A Series of New Pyrrole Alkaloids with ALR2 Inhibitory Activities from the Sponge Stylissa massa

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Abstract: Twelve new and four known alkaloids including five different structural scaffolds were isolated from the sponge Stylissa massa collected in the South China Sea. Compound 1 is the first identified precursor metabolite of the classic 5/7/5 tricyclic skeleton with unesterified guanidine and carboxyl groups, compounds 2–5 and 13–15 belong to the spongiacidin-type pyrrole imidazole alkaloids (PIAs). Z- and E-configurations of the spongiacidin-type PIAs often appeared concomitantly and were distinguished by the chemical shift analysis of 13C NMR spectra. The structures of all twelve new compounds were determined by NMR, MS, and ECD analysis combined with single-crystal data of compounds 1, 5, and 10. In the aldose reductase (ALR2) inhibitory assay, six 5/7/5 tricyclic compounds (2–5, 13–15) displayed significant activities. Compounds 13 and 14, as the representative members of spongiacidin-PIAs, demonstrated their ALR2-targeted activities in SPR experiments with KD values of 12.5 and 6.9 μM, respectively.

Keywords: pyrrole-imidazole alkaloids; ALR2 inhibitory activities; sponge; Stylissa massa; spongiacidin

1. Introduction

Pyrrole-imidazole alkaloids (PIAs) and simple pyrrole alkaloids represent a specific structural class of compounds isolated from sponges including those from the genus Agelas, Axinella, Hymeniacidon, Phakellia and Stylissa [1–5]. PIAs can be divided into monomeric and polymeric groups. Like sceptrin 1, palau’amine 6, ageliferin 7, and stylissadine 8 represent members of polymeric PIAs. Biosynthesis of mono-PIAs originates from proline and lysine [9], evolving to form several skeletons such as oroidin [10], phakellin [11], ugibohlin [12], and spongiacidin types [13], which have 5/5 bicyclic, 5/6/5/5 tetracyclic, 5/6/5 tricyclic, and 5/7/5 tricyclic systems, respectively. Currently, although hundreds of PIAs have been discovered from sponges, the structural diversity of this alkaloid family, especially for the monomeric ones, is relatively conservative.

We collected the Stylissa massa sponge from the Xisha Islands (Paracel island) in the South China Sea. Targeted isolation for methanol extraction yielded twelve new and four known compounds. Five biosynthetic-related PIA skeletons including the 5/7 imidazole-acyclic compound 1, 5/7/5 spongiacidins (2–5, and 13–15), 5/6/5/5 phakellins (6 and 16), 5/7 bicyclic (7–9), and pyrrole single ring alkaloids (10–12) were obtained from Stylissa massa. Compound 1 is the first identified precursor metabolite of the classic 5/7/5 tricyclic skeleton with unesterified guanidine and carboxyl groups. Compounds 2a and 2b are a scalemic mixture with a hydroxyl group positioned at C-9. Compounds 3a and 3b are also scalemic mixture compounds with the E-configuration of Δ10,11 versus the
Z-configuration of 2. Compound 4 has a hydroxide at C-9 with a double bond $\Delta^9,10$ at a different position, and the new compound 5 has an extra methyl group at 13-NH.

Guanidine compounds often exhibit effective diabetes-related activities [14]. ALR2 is a key limiting enzyme of the glucose polyol metabolic pathway, which is the key target for the treatment of diabetes complications [15]. In the ALR2 enzyme activity assay in vitro, spongiacidin-type PIAs (compounds 2–5, and 13–15) presented superior inhibitory activities than the other skeletons at 20 µM. The IC$_{50}$ values ranged from 8.6 to 13.6 µM, respectively (Figure S23). The SPR experiments verified the interaction between ALR2 and compound 14, with the K$_D$ value of 6.9 µM. In summary, spongiacidin-PIAs are efficient ALR2-targeted inhibitors with 5/7/5 tricyclic skeletons. Thus, the structural elucidation and the ALR2 inhibitory activities of these PIAs are concluded below (Figure 1).

Figure 1. The structures of compounds 1–16.

2. Results

Compound 1 was obtained as a colorless bulk crystal. Its molecular formula C$_{11}$H$_{12}$BrN$_5$O$_3$ was determined by HRESIMS with the ion peak at $m/z$ 342.0197, and 344.0170 with the proportion of 1:1 (calcd. for [M + H]$^+$ $m/z$ 342.0196, and 344.0176), with the unsaturation degree of 8. The $^{13}$C NMR and DEPT spectra revealed 11 resonances (Table S1) including one methylene, three methines, and seven non-protonated carbons. The chemical shifts ranging from $\delta^C$ 104.3 to 133.6 ppm showed six olefinic carbons, which included two methines and four non-protonated carbons. In the low field of the $^{13}$C NMR spectrum with the chemical shifts of $\delta^C$ 170.5, 162.1, and 156.4, there could be two carbonyl groups and one guanidyl group that exist in this molecule. The $^1$H NMR and HSQC spectra (Table S2) showed the presence of two olefinic protons at $\delta^H$ 6.36 (1H, d, $J$ = 2.3 Hz), and $\delta^H$ 6.04 (1H, t, $J$ = 6.8 Hz), one methine proton at $\delta^H$ 5.21 (1H, d, $J$ = 8.5 Hz), and one methylene proton at $\delta^H$ 3.42 (2H, m), and there were three additional heteroatomic protons at $\delta^H$ 12.71 (brs), $\delta^H$ 7.80 (1H, d, $J$ = 8.3 Hz), and $\delta^H$ 7.79 (1H, q, $J$ = 4.7 Hz). The $^3$H-$^1$H COSY spectrum (Figure 2) revealed a pyrrole conjugate ring system and another spin system with the correlations between 1-NH and H-3, between 15-NH and H-11, between 7-NH and H$_2$-8, and between H$_2$-8 and H-9. Key HMBC correlations (Figure 2) of 1-NH/C-3, C-4, and C-5, H-3/C-2, and C-4, 7-NH/C-5, and C-9, H$^2$-8/C-6, C-9, and C-10, H-9/C-4, C-8, and C-10 determined the existence of a 5/7 bicyclic 2-bromo-6,7-dihydropyrrolo-[2,3-c]azepin-8(1H)-one skeleton (Figure 2). The already existential bicyclic skeleton and four double bonds occupied six degrees of unsaturation, and the remaining two degrees pointed out that there was no other ring systems in compound 1. The final planar structure of compound 1 was settled down by the HMBC correlations from H-11 to C-4, C-9, C-10, C-12, and C-14, together with the correlations from H-9 to C-11 as well as 15-NH to C-12. A suitable bulk single crystal of compound 1 was obtained to perform the X-ray diffraction experiment, which ensured the planar structure of 1 (Figure 3). The space group of 1 indicated that it was a scalemic mixture, and ECD calculation finally determined the absolute configurations of 1a and 1b isolated through chiral HPLC by comparison with each of their ECD spectra (Figures S1 and S2).
Compound 2 was obtained as a yellow oil and its molecular formula was determined as \( \text{C}_{11}\text{H}_{11}\text{N}_{5}\text{O}_3 \) by the \([\text{M} + \text{H}]^+ \) ion peak presented at \( m/z \) 262.0936 (calcd. \( \text{C}_{11}\text{H}_{12}\text{N}_{5}\text{O}_3 \) for \( m/z \) 262.0935) in the HRESIMS spectrum with nine unsaturation degrees. One more unsaturation degree and the similar chemical shifts of the carbons (Table S1) with 1 indicated that compound 2 may have a 5/7/5 tricyclic spongiacidin-PIA skeleton, close to the known compound 13. The HMBC correlations (Figure 2) of 1-NH/C-3, and C-4, H-2/C-3, C-4, and C-5, H-3/C-2, C-4, and C-5, 7-NH/C-5, C-8, and C-9, H-8/C-6, C-9, and C-10, and H-9/C-2, C-4, C-10, and C-11, together with the COSY correlations of 1-NH/H-2/H-3, and 7-NH/H-8/H-9, constructed the classic 5/7/5 tricyclic structure with the hydroxyl group substituted at C-9, taking the chemical shift of C-9 (\( \delta_C \) 62.8) into consideration. Thus, the planar structure of 2 was established.

The 1D and 2D NMR spectra as well as the HRESIMS spectrum indicated that compound 3 possessed the same planar structure with 2 (Figure 2). The Z- and E-configuration of double bond \( \Delta^{10,11} \) in spongiacidin-type PIAs often concomitantly appeared and their differences can be attributed to the anisotropic effect of the carbonyl at C-12 [16]. Two configurations of known compounds 13 and 14 could be distinguished by the chemical shift values of H-3 and H-2-9, but the existence of 9-OH substituted in compounds 2 and 3 made it so that the judgement rule did not work [compound 2: \( \delta_H \) 6.45 (H-3), 5.80 (H-9); compound 3: \( \delta_H \) 6.43 (H-3), 5.83 (H-9)]. Through careful analysis of their \( ^{13} \text{C} \) NMR spectra, we found the double bonds of compounds 2 and 13 could be in the Z-configuration [16] because the signals for C-4, C-10, C-11, C-12, and C-14 were weak compared with the stronger signals for C-2, C-3, C-5, C-6, C-8, and C-9, while the carbon signals of compounds 3 and 14 were distributed on average comparatively (Figure 4). Known compound 15 co-isolated was also determined to be of a Z-configuration with the evidence of its carbon signal analysis in the \( ^{13} \text{C} \) NMR spectrum. Thus, the double bond of \( \Delta^{10,11} \) in compound 2
was determined as the Z-configuration and compound 3 was the E-configuration. C-9’s absolute configurations of compounds 2a, 2b and 3a, 3b were all identified based on the ECD calculations together with the chiral HPLC method (Figures S3–S6).

Figure 4. The comparison of the $^{13}$C NMR spectra of compounds 2, 3, 13, 14, and 15 (125 MHz, DMSO-$d_6$).

Compound 4 is a molecule similar to compounds 2 and 3, with the same molecular formula C$_{11}$H$_{11}$N$_5$O$_3$ by the [M + H]$^+$ ion peak presented at $m/z$ 262.0931 (calcd. for $m/z$ C$_{11}$H$_{12}$N$_5$O$_3$ 262.0935) in the HRESIMS spectrum. The key $^1$H-$^1$H COSY correlations between 7-NH and H$_2$-8, and between H-11 and 15-NH uncovered the different structure of 4, and its final planar structure was determined by the HMBC correlations from 1-NH to C-2, C-3, C-4, and C-5, from H-2 to C-3, C-4, and C-5, from H-3 to C-2, C-4, C-5, and C-10, from 7-NH to C-5, C-8, and C-9, from H-8 to C-6, C-9, and C-10, from H-11 to C-4, C-9, C-10, C-12, and C-14, from 15-NH to C-10, and from 9-OH to C-8, C-9, and C-10 (Figure 2). ECD calculation was also carried out to determine the absolute configurations of 4a and 4b (Figures S7 and S8).

Compound 5 was obtained as colorless bulk crystals. Its molecular formula was determined to be C$_{12}$H$_{13}$N$_5$O$_2$ by HRESIMS ($m/z$ 260.1148, calcd. [M + H]$^+$ for $m/z$ 260.1142), which required nine degrees of unsaturation. The 1D and 2D NMR data revealed its similarity with the known compound 13, with the only difference at 13-NMe with the extra signals of $\delta^H$ 3.11 and $\delta^C$ 25.8 in the $^1$H and $^{13}$C NMR spectra (Tables S1 and S2). The HMBC correlations from 13-NMe to C-12 and C-14 further confirmed the planar structure of 5 (Figure 2). Fortunately, a suitable bulk crystal was obtained followed by X-ray diffraction (Figure 3), and the result showed that the previously proposed rule of distinguishing Z/E configurations of double bond $\Delta^{10,11}$ in 5/7/5 spongiacidin-type PIAs was trustworthy.

The molecular formula of compound 6, obtained as a yellow oil, was determined to be C$_{12}$H$_{14}$BrN$_5$O$_3$, according to the HRESIMS results, which showed a protonated molecular ion at $m/z$ 356.0357, 358.0329 (calcd. for [M + H]$^+$, $m/z$ 356.0353, 358.0332). The analysis of the 1D and 2D NMR spectra (Tables S3 and S4) of 6 indicated that it had a very similar structure with the reported compound (10) obtained by organic synthesis [17], with the only difference of 2-OMe rather than 2-OH. The relative configuration of 6 was determined by DP4+ analysis, where the results showed that the only possibility was 2R$^*$6R$^*$10S$^*$, and further ECD calculations confirmed the absolute configuration of 6 was 2S6S10R (Figures S9 and S10).
Compounds 7–9 were 5/7 bicyclic pyrrole alkaloids, in which compounds 8 and 9 were 2-bromo substituted ones. Their structures were confirmed by HRESIMS and NMR data (Tables S3 and S4). Compounds 7 and 8 were two pairs of scalemic mixture, and their absolute configurations were solved by the chiral HPLC and ECD calculation method (Figures S11–S14).

Compounds 10–12 were simple pyrrole alkaloids with the 2-carboxyl and 3-bromo characteristic, which were pairs of scalemic mixture. Their planar structures were determined by HRESIMS, NMR (Tables S3 and S4), and single crystal X-ray diffraction (Figure 3), and the absolute configurations were confirmed by chiral HPLC and ECD calculations (Figures S15–S20).

Five characteristic skeletons of the alkaloids (1–12) above-mentioned were isolated from the sponge *Stylissa massa*. Compound 1 was the first identified precursor metabolite of the classic 5/7/5 tricyclic skeleton with unesterified guanidine and carboxyl groups. Through the NMR data analysis of compounds 2, 3, 13, 14, and 15, an experience rule to determine the Z/E configurations of double bond $\Delta_{10,11}$ was summarized based on the signal intensity in the $^{13}$C NMR spectra (Figure 4).

Some guanidine compounds were reported to exhibit advantageous biological activities on diabetes [14], which indicated the following aldose reductase (ALR2) assay in vitro. We successfully obtained the protein AKR1B1 (ALR2) by genetic engineering methods (Figure S21) and compounds 1–16 were tested. Compounds 2–5 and 13–15, representative of 5/7/5 tricyclic spogiacidin-type PIA compounds, displayed superior inhibitory activities compared with other compounds (Figure S22) with epalrestat as the positive control. Further concentration gradient experiments carried out to calculate their IC$_{50}$ values showed results that ranged from 8.6 to 13.6 $\mu$M (Figure S23). The analysis of the structure–activity relationships indicated that 9-OH and 13-NMe may enhance the ALR2 inhibitory activities of this spogiacidin-alkaloid family. Compounds 13 and 14 with the basic 5/7/5 spogiacidin skeleton without stereoconfiguration were isolated as the major metabolites in sponge *Stylissa massa*. In order to research the interaction mechanism between spogiacidin-skeleton compounds and ALR2, we carried out surface plasmon resonance (SPR) binding assays of compounds 13 and 14 under the Biacore T200 instrument, where the results showed the binding power ($K_D$ value) between ALR2 and compound 13 was 12.5 $\mu$M (Figure S24), and 14 was 6.9 $\mu$M (Figures 5 and S25). Molecular docking using the GBVI/WSA $\Delta G$ rescoring method was applied to screen the best docking pose between ALR2 and compound 14. The results showed that the pyrrole and imidazole ring systems were binding to the pocket of ALR2 by H–π and π–π conjugate bonds, respectively (Figure 5).

![Figure 5. The ALR2 inhibitory activity for compound 14. (A) Concentration dependent curve of the ALR2 inhibitory assay for 14. (B) The result of the surface plasmon resonance (SPR) binding assay of 14 and ALR2 with a $K_D$ value of 6.89 $\mu$M. (C) Ligand interactions between ALR2 and 14. (D) The 3D binding model of compound 14 with ALR2, the surface of the protein is shown in grey, and the interaction bond is shown in the red dotted line.](image-url)
3. Materials and Methods
3.1. General Experimental Procedures

Optical rotations were measured on a JASCO P-1020 digital polarimeter. UV and ECD spectra were obtained on a Jasco J-810 spectropolarimeter (Tokyo, Japan). The NMR spectra were measured by a Bruker AVANCE III 500 MHz spectrometer (Bruker company, Fällanden, Switzerland). The 2.50 ppm and 39.5 ppm resonances of DMSO-d$_6$ were used as internal references for the $^1$H and $^{13}$C NMR spectra, respectively. HRESIMS data were measured on Micromass Q-Tof Ultima Global GAA076LC (Waters, Milford, CT, USA) and Thermo Scientific LTQ Orbitrap Exploris 480 mass spectrometers (Waltham, MA, USA). X-ray data were obtained by a Rigaku Xtalab Synergy using Cu-Kα radiation (Tokyo, Japan). Semi-preparative HPLC utilized an ODS column (Agilent XDB C-18, 9.6 mm × 250 mm, 5 μm).

3.2. Sponge Material

The marine sponge *Stylissa massa* was collected from the Xisha Islands of the South China Sea in June 2013, and was frozen immediately after collection. The specimen was identified by Nicole J. de Voogd, National Museum of Natural History, Leiden, The Netherlands. The voucher specimen (No. XS-2013-07) was deposited at lab A1007, School of Pharmacy, Qingdao University, P. R. China.

3.3. Extraction and Isolation

*Stylissa massa* (8.0 kg, wet weight) was crushed and then extracted with MeOH four times (3 days each time) at room temperature. The combined solutions were concentrated in vacuo and the residue was subsequently desalted to yield the organic extract (191.0 g). The extract was subjected to silica gel vacuum liquid chromatography (VLC), eluting with a gradient of petroleum ether/EtOAc (from 10:1 to 0:1, v/v) to give nine fractions Fr.1–Fr.9. Fr.1 (8.5 g) was then subjected to a silica gel CC (petroleum ether/EtOAc, from 5:1 to 1:1, v/v) to give six fractions Fr.1–Fr.6. Fr.1 (200 mg) was then purified by semi-preparative HPLC (ODS, 5 μm, 250 × 9.6 mm; MeOH/H$_2$O, 25:75, v/v; 2.0 mL/min, 48 min) to afford compound 1 (R$_f$ = 21.8 min, 1.0 mg) and compound 2 (R$_f$ = 24.3 min, 1.0 mg). Fr.2 (3.0 g) was subjected to a silica gel CC (petroleum ether/EtOAc, from 5:1 to 1:1, v/v) to give five fractions Fr.2-1–Fr.2-5. Fr.2-2 (4.0 g) was then purified by semi-preparative HPLC (ODS, 5 μm, 250 × 9.6 mm; MeOH/H$_2$O, 25:75, v/v; 2.0 mL/min, 33 min) to afford compound 3 (R$_f$ = 45.0 min, 4.0 mg). Fr.8 (14.5 g) was subjected to a silica gel CC (CH$_2$Cl$_2$/MeOH, 50:1–1:1, v/v) to give nine fractions Fr.8-1–Fr.8-9. Fr.8-1 (5.0 g) was then subjected to silica gel CC (CH$_2$Cl$_2$/MeOH, 20:1–1:1, v/v) to give three fractions Fr.8-1–Fr.8-3. Fr.8-2 (2.0 mg) was then purified by semi-preparative HPLC (ODS, 5 μm, 250 × 9.6 mm; MeOH/H$_2$O, 25:75, v/v; 2.0 mL/min, 20 min) to afford compound 4 (R$_f$ = 21.1 min, 2.6 mg), compound 5 (R$_f$ = 26.1 min, 3.7 mg), and compound 6 (R$_f$ = 23.8 min, 3.7 mg) and compound 7 (R$_f$ = 23.9 min, 3.0 mg). Fr.8-1-5 (4.0 g) was purified subjected to an ODS CC (MeOH/H$_2$O, from 5:95 to 100:0, v/v) to give six fractions Fr.8-1-5-1–Fr.8-1-5-6. Fr.8-1-5-4 (20 mg) was then purified by semi-preparative HPLC (ODS, 5 μm, 250 × 9.6 mm; MeOH/H$_2$O, 5:95–100:0, v/v; 2.0 mL/min, 36 min) to afford compound 8 (R$_f$ = 21.1 min, 2.6 mg), compound 9 (R$_f$ = 26.1 min, 3.7 mg), and compound 10 (R$_f$ = 24.3 min, 1.0 mg). Fr.7 (3.0 g) was subjected to a silica gel CC (Petroleum ether/EtOAc, from 5:95 to 100:0, v/v) to give five fractions Fr.7-1–Fr.7-5. Fr.7-2 (2.0 mg) was then purified by semi-preparative HPLC (ODS, 5 μm, 250 × 9.6 mm; MeOH/H$_2$O, 25:75, v/v; 2.0 mL/min, 48 min) to afford compound 11 (R$_f$ = 40.3 min, 5.8 mg) and compound 12 (R$_f$ = 45.0 min, 4.0 mg). Fr.15 (17.2 g) was subjected to a silica gel CC (CH$_2$Cl$_2$/MeOH, 50:1–1:1, v/v) to give nine fractions Fr.15-1–Fr.15-9. Fr.15-1 (200 mg) was then subjected to a silica gel CC (CH$_2$Cl$_2$/MeOH, 20:1–1:1, v/v) to give three fractions Fr.15-1–Fr.15-3. Fr.15-2 (1.1 g) was then subjected to an ODS CC (MeOH/H$_2$O, from 5:95 to 100:0, v/v) to give six fractions Fr.15-2-1–Fr.15-2-6. Fr.15-2-4 (300 mg) was then purified by semi-preparative HPLC (ODS, 5 μm, 250 × 9.6 mm; MeOH/H$_2$O, 10:90, v/v; 2.0 mL/min, 55 min) to afford...
compound 2 (tR = 29.1 min, 16.0 mg), compound 15 (tR = 44.3 min, 6.0 mg), and compound 14 (tR = 47.5 min, 150 mg). Fr.17 (20.0 g) was subjected to an ODS CC (MeOH/H2O, from 5:95 to 100:0; v/v) to give seven fractions Fr.17-1–Fr.17-7. Fr.17-3 (800 mg) was then subjected to a silica gel CC (CH2Cl2/MeOH, from 1:10 to 1:1, v/v) to give six fractions Fr.17-3-1–Fr.17-3-6. Fr.17-3-3 was then purified by semi-preparative HPLC (ODS, 5 μm, 250 × 9.6 mm; MeOH/H2O, 5:90–100:0, v/v; 2.0 mL/min, 40 min) to afford compound 4 (tR = 18.5 min, 6.8 mg). Fr.17-3-4 was then purified by semi-preparative HPLC (ODS, 5 μm, 250 × 9.6 mm; MeOH/H2O, 5:90–100:0, v/v; 2.0 mL/min, 38 min) to afford compound 1 (tR = 21.2 min, 5.9 mg).

**Compound 1:** Colorless crystals; UV (MeOH) λmax 226 nm; 1H and 13C NMR (DMSO-d6) data, see Tables S1 and S2; HRESIMS m/z 342.0184, 344.0162 ([M + H]+ (calcd. for C11H12BrΝ2O5, 342.0184, 344.0165); compound 1a: [α]20D = 19.7 (c 0.1, MeOH), compound 1b: [α]20D = 27.3 (c 0.1, MeOH).

**Compound 2:** Yellow oil; UV (MeOH) λmax 354 nm; 1H and 13C NMR (DMSO-d6) data, see Tables S1 and S2; HRESIMS m/z 262.0936 ([M + H]+ (calcd. for C11H12Ν2O5, 262.0935); compound 2a: [α]20D = −40.3 (c 0.1, MeOH), compound 2b: [α]20D = 34.5 (c 0.1, MeOH).

**Compound 3:** Yellow oil; UV (MeOH) λmax 346 nm; 1H and 13C NMR (DMSO-d6) data, see Tables S1 and S2; HRESIMS m/z 262.0933 ([M + H]+ (calcd. for C11H12Ν2O5, 262.0935); compound 3a: [α]20D = −29.8 (c 0.1, MeOH), compound 3b: [α]20D = 36.3 (c 0.1, MeOH).

**Compound 4:** Yellow oil; UV (MeOH) λmax 346 nm; 1H and 13C NMR (DMSO-d6) data, see Tables S1 and S2; HRESIMS m/z 262.0931 ([M + H]+ (calcd. for C11H12Ν2O5, 262.0935); compound 4a: [α]20D = 30.1 (c 0.1, MeOH), compound 4b: [α]20D = −18.4 (c 0.1, MeOH).

**Compound 5:** Colorless crystals; UV (MeOH) λmax 354 nm; 1H and 13C NMR (DMSO-d6) data, see Tables S1 and S2; HRESIMS m/z 260.1148 ([M + H]+ (calcd. for C12H14Ν2O5, 260.1142).

**Compound 6:** Yellow oil; UV (MeOH) λmax 216 nm; 1H and 13C NMR (DMSO-d6) data, see Tables S3 and S4; HRESIMS m/z 356.0357, 358.0353 ([M + H]+ (calcd. for C12H15BrΝ2O5, 356.0353, 358.0332); [α]20D = −19.5 (c 0.1, MeOH).

**Compound 7:** Yellow oil; UV (MeOH) λmax 216 nm; 1H and 13C NMR (DMSO-d6) data, see Tables S3 and S4; HRESIMS m/z 195.0770 ([M + H]+ (calcd. for C9H11Ν2O3, 195.0764); compound 7a: [α]20D = 27.7 (c 0.1, MeOH), compound 7b: [α]20D = −24.1 (c 0.1, MeOH).

**Compound 8:** Yellow oil; UV (MeOH) λmax 276 nm; 1H and 13C NMR (DMSO-d6) data, see Tables S3 and S4; HRESIMS m/z 287.0025, 289.0003 ([M + H]+ (calcd. for C10H12BrΝ2O5, 287.0026, 289.0005); compound 8a: [α]20D = 23.3 (c 0.1, MeOH), compound 8b: [α]20D = −20.8 (c 0.1, MeOH).

**Compound 9:** Yellow oil; UV (MeOH) λmax 250 nm; 1H and 13C NMR (DMSO-d6) data, see Tables S3 and S4; HRESIMS m/z 284.9867, 286.9845 ([M + H]+ (calcd. for C10H10BrΝ2O3, 284.9869, 286.9849).

**Compound 10:** Colorless crystals; UV (MeOH) λmax 240 nm; 1H and 13C NMR (DMSO-d6) data, see Tables S3 and S4; HRESIMS m/z 234.9715, 236.9695 ([M + H]+ (calcd. for C9H12BrΝ2O3, 234.9713, 236.9692); compound 10a: [α]20D = 26.8 (c 0.1, MeOH), compound 10b: [α]20D = −32.4 (c 0.1, MeOH).

**Compound 11:** Yellow oil; UV (MeOH) λmax 216 nm; 1H and 13C NMR (DMSO-d6) data, see Tables S3 and S4; HRESIMS m/z 235.9920, 237.9899 ([M + H]+ (calcd. for C9H11BrΝO3, 235.9920, 237.9899); compound 11a: [α]20D = 52.9 (c 0.1, MeOH), compound 11b: [α]20D = −46.5 (c 0.1, MeOH).

**Compound 12:** Yellow oil; UV (MeOH) λmax 212 nm; 1H and 13C NMR (DMSO-d6) data, see Tables S3 and S4; HRESIMS m/z 321.0073, 323.0053 ([M + H]+ (calcd. for C10H14BrΝ2O5, 321.0081, 323.0060); compound 12a: [α]20D = 20.0 (c 0.1, MeOH), compound 12b: [α]20D = −13.3 (c 0.1, MeOH).

**Compound 13:** Yellow oil; UV (MeOH) λmax 352 nm; C11H11Ν2O2; 1H NMR (DMSO-d6) data, δH 12.1 (brs, 1-NH), 8.04 (t, J = 4.3, 7-NH), 7.11 (t, J = 2.4, H-2), 6.54 (t, J = 2.4, H-3), 3.28 (m, H-2 and H-2); 13C NMR (DMSO-d6) data, δC 164.4, 163.0, 154.9, 129.6, 126.6, 122.6, 121.0, 120.4, 109.6, 39.1, and 31.4. [18]
Compound 14: Yellow oil; UV (MeOH) λ_{max} 352 nm; C_{11}H_{11}N_{5}O_{2}; 1H NMR (DMSO-d_{6}) data, δ_H 11.9 (brs, 1-NH), 7.98 (t, J = 4.3, 7-NH), 6.91 (t, J = 2.3, H-2), 6.79 (t, J = 2.3, H-3), 3.26 (q, J = 4.5, H-8), 2.85 (q, J = 4.5, H-9); 13C NMR (DMSO-d_{6}) data, δ_C 163.9, 161.2, 153.4, 130.5, 126.1, 122.3, 120.4, 118.6, 112.6, 38.3, and 36.6. [16]

Compound 15: Yellow oil; UV (MeOH) λ_{max} 360 nm; C_{12}H_{13}N_{5}O_{3}; 1H NMR (DMSO-d_{6}) data, δ_H 12.0 (brs, 1-NH), 7.76 (dd, J = 6.5, 1.7, 7-NH), 7.09 (t, J = 2.8, H-2), 6.52 (m, H-3), 5.73 (d, J = 6.7, H-9), 3.57, 3.28 (m, H-2 and H-8), 3.21 (s, 9-OMe); 13C NMR (DMSO-d_{6}) data, δ_C 164.8, 162.6, 155.8, 130.3, 123.3, 122.9, 127.1, 123.3, 117.5, 110.6, 69.0, and 43.2. [19]

Compound 16: Yellow oil; UV (MeOH) λ_{max} 214 nm; C_{12}H_{14}BrN_{5}O_{3}; 1H NMR (DMSO-d_{6}) data, δ_H 9.78 (brs, 9-NH), 9.44 (brs, 7-NH), 8.10 (brs, 16-NH), 7.68 (s, H-3), 5.74 (s, H-6), 3.47, 3.36 (m, H-2 and H-13), 2.24 (m, H-2 and H-11), 1.98 (m, H-2 and H-12); 13C NMR (DMSO-d_{6}) data, δ_C 163.6, 163.1, 156.5, 146.0, 118.4, 85.8, 81.4, 63.9, 44.8, 39.5, 19.7 [17].

3.4. Computational Section

Conformational analyses were carried out in the MMFF minimization force field by the Spartan 10 v1.2.4 software package (Microsoft, Redmond, WA, USA). The resulting conformers were optimized using DFT at the B3LYP/6-31+G(d,p) level in the gas phase by the GAUSSIAN 09 C.03 program (Gaussian, Inc. Wallingford, CT, USA). The optimized conformations, whose Boltzmann distributions of Gibbs free energies were more than 1.0 percent, were used for the ECD calculations using the TD-DFT method with the basis set RB3LYP/DGDZVP, or the NMR calculations using the GIAO method at the PCM/b3lyp/6-311+G(d,p) level.

3.5. Molecular Docking

The initial receptor structure was constructed based on the crystal structure of aldose reductase in complex with cofactor NADP+ and the inhibitor idd594 (PDB code: 1US0) from the Protein Data Bank [20]. All nonstandard groups (HETATM) were deleted except for the inhibitor and cofactor when preparing for the receptor structure. The Protonate 3D module in the Molecular Operating Environment (MOE) program was used to estimate the protonated state of titratable residues and add hydrogen atoms. Meanwhile, MOE was also used to construct the ligand structures (compound 14). The subsequent molecular docking was performed with an induced fit protocol. During the process, the protein–cofactor complex was defined as the receptor and the position of the inhibitor idd594 was defined as the docking site. The triangle matcher placement with the London ΔG initial scoring methodology was set for conformational sampling and 100 poses were recorded, then the forcefield post-placement refinement with GBVI/WSA ΔG rescoring methodology was utilized to further screen the best docking pose. In addition, the MMFF94x force field was adopted for the whole process.

3.6. ALR2 Expression and Purification

For the AKR1B1 enzyme assay and SPR measurements, the protein was expressed and purified using the protocol related to the one reported previously [15]. The plasmid (pET28b, Novagen) containing the open reading frame of the human ALR2 gene was kindly provided by Atagenix, Wuhan, Hubei, China. The E. coli strain BL21 gold (DE3) (Novagen) was used to express the hexa-histidine tagged protein after induction with IPTG (Roth) for 16 h at 293 K. The pellet from a 4 L culture was resuspended in a buffer containing 20 mM Tris and 500 mM NaCl (pH 8.0) before being sonicated and centrifuged. A HiTrap chelating HP column (GE Healthcare) was loaded with the supernatant. After a short washing step with a low imidazole concentration, the fusion protein was eluted by applying a gradient of imidazole. The buffer was exchanged with 20 mM Tris-HCl, 10 mM NaCl, 1 mM EDTA, and the tag cleaved by thrombin (yuanyeBio-Technology Co. Ltd. Shanghai, China). A HiPrep DEAE FastFlow 16/10 column (GE Healthcare) was loaded with the remaining solution. A NaCl gradient was used to elute the ALR2 from the column.
3.7. ALR2 Enzyme Assay (In Vitro)

In the first step of the polyol pathway, glucose reduction is accompanied by the conversion of NADPH to NADP, where only NADPH has an obvious spectral absorption around 340 nm and NADP has none. Thus, the decrease in OD$_{340}$ nm can represent the consumption of NADPH. Therefore, we detect the change of OD$_{340}$ nm before and after reaction to screen the effective ARIs or evaluate the AR activity; assays with epalrestat were used as positive controls. Briefly, the incubation system contains 10 µL ALR2 enzyme (20 µg/mL), 80 µL DL-glyceraldehyde (0.16 mmol/L) as the substrate, 1.0 mmol/L NADPH·4Na as a coenzyme, and 0.1 mol/L PBS (pH = 6.2). The incubation mixture was minimized to a total volume of 100 µL and ongoing in a 96-well ultraviolet plate. Then, test wells were treated with the tested compounds for 10 min at 25 °C. Then, the absorbance was measured using FlexStation 3 Reader (Molecular Devices, San Francisco, CA, USA) at 340 nm. The absorbance of wells treated with PBS and NADPH was considered as 100% (OD$_1$) and the absorbance of wells treated with PBS and DL-glyceraldehyde was considered as 0% (OD$_2$); the inhibitory rate was calculated by the formula OD$_{compounds}$ − OD$_2$/OD$_1$ − OD$_2$. IC$_{50}$ represents the concentration that inhibits the ALR2 enzyme by 50%.

3.8. Surface Plasmon Resonance (SPR) Binding Assay

SPR binding analysis methodology can be used to study molecular interactions. Herein, SPR was applied to measure the interactions between compounds 13/14 and ALR2. Initially, ALR2 was prepared in 10 mM sodium acetate (pH 5.0) and then immobilized covalently by an amine-coupling reaction on a CM5 sensor chip. The remaining binding sites of the sensor chip were then blocked by ethanolamine. The addition of compound 13/14, the flow-through analyte, to the chamber resulted in binding to the immobilized protein ligand, producing a small change in the refractive index at the gold surface. In this step, the compound was diluted in PBS-P$^+$ buffer to the desired concentration and was injected over the chip with a flow rate of 10 µL/min. All of the above buffers, solutions, and sensor chips were placed at room temperature before the run. The association time and dissociation time were both set at 60 s. Binding affinities were obtained from the ratio of rate constants to directly characterize the protein-molecular interactions. Data analysis was completed via the state model in T200 evaluation software (Cytiva Danaher, Marlborough, MA, USA).

4. Conclusions

In conclusion, a series of new pyrrole alkaloids including different structural scaffolds were isolated from the sponge *Stylissa massa* collected in the South China Sea, which enriched the structural diversity of this alkaloid family. Aldose reductase (ALR2), which participates in the glucose polyol metabolic pathway and cell inflammatory reaction, is an important target for the treatment of diabetes complications, and 5/7/5 tricyclic spongiacidin-PIAs isolated from sponge *Stylissa massa* provided a new skeleton targeted to ALR2 which have never been previously reported.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/md20070454/s1, Tables S1–S4: 1H (500 MHz) and 13C (125 MHz) NMR data for 1–14 acquired in DMSO-d6. Tables S5–S7: X-ray diffraction analysis of compounds 1, 5, and 10. Figures S1–S20: The chiral HPLC data and ECD spectra of compounds 1–12; Figures S21–S25: The ALR2 inhibitory activities of related compounds. Figures S26–S117: The spectroscopic data (including HRESIMS, 1H NMR, 13C NMR, and 2D NMR) of compounds 1–16.

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