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Eryloside F, a Novel Penasterol Disaccharide Possessing Potent Thrombin Receptor Antagonist Activity

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Abstract—We report the discovery of Eryloside F, a novel disaccharide of the steroidal carboxylic acid penasterol, isolated from an extract of the marine sponge *Erylus formosus*. The compound is a potent thrombin receptor antagonist, and furthermore inhibits human platelet aggregation in vitro.

Introduction

Penasterol (**1**, Fig. 1), an acidic steroidal metabolite closely related to lanosterol (**2**, Fig. 1) and possessing potent antileukemic activity, was originally isolated from *Penares* sp. by Cheng, Kobayashi and co-workers[1,2] in 1988. The compound together with its close analogues penasterone and acetylpenasterol, isolated from *Penares incrustans*, have been shown to inhibit IgE-dependent histamine release from rat mast cells.[3,4] The erylosides,[5–7] isolated from various *Erylus* spp. constitute a family of glycosides of penasterol and related aglycones. Eryloside A[5] possesses antitumour and antifungal activity, whilst Eryloside E[7] is an antagonist of C5a-receptor binding. The penasterol tetrasaccharide formoside[8] was recently isolated by Jaspars et al. from *Erylus formosus*. We describe here the discovery of a novel penasterol disaccharide Eryloside F (**3**, Fig. 1). The compound is closely related to formoside, though lacks the terminal Ara-Gal-disaccharide moiety. Eryloside F was discovered during a screen for thrombin receptor antagonists. It has potent receptor antagonist activity, and furthermore inhibits platelet aggregation in vitro. The compound possesses low toxicity against hepatocyte (HepG2) cells.

Materials and Methods

Animal material

Two samples of the sponge *E. formosus* Sollas, 1886 (Class, Demospongiae, Order Choristida, Family Geodiidae), were analyzed in this study. The first specimen (HBOI No. 5-VI-86-4-013) was collected by scuba at a depth of 60 feet in the Bahamas off Black Rock in the Little Bahama Bank (latitude 26 15.3° N, longitude 79 39.3° W). The second specimen (HBOI No. 16-XI-87-2-019) was collected by scuba at a depth of 55 feet in the Bahamas at Wood Cay, northwest of Grand Bahama Island (latitude 26 44.5° N, longitude 79 01.5° W). Both specimens are described as growing in a thick encrusting form with a smooth thin ectosome and very small pores distributed over the surface. The exterior of the sponge is black while the interior is tan in colour. All features of the sponge conform to the taxonomic assignment of *E. formosus* as described in Widenmeyer.[10] Voucher specimens have been deposited at the Harbor Branch Oceanographic Museum with catalog acquisition numbers of 003:00937 and 003:00938, respectively.

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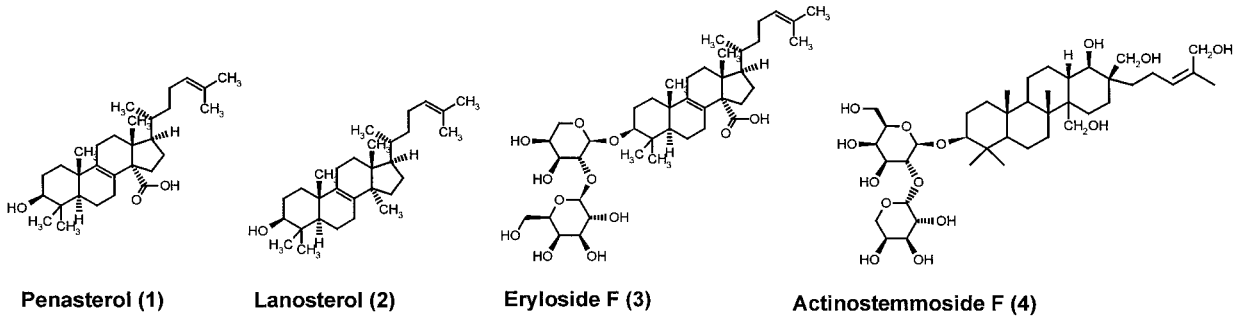

Figure 1. Structure of Eryloside F and related compounds.

Extraction and isolation

To generate samples for screening, extracts were prepared by grinding 8 g of frozen sponge with ethanol (20 mL). After standing for 24 h at −20 °C, solid material was removed by filtration. The filtrate was dried, and a proportion was dissolved in DMSO to give a 5 mg/mL solution. After detection of activity, a larger extract was prepared for sample HBOI No. 5-VI-86-4. Seventy-five (75) g of the frozen sponge was extracted exhaustively with ethanol using a Waring blender. The extract was filtered and the solvent removed by distillation under reduced pressure to yield 3.5 g of a crude ethanol extract. A portion of this (350 mg) was taken and dissolved in aqueous buffer (100 mM $NH_4H_2PO_4$ in water, pH 2.5) then chromatographed using preparative HPLC as follows: column Kromasil 7 μm C8 15×2 cm. Mobile phase 50% v/v acetonitrile, 50 mM $NH_4H_2PO_4$ in water with 3 mL/L H_3PO_4 added. Flow rate 20 mL/min. Injection volume 5 mL. Eryloside F eluted after 30 min approx. Relevant fractions were pooled and acetonitrile was removed by rotary evaporation. The aqueous solution was de-salted by adsorption onto C18 silica (500 mg Bond-Elut cartridge, Varian Ltd.), resin washed with water then adsorbed material eluted with methanol (2 mL). The eluate was concentrated to dryness using a Speedivac concentrator to yield eryloside F (2.0 mg) as a white solid.

Acquisition of NMR and MS data

NMR spectra were recorded using a Varian INOVA-750 spectrometer, with ^1H observation at 750 MHz, using a 5 mm inverse $^1H/^{13}C/^{15}N$ triple probe fitted with a z gradient coil. All data were acquired using pulse sequences supplied by Varian Instruments Ltd. These included 1D-TOCSY, DQFCOSY, HMQC, and gradient-assisted HMBC. The 1-D-TOCSY spectra were acquired using an eburp excitation pulse over 50 Hz and a mix time of 120 ms. Typically 64 transients were acquired into 96 K data points over a spectral width of 20 ppm and a pulse repetition time of 3.4 s. The DQFCOSY spectrum was acquired using 512 increments of 16 transients. FIDs were collected into 2 K data points with a spectral width of 4.4 KHz and a pulse repetition time of 1.3 s. The HMQC spectrum was acquired using 256 increments of 32 transients. FIDs were collected into 1 K data points with a spectral width of 4.4 KHz and a pulse repetition time of 0.6 s. The gradient assisted HMBC spectrum was acquired using 256 increments of 96 transients. FIDs were collected into 2 K data points with a spectral width of 4.0 KHz and a pulse repetition time of 1.8 s. Mass spectra were measured on a Finnigan Mat LCQ ion trap mass spectrometer, fitted with an electrospray interface, operated in both positive and negative ionisation modes. Accurate mass measurements were obtained using a Micromass QTOF instrument with an electrospray source and operated in negative ion mode. The instrument was calibrated using poly-alanine (Sigma, P-9003) with corrections for drift made with a loc K mass of reserpine.

Thrombin receptor antagonist assay

HeLa cells were suspended complete media and the cell density measured was adjusted to give a final cell density of 2.0×10^5/mL. One hundred microlitres of cell suspension was dispensed into 96-well microtitre plates using a Multidrop 96/384 well plate dispenser. Plates were incubated overnight at 37 °C/5% CO_2. One hundred microlitres of a solution of fluo-3 (Cambridge Biosciences, UK) was added to all wells giving 10 μg/mL final concentration. The plates were then placed in a tissue culture incubator in stacks of no more than three high. Test samples (in DMSO) were added to wells (to give 1%v/v final DMSO concentration) followed by thrombin (final concentration 0.3 U/mL), then plates placed on the FLIPR. Fluorescence output was read every 1 s for the first 20 s then every 5 s for a further 30 s.

Platelet aggregation assay

Blood was withdrawn from the antecubal vein of healthy human volunteers who had taken no medication for 14 days. The blood (9 volumes) was anti-coagulated with 1 volume of 3.8% w/v tri-sodium citrate, to give a final citrate concentration of 12.9 mM. To each 20 mL volume of anti-coagulated blood 50 μL of a 10 μg/mL prostacyclin (PGI_2) solution was added to prevent activation during the platelet isolation process. The prostacyclin-treated blood was then centrifuged (1280×*g*; 4 min) to yield platelet-rich plasma (PRP). The PRP was carefully removed from above the red and white blood cells with a plastic Pasteur pipette and transferred to a clean Sterilin® (30 mL) tube. This PRP was then centrifuged (1280×*g*; 10 min) to pellet the platelets. The

supernatant (platelet-poor plasma) was discarded and the platelets resuspended in a Hepes buffer (NaCl (8 g/L); $NaHCO_3$ (1 g/L); KCl (0.21 g/L); KH_2PO_4 (0.1 g/L); Hepes (1.2 g/L); D-glucose (1 g/L)) adjusted to a pH of 6.4. The resultant platelet suspension was again centrifuged (1280×*g*; 8 min) to pellet the platelets. The supernatant was discarded and the platelets resuspened in a Hepes buffer of the same composition, but adjusted to a pH of 7.4. The platelet count was then adjusted to 300,000 platelets/μL, following quantification on a Sysmex K1000 Haematological analyser and $CaCl_2$ (1 mM) and $mgCl_2$ (0.5 mM) were added back. The platelet preparation was then left for at least 30 min before aggregation studies were performed.

Results and Discussion

Several extracts of marine organisms were found to have activity in a thrombin receptor antagonist high throughput screen. The screen was based upon measurement of inhibition of thrombin-induced calcium mobilisation in HeLa cells. Intracellular calcium flux was measured using the fluorescent dye 'fluo-3' which in the absence of calcium ions fluoresces only weakly, but in the presence of calcium increases fluorescence by approx. 100-fold. Fluorescence was measured in real time using a fluorescence imaging plate reader. Two highly active marine samples—both extracts of *E. formosus*—were fractionated by HPLC, using the calcium mobilisation assay to guide the fractionations. The HPLC profiles of the extracts were very similar, and in both cases the biological activity resided in the same chromatographic fractions indicating common active principles. One of these was fully deconvoluted, and several active components were isolated. These were all glycosides of penasterol though differing in the number of sugar residues attached at the C-1 position (data not shown). It is notable that from a screen of over 50,000 compound and natural product samples, only Eryloside F (and related compounds isolated from the sponge extract — data not shown), demonstrated both selective thrombin receptor antagonist activity and functional activity in a pletelet aggregation assay.

The structure of Eryloside F was determined using a combination of MS and 1-D and 2-D-NMR data. The observations of positive ions at *m/z* 768 $[M+NH_4]^+$ and 1518 $[dimer+NH_4]^+$ and negative ions at *m/z* 749 $[M\text{-}H]^-$ and 1499 $[dimer\text{-}H]^-$ were consistent with a molecular weight of 750 amu. Accurate mass experiments in negative ion mode showed a molecular ion at *m/z* 749.4466 with a 1.3 ppm error from the calculated mass for the formula $C_{41}H_{65}O_{12}$ ($M\text{-}H^-$). Initial examination of the 1-D proton and one bond ^1H-^{13}C correlation NMR data suggested the presence of two sugars (anomeric signals at δ^1H 4.55→$\delta^{13}C$ 104.9 and δ^1H 4.47→$\delta^{13}C$ 105.6). This was supported by fragment ions in the positive ion mass spectrum which were consistent with the loss of a C6 sugar (*m/z* 589 $[M+NH_4\text{-sugar}]^+$, 571 $[M+H\text{-sugar}]^+$) and with the loss of both a C6 and a C5 sugar (*m/z* 457 $[M+NH_4\text{-2 sugars}]^+$). Careful analysis of the ^1H-^1H, one bond and long range ^1H-^{13}C correlation data served to identify the aglycone part of the molecule as penasterol. The observed chemical shifts (Table 1) for the aglycone are in good agreement with those reported for the closely related penasterol glycoside, formoside.[8] However, some of the reported assignments, which appear to be based on earlier work on penasterol itself,[1] clearly differ from ours. In the light of the 2D data supporting our assignments (Table 2) the earlier literature assignments for both penasterol and formoside[1,8] must be considered suspect. The linking points between the sugars and to the aglycone followed from the long range ^1H-^{13}C correlations (δ^1H 4.55→$\delta^{13}C$ 90.3 and δ^1H 4.47→$\delta^{13}C$ 79.7) observed from the anomeric protons. The stereochemistry of the sugars was identified from an analysis of the ^1H-^1H coupling constants (Table 1). These are very similar to those previously observed for this disaccharide group in actinostemmoside F[9] (**4**, Fig. 1). The measurement of these couplings was facilitated by the acquisition of selective 1-D-TOCSY spectra starting from each anomeric proton.

The biological activity of Eryloside F and its analogues in the FLIPR assay varied inversely with the length of the oligosaccharide chain; Eryloside F which was the shortest chain oligosaccharide isolated from the active extract showed the most potent activity, and so its chemical structure was fully elucidated. Eryloside F was also evaluated in a platelet aggregation assay. Platelet aggregation, in a human washed platelet preparation, was quantified using an optical aggregometer. Platelets were isolated from citrated whole blood by a series of centrifugation steps and resuspended in a physiological Hepes buffer. The compounds were tested against

Table 1. ^{13}C (188.6 MHz) and ^1H (750 MHz) NMR data in CD_3OD for Eryloside F

$\delta^{13}C$[a]		δ^1H[b]	$\delta^{13}C$		δ^1H
36.1	1	1.78; 1.29	125.7	24	5.09 (m)
27.2	2	1.91; 1.74	131.6	25	—
90.3	3	3.14 (dd,12.0,4.5)	17.3	26	1.60 (brs)
40.1	4	—	25.5	27	1.67 (brs)
51.3	5	1.12 (dd,12.5,2)	179.6	28	—
18.9	6	1.71; 1.54	28.0	29	1.04 (s)
28.3	7	2.11; 1.97	16.4	30	0.88 (s)
128.6	8	—			
140.7	9	—		Ara	
38.2	10	—	104.9	1′	4.55 (d,5.5)
22.9	11	2.18 (m); 2.11	79.7	2′	3.86 (dd,7.5,5.5)
32.3	12	2.23 (m); 1.71	72.8	3′	3.78 (dd,7.5,3.5)
47.6	13	—	68.1	4′	3.87 (m)
63.6	14	—	64.2	5′	3.84 (dd,11.5,5.0)
					3.51 (dd,11.5,2.0)
28.4	15	2.08; 1.59			
29.9	16	2.07; 1.39		Gal	
51.7	17	1.54	105.6	1′	4.47 (d,7.5)
17.9	18	0.80 (s)	73.1	2′	3.56 (dd,9.5,7.5)
19.6	19	1.06 (s)	74.6	3′	3.48 (dd,9.5,3.5)
36.6	20	1.45	69.6	4′	3.86 (dd,3.0,1.0)
18.7	21	0.94 (d, 6.5)	76.4	5′	3.49 (td,6.5,1.0)
36.9	22	1.43; 1.04	61.6	6′	3.71 (d,6.5)
25.4	23	2.02; 1.88			

[a]Referenced to residual CHD_2OD at 48.9 ppm.
[b]Referenced to residual CHD_2OD at 3.31 ppm. Multiplicities and coupling constants (±0.5 Hz) are given in parentheses.

Table 2. Key long-range $^{1}H \rightarrow ^{13}C$ correlations for Eryloside F

$\delta^{1}H$	$\delta^{13}C$
0.80	32.3; 47.6; 51.7; 63.6
0.88	28.0; 40.1; 51.3; 90.3
0.94	36.6; 36.9; 51.7
1.04	16.4; 40.1; 51.3; 90.3
1.06	36.1; 38.2; 51.3; 140.7
1.59	179.6
1.60	25.5; 125.7; 131.6
1.67	17.3; 125.7; 131.6
2.08	179.6
4.47	79.7
4.55	90.3

thrombin, SFLLRN (a thrombin receptor activating peptide) and U-46619 (a stable thromboxane A2 mimetic) in order to determine if they were selective at inhibiting protease-activated receptor 1 (PAR-1) mediated platelet aggregation. Eryloside F inhibited both SFLLRN (3 μM)- and U-46619 (3 μM)-induced platelet aggregation in a concentration related manner. Eryloside F was approximately 6-fold more potent against SFLLRN than against U-46619-induced platelet aggregation, producing IC_{50} values of 0.3 and 1.7 μg/mL respectively. Eryloside F was also tested against thrombin (0.1 U/mL)-induced platelet aggregation, however its potency relative to that against SFLLRN-induced platelet aggregation was approx. 20-fold lower (data not shown). In conclusion therefore, Eryloside F is a novel steroidal disaccharide metabolite of *E. formosus* which we have discovered to possess potent and relatively selective thrombin receptor antagonist activity. Activity in the high throughput calcium mobilisation FLIPR assay was correctly predictive of functional activity in a platelet aggregation assay.

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