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Discovery of Amantamide, a Selective CXCR7 Agonist from Marine Cyanobacteria

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Abstract

CXCR7 plays an emerging role in several physiological processes. A linear peptide, amantamide (**1**), was isolated from marine cyanobacteria and the structure determined by NMR and mass spectrometry. The total synthesis was achieved by solid-phase method. After screening two biological target libraries, **1** was identified as a selective CXCR7 agonist. The selective activation of CXCR7 by **1** could provide the basis for developing CXCR7 targeted therapeutics and deciphering the role of CXCR7 in different diseases.

Graphical Abstract

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Author Contributions

X.L., structure elucidation, further purification through hydrolysis-re-esterification, cell-based biological experiments, experimental design and manuscript writing; D.L., structure elucidation; J.Y., total synthesis; M.R., molecular modeling; L.A.S. and S.P.G., natural product isolation; C.L., supervision of molecular modeling; T.Y., supervision of total synthesis; V.J.P., sample collection and identification and manuscript editing; H.L., supervision of experimental design and conduction and manuscript editing. All authors have given approval to the final version of the manuscript.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Experimental procedures, spectral data and biological investigation results (PDF).

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G protein-coupled receptors (GPCRs) are the largest family of membrane receptors and represent the most commonly used signal-transduction system in many different species.¹ GPCRs are the most intensively studied targets for therapeutic agents since they are involved in diverse pharmacological activities and they possess easily accessed druggable sites at the cell surface.^{2,3} More than 100 GPCR targeted drugs have been approved by the Food and Drug Administration (FDA) and even more agents are currently in clinical trials.⁴ Therefore, as potential drug targets, GPCRs present a promising biological matrix for the discovery of therapeutics against various diseases. In addition to the established role of GPCRs in inflammatory processes, GPCRs are reported to function during tumor growth and metastasis.⁵ For example, C-X-C chemokine receptor type 7 (CXCR7) is demonstrated to be a biological target in cancer progression. CXCR7 promotes cell growth and metastasis in various cancers, including lung, hepatocellular, colorectal and breast cancers.⁶ Moreover, CXCR7 expression is enhanced during pathological inflammation and tumor development, and emerging data suggests this receptor is an attractive therapeutic target for autoimmune diseases and cancer.⁷ Herein, we describe the isolation, structure elucidation, total synthesis, preliminary biological investigation and target elucidation study of the first selective CXCR7 agonist from marine cyanobacteria, which might assist in illuminating the biological role of CXCR7 and also serve as a template for the development of therapeutic agents targeting CXCR7.

Marine cyanobacteria have been a valuable source for the discovery of structurally diverse natural products as a result of their prolific biosynthetic machinery. The diversity of the secondary metabolites from marine cyanobacteria translates into various biological activities.⁸ One important group of these metabolites is ribosomal or non-ribosomally synthesized peptides or depsipeptides, which can function as GPCR modulators and/or inhibitors of different proteases. For instance, a group of modified linear peptides, brintonamides A and D (Figure 1), were reported to be dual protease and GPCR modulators.⁹ Now, we have isolated a linear peptide amantamide (**1**, Figure 1) from two marine cyanobacterial samples, which was then identified as a selective CXCR7 agonist.

One gray filamentous cyanobacteria sample (Oscillatoriiales) was collected from Two Lover's Point (Puntan dos Amantes), Guam and two natural products, amantelides A and B, were previously isolated from that sample.¹⁰ Although molecular methods are required for taxonomic characterization, based on microscopy this cyanobacterium may fall within the genus *Okeania*.¹¹ The non-polar extract (EtOAc/MeOH 1:1) was partitioned and then fractionated by silica column chromatography, followed by reversed-phase HPLC to yield amantamide (**1**) {white solid, $[\alpha]_D^{20}$ -9.0 (c 0.1, MeOH)}. Amantamide (**1**) was also isolated and purified from another cyanobacterial sample, a similar gray filamentous mat,

collected from Anae Island, Guam after an additional hydrolysis–re-esterification process to get rid of a co-eluting impurity (Scheme S1). The HRESIMS of **1** in the positive mode exhibited a $[M+Na]^+$ peak at m/z 1102.6918, suggesting a molecular formula of $C_{57}H_{93}N_9O_{11}$ with sixteen degrees of unsaturation. The structure of **1** was determined using a combination of 1D and 2D NMR techniques. The 1H and ^{13}C NMR spectra (Figures S1, S2) indicated the presence of several characteristic signals corresponding to α -protons (δ_H 4–5 ppm), exchangeable amide protons (δ_H 7–8 ppm), three N-methyl groups (δ_H 2.9–3.1 ppm, δ_C 29–31 ppm) and one *O*-methyl group (δ_H 3.69 ppm, δ_C 52.0 ppm), suggesting a peptidic structure. Examination of the 2D NMR spectra (COSY, TOCSY, HSQC, HMBC and NOESY) of **1** in $CDCl_3$ (Figures S3–7) revealed the presence of proteinogenic amino acids Gly, Ile, Pro, Leu and Val as well as *O*-CH₃-Pro, *N*-CH₃-D-Phe, *N*-CH₃-D-Val, *N*-CH₃-L-Leu and a butyric acid (BA). The sequence of these units was confirmed by ESI-MS/MS fragmentation (Figure 2). To establish the absolute configuration, a small portion of **1** (100 μ g) was hydrolyzed using 6 N HCl (110 °C, 24 h) and the hydrolyzate was analyzed using chiral HPLC-MS. The resulting residues revealed retention times corresponding to L-Pro, *N*-CH₃-D-Phe, Gly, L-Ile, *N*-CH₃-D-Val, L-Pro, L-Leu, *N*-CH₃-L-Leu and L-Val by comparison with authentic amino acid standards. Of note, since **1** is a methyl ester and we used MeOH during the isolation, we cannot fully exclude the possibility that the corresponding carboxylic acid was esterified during that process; however, the whole isolation was conducted under neutral conditions where the esterification is unlikely to have occurred.

Due to the limited amount of **1** obtained from isolation, the total synthesis of **1** was developed to provide sufficient material for subsequent biological studies. The synthesis of this nonapeptide was accomplished by use of the standard Fmoc-based solid-phase synthetic method (Scheme 1). Elongation of the peptide chain was accomplished by stepwise coupling of the required amino acids using *O*-(7-azabenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU). After coupling with butyric acid, the peptide was cleaved from the resin mediated by TFA, followed by esterification of the resulting acid with tetramethylsilyldiazomethane to afford **1** {45% overall yield, white solid, $[\alpha]_D^{20}$ –9.0 (*c* 0.1, MeOH)}. Comparison of the 1H and ^{13}C NMR spectrum of the synthetic compound with that of the isolated natural product confirmed the correct assignment of the structure (Figures S8, S9).

Marine cyanobacteria are known to produce various protease inhibitors. For example, tasiamide B was reported to display selective inhibitory activity against BACE1, a potential therapeutic target for Alzheimer's disease,¹² while tasiamide F is a potent inhibitor of cathepsins D and E with greater selectivity over BACE1.⁸ Considering the similarity of **1** with these reported tasiamides as well as brintonamides (Figure 1) and to probe its activity and selectivity, **1** was profiled against a panel of 63 proteases in a dose-response format starting at 10 μ M. As a result, **1** is identified to display modest inhibitory activity against caspase 9 with IC₅₀ value of 1.95 μ M. For the other proteases included in the screen, **1** either displayed little inhibitory effect or exhibited IC₅₀ values over 10 μ M. The screening results were shown in Figure S10.

As previously mentioned, we have recently shown that marine cyanobacteria also produce peptidic GPCR modulators. Thus, **1** was also profiled against a panel of 168 GPCR targets in both agonist and antagonist mode at 10 μ M final concentration using PathHunter β -arrestin assays. The screen identified CXCR7 to be the only GPCR target of **1** in agonist mode, while **1** had no antagonistic effects on any of these targets (Figures 3, S11). Follow-up dose-response test indicates the EC₅₀ of **1** against CXCR7 is 2.5 μ M (Figure 3). Recently, it is reported that CXCR7 in human glioma cells functions as a regulator to mediate cellular antiapoptotic responses and exacerbate drug-induced apoptosis.¹³ CXCR7 activation is also reported to stimulate angiogenesis *in vitro* and *in vivo*¹⁴ and promote alveolar repair and reduce fibrosis after repetitive lung injury.¹⁵ Additionally, CXCR7 is demonstrated to be a novel target for therapeutic vasculogenesis in patients with coronary artery disease and can influence *VEGFA* expression in endothelial outgrowth cells,¹⁶ which altogether demonstrates the potential of CXCR7 as a promising therapeutic target for drug discovery and pharmacological study.

To validate the cellular activity of amantamide (**1**) as a CXCR7 agonist, the effect of amantamide (**1**) on Erk1/2 phosphorylation was monitored in A549 human lung cancer cells, which is an important factor in the downstream pathway of CXCR7 activation¹⁶. The production of phosphorylated Erk1/2 (pErk1/2) was significantly increased after 3 hours in all agonist treatment groups with an unchanged production of total Erk1/2 (tErk1/2) (Figure 4A). Furthermore, the effect of amantamide (**1**) on mRNA expression of the angiogenesis-related gene *VEGFA* in A549 cells was measured after 3 h treatment. *VEGFA* is one of the most studied angiogenic growth factors of the *VEGF* family, which promotes all steps in the angiogenic cascade.¹⁷ As shown in Figure 4B, after treatment with amantamide (**1**), the transcript level of *VEGFA* in A549 cells was elevated approximately 3-fold compared to solvent (DMSO) control. Interestingly, we found that CXCL12, the endogenous ligand of CXCR7 and also CXCR4, induced transcript expression of *VEGFA* like amantamide (**1**), while TC14012 (Figure S12), a known CXCR7 agonist and a CXCR4 antagonist, decreased *VEGFA* expression instead. The involvement of CXCR4 might contribute to such different results. In addition, the structural difference of TC14012 with amantamide (**1**) may suggest different modes of action for these agonists. Further investigation is ongoing. It is noteworthy that when CXCR4 and CXCR7 are co-expressed, receptor heterodimers form as much as receptor homodimers.¹⁸ Considering the confirmed expression of CXCR7 as well as CXCR4 in A549 cells,^{19, 20} our results indicate that amantamide (**1**) would be able to serve as a selective agonist to help decipher the biological role of CXCR7 in different disease models.

To decipher the molecular interaction of amantamide (**1**) against CXCR7, molecular modeling experiments were performed. Brintonamide D was also identified as a CXCR7 agonist (EC₅₀ 3.97 μ M), although it is a more potent CCR10 antagonist,⁹ so it was also included in the modeling process. The sequence of CXCR7 protein was retrieved from UniProt Knowledge Base with 362 residues²⁰. Due to lack of available CXCR7 protein structure in the Protein Data Bank,²¹ we developed a homology 3D model template for molecular docking. Compared to a previous homology model published by Montpas *et al.*,²² we expect our models be more reliable, because they possess much lower E-values and the

aligned parts lie on a single segment (see Supporting Information for details). The docking results (Figures 5, S13) indicate the main contribution to binding energies for both compounds arises from hydrophobic interactions. Only a small number of hydrogen bonds are formed, three for each compound. In addition, π - π stacking occurs between Tyr268A and the aromatic ring in brintonamide D. While the best docking poses for the two peptides do not interact directly with the key Aspartate residues (D179 and D275) reported by Montpas *et al.*,²² both amantamide (**1**) and brintonamide D occupy the major binding pocket between TM3 and TM7 trans-membrane helices, which is consistent with their finding regarding the binding mode of TC14012 (see Supporting Information for details). However, ligand displacement assays and mutational studies would be necessary to validate the binding mode of amantamide (**1**) with CXCR7.

In summary, we isolated amantamide (**1**), a new marine cyanobacterial natural product, synthesized the compound and characterized it as a new CXCR7 agonist with confirmed cellular activity. SAR studies to produce a diverse amantamide analog library and optimize the agonistic activity of amantamide is ongoing. The selective activation of CXCR7 by **1** could provide the basis for developing CXCR7 targeted therapeutics and deciphering the biological role of CXCR7 without interference with CXCR4.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

- (1). Bouvier M, Nat Rev Neurosci 2001, 2 (4), 274–86. [PubMed: 11283750]
- (2). Hauser AS; Attwood MM; Rask-Andersen M; Schiöth HB; Gloriam DE, Nat Rev Drug Discov 2017, 16 (12), 829–842. [PubMed: 29075003]
- (3). Lu S; Zhang J, J Med Chem 2019, 62 (1), 24–45. [PubMed: 29457894]
- (4). Hauser AS; Chavali S; Masuho I; Jahn LJ; Martemyanov KA; Gloriam DE; Babu MM, Cell 2018, 172 (1–2), 41–54.e19. [PubMed: 29249361]
- (5). Zlotnik A; Burkhardt AM; Homey B, Nat Rev Immunol 2011, 11 (9), 597–606. [PubMed: 21866172]
- (6). Xu D; Li R; Wu J; Jiang L; Zhong HA, Curr Top Med Chem 2016, 16 (13), 1441–51. [PubMed: 26369824]
- (7). Sánchez-Martín L; Sánchez-Mateos P; Cabañas C, Trends Mol Med 2013, 19 (1), 12–22. [PubMed: 23153575]
- (8). Al-Awadhi FH; Ratnayake R; Paul VJ; Luesch H, Bioorg Med Chem 2016, 24 (15), 3276–82. [PubMed: 27211244]
- (9). Al-Awadhi FH; Gao B; Rezaei MA; Kwan JC; Li C; Ye T; Paul VJ; Luesch H, J Med Chem 2018, 61 (14), 6364–6378. [PubMed: 30015488]
- (10). Salvador-Reyes LA; Sneed J; Paul VJ; Luesch H, J Nat Prod 2015, 78 (8), 1957–62. [PubMed: 26204500]

- (11). Engene N; Paul VJ; Byrum T; Gerwick WH; Thor A; Ellisman MH, J Phycol 2013, 49 (6), 1095–106. [PubMed: 27007630]
- (12). Liu J; Chen W; Xu Y; Ren S; Zhang W; Li Y, Bioorg Med Chem 2015, 23 (9), 1963–74. [PubMed: 25842365]
- (13). Hattermann K; Held-Feindt J; Lucius R; Mürköster SS; Penfold ME; Schall TJ; Mentlein R, Cancer Res 2010, 70 (8), 3299–308. [PubMed: 20388803]
- (14). Zhang M; Qiu L; Zhang Y; Xu D; Zheng JC; Jiang L, Sci Rep 2017, 7 (1), 8289. [PubMed: 28811579]
- (15). Cully M, Nat Rev Drug Discov 2016, 15 (3), 160. [PubMed: 26915452]
- (16). Cao Z; Tong X; Xia W; Chen L; Zhang X; Yu B; Yang Z; Tao J, PLoS One 2016, 11 (9), e0161255. [PubMed: 27612090]
- (17). Cai W; Salvador-Reyes L; Zhang W; Chen Q; Matthew S; Ratnayake R; Seo S; Dolles S; Gibson D; Paul V; Luesch H, ACS Chemical Biology 2018, 13 (1), 91–99. [PubMed: 29205032]
- (18). Levoe A; Balabanian K; Baleux F; Bachelerie F; Lagane B, Blood 2009, 113 (24), 6085–93. [PubMed: 19380869]
- (19). Puchert M; Koch C; Engele J, Exp Cell Res 2018, 364 (2), 175–183. [PubMed: 29408206]
- (20). The UniProt Consortium Nucleic Acids Res 2017, 45 (D1), D158–D169. [PubMed: 27899622]
- (21). Berman HM; Westbrook J; Feng Z; Gilliland G; Bhat TN; Weissig H; Shindyalov IN; Bourne PE, Nucleic Acids Res 2000, 28 (1), 235–42. [PubMed: 10592235]
- (22). Montpas N; Cabana J; St-Onge G; Gravel S; Morin G; Kuroyanagi T; Lavigne P; Fujii N; Oishi S; Heveker N, Biochemistry 2015, 54, 1505–1515. [PubMed: 25669416]

Figure 1.
Structure of amantamide (1) and related natural products.

Figure 2.
ESI-MS/MS fragmentation pattern of amantamide (1).

Figure 3.

GPCR profiling of amantamide (**1**) using cell-based PathHunter β -arrestin assays under agonist mode and antagonist mode at 10 μ M final concentration. Compound % activation or inhibition is calculated relative to basal and max activity values obtained through treatment of corresponding target ligand (100% at EC80). (A) Profiling results shown in scatter plot. The hit identified with >50% activation or inhibition is labeled. (B) Dose-response curve of **1** against CXCR7 (EC₅₀ 2.5 μ M). CXCL12, a CXCR7 ligand, was used as positive control (EC₅₀ 0.017 μ M). Data is presented as mean $\pm$ SD (n=2).

Figure 4.

(A) Effects of amantamide (**1**) as well as CXCL12 (100 ng/mL) and TC14012 (30 μ M) as positive control on Erk1/2 phosphorylation (pErk1/2) are shown in western blots, with total Erk1/2 (tErk1/2) and GAPDH as loading controls. The corresponding quantitative analysis of pErk1/2 expression is also shown. (B) Transcript expression of VEGFA in A549 cells after 3 h treatment with **1** as well as positive controls CXCL12 (100 ng/mL) and TC14012 (30 μ M). GAPDH was used as endogenous control. Data are presented as mean $\pm$ SD, * P < 0.05, ** P < 0.01, **** P < 0.0001, compared to control using Two-way ANOVA (n = 3).

Figure 5.

Top view (A) and side view (B) of the binding sites with the best docking poses for amantamide (**1**, magenta) are shown. The interactions of each compound with key amino acid residues in the binding site are indicated. The compounds are displayed as sticks.

Scheme 1.
Total synthetic route of amantamide (1).