Supporting Information

Identification of a potent and selective cannabinoid CB_1 receptor antagonist from *Auxarthron reticulatum*

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Contents:

General experimental procedures. Melting points were measured on a Büchi 535 apparatus. UV and IR spectra were obtained on Perkin-Elmer Lambda 40, and Perkin-Elmer Spectrum BX instruments, respectively. VLC grade (40-63 μm) Si gel was used for vacuum liquid chromatography. All organic solvents were distilled prior to use. HPLC was carried out using a Waters system, controlled by Waters Millenium software, consisting of a 600E pump, a 996 PDA, and a 717 plus autosampler. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance 300 DPX spectrometer. NMR spectra were referenced to residual solvent signals of $CDCl_3$ and DMSO-d_6 at $\delta_{H/C}$ 7.26 / 77.0 and 2.50 / 39.5, respectively. Optical rotations were measured with a Jasco DIP 140 polarimeter. ESI mass spectra were obtained on an Applied Biosystems/MDS Sciex API 2000 MS spectrometer. Dark brown single crystals of **2** were grown using an equal mixture of water and methanol. The data collection was performed on a Bruker X8-KappaApexII diffractometer (area detector) using graphite monochromated Mo-Kα radiation (λ = 0.71073 Å). The diffractometer was equipped with a low temperature device (Kryoflex I, Bruker AXS GmbH, 100K). Intensities were measured by fine-slicing ω and φ-scans and corrected for background, polarization and Lorentz effects.

Amino acid hydrolysis of compound 1 by chiral HPLC: A suspension of **1** (2 mg) in 6N HCl (1 ml) was heated at 110°C for 4 h under an argon atmosphere. The hydrolysate was dried with a stream of nitrogen. The residue was dissolved in the mobile phase used for the chiral HPLC (2 mM $CuSO_4$ in H_2O/MeCN, 95:5). The chiral HPLC was performed by using a Chirex 3126 (D)-penicillamine column (Phenomenex; 250 x 4.60 mm; flow rate 1 $mL \cdot min^{-1}$).

Origin of the sponge sample and isolation and taxonomy of the fungus: A sponge sample of *Ircinia variabilis* was collected from Malta. The isolation of the fungus was carried out using an indirect isolation method. Sponge samples were rinsed three times with sterile H_2O. After surface sterilization with 70% EtOH for 15 s the sponge was rinsed in sterile artificial seawater (ASW). Subsequently, the sponge material was aseptically cut into small pieces and placed on agar plates containing the isolation medium: agar 15 gL^{-1}, ASW 800 mLL^{-1}, glucose 1 gL^{-1}, peptone from soymeal 0.5 gL^{-1}, yeast extract 0.1 gL^{-1}, benzylpenicillin 250 mgL^{-1}, and streptomycin sulfate 250 mgL^{-1}. The fungus growing out of the spongeal tissue was separated on biomalt medium (biomalt 20 gL^{-1}, agar 10 gL^{-1}, ASW 800 mLL^{-1}) until the culture was pure. The

fungal strain (number 251) was identified as *Auxarthron reticulatum*. The identification of the fungus was done by Prof. Imhoff, Kiel, Kiel Centre of Marine Natural Products (Kieler Wirkstoffzentrum), Germany.

Cultivation: The fungal strain *Auxarthron reticulatum* was cultivated for 40 days on 10 L of solid BMS (Gesundheitsprodukte GmbH, Kirn, Germany) with agar (15 gL^{-1}) at room temperature in 40 Fernbach flasks.

Extraction and isolation: Fungal biomass and media were homogenized using an Ultra-Turrax apparatus and extracted with 8 L EtOAc to yield 920 mg of crude extract. This material was fractionated by Si VLC using a stepwise gradient solvent system of increasing polarity starting from 20% acetone in petroleum ether to 100% acetone to yield 11 fractions. RP-HPLC separation of the subfraction 3 (column: Macherey-Nagel Isis-100 C_{18}, Ec250 x 4.6 mm, 5 µm; acetonitrile/H_2O (65:35), 1.5 $mLmin^{-1}$) afforded compound **1**; compound **2** was isolated from subfraction 11 (column: Phenomenex C_{18} Luna-100, 250 x 10 mm, 5µm; MeOH/H_2O (50:50), 2 $mLmin^{-1}$).

Compound characterization

Amauromine (compound 1): brownish white amorphous compound (50 mg; 5 mgL^{-1}). $[\alpha]_D^{23}$ -580 (*c* 0.50, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 214 nm (4.3), 243 nm (4.1), 300 nm (3.7); IR (ATR) ν_{max} 3366, 2966, 2359, 2341, 1662, 1606, 1466, 1304, 1060 cm^{-1}. HRESIMS *m/z* 509.2911 $[M+H]^+$ (calcd. for $C_{32}H_{37}N_4O_2$, *m/z* 509.2917).

Methyl-penicinoline (compound 2): brownish crystals (15.5 mg; 1.5 mgL^{-1}). m.p. > 300°C; UV (MeOH) λ_{max} (log ε) 207 nm (4.5), 284 nm (4.0), 332 nm (4.1); IR (ATR) ν_{max} 3330, 3212, 2924, 2359, 2341, 1711, 1631, 1587, 1555, 1454, 1354, 1202, 758 cm^{-1}. HRESIMS *m/z* 291.0740 $[M+Na]^+$ (calcd. for $C_{15}H_{12}NaN_2O_3$, *m/z* 291.0733).

X-ray diffraction structure determination for compound 2:

Crystal data: $C_{15}H_{12}N_2O_3$; crystal size (mm) 0.20 x 0.06 x 0.02; crystal system triclinic; space group P -1; unit cell dimensions a = 8.5613(9) Å, b = 12.7976(13), c = 13.1334(14) Å, α = 63.810(3)°, β = 75.110(3)°, γ = 80.264(3)°; volume 1245.3(2) $Å^3$; Z = 4, density (calcd) 1.431 gcm^{-3}; absorption coefficient 0.102 mm^{-1}; F(000) 560; Theta range for data collection 1.91 to 26.00°; reflections collected /unique 11544/4880 [R_{int} = 0.0468]; completeness to theta 26.00° 99.7%; empirical absorption correction; full-matrix least-squares on F^2; final R indices [I>2σ(I)] R_1 = 0.0658, wR_2 = 0.1593; [all data] R_1 = 0.1189, wR_2 = 0.1840; largest diff. peak and hole 0.861 and -0.484 e Å-3.

CCDC-837283 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

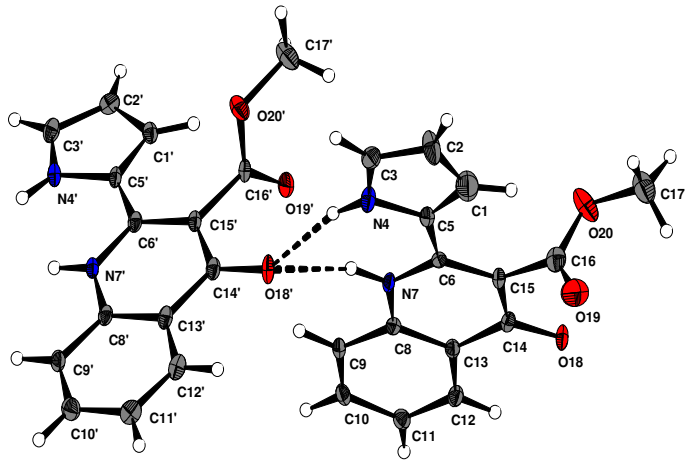


Figure **S1**: X-ray crystallographic structure of compound **2**

Radioligand binding studies at CB_1 and CB_2 receptors:

Competition binding assays were performed versus the cannabinoid receptor agonist radioligand [^{3}H](-)-cis-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-trans-4-(3-hydroxy-propyl)cyclohexanol (CP55,940) as described before with some modifications (Behrenswerth, A.; Volz, N.; Torang, J.; Hinz, S.; Bräse, S.; Müller, C. E. Synthesis and pharmacological evaluation of coumarin derivatives as cannabinoid receptor antagonists and inverse agonists. *Bioorg. Med. Chem.* **2009**, *17*, 2842-2851). [^{3}H]CP55,940 was used in a concentration of 0.1 nM. Membrane preparations of Chinese hamster ovary (CHO) cells stably expressing either human CB_1 or human CB_2 receptors were used (ca. 25 µg of protein per vial). The CHO cells had been retrovirally transfected (Hinz, S.; Karcz, T.; Müller, C.E., unpublished) in analogy to a procedure previously described for adenosine A_{2B} receptors (Schiedel, A.; Hinz, S.; Thimm, D.; Sherbiny, F.; Borrmann, T.; Maass, A.; Müller, C.E. The four cysteine residues in the second extracellular loop of the human adenosine A_{2B} receptor: role in ligand binding and receptor function. Biochem. Pharmacol. 2011, 82, 389-399). Stock solutions of the test compounds were prepared in DMSO. The final DMSO concentration in the assay was 2.5%. After the addition of 60 µL ^{3}[H]CP55,940, 60 µL membrane preparation and 465 µL assay buffer (50 mM TRIS, 3 mM $MgCl_2$, 0.1% BSA, pH 7.4) to 15 µL test compound, the suspension was incubated for 2 h at room temperature. Total binding was determined by adding DMSO without the test compound. Nonspecific binding was determined in the presence of 10 µM of unlabeled CP55,940. Incubation was terminated by rapid filtration through GF/C glass fiber filters presoaked with 0.3% polyethyleneimine, using a Brandel 48-channel cell harvester (Brandel, Gaithersburg, Maryland, USA). Filters were washed three times with ice-cold washing buffer (50 mM TRIS, 0.1% BSA, pH 7.4) and then dried for 1.5 h at 50°C. Radioactivity on the filters was determined in a liquid scintillation counter (TRICARB 2900TR, Packard / Perkin-Elmer) after 6 h of preincubation with 3 mL scintillation cocktail (LumaSafe plus, Perkin-Elmer). Data were obtained from three independent experiments, performed in duplicates.

Data were analyzed using Graph Pad Prism Version 4.02 (San Diego, CA, USA). For the calculation of K_i values the Cheng-Prusoff equation and a K_D value of 2.4 nM ([^{3}H]CP55,940 at hCB_1) were used.

cAMP accumulation assays:

The measurement of intracellular cAMP concentration was performed as described before with some modifications (Drabczynska, A.; Yuzlenko, O.; Köse, M.; Paskaleva, M.; Schiedel, A. C.; Karolak-Wojciechowska, J.; Handzlik, J.; Karcz, T.; Kuder, K.; Müller, C. E.; Kiec-Kononowicz, K. Synthesis and biological activity of tricyclic cycloalkylimidazo-, pyrimido- and diazepinopurinediones. *Eur. J. Med. Chem.* **2011**, *46*, 3590-3607). Inhibition of adenylate cyclase activity was determined in CHO cells stably expressing CB_1 or CB_2 receptor subtypes using a competition binding assay for cAMP. Cells were seeded into a 24-well plate at a density of 200,000 cells per well. After an incubation of 24 h the medium was removed and cells were washed with Hank's buffered saline solution (HBSS) consisting of NaCl (13 mM), HEPES (20 mM), glucose (5.5 mM), KCl (5.4 mM), $NaHCO_3$ (4.2 mM), $CaCl_2$ x 2 H_2O (1.25 mM), $MgSO_4$ (0.8 mM), $MgCl_2$ (1 mM), KH_2PO_4 (0.44 mM), Na_2HPO_4 (0.34 mM) dissolved in deionized, autoclaved water. After adding 190 µL HBSS per well cells were incubated for 2 h at 37°C. After this period the phosphodiesterase (PDE) inhibitor Ro-20-1724 (40 µM), the test compound, and forskolin (10 µM), all dissolved in HBSS containing 10% DMSO, were added to each well. The final DMSO amount was 1.9%. The suspension was incubated for 10 min after the addition of Ro-20-1724, for 5 min after the addition of the test compound and for another 15 min after adding forskolin. For testing the antagonistic potency of compound **1** cAMP accumulation was determined in the presence of the agonist CP55,940. Therefore cells were incubated for 20 min with compound **1** before the agonist was added to the assay. cAMP accumulation was stopped by removing the supernatant from the cell suspension and subsequently lysing the cells with 500 µL of hot lysis buffer (100°C; 4 mM EDTA, 0.01% trition X-100). Aliquots of 50 µL of the cell suspension were transferred to 2.5 mL tubes, and 30 µL [^{3}H]cAMP and 40 µL cAMP-binding protein were added, followed by 1 h of incubation on ice. The cAMP binding protein was obtained from bovine adrenal cortex as previously described (Nordstedt, C; Fredholm, B.B. A modification of a protein-binding method for rapid quantification of cAMP in cell-culture supernatants and body fluid. *Anal. Biochem.* **1990**, *89*, 231-234). For determining intracellular cAMP concentrations 50 µl of various cAMP concentrations were measured instead of cell lysates, to obtain a standard curve. Total binding was determined by adding radioligand and binding protein to lysis buffer, and the background was determined without

addition of the binding protein. Bound and free radioligand were separated by rapid filtration through GF/B glass fiber filter. Radioactivity on the filters was determined in a liquid scintillation counter (TRICARB 2900TR, Packard / Perkin-Elmer) after 6 h of preincubation with 3 mL scintillation cocktail (LumaSafe plus, Perkin-Elmer). Data were obtained from three independent experiments, performed in duplicates.

Data were analyzed using Graph Pad PRISM Version 4.02 (San Diego, CA, USA). The amount of intracellular cAMP was determined by comparison to a standard curve generated for each experiment and normalized to the effect of forskolin (10 µM).

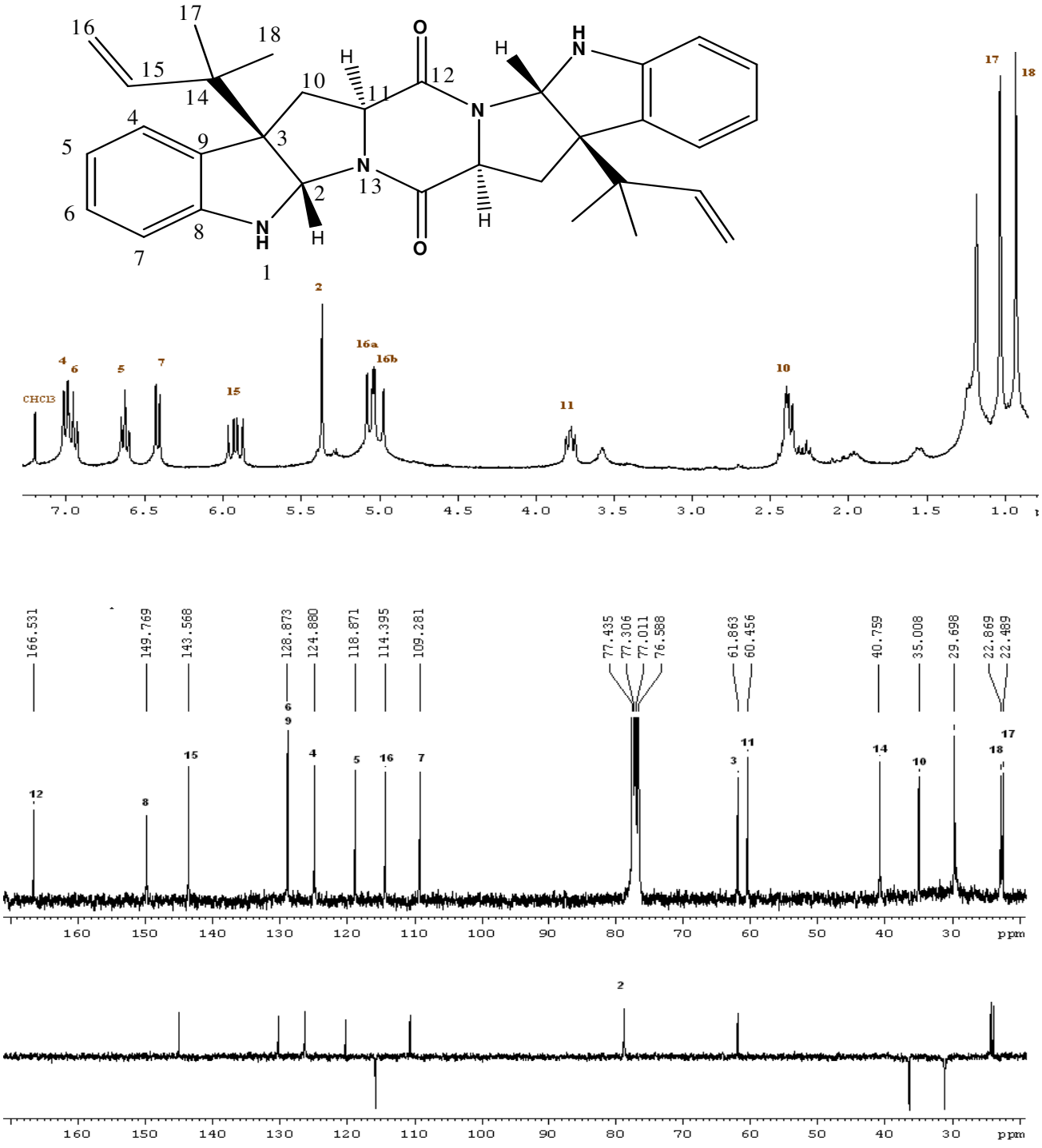


Figure **S2**. ^{1}H, ^{13}C and DEPT 135 NMR spectra ($CDCl_3$) of compound **1.**

Table **S3**: ^{1}H- and ^{13}C-NMR ($CDCl_3$) chemical shifts for compound **1** and ^{13}C-NMR ($CDCl_3$) chemical shifts of amauromine alkaloid.

No.	mult.[a]	δ_H, (mult., *J* in Hz)	δ_C of compound **1**	δ_C of **amauromine**[b]
2/2´	CH	5.43, s	77.2	77.1
3/3´	C	-	61.8	61.8
4/4´	CH	7.06, d (7.7)	124.8	124.7
5/5´	CH	6.68, t (7.7)	118.8	118.6
6/6´	CH	6.99, t (7.7)	128.8	128.7
7/7´	CH	6.47, d (7.7)	109.2	109.2
8/8´	C	-	149.8	149.9
9/9´	C	-	128.8	128.8
10a/10a´	CH_2	2.48, dd, (6.6, 12.8)	35.0	35.0
10b/10b´		2.42, dd, (10.6, 12.8)		
11/11´	CH	3.83, dd (6.6, 10.6)	60.4	60.3
12/12´	C	-	166.4	166.1
14/14´	C	-	40.7	40.7
15/15´	CH	5.98, dd (11.0, 17.2)	143.5	143.5
16a/16a´	CH_2	5.12, d (11.0)	114.4	114.3
16b/16b´		5.06, d (17.2)		
17/17´	CH_3	1.10, s	22.4	22.5
18/18´	CH_3	0.99, s	22.8	22.8

[a] Implied multiplicities determined by DEPT 135.

[b] Data from: Takase, S.; Kawai, Y.; Uchida, I.; Tanaka, H.; Aoki, H. Structure of amauromine, a new alkaloid with vasodilating activity produced by *Amauroascus* sp. *Tetrahedron Lett.* **1984**, *25*, 4673-4676.

Figure **S4**. ^{1}H, ^{13}C and dept 135 NMR spectra of compound **2**

Table **S5**: NMR spectroscopic data (DMSO-d_6) for compound **2**

No.	δ_C	mult. [a]	δ_H (mult., J in Hz)	^{1}H-^{1}H COSY	^{1}H-^{13}C HMBC (H to C)	2D-NOESY
1	112.1	CH	6.47 (dd, 1.5, 3.7)	2, 3	2, 3, 5	2
2	110.1	CH	6.25 (dd, 2.6, 3.7)	1, 3	1, 3, 5	1, 3
3	122.8	CH	7.13 (dd, 1.5, 2.6)	1, 2	1, 2, 5	2
NH-4			11.63, s	1, 2, 3		3
5	123.0	C				
6	140.9	C				
NH-7			11.70, s		5, 6, 9, 13, 15	1, 9
8	139.7	C				
9	118.7	CH	7.66 (d, 7.7)	10	11, 13	10, NH-7
10	132.6	CH	7.70 (t, 7.7)	9, 11	8, 12	9, 11
11	123.9	CH	7.36 (br t, 7.7)	10, 12	9, 13	10, 11
12	124.9	CH	8.07 (d, 7.7)	11	8, 10, 14	11
13	124.3	C				
14	173.8	C				
15	113.6	C				
16	167.8	C				
17	52.1	CH_3	3.67, s		15, 16	1

[a] Implied multiplicities determined by DEPT 135.

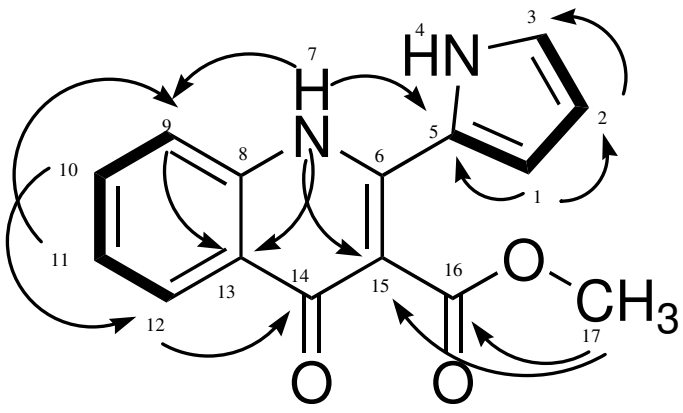


Figure **S6**: ^{1}H-^{1}H COSY correlations (bold lines) and ^{1}H-^{13}C HMBC correlations (arrows from H to C) of compound **2**.

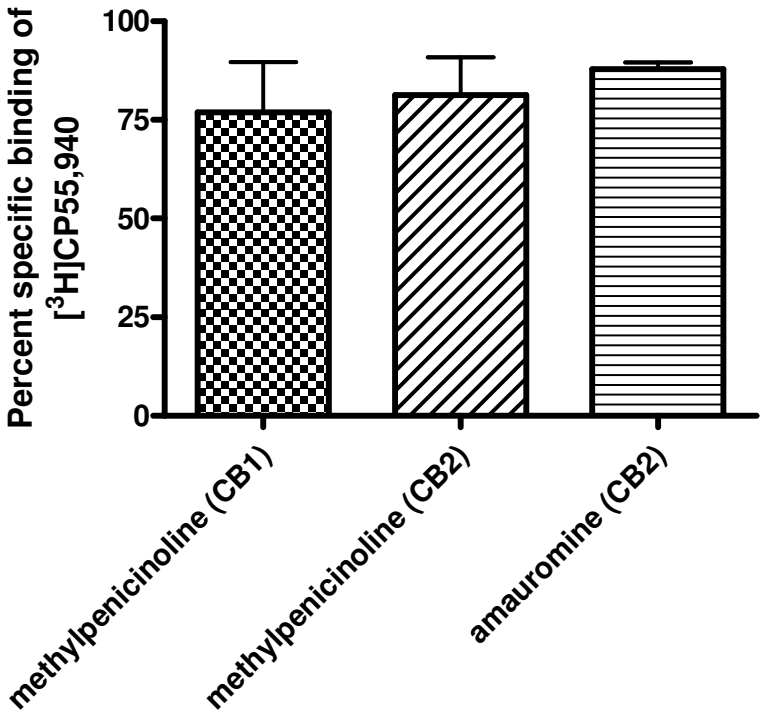


Figure **S7.** Inhibition of specific [³H]CP55,940 binding (0.1 nM) by 10 μM of methylpenicinoline (**2**) or 10 μM of amauromine (**1**) to membrane preparations of CHO cells expressing either human CB_1 or CB_2 receptors. Data represent means ± SEM of three separate experiments, performed in duplicates.

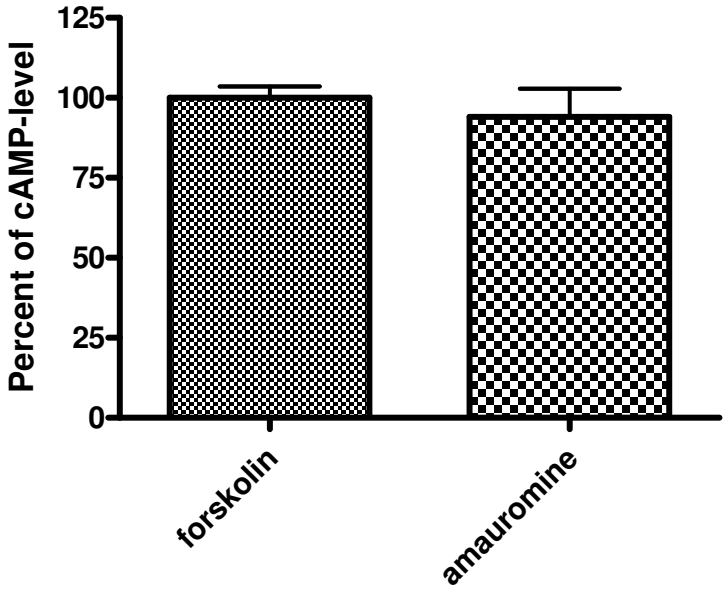


Figure **S8**. Amauromine (10 μM) showed no effect on forskolin- (10 μM) induced cAMP production in CHO cells transfected with the human CB_1 receptor. The results are means of three independent experiments, performed in duplicates. The effect of 10 μM forskolin is set at 100%.

Purity data for amauromine (1):

The purity of amauromine was assessed by three different HPLC methods:

Method A:

A Waters HPLC system equipped with a 996 PDA detector, a 600 pump and 717plus autosampler; column: Macherey-Nagel, EC 250 x 4.6 mm Nucleodur Sphinx; RP-18, 5 µm, mobile phase: MeOH/H_2O (80/20); flow: 2.0 $mLmin^{-1}$.

Method B:

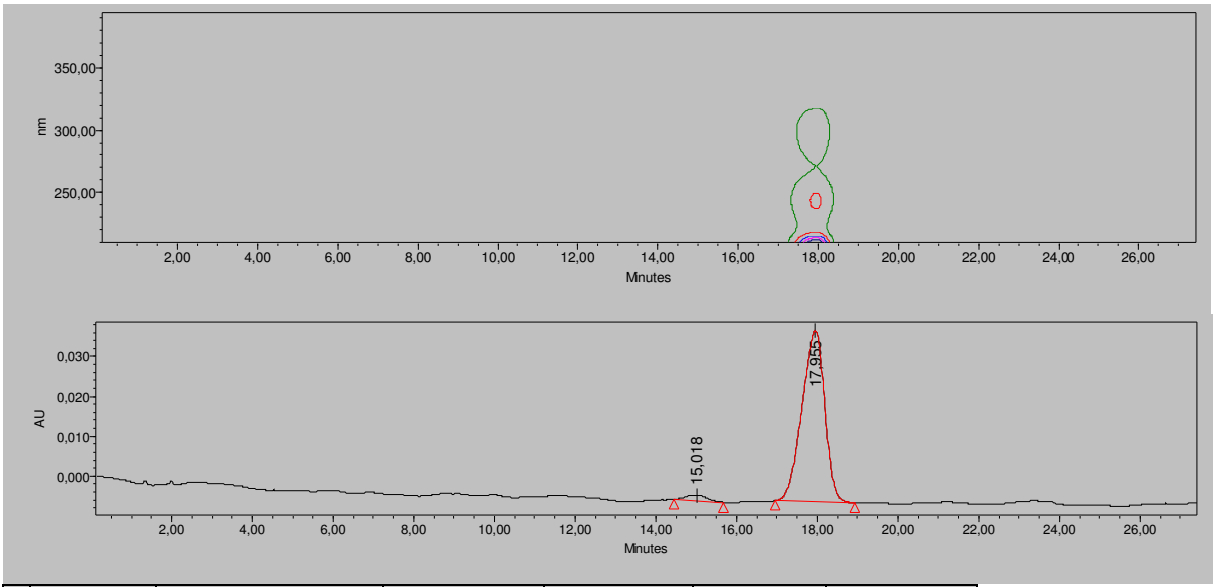
A Waters HPLC system equipped with a 996 PDA detector, a 600 pump and 717plus autosampler; column: Atlantis RP-18, 5 um, EC 250 x 4.6 mm; mobile phase: MeOH/H_2O (80/20); flow: 2.0 $mLmin^{-1}$.

Method C:

A Waters HPLC system equipped with a 996 PDA detector, a 600 pump and 717 plus autosampler; column: Macherey-Nagel; EC 250 x 4.6 mm; Nucleodur Isis, RP-18, 5 um, mobile phase: MeOH/H_2O (80/20); flow: 2.0 $mLmin^{-1}$.

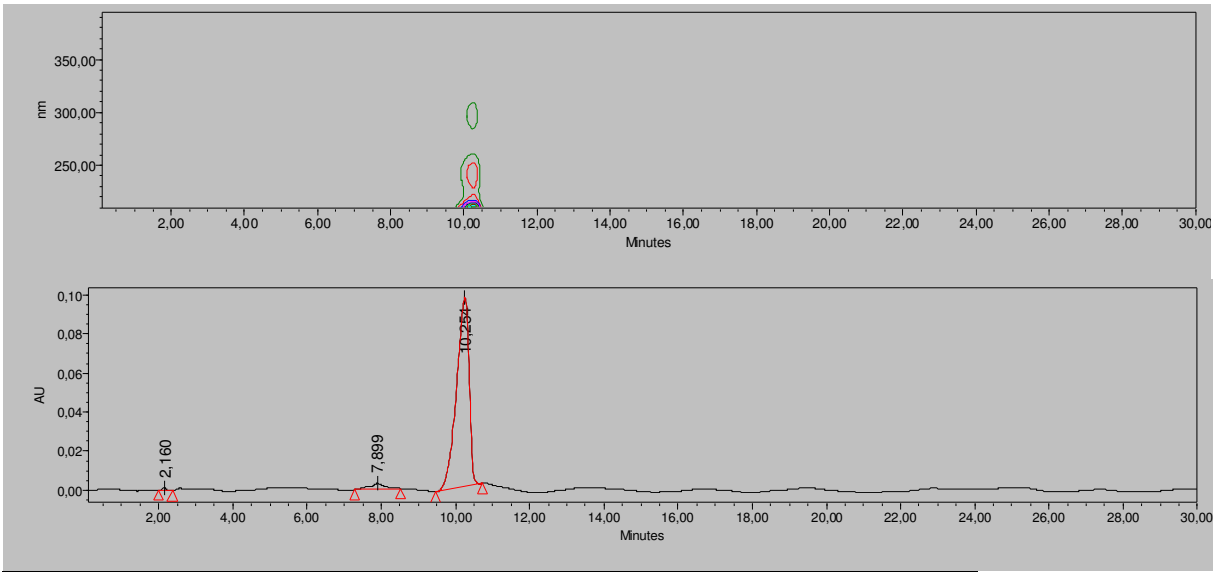
Table S9: Purity analysis of amauromine (**1**) with HPLC

Method	**Wavelength**	**Retention Time**	**Purity**
A	240 nm	17.95 min	97%
B	240 nm	10.25 min	96%
C	240 nm	9.28 min	97%



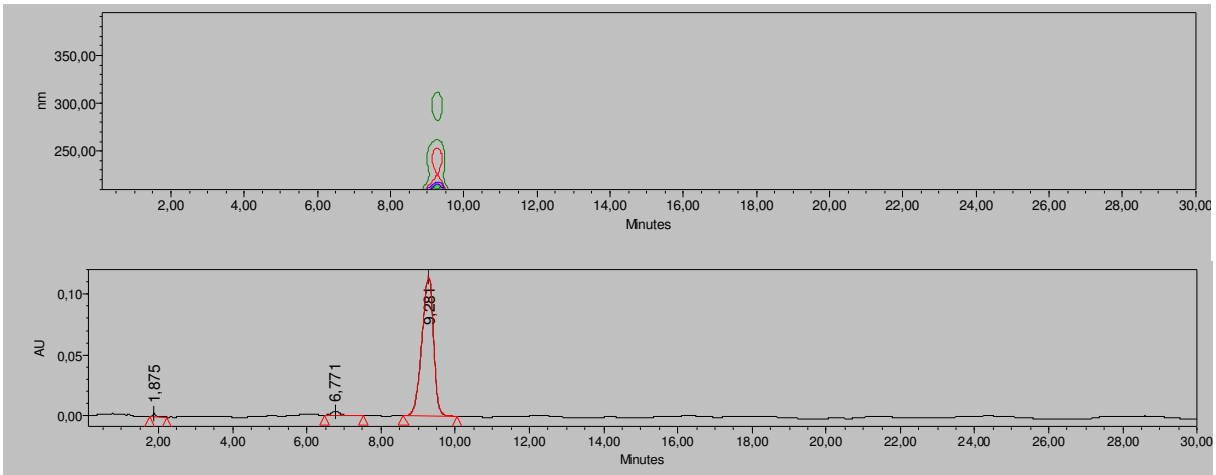
	Name	Retention Time	Area	% Area	Height	Int Type
2		17,955	1682501	97,19	42769	bb
1		15,018	48609	2,81	1381	bb

Figure **S10**. HPLC chromatogram (contour plot and detection at 240 nm) of compound **1** using method A.



	Name	Retention Time	Area	% Area	Height	Int Type
3		10,254	2312829	96,24	96897	bb
2		7,899	79277	3,30	2880	bb
1		2,160	11205	0,47	1616	bb

Figure **S11**. HPLC chromatogram (contour plot and detection at 240 nm) of compound **1** using method B.



	Name	Retention Time	Area	% Area	Height	Int Type
3		9,281	2452727	97,26	114947	bb
2		6,771	45149	1,79	2976	bb
1		1,875	24078	0,95	3003	bb

Figure **S12**. HPLC chromatogram (contour plot and detection at 240 nm) of compound **1** using method C.

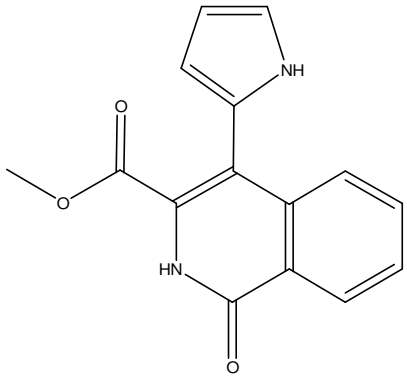
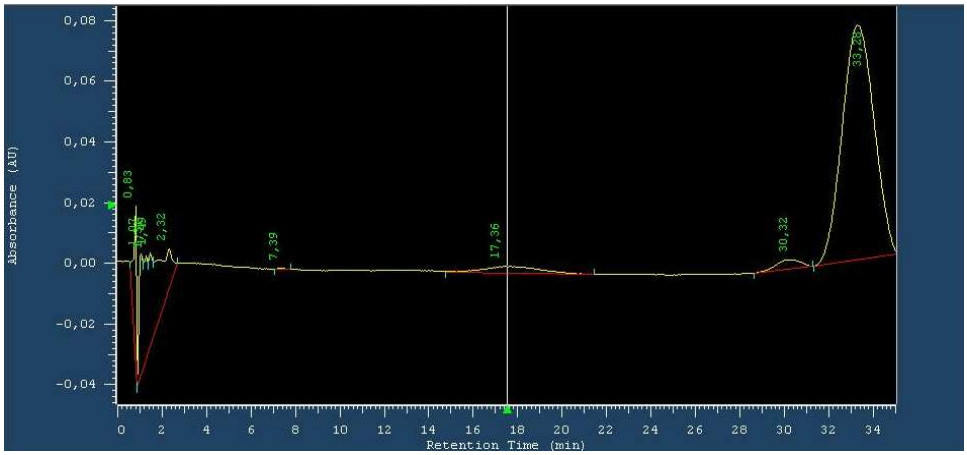
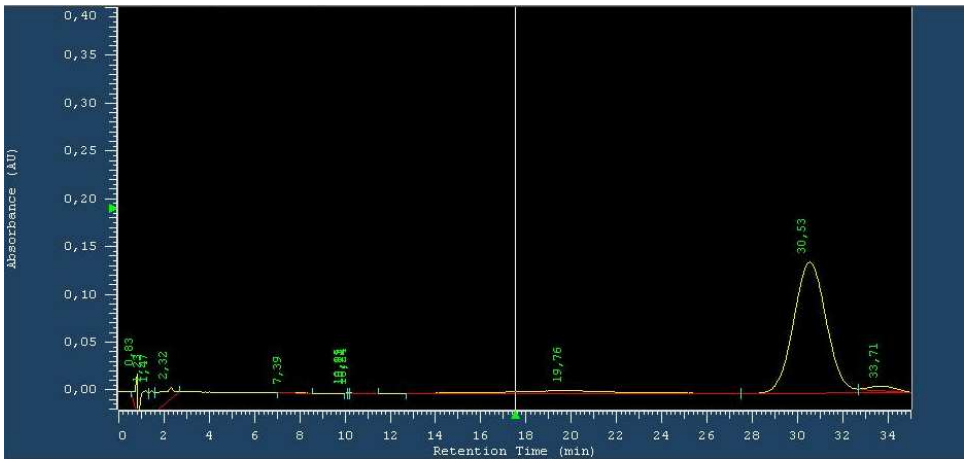


Figure **S13**. Reported structure of methyl-marinamide.

a)



b)



c)

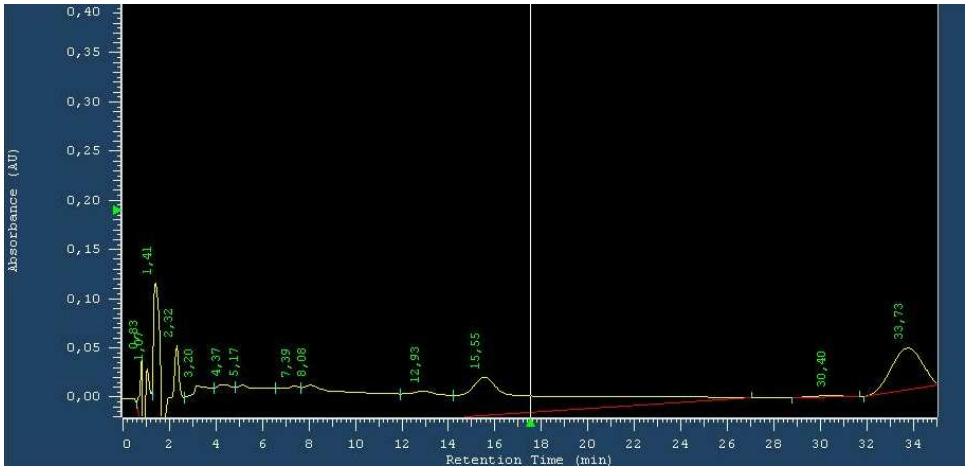


Figure **S14.** HPLC chromatograms of a) *L*-tryptophan, b) *D*-tryptophan, and c) hydrolyzed sample.