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Identification of a Potent and Selective Cannabinoid CB_1 Receptor Antagonist from *Auxarthron reticulatum*

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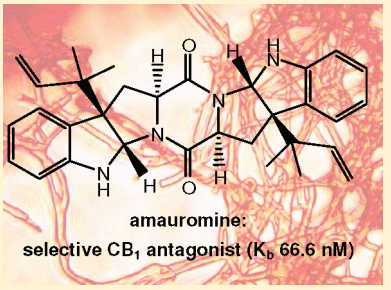
Supporting Information

ABSTRACT: The fungus *Auxarthron reticulatum* derived from the marine sponge *Ircinia variabilis* produced the diketopiperazine alkaloid amauromine (**1**) and the quinolinone methyl-penicinoline (**2**). Compound **2** is identical to the reported methyl-marinamide, whose structure is herewith revised. In radioligand binding studies at human cannabinoid CB_1 and CB_2 receptors recombinantly expressed in Chinese hamster ovary (CHO) cells, amauromine (**1**) was found to exhibit high affinity and selectivity for the CB_1 receptor (K_i = 178 nM). The compound was shown to be a neutral CB_1 antagonist with a K_b value of 66.6 nM determined in cAMP assays. Compound **2** exhibited only weak or no effects at CB receptors. To the best of our knowledge, compound **1** is the first fungal natural product that shows affinity for cannabinoid CB_1 receptors. Because of its high antagonistic potency and selectivity, it may have potential for use as a drug and/or serve as a lead structure for drug development.



KEYWORDS: *Auxarthron reticulatum*, amauromine, cannabinoid receptors, CB_1, CB_2, natural products

Cannabinoid receptors are located in the cell membrane and belong to the G protein-coupled receptor (GPCR) superfamily.[1] They are divided into two distinct subtypes designated CB_1 and CB_2, both of which are G_i protein-coupled mediating inhibition of adenylate cyclase, and thus, their activation results in reduced intracellular cAMP levels.[2] The CB_1 receptor is expressed in the central nervous system (CNS) in high density but also is present in peripheral tissues, including the lungs, liver, kidneys, and adipocytes.[3] CB_1 activation mediates analgesia, stimulation of appetite, and euphoria, among other effects. CB_1 antagonists show appetite-suppressing and antischizopathic properties.[3] The CB_2 receptor is mainly present in organs and cells of the immune system including spleen, tonsils, and thymus, and its activation results in analgesic and antiinflammatory effects.[3,4]

Regarding the origin of the ligands of cannabinoid receptors, they can be classified into three groups: (1) endocannabinoids, such as *N*-arachidonoylethanolamine, which are found in the mammalian nervous and immune system;[5] (2) phytocannabinoids, such as Δ^9-tetrahydrocannabinol (Δ^9-THC), which are produced by the plant *Cannabis sativa*;[6] and (3) synthetic cannabinoids such as the agonist nabilone, a synthetic analogue of Δ^9-THC, and the antagonist rimonabant, which is synthetically produced. Nabilone (Cesamet) is therapeutically used for the suppression of chemotherapy-induced nausea and vomiting,[7] for the treatment of neuropathic pain, and for the therapy of anorexia in patients with AIDS.[3] Further indications include chronic pain, fibromyalgia, and multiple sclerosis.[3] CB_1 receptor antagonists are potential drugs for the treatment of schizophrenia, especially for treating the negative symptoms of the disease, and for the therapy of drug and alcohol addiction.[3] Another indication for CB_1 receptor antagonists is the treatment of obesity, due to an appetite-suppressing effect mediated via central CB_1 receptors in the hypothalamus. Besides weight loss, a reduction in HbA_{1c} and triglyceride levels and an increase in high-density lipoprotein levels can be observed. Investigations have shown that these metabolic improvements are based on peripheral CB_1 receptor blockade. Thus, peripherally acting CB_1 receptor antagonists without CNS penetration would be promising drugs for the treatment of metabolic disorders associated with abdominal obesity, as they would be devoid of side effects caused by central CB_1 receptor activation, for example, depression and anxiety.[3,4,8] CB_2 receptor inverse agonists/antagonists are potential drugs for the treatment of osteoporosis and were also shown to have beneficial effects in animal models of dermatitis.[3,7] In the present study, we isolated compounds **1** and **2** from the marine sponge-derived fungus *Auxarthron reticulatum* and investigated the natural products for their interaction with CB receptors.

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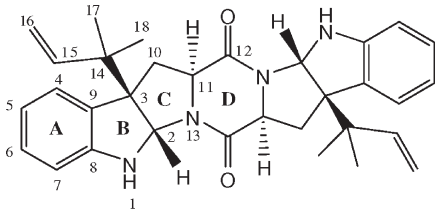
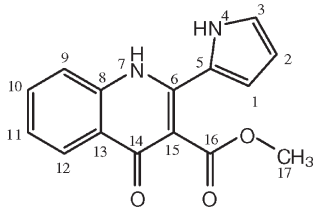


1 amauromine



2 methyl-penicinoline

Figure 1. Structures of amauromine (**1**) and methyl-penicinoline (**2**).

The fungal metabolite **1** is a dipeptide composed of two modified tryptophan units, which are cyclized forming a central diketopiperazine ring. Compound **1** is further characterized by the presence of two prenyl moieties, which are attached to the core structure through "reversed prenylation" (Figure 1). The molecular formula of compound **1** was deduced by accurate mass measurement [high-resolution electrospray ionization mass spectrometry (HRESIMS), m/z = 509.2911 $[M + H]^+$] to be $C_{32}H_{36}N_4O_2$. The NMR spectroscopic data showed only resonances for $C_{16}H_{18}$, and thus, it was concluded that a homodimeric structure was present and that each resonance signal in the ^1H and ^{13}C NMR spectra represented at least two magnetically equivalent nuclei. Indeed, compound **1** is characterized by the presence of a *C*2-axis of symmetry (Supporting Information, Figure S2 and Table S3).

Because several stereoisomers of compound **1** are possible, it is difficult to delineate the absolute configuration of the molecule. Thus, for the determination of the absolute configuration, acid hydrolysis was performed followed by chiral HPLC chromatography (Supporting Information, Figure S14). This resulted in the detection of L-tryptophan indicating the *S*-configuration of the α-carbon atom of compound **1** (C-11/11′). Our data therefore showed that compound **1** is the alkaloid amauromine[9−11] (=nigrifortine[12]). Compound **1** was previously investigated for its pharmacological effects and reported to be a vasodilator acting as a calcium entry blocker.[9−11]

HRESIMS analysis of compound **2** (Figure 1) yielded the molecular formula $C_{15}H_{12}N_2O_3$ with 11 degrees of unsaturation. This molecular formula was supported by ^1H and ^{13}C NMR spectra (Supporting Information, Figure S4 and Table S5), which showed resonance signals for two carbonyl groups (δ_c 173.8 for C-14 and 167.8 for C-16), five unsaturated quaternary carbon atoms (δ_c 123.0 for C-5, 140.9 for C-6, 139.7 for C-8, 124.3 for C-13, and 113.6 for C-15), seven unsaturated methine carbon atoms ($\delta_{H/C}$ 6.47/112.1 for CH-1, 6.25/110.1 for CH-2, 7.13/122.8 for CH-3, 7.66/118.7 for CH-9, 7.70/132.6 for CH-10, 7.36/123.9 for CH-11, and 8.07/124.9 for CH-12), and one methoxyl group (δ_H/c 3.67/52.1 for CH_3-17). In addition, in the ^1H and ^{13}C NMR spectra, there are two downfield shifted resonances at δ_H 11.63 and 11.70, which were determined to arise from NH-4 and NH-7, respectively. The downfield shift may be explained by intermolecular hydrogen bonds of the NH groups with the carbonyl group at C-14 as seen in the X-ray derived structure (Supporting Information, Figure S1).

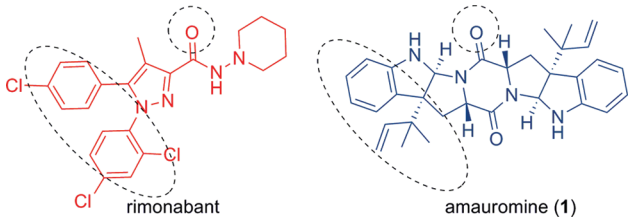


Figure 2. Comparison of the structure of amauromine (**1**) with that of the CB_1 antagonist/inverse agonist rimonabant. Essential structural features (carbonyl group, lipophilic, and aromatic residues) of rimonabant and corresponding features in amauromine are circled by dashed lines.

The methine carbons CH-9 to CH-12 are connected due to the mutual cross-peak correlations in both $^1H-^1H$ correlation spectroscopy (COSY) and $^1H-^{13}C$ heteronuclear multiple bond correlation (HMBC) spectra (Supporting Information, Figure S6). They are also bound to the quaternary aromatic carbons C-13 and C-8, as confirmed by $^1H-^{13}C$ HMBC correlations from H-9 and from H-11 to C-13 and from H-10 and H-12 to C-8. Compound **2** is thus an ortho-disubstituted benzene derivative. C-13 is attached to the carbonyl carbon C-14 due to the $^1H-^{13}C$ HMBC correlation from H-12 to C-14, and C-8 is attached to NH-7 due to $^1H-^{13}C$ HMBC correlations from NH-7 to both C-9 and C-13. Also, in the $^1H-^{13}C$ HMBC spectrum, NH-7 exhibits correlations to C-6 and C-15, which results in a γ-pyridone ring including carbons C-8, C-13, and C-14. Such an arrangement forms a 4-quinolinone ring, which is substituted at C-15 and C-6. The substitution at C-15 is a methyl carboxylate residue due to the $^1H-^{13}C$ HMBC cross-peak correlations from the methoxyl group CH_3-17 to both C-15 and C-16, whereas the substitution at C-6 is an α-pyrrolyl moiety. The latter was proven from the data acquired in $^1H-^1H$ COSY and $^1H-^{13}C$ HMBC experiments. Thus, H-1 to H-3 are forming a spin system, which together with the key HMBC correlations between them and the carbons C-1 to C-5 implies the presence of a pyrrol ring. The nuclear Overhauser effect spectroscopy correlation of NH-4 to H-3 confirmed this conclusion. The pyrrol ring is linked to the quinolinone nucleus at C-6 due to the $^1H-^{13}C$ HMBC cross-peak correlation from NH-7 to C-5. X-ray diffraction analysis allowed the structure of **2** to be unambiguously established. It is a structurally unique 4-quinolinone, linked to a pyrrole ring on one side and a methyl carboxylic acid ester moiety at the other side.

A similar compound, that is, methyl-marinamide,[13] which was described as an isoquinolinone analogue of **2**, was already described in the literature (Supporting Information, Figure S13). Surprisingly, this compound has the same NMR chemical shifts as compound **2**, and thus, we assume that the structures of methyl-marinamide and also of the related marinamide are wrong. This is further confirmed by the recently reported compound penicinoline,[14] which shows the same structural features as compound **2**, except that it is not methylated (i.e., CH_3-17). Penicinoline, according to our analysis, is identical to marinamide.

Because of the structural similarity between amauromine and the CB_1 antagonist rimonabant (Figure 2), we investigated the isolated natural products **1** and **2** in radioligand binding studies[15] at human cannabinoid CB_1 and CB_2 receptors recombinantly expressed in Chinese hamster ovary (CHO-K1) cells obtained by

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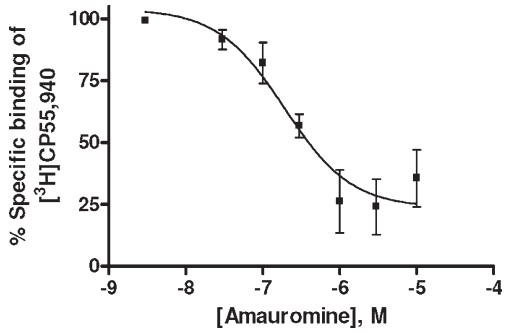

Figure 3. Concentration-dependent inhibition of specific [^{3}H]CP55,940 binding by amauromine at membrane preparations of CHO cells recombinantly expressing human CB_1 receptors. Data points represent means ± SEMs of three independent experiments, performed in duplicate.

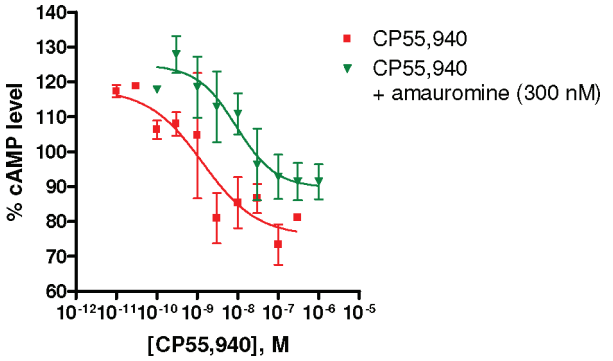

Figure 4. Concentration-dependent inhibition of forskolin (10 μM)-induced cAMP accumulation by the CB agonist CP55,940 in CHO cells recombinantly expressing the human CB_1 receptor in the absence and in the presence of amauromine (300 nM). Data points are means ± SEMs of three independent experiments performed in duplicate. A K_B value of 66.6 ± 57.0 nM was determined for amauromine (**1**).

retroviral transfection (Hinz, S.; Karcz, T.; Müller, C. E. Unpublished results). Compound **1** showed very high affinity for CB_1 receptors exhibiting a K_i value of 178 nM (Figure 3), while it did not show any affinity at a high concentration of 10 μM at CB_2 receptors (Figure S7, Supporting Information). In functional assays measuring forskolin-induced cAMP accumulation in CHO cells,[16] expressing the human CB_1 receptor amauromine (**1**) did not show agonistic effects (Figure S8, Supporting Information). However, compound **1** (300 nM) led to a significant rightward shift of the concentration−response curve for the potent CB receptor agonist CP55,940 in inhibiting forskolin-induced cAMP accumulation at the G_i protein-coupled CB_1 receptor (Figure 4). A K_b value of 66.6 nM was determined for **1**. To the best of our knowledge, compound **1** is the first compound of fungal origin to show affinity toward cannabinoid receptors. Compound **2** exhibited only weak affinity for CB_1 and CB_2 receptors (Figure S7, Supporting Information).

In light of our results, it is interesting to note that many synthetic indole derivatives have already been examined with regard to their affinity for CB receptors.[7,17−19] In a number of studies, indole derivatives were identified that exhibit potent agonistic, cannabimimetic effects on CB_2, but only weak or no affinity for CB_1 receptors. In contrast to those previous results, compound **1** functions as a selective antagonist at the CB_1 receptor. Only very recently, the first natural alkaloids with CB_1-antagonistic activity isolated from the plant *Voacanga africana* were described.[19]

Functional studies indicate that compound **1** appears to be a neutral competitive antagonist, as no increased cAMP levels could be detected in cAMP accumulation assays. There is evidence that neutral antagonists may be preferable over inverse agonists when used as drugs, because they do not alter basic activity arising from spontaneous receptor signaling and, thus, may cause less side effects. For example, it has been reported that the neutral CB_1 receptor antagonist AM4113 had effects comparable to that of the CB_1 inverse agonist AM251 regarding food intake and food-reinforcement behavior in rats, but in contrast to AM251, it failed to induce nausea.[7,20] Furthermore, neutral antagonists are useful experimental tools as they show no effect on their own and, thus, will facilitate the investigation of the physiological role of the endocannabinoid system.[7,21]

Recent studies have emphasized important roles for the endocannabinoid system, that is, receptors and endogenous ligands, in many pathophysiological processes. Therefore, CB_1 and CB_2 receptors are regarded as important drug targets for a number of common diseases for which new drugs are urgently needed, including Parkinson's and Alzheimer's disease, major depression, inflammation, neuropathic pain, and metabolic syndrome.[22] The identification of the structurally novel cannabinoid receptor ligand amauromine (compound **1**) provides an opportunity for the development of a new chemical class of therapeutic agents for the treatment of a number of disorders involving CB_1 cannabinoid receptors.

■ ASSOCIATED CONTENT

Supporting Information. Full experimental procedures, complete crystallographic data for compound **2**, compound characterization for compounds **1** and **2**, ^{1}H and ^{13}C NMR spectra for compounds **1** and **2**, purity data of compound **1**, diagrams of affinities of compounds **1** and **2** toward CB receptors, and results for compound **1** in cAMP accumulation assays at CB_1 receptor. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ DEDICATION

This paper is dedicated to Professors Hassan Elrady A. Saad, Sahar Gedara, and Ahmed Gohar.

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