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Chembiochem. Author manuscript; available in PMC 2013 December 21.

Published in final edited form as:
Chembiochem. 2012 December 21; 13(18): 2676–2681. doi:10.1002/cbic.201200502.

Marine Cyanobacterial Fatty Acid Amides Acting on Cannabinoid Receptors

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Keywords

Anti-inflammatory; cannabimimetic; fatty acid amides; malyngamides; marine cyanobacteria

Lipids are utilized by diverse organisms, which suggests an evolutionarily conserved role of this class of compounds.[1] Indeed, lipidomics is emerging as a crucial field of research because of the key role of lipid biomolecules in a wide array of physiological functions.[2] Research has also uncovered some vital lipid-protein interactions where lipid molecules can bind to specific protein domains to mediate physiological effects. The endocannabinoid system is a representative example, where two characterized G-protein coupled cannabinoid receptors CB_1 and CB_2 are modulated by endogenous lipids known as endocannabinoids. Importantly, this system has been implicated in various pathophysiologies, including neurodegenerative diseases, eating disorders, pain, inflammation and cancer.[3–7] Therefore, a better understanding of this system has become of significant interest, and the cannabinoid receptors have been viewed as possible targets for different diseases.[1, 5] The classical concept that all agonists at a given GPCR induce a similar repertoire of downstream events is now uncertain, and the latest experimental evidence supports the existence of ligand-specific functional selectivity at the cannabinoid receptors.[3] Consequently, the identification of new structural scaffolds that can bind to the cannabinoid receptors remains an essential tool to dig further into this complex system.

Anandamide (*N*-arachidonoylethanolamine) (**1**) (Scheme 1) was the first endogenous ligand to be identified among the endocannabinoid family.[8] Influenced by its structure, the discovery of this fatty acid amide suggested that other natural and synthetic fatty acid amides might function as cannabinoid receptor ligands as well. In that context, marine cyanobacteria of the genus *Lyngbya* have a characteristic metabolic profile that is rich in fatty acid amides (in addition to peptides),[9] and therefore represent a potential source of new model compounds acting on the cannabinoid receptors. Support for this assumption arises from reports of metabolites isolated from *Lyngbya* samples that can interact with the cannabinoid receptors. To the best of our knowledge, there are only five marine cyanobacterial fatty acid amides identified to date with binding affinities to the cannabinoid receptors; grenadamide (**2**)[10], semiplenamides A (**3**), B, and G[11] and the recently reported metabolite serinolamide A (**4**)[12] (Scheme 1). However, none of them have been tested in

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functional assays before, so it remains unknown whether those metabolites act as receptor agonists or antagonists.

Exploring a *Lyngbya* sample from Piti Bomb Holes from Guam led us to isolate and characterize the new fatty acid amide serinolamide B (**5**), a closely related analogue to the recently reported lipid serinolamide A (**4**) (Scheme 1). Based on structural features, we evaluated the ability of compound **5** to bind to both human cannabinoid receptors CB_1 and CB_2 with a functional outcome. Additionally, the marine cyanobacteria *Lyngbya* spp. are well-known for the production of a large class of fatty acid amides known as malyngamides.[13] To date, there are more than 30 known malyngamide analogues with a broad spectrum of bioactivities. Although malyngamides usually contain different amine portions and sometimes vary in the length of the fatty acyl chain, they generally have a unique and characteristic structural scaffold of an *N*-substituted amide of a long chain 7-methoxy fatty acid with mono-unsaturation at C4 (Scheme 2). Since malyngamides are the most abundant fatty acid amides found in *Lyngbya* spp., we were interested in examining whether the malyngamide-type structural features could also bind to the cannabinoid receptors, although they usually possess more complicated amine portions and somewhat different fatty acid side chains compared to the known endogenous cannabinoids. For this, we tested malyngamide B (**6**)[14] (Scheme 2) for its potential as a cannabimimetic compound. Interestingly, malyngamide B (**6**) can also bind to both receptors CB_1 and CB_2 with moderate potencies. Here, we wish to report the results of our studies on two marine cyanobacterial metabolites; the newly identified analogue serinolamide B (**5**) and a member of a large group of cyanobacterial fatty acid amides, malyngamide B (**6**).

A cyanobacterial sample from Guam was extracted three times with EtOAc–MeOH mixtures. Solvent partitioning of the organic extract yielded 7.2 g of a semi-polar *n*-BuOH fraction, which was then fractionated by silica gel chromatography. Compound **5** was purified by reversed-phase HPLC from a silica column fraction that also contained pitipeptolides.[15] The molecular formula $C_{22}H_{43}NO_3$ was established by HRESIMS (*m*/*z* 370.3316 for $[M + H]^+$). NMR profiles of this compound were characteristic of a fatty acid derivative, where typical chemical shifts of a monounsaturated fatty acid chain were prominent (Table 1): ^1H and ^{13}C NMR spectra showed a number of overlapping methylene groups ($\delta_H \approx 1.2$ ppm and $\delta_C \approx 23$ ppm), a carbonyl (δ_C 172.9 ppm), an α-methylene group (δ_H 2.27 ppm and δ_C 36.4 ppm), a terminal methyl group (δ_H 0.88 ppm and δ_C 13.9 ppm) and characteristic olefinic methines (δ_H 5.39 ppm, δ_C 127.8 ppm and δ_H 5.49, δ_C 132.1 ppm). Additionally, the compound appeared to be a fatty acid amide; where the amide proton at δ_H 6.16 ppm showed HMBC correlation to the carbonyl carbon as well as a COSY correlation to a methine proton at 4.07 ppm (δ_C 50.3 ppm). Three additional oxygenated groups were also identified based on their chemical shifts; two methylene groups (δ_H 3.58, 3.53 ppm, δ_C 73.6 ppm; and δ_H 3.66, 3.82 ppm, δ_C 64.2 ppm) and a methoxy group (δ_H 3.36 ppm and δ_C 59.2 ppm), where the latter showed HMBC correlation to the first oxygenated methylene group (δ_C 73.6 ppm) (Table 1). Further analysis of COSY, TOCSY and HMBC data allowed for the construction of the amine part of this molecule as a mono-methyl serinol and located the olefinic system in the fatty acid chain between C4-C5 (Scheme 1, Table 1). Finally, to complete the molecular formula suggested by MS data, the number of methylene groups forming the fatty acid chain was assigned to construct an 18-carbon monounsaturated fatty acid.

The absolute configuration of the chiral center in the serinol moiety was determined through Jones oxidation of the primary alcohol to its corresponding carboxylic acid, followed by acid hydrolysis to liberate *O*-Me serine. Enantioselective analysis revealed *S* configuration of the amino acid and consequently *R* configuration in the parent compound. Additionally, the double bond geometry was assigned as *trans* based on the chemical shifts of the adjacent

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methylene groups C3 and C6 [12, 16] and the absence of NOESY correlations between the two olefinic protons. While we were investigating the biological activity of this metabolite, the Gerwick group reported the closely related analogue **4** (Scheme 1).[12] Notably, ^{1}H and ^{13}C chemical shifts and the stereochemical assignments for **5** match those reported for **4**.

Based on the structural similarity of serinolamide A (**4**) to the endocannabinoids anandamide (**1**) (Scheme 1) and 2-arachidonoyl glycerol, it was tested for binding to the human cannabinoid receptors CB_1 and CB_2, where it appeared to possess more than 5-fold selectively for the CB_1 receptor, with a moderate binding affinity (K_i = 1.3 μM).[12] As the only structural difference between the two serinolamide analogues is a secondary amide NH group in **5** instead of the tertiary amide *N*-Me group in **4**, we evaluated the cannabimimetic activity of serinolamide B (**5**). Compound **5** can also bind to both CB_1 and CB_2 receptors with moderate to weak binding affinities (Figure 1a, Table 2). However, **5** showed an opposite trend in binding affinities compared to **4**; where it exhibited a moderate affinity and higher selectivity for CB_2 (K_i = 5.2 μM) over CB_1 receptor (K_i = 16.4 μM). Notably, the endocannabinoid anandamide (**1**) shows higher selectivity for CB_1 receptor (K_i for CB_1 = 32 nM; K_i for CB_2 = 1.9 μM)[17], suggesting that the presence of a secondary amide versus a tertiary amide is not the main determinant for receptor selectivity.

Malyngamides have been reported with different biological activities, including cytotoxic, anti-inflammatory and quorum sensing actions.[18–20] Yet, to the best of our knowledge, the malyngamide structural features were not probed before for cannabinoid receptor interactions. Accordingly, we were interested in testing a representative analogue to determine whether this molecular architecture is capable of possessing cannabimimetic effects. We obtained malyngamide B (**6**) from our marine natural products library and tested it for CB_1 and CB_2 binding. Interestingly, **6** appeared to possess moderate binding affinities to both receptors with K_i values of 3.6 μM for CB_1 and 2.6 μM for CB_2 (Figure 1a, Table 2). Those results are noteworthy, since **6** shows a more complex amine portion compared to the endocannabinoids and other cyanobacterial fatty acid amides which possess the same biological activity (Scheme 1, Scheme 2).

It has not yet been determined whether the marine cyanobacterial fatty acid amides that can bind to the cannabinoid receptors act as agonists or antagonists. In order to clarify this, we tested the functional response induced by the binding of **5** and **6** to the cannabinoid receptors. Cannabinoid receptors are G-protein coupled receptors that are functionally coupled to the inhibition of adenylyl cyclase and subsequent inhibition of cAMP accumulation (Figure 1b).[6] Compounds **5** and **6** were able to inhibit forskolin-stimulated cAMP accumulation through both CB_1 and CB_2 receptors with moderate potencies (Figure 1c, Table 2), which proves that those metabolites act as cannabinoid receptor agonists. Intriguingly, serinolamide B (**5**) appeared to be more CB_2 receptor selective in the binding as well as the functional assays, but malyngamide B (**6**) appeared to bind to both receptors similarly with comparable functional outcomes (Figure 1c, Table 2).

It is also known that anandamide signaling is terminated by the enzyme fatty acid amide hydrolase (FAAH), which catalyzes anandamide hydrolysis. Therefore, one emerging pharmacological approach to augment the endocannabinoid activity is directed towards FAAH inhibition.[21] Thus, we tested the ability of metabolites **5** and **6** to inhibit this enzyme. However, no considerable inhibitory effects were detected for either compound when tested at 10 and 100 μM.

It has been repeatedly shown that cannabimimetic compounds could also mediate anti-inflammatory responses. [1, 7, 22] From that perspective, we tested the ability of compounds

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5 and **6** to exert anti-inflammatory effects in lipopolysaccharide (LPS)-induced murine macrophages RAW 264.7. Serinolamide B (**5**) showed a weak effect with an $IC_{50} > 25\ \mu M$. However, malyngamide B (**6**) was more potent, where it inhibited NO production with an IC_{50} of 6.2 μM without affecting cellular viability up to 25 μM. Although evidence suggests that the anti-inflammatory effects of some cannabimimetic compounds are mediated through cannabinoid receptors, particularly CB_2, it is questionable if this is also the case with malyngamide B (**6**). Notably, Mukhopadhyay et al. showed that no detectable CB_2 receptors were apparent in RAW 264.7 cells unless they are stimulated by LPS.[23] Therefore, the effect of **6** on LPS-induced inflammation is probably not totally mediated through the cannabinoid receptors, since it was able to prevent the early stimulation by LPS. Further experiments using CB_2 receptor selective antagonists or CB_2 receptor deficient cells will help ascertain the presence or absence of a cannabinoid receptor mediated anti-inflammatory effect for **6**.

Some malyngamides can also reduce NO accumulation under similar anti-inflammatory assay conditions, where malyngamide F acetate (**7**) possesses an IC_{50} value of 7.1 μM[20] and malyngamide 2 (**8**) has an IC_{50} value of 8 μM (Scheme 2).[19] Malyngamide F acetate (**7**) was shown to possess a distinctive cytokine profile and appeared to be selectively inhibiting the MyD88 dependent pathway.[20] Notably, **7** and **8** share common structural features including oxidized cyclo-hexyl rings (Scheme 2). Instead, malyngamide B (**6**) has a significantly different amine entity (Scheme 2). To our knowledge, this is the first report of such activity for an anti-inflammatory malyngamide with the pyrrolidone ring in the amine portion rather than the common six-membered cyclic ketone or lactone.

We also tested the cytotoxic effects of compounds **5** and **6** against cancer cells. Serinolamide B (**5**) failed to show significant cytotoxicity against HT-29 colon adenocarcinoma and MCF7 breast cancer cell lines up to 100 μM. It is important to point out here that serinolamide A (**4**) showed some cytotoxic properties in a different cell line.[12] In contrast, malyngamide B (**6**) is known as a feeding deterrent,[24] and in our hands, **6** was cytotoxic to HT-29 cells with an IC_{50} value of 26 μM, but it remains unclear if its cannabimimetic activity contributes to this cytotoxic effect.

In this report, we identified the new cannabimimetic marine cyanobacterial fatty acid amide serinolamide B (**5**). In our hands, serinolamide B with a secondary amide had higher CB_2 receptor selectivity and lower cytotoxicity compared to the data published for its analogue with a tertiary amide. In agreement, other reports showed that several structural features can increase the affinity for CB_2 receptor, including 4*E* double bond, an amide proton and additional substituents in the amine part.[1, 22] Yet, testing the two analogues **4** and **5** side by side under the same experimental conditions will unequivocally clarify this comparison. We also show that malyngamide B (**6**) possesses cannabimimetic properties as well, which provides new insight into the biological activities of malyngamides, the most abundant marine fatty acid amide class in *Lyngbya* spp. This finding introduces a new structural lead to the cannabimimetic field from the marine environment, and should foster the cannabimimetic evaluation of further analogues. Additionally, our finding that both metabolites act as receptor agonists proposes that they can mediate certain physiological effects through this pathway, and therefore opens more research avenues. Several malyngamides have been subjected to total chemical syntheses and some well-established synthetic routes are already available,[25] which can assist structural optimization efforts towards more potent analogues.

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Experimental Section

General Experimental Procedures

The optical rotation was measured on a Perkin-Elmer 341 polarimeter. UV and optical activity were measured on a SpectraMax M5 (Molecular Devices), and IR data were obtained on a Perkin-Elmer Spectrum One FT-IR Spectrometer. The ^{1}H and 2D NMR spectra were recorded on a Bruker Avance II 600 MHz spectrometer. All spectra were obtained in $CDCl_3$ using residual solvent signals (δ_H 7.26, δ_C 77.16 ppm) as internal standards. HSQC and HMBC experiments were optimized for $^1J_{CH}$= 145 and $^1J_{CH}$ =7 Hz, respectively. HRMS data was recorded on an Agilent LC-TOF mass spectrometer equipped with an APCI/ESI multimode ion source detector in positive ion mode. LC-MS data were obtained using an API 3200 triple quadrupole MS (Applied Biosystems) equipped with a Shimadzu LC system.

Extraction and Isolation

The sample of the marine cyanobacterium *Lyngbya majuscula* (recollection of UOG strain VP627) was collected at Piti Bomb Holes, Guam, in February 2000. A voucher sample (voucher specimen number EC025) has been preserved at the Smithsonian Marine Station at Fort Pierce, FL. The freeze-dried organism was extracted three times with EtOAc-MeOH (1:1) to give a crude organic extract (35.5 g), which was partitioned between hexanes and 80% aqueous MeOH. After drying the methanolic phase, the residue was partitioned between *n*-BuOH and H_2O. The concentrated *n*-BuOH residue (7.2 g) was subjected to flash chromatography over silica gel, eluting with increasing gradients of *i*-PrOH in CH_2Cl_2, and finally with MeOH. The fraction eluting with 4% *i*-PrOH/ CH_2Cl_2 was fractionated on a semi-preparative reversed-phase HPLC column (YMC-Pack ODS-AQ, 250 × 10 mm, 5 μm, 2 mL/min; UV detection at 220/254 nm) using a MeOH/H_2O linear gradient (75–100% aqueous MeOH over 30 min, and then 100% MeOH for 10 min) to afford 10 fractions. Re-purification of five fractions yielded pitiprolamide[26] and pitipeptolides.[15] Compound **5** eluted as a single peak (fraction 10) at t_R 28.8 min.

Serinolamide B (**5**): colorless, amorphous solid; $[\alpha]^{20}_D$ – 7.9 (c 0.075, $CHCl_3$); IR (film) ν_{max} 3290, 3077, 2955, 2920, 2851, 1641, 1542, 1465, 1377 cm^{-1}; ^{1}H NMR, ^{13}C NMR and HMBC data, see Table 1; HRESI/APCIMS *m/z* 370.3324 $[M+H]^+$ (calcd $C_{22}H_{44}NO_3$ 370.3316).

Jones Oxidation and Enantioselective Amino Acid Analysis by HPLC/MS

Compound **5** (1 mg) was dissolved in acetone (1 mL) followed by the addition of freshly prepared Jones reagent (CrO_3 in diluted H_2SO_4, 50 μL). The reaction mixture was stirred at room temperature for 1h. The reaction was then quenched by the addition of a few drops of *i*-PrOH and filtered through a pad of celite. The reaction mixture was dried down under nitrogen and the residue was re-dissolved in water and partitioned between water and EtOAc three times. The organic layer was dried under nitrogen and the crude product was then purified using HPLC (YMC-Pack ODS-AQ, 250 ×10 mm, 5 μm, 2 mL/min; UV detection at 220/200 nm) using a MeOH/0.05% aqueous TFA linear gradient (75–100% aqueous MeOH over 20 min, then 100% MeOH for 10 min) to give the oxidized compound (0.7 mg) at t_R 27.7 min (68% yield). Then, 6N HCl (400 μL) was added to the product (100 μL) and stirred at 110°C overnight. The reaction mixture was dried and reconstituted in water (100 μL) and subjected to HPLC/MS enantioselective analysis. For the standards, (*S*)-*O*-Me-Ser standard (0.3 mg) (Waterstone Technology) was subjected to partial epimerization to obtain the (*R*)-*O*-Me Ser standard. The compound was dissolved in water (80 μL) followed by the addition of triethylamine (32 μL) and acetic anhydride (32 μL). The reaction mixture was stirred at 60°C for 1 h and then dried down. The residue was re-dissolved in 6N HCl (100

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μL) and stirred at 110°C overnight and then dried again. The ratio of *S:R* enantiomers obtained from the partial epimerization reaction was 9:1. Standards as well as the test compound were subjected to HPLC/MS chiral analysis (MRM monitoring) under the following conditions: CUR 10, CAD medium, IS 5500, TEMP 600, GS1 55, GS2 55, positive ion mode, MRM pair [120→74], t_R: (*S*)-*O*-Me-Ser (10.6 min), (*R*)-*O*-Me-Ser (15.4 min), oxidized moiety from compound **5** (10.6 min).

Cannabinoid CB_1/CB_2 Receptor Binding Assays

Assays were done by Caliper Life Sciences. Human recombinant CB_1 (B_{max} = 1.5 pmol/mg protein) or CB_2 (B_{max} = 8 pmol/mg protein) receptors were expressed in HEK-293 cells. [^{3}H]CP-55940 was used as the radioligand with a final concentration of 0.5 nM (K_d for CB_1 = 0.6 nM and for CB_2 = 4.2 nM). HU-210 (1 μM) was used as non-specific binding determinant (K_i values of 1.1 and 3.0 nM for CB_1 and CB_2 receptors, respectively). Reactions were carried out in TRIS HCl buffer (50 mM, pH 7.4) containing EDTA (2.5 mM), $MgCl_2$ (5 mM), and BSA (0.1%) at 30 °C for 90 minutes. The reaction was terminated by rapid vacuum filtration onto glass fiber filters. Radioactivity trapped onto the filters was determined using liquid scintillation spectrometry and compared to control values in order to ascertain any interactions of test compound with the CB_1 or CB_2 binding sites.

cAMP Functional Assay

CHO-K1 cells expressing CB_1 or CB_2 receptors were used, and cAMP levels were determined after forskolin stimulation using cAMP Hunter express GPCR assay kits (DiscoverX) according to the manufacturer's procedures. Briefly, cells were seeded in 96-well plates (3×10^4 cells/well) and incubated at 37 °C humidified air with 5% CO_2. After 24 h, the medium was aspirated and cell assay buffer with cAMP antibody reagent were added to the wells. Test compound and forskolin were dissolved in DMSO. The cells were then stimulated with different concentrations of the test compound in the presence of forskolin (20 μM) for 30 min at 37 °C. Cell lysis and chemiluminescent signal detection were done using the detection reagents according to the recommended protocol.

FAAH Inhibitor Enzyme Assay

FAAH inhibitor screening assay kit was purchased from Cayman Chemical and used as recommended. In a black 96-well plate, FAAH enzyme (10 μL) was added to the assay buffer (170 μL), followed by the addition of the test compound or the solvent control (10 μL). The reactions were initiated by adding the substrate AMC arachidonoyl amide (10 μL, 20 μM final concentration), and the plate was incubated for 30 minutes at 37 °C. After incubation the signal was detected at an excitation wavelength of 350 nm and an emission wavelength of 455 nm using a microplate reader.

NO Assay

RAW 264.7 mouse macrophage cells were cultured and maintained in DMEM supplemented with 10% FBS in a humidified environment with 5% CO_2. Cells were seeded in 96-well plates (2×10^4 cells/well), and after 24 h the cells were treated with different concentrations of the test compound or solvent control (1% EtOH), followed by LPS (0.5 μg/mL) to stimulate an inflammatory response. The production of NO was assessed by measuring the nitrite concentration in the culture medium after 24 h using Griess reagent. Briefly, sulfanilamide (1% w/v) in phosphoric acid (5% v/v) (50 μL) was added to the cell culture supernatant (50 μL) and incubated for 5 min at room temperature in the dark, followed by the addition of naphthylethylenediamide-HCl (0.1% w/v, 50 μL). After 5 min incubation at room temperature in the dark, the absorbance of the reaction mixture was

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measured at 540 nm using a microplate reader. Assays were run in duplicates. Nitrite quantification was determined relative to a nitrite standard curve (0–100 μM).

Cell Viability Assays

Cells were propagated and maintained in Dulbecco's modified Eagle medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT) at 37 °C humidified air and 5% CO_2. Cells were seeded in 96-well plates (MCF7 10,500 cells/well; HT-29 11,000 cells/well). After 24 h, cells were treated with various concentrations of the test compound, or solvent control (1% EtOH). After 48 h of incubation, cell viability was measured using MTT according to the manufacturer's instructions (Promega, Madison, WI).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was supported by the National Institutes of Health, NIGMS Grant P41GM086210. This is contribution 893 from the Smithsonian Marine Station.

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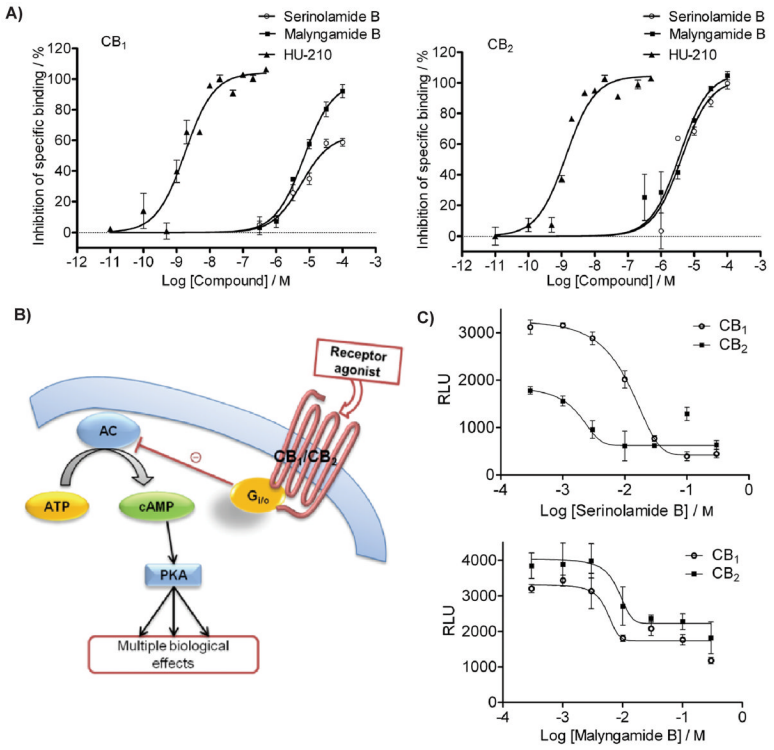

Figure 1.

A) Binding of compounds **5** and **6** to CB_1 (left) and CB_2 (right) receptors, represented as percent inhibition of the binding of a radioactive ligand; B) Simplified diagrammatic representation of the cannabinoid receptors and the consequences of agonist binding; C) Effect of compounds **5** (upper) and **6** (lower) on forskolin-induced cAMP accumulation. A higher level of cAMP produces a higher luminescence reading (RLU).

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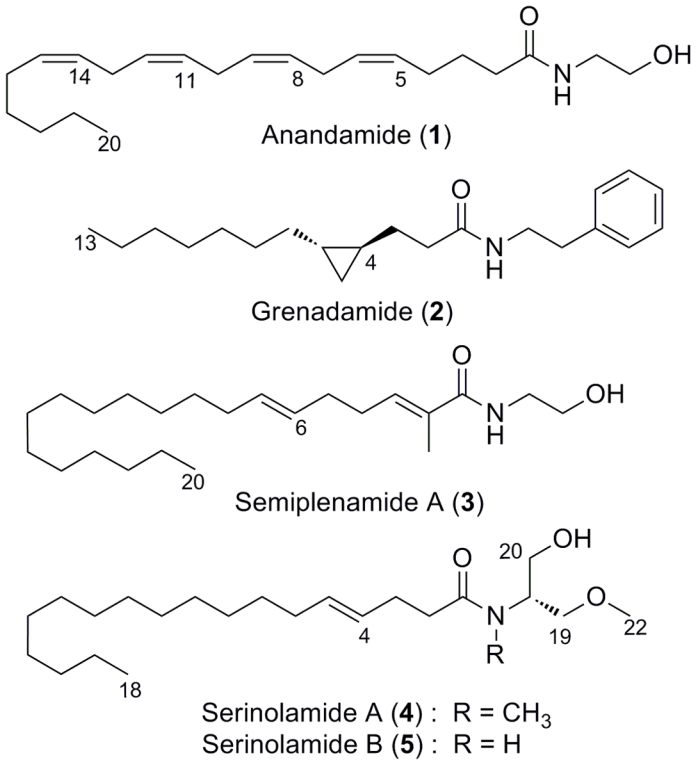

Scheme 1.
Structures of the endocannabinoid anandamide and fatty acid amides from marine cyanobacteria with binding affinities to the cannabinoid receptors

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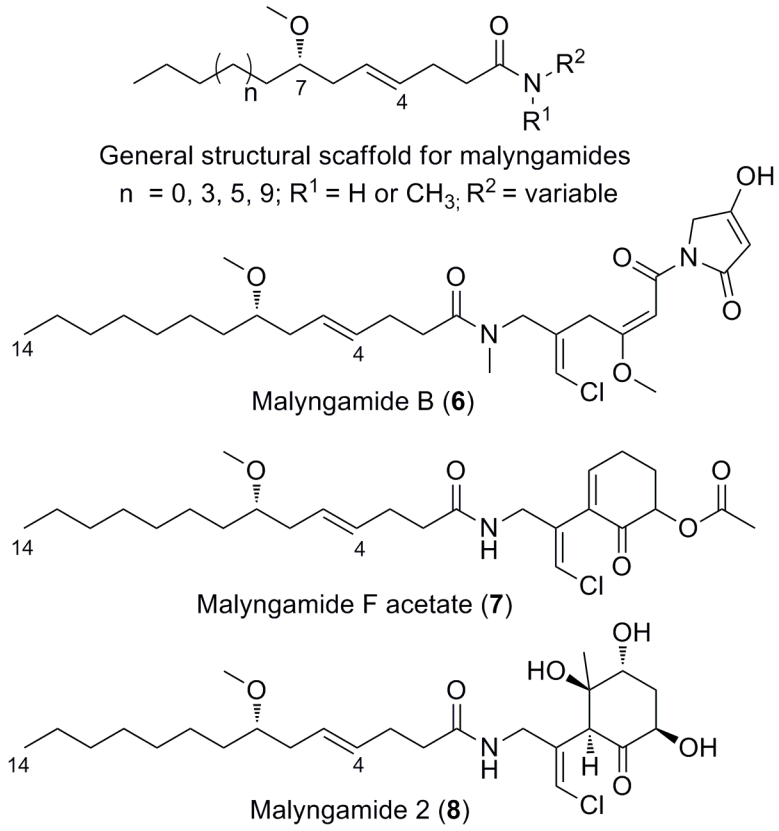

Scheme 2.
General structural scaffold of malyngamides and the structures of three malyngamides.

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Table 1

^{1}H and ^{13}C NMR Spectroscopic Data for Serinolamide B (**5**) in $CDCl_3$ (δ in ppm, J in Hz) at 600 MHz

Unit	C/H #	δ_C	δ_H (J)	HMBC[a]
Fatty acid	1	172.9 qC		2, 3, 19, NH
	2	36.4 CH_2	2.27 dd (6.6, 2.1)	3b, 4
	3 a	28.4 CH_2	2.28 d (6.9)	2, 4, 5
	b		2.23 m	
	4	127.8 CH	5.39 m	3a, 3b, 5, 6
	5	132.1 CH	5.49 m	3b, 4, 6, 7
	6	32.3 CH_2	1.96 ddd (7.7,7.5, 6.8)	4, 5, 7, 8
	7	29.3 CH_2	1.32 m	6, 8
	8–15[b]	29.4 CH_2	1.25 m	6, 7
	16	31.8 CH_2	1.25 m	17, 18
	17	22.4 CH_2	1.29 m	16, 18
	18	13.9 CH_3	0.88 t (6.8)	16, 17
Serinol ether	19	50.3 CH	4.07 m	20a, 21a, 21b, NH
	20 a	64.2 CH_2	3.82 dd (11.2, 4.1)	19, 21a, 21b
	b		3.66 d br (11.2)	19, 21a, 20b, 22
	21 a	73.6 CH_2	3.58 dd (9.3, 4.2)	21a, 21b
	b		3.53 dd (9.3, 4.2)	
	22	59.2 CH_3	3.36 s	
	NH		6.16 d (6.8)	

[a] Protons showing long-range correlation to indicated carbon.

[b] Overlapping peaks.

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Table 2

Cannabinoid receptors affinities (K_i) and consequent functional effects on cAMP accumulation (EC_{50}) by compounds **5** and **6**

	CB_1		CB_2	
Compound	**K_i (μM)**	**EC_{50} (μM) [a]**	**K_i (μM)**	**EC_{50} (μM) [a]**
HU-210[b]	0.00069	-	0.0011	-
CP 55940[c]	-	0.00024	-	0.00036
5	16.4	11.8	5.2	1.8
6	3.6	5.3	2.6	8.8

[a] Results from cAMP functional assays. EC_{50} is the agonist concentration to cause half maximal inhibition of forskolin-induced cAMP accumulation.

[b] Positive control for binding assays.

[c] Positive control for functional assays.