	27.4	27.2		2^2	77.5	76.7
3	3.18 (dd, 1, 4 Hz)		3′	4.26 (m)		3	88.9	89.1	88.7	88.4		3^2	78.1	76.2
4			4′	4.32 (m)		4	40.4	40.4	40.0	39.6		4^2	77.1	88.0
5	1.01 (d, 11 Hz)		5′	4.40 (dd, J=5, 11.5 Hz)		5	53.1	53.2	52.9	52.3		5^2	73.9	72.1
6	1.59 (m), 1.77 (m)	Sugar B	1″	5.20 (d, 7.5 Hz)		6	21.6	21.6	20.3	20.8		6^2	19.0	18.6
7	1.52 (m), 1.79 (m)		2″	4.08 (dd, 7.5, 9 Hz)		7	28.7	29.0	28.2	28.0	Sugar C	1^3	103.8	103.2
8	3.38 (dd, 12, 5 Hz)		3″	4.16 (J=9, 9 Hz)		8	41.2	40.5	41.0	40.5		2^3	74.8	73.4
9			4″	3.75 (t, 9, 9 Hz)		9	154.2	153.5	153.7	153.6		3^3	78.6	88.3
10			5″	3.81 (m)		10	40.1	40.0	39.7	39.3		4^3	72.0	70.0
11	5.65 (d, 5.5 Hz)		6″	1.69 (d, 5 Hz)		11	115.9	116.6	115.6	115.3		5^3	79.2	78.3
12	5.0 (d, 5.5 Hz)	Sugar C	1″	5.04 (d, 7.5 Hz)		12	71.9	68.5	71.7	71.1		6^3	62.9	62.6
13			2″	4.04 (dd, 7.5, 9 Hz)		13	59.1	64.5	59.0	58.4	Sugar F	1^4		106.1
14			3″	4.23 (m)		14	46.3	47.0	46.0	46.5		2^4		75.5
15	1.84 (m), 1.44 (m)		4″	4.24 (m)		15	37.2	37.6	27.2	26.6		3^4		88.4
16	2.98 (m), 2.43 (m)		5″	4.00 (m)		16	35.9	24.5	38.6	35.4		4^4		70.8
17			6″	4.34 (dd), 4.56 (m)		17	90.1	47.4	89.7	89.0		5^4		78.7
18						18	174.9	177.7	174.3	174.4	–OMe	6^4		62.5
19	1.41 (s)					19	22.9	22.9	19.0	19.9		—		61.2
20						20	87.0	85.2	86.5	87.0	Sugar D	1^5		105.4
21	1.77 (s)					21	19.2	26.8	22.5	22.2		2^5		74.0
22	4.35 (m)					22	81.0	40.0	80.7	36.4		3^5		88.4
23	2.03 (m)					23	28.5	22.7	36.6	22.3		4^5		70.3
24	1.64 (t, J=7.5 Hz)					24	38.8	39.8	28.7	38.5		5^5		78.7
25						25	81.8	28.4	81.3	27.7		6^5		62.4
26	1.19 (s)					26	27.8	23.0	28.7	22.4	Sugar E	1^6		106.1
27	1.20 (s)					27	29.0	23.0	28.2	22.4		2^6		75.4
28	1.16 (s)					28	28.5	28.4	21.4	28.0		3^6		88.4
29	1.3 (s)					29	17.1	17.1	27.4	16.4		4^6		70.9
30	1.69 (s)					30	20.7	22.4	16.7	22.2		5^6		78.7
					Sugar A	1^1	105.8	105.7				6^6		62.5
						2^1	83.8	84.0				–OMe		61.2
						3^1	76.2	76.1						
						4^1	77.9	77.7						
						5^1	64.5	64.4						

NMR spectra were run in $CDCl_3 + CD_3OD$.

The literature search on possible types of compounds produced by these sea animals revealed several reports about the isolation of lanostane-type triterpenes from sea cucumbers.[5–11] The structures were deduced from the 2-D NMR spectra. Comparison of carbon chemical shift values of **1** with the literature values of known compounds (aglycone of holothurin A[10] and echinoside glycoside[9]) suggested that the aglycone of **1** to be identical to the values reported in the literature for the compound holothurigenol.[8] This was further confirmed using extensive two dimensional NMR experiments.

HMQC was used to establish one-bond carbon–proton connectivities. Proton–proton connectivities were established using HMQC-TOCSY at two different mixing times (15 and 30 ms). The HMQC-TOCSY spectra at the two mixing times yielded information similar to the COSY and TOCSY spectra, respectively, but are more useful in crowded regions of the spectra since the correlations are separated in the second dimension by the chemical shifts of the carbon resonances. HMBC was used to establish long-range (2- or 3-bond) carbon–proton connectivities. This experiment yielded complementary results to the HMQC, HMQC-TOCSY experiments by connecting the non-protonated carbons to the protons. All these experiments confirmed the structure as in **1**. No hydrolysis experiments were performed to identify the sugars separately, due to the lack of material. Their structures were deduced mainly from 2-D experiments. In compound **1**, sugars A and B have carbon chemical shifts identical to xylo-pyranoside and quinovo-pyranoside as in holothurin B. However, the third sugar, a glucose unit is linked to the C4 of the xylo-pyranoside unit as evident by a cross peak in the HMBC spectrum. The C4 of the first sugar is also shifted downfield by about 7 ppm, which is consistent with the structure. The NaO_3S- group in holothurigenol has been replaced by a gluco-pyranoside in **1**.

In compound **2**, the carbon chemical shifts of the aglycone were similar to that of holothurigenol, however the five membered ring including carbons C21–C25 were open and C25 was not oxygenated and was a methine carbon. Further the C17 carbon does not have hydroxyl group attached to it. The ^{13}C chemical shifts of 2 are compared with the aglycones of 3-*O*-[α-L-rhamnopyranosyl (1→2)-β-D-xylopyranosyl]-3β, 12α, 17α, 20(*S*)-tetrahydroxylanost-9(11)-en-18, 20-olide(**4**)[7] and aglycone of holothurin A(**3**).[10] Aglycone of **2** was identical to 17-deoxy echinoside B.[9a] We have previously isolated echinoside B from another sea cucumber *Actinopyga mauritiana*.[9b] The carbon chemical shifts of first three sugars xylo-pyranoside, quinovo-pyranoside and gluco-pyranoside of **2** are consistent with the same three sugars in **1** and the additional three sugars were gluco-pyranoside units. The linkages between these gluco-pyranoside units and the positions of the *O*-methyl groups were established from the HMBC spectra. The carbon chemical shift data are consistent with the linkage. For example, the C4 of sugar B in compound **2** is shifted downfield by about 11 ppm, while the C3 of the sugar C, D, E and F are all shifted downfield by about 10 ppm compared to glucopyranoside in compound **1**.

Compounds **1** and **2** showed a K_i value of 30 and 5.1 μM, respectively, in the CCR5[12] assay. However they did not show any inhibition at 50 μM in a CXCR2 assay suggesting that these compounds exhibit some selectivity for CCR5 inhibition. This family of compounds has been reported from different groups,[5–11] however no biological activity has been reported.

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12. A screening assay utilizing a membrane-binding assay was developed to identify antagonists of the ligand RANTES binding to the CCR5 receptor. Cell membranes were prepared from CHO cells (BioSignal Inc.) transduced to express the human CCR5 chemokine receptor. These membrane preparations were incubated with ^{125}I-RANTES in the presence or absence of compound for one hour at 25 °C. Compounds were serially diluted over a range of 0.3 nM–3 μM on a nine-point curve and tested in replicates of four. Reaction cocktails were harvested through glass fiber filters, and washed thoroughly. Total replicate counts were averaged and IC_{50} values calculated as the amount of compound required to inhibit 50% of total ^{125}I-RANTES binding. The binding affinity constant, K_i, was determined using the Graph Pad PRISM software.