HPLC-Based Activity Profiling for $GABA_A$ Receptor Modulators: A New Dihydroisocoumarin from *Haloxylon scoparium*

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A new dihydroisocoumarin was isolated from a dichloromethane extract of *Haloxylon scoparium* with the aid of a functional assay with *Xenopus* oocytes transiently expressing $GABA_A$ receptors of defined subunit composition ($\alpha_1\beta_2\gamma_{2S}$). Compound **1** induced a maximum potentiation of the chloride currents by 144.6 ± 35.3% with an EC_{50} of 140.2 ± 51.2 μM.

Gamma-aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the brain and binds to $GABA_A$ and $GABA_B$ receptors. The $GABA_A$ receptor is a heteropentameric ligand gated chloride channel, for which 19 different subunit isoforms have been identified in the human genome.[1] The most abundant $GABA_A$ receptor subtype consists of two α_1, two β_2, and one γ_2 subunit.[2] Ten additional subtypes have been identified in vivo, which differ in tissular distribution and functional properties.[3,4] $GABA_A$ receptors are targets for various drugs used in the treatment of anxiety, panic disorders, insomnia, and epilepsy.[5] Due to their unique structural diversity, natural products play a major role in pharmaceutical drug design.[6]

We have initiated a project on the discovery of $GABA_A$ receptor modulators of plant and fungal origin possessing scaffolds new for the target.[7,8] In the course of this project we investigated a dichloromethane extract of *Haloxylon scoparium* Pomel (syn. *Hammada scoparia* (Pomel) Iljin.) (Chenopodiaceae).[9] The genus *Haloxylon* consists of 25 species that grow in arid and saline areas. *H. scoparium* is a small, highly branched halophytic shrub distributed in sandy waste places of North Africa and the Middle East. *Haloxylon* species contain numerous alkaloids,[10−14] sterols,[15,16] flavonoid glycosides,[17] and pyranones,[18] for which cholinesterase and chymotrypsin inhibitory, antifungal, and nicotinergic activities have been described. We here report on a new dihydroisocoumarin (**1**) acting as a positive allosteric modulator of the $GABA_A$ receptor.

The dichloromethane extract of *H. scoparium* was submitted to LC-PDA-MS analysis, and the time window of 27 to 37.5 min to HPLC-based activity profiling using a previously validated protocol.[7] This time window had previously shown a potentiating effect on the GABA-induced chloride ion current (I_{GABA}) by 66.7 ± 9.2% at a test concentration of 100 μg/mL. The critical time window of a semipreparative HPLC separation of 10 mg of extract and the corresponding activity profile of the time-based fractionation (microfractions of 90 s each) are shown in Figure 1. The strongest activity was seen in fraction 1 (potentiation of I_{GABA} by 185.1 ± 3.7%), which corresponded to a peak eluted at t_R 28 min. The less active fractions 3−6 corresponded to minor HPLC peaks at t_R 31, and 35 to 36 min. Due to the limited amount of extract at our disposition and the anticipated small concentration of active constituents, we focused our efforts on the major compound **1** in this time window.

Compound **1** was obtained as pale yellow solid. The positive-ion HRESIMS of **1** showed a quasimolecular ion peak at *m*/*z* 321.0725 $[M + Na]^+$, indicating a molecular formula of $C_{17}H_{14}O_5$ (calcd for $C_{17}H_{14}O_5Na$, 321.0733). The ^1H NMR spectrum indicated the presence of a methoxy group [δ_H 4.06 (s)] and a monosubstituted benzene ring [δ_H 7.29 (dd), 7.32 (dd), 7.37 (d)]. Two singlets [δ_H 5.94, 5.97] were assigned to a methylenedioxy group with the aid of an HSQC spectrum. A second methylene group [δ_H 3.12 (dd), 2.93 (dd)] showed ^3J-coupling as well as correlation in the COSY-NMR with an oxygen-bearing methine group [δ_H 5.32 (dd)]. The remaining aromatic methine signal [δ_H 6.39 (s)] showed extensive correlations in the HMBC spectrum to quaternary aromatic carbon atoms [δ_C 111.5, 137.0, 137.0, 153.2]. Two of these carbon resonances [δ_C 137.0, 153.2] showed also correlation to the methylenedioxy signals. Weaker correlations were observed from the aromatic singlet to two further carbon signals. One of them, by chemical shift, was assigned to a lactone carbonyl [δ_C 162.2], and the other to a quaternary aromatic carbon [δ_C 145.8] bearing a methoxy group (Table 1). The structural elements described above were assembled for dihydroisocoumarin **1** (Figure 1) with the aid of HMBC and NOESY data. Key correlations are given in Table 1 and Figure 2. The chemical shifts of all relevant signals are in good agreement with the structurally similar dihydroisocoumarin tunberginol C.[19]

The CD spectrum showed a negative Cotton effect at 233 nm and a positive Cotton effect at 253 nm. The absolute configuration of **1** was established by comparison with CD spectra of various 3-substituted dihydroisocoumarins,[20−23] and the absolute configuration at C-3 was deduced to be *S*. Thus, the structure of **1** was established as (*S*)-4-methoxy-7-phenyl-7,8-dihydro[1,3]dioxolo[4,5-*g*]isochromen-5-one, a new dihydroisocoumarin.

In the functional assay with *Xenopus* oocytes transiently expressing $GABA_A$ receptors of the subtype $\alpha_1\beta_2\gamma_{2S}$, **1** enhanced I_{GABA} at a GABA EC_{5-10} in a dose-dependent manner. The currents were stimulated at concentrations ≥ 3 μM, and maximum stimulation of receptors (144.6 ± 35.3%, $n = 4$) occurred at ∼500 μM. The estimated EC_{50} value (140.2 ± 51.2 μM, Figure 3) is lower than that of (+)/(−)-borneol (EC_{50} between 248 and 237 μM),[24] but somewhat higher than EC_{50} values estimated in functional studies using the same receptor subunit composition for other natural products such as flavonoids and their derivatives (EC_{50}'s between 24 nM and 22 μM)[25−27] and ginsenoside Rc ($EC_{50} = 53.2$ μM).[28] In the absence of GABA, application of **1** at the same concentrations did not elicit chloride currents and thus indicated allosteric $GABA_A$ receptor modulation (data not shown).

Dihydroisocoumarins have been identified from microbial, plant, and insect sources,[29] and 3-phenyl-substituted dihydroisocoumarins occur in plants of the families Asteraceae,[30,31] Guttiferae,[22] and

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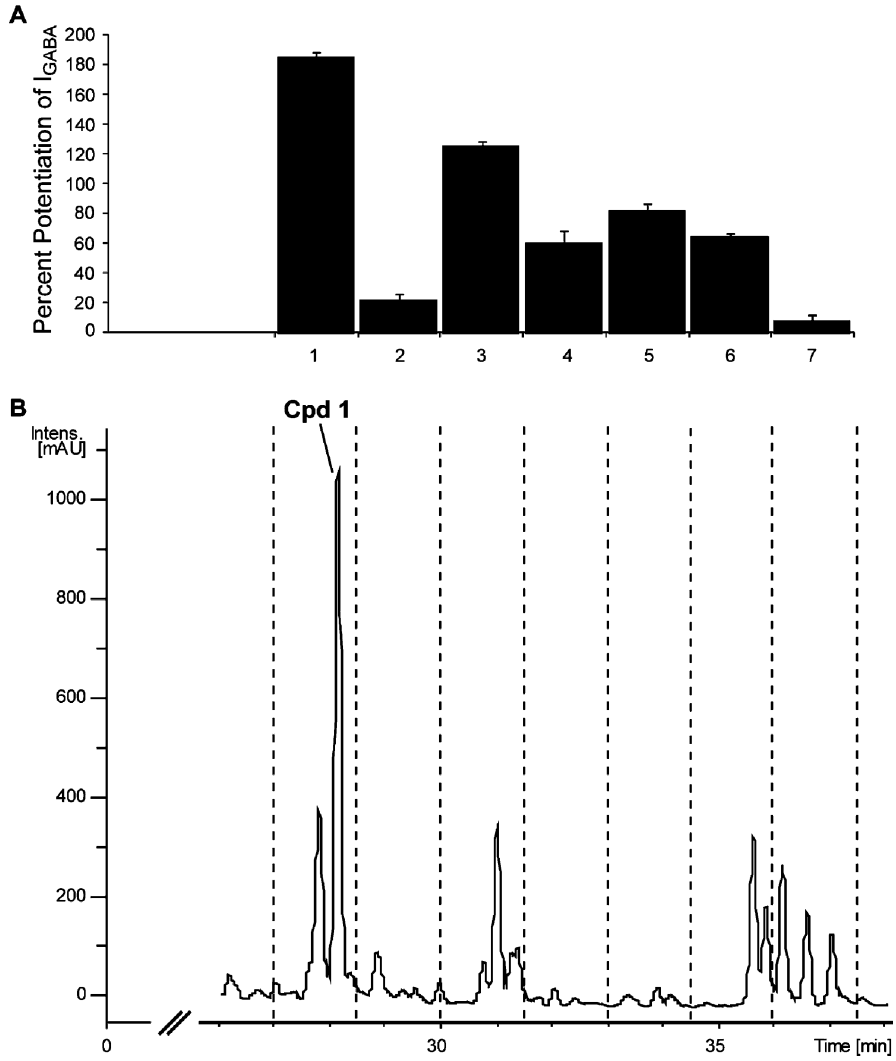

Figure 1. Critical time window of semipreparative HPLC separation of *Haloxylon scoparium* dichloromethane extract (10 mg injected), with chromatogram recorded at 280 nm (B) and corresponding activity profile of the time-based fractionation (microfractions of 90 s each) (A). The bars represent potentiation [%] of the GABA-induced chloride current in *Xenopus* oocytes (I_{GABA}).

Table 1. ^{1}H, ^{13}C, and 2D-NMR Data (*d*-Chloroform) of (*S*)-4-Methoxy-7-phenyl-7,8-dihydro[1,3]dioxolo[4,5-*g*]isochromen-5-one (**1**)

position	δ_H	δ_C	HMBC (H→C)	NOESY
1		162.2		
3	5.32 dd (11.5, 2.5)	79.1	1′, 2′, 6′	4, 5
4	3.12 dd (16.5, 12.0) 2.93 dd (16.5, 2.5)	37.0	3, 5, 6, 4a, 8, 8a	
5	6.39 s	102.1	1, 4, 4a, 6, 7, 8, 8a	3
6		153.2		
7		137.0		
8		145.8		
4a		137.0		
8a		111.5		
1′		138.8		
2′, 6′	7.37 d (7.0)	126.3	3, 3′, 4′, 5′	3, 4
3′, 5′	7.32 dd (7.5)	128.7	1′, 4′,	
4′	7.29 dd (7.5)	128.8	2′, 6′	
OCH_3	4.06 s	60.8	8	
O-CH_2-O	5.94 s, 5.97 s	101.9	6, 7	

Saxifragaceae.[29,32−34] To our knowledge, this is the first report of a dihydroisocoumarin from the family Chenopodiaceae. Dihydroisocoumarins possess an array of biological activities.[29] Interestingly, a fungal isocoumarin, PF1223, was recently reported as a ligand of the insect $GABA_A$ receptor with presumed inhibitory activity at the allosteric antagonist site.[35] Compound **1** is thus the first dihydroisocoumarin potentiating GABA-induced chloride current at the $GABA_A$ receptor.

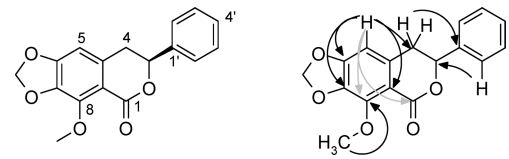

Figure 2. Structure of compound **1** and selected HMBC correlations (gray lines indicate weak correlations).

Experimental Section

General Experimental Procedures. HRMS data were recorded on a microTOF ESI-MS system (Bruker Daltonics) connected to an 1100 series HPLC (Agilent). Parallel evaporation of HPLC fractions was performed with an EZ-2 Plus vacuum centrifuge (Genevac). The NMR spectra were recorded in *d*-chloroform on an AVANCE III spectrometer (500.13 MHz for ^{1}H, 125.77 MHz for ^{13}C) equipped with a 1 mm TXI microprobe and 5 mm BBO probe (Bruker BioSpin). Semipreparative HPLC was carried out on an Agilent 1100 system consisting of a quaternary pump, degasser, autosampler, column oven, and diode array detector. SunFire C18 (3.5 μm, 3.0 × 150 mm) and SunFire Prep C18 (5 μm, 10 × 150 mm) columns (Waters) were used for analytical and semipreparative HPLC separations. HPLC-grade acetonitrile and methanol (Scharlau Chemie S.A.) were used. HPLC-grade water was obtained from a water-purifying unit (EASYpure II, Barnstead). Solvents used for extraction were of analytical grade.

Plant Material. Roots of *Haloxylon scoparium* were collected by T. Friedrich in Beni, Morocco, in April 2000 and identified by T.

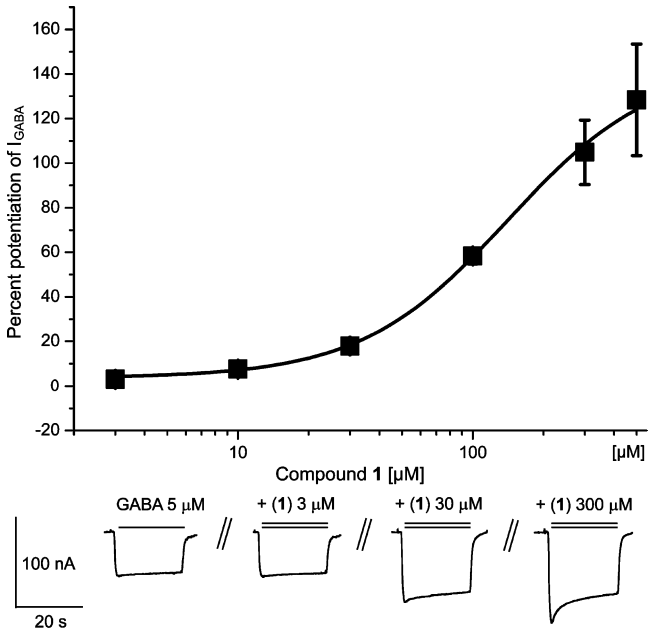

Figure 3. Concentration−response curve for I_{GABA} modulation by compound **1** using a GABA EC_{5-10}. $GABA_A$ receptors were composed of α_1, β_2, and γ_{2S} subunits. Typical traces for I_{GABA} modulation by **1** are shown.

Friedrich. A voucher specimen (00132) of the plant is deposited at the Institute of Pharmaceutical Biology, University of Basel, Basel, Switzerland.

Extraction. Roots of *H. scoparium* (200 g) were milled and extracted at room temperature with CH_2Cl_2 (3 × 1 L) over 3 days. Evaporation gave a dark residue (650 mg), which was stored at −20 °C until use.

Microfractionation. Microfractionation for $GABA_A$ receptor activity profiling was performed as previously described,[7] with minor modifications: Separation was carried out on a semipreparative HPLC column with acetonitrile (solvent A) and water (solvent B) using the following gradient: 5% A for 7 min, 5% A to 100% A over 38 min, 100% A for 8 min. The flow rate was 5 mL/min, and detection was at 280 nm. Injection volume was 500 μL (20 mg/mL in dimethyl sulfoxide). Microfractions of 90 s each were collected into glass tubes followed by parallel evaporation. The $GABA_A$ receptor assay was carried out according to previously published protocols: microfractions were dissolved in 30 μL of dimethyl sulfoxide and subsequently mixed with 2.97 mL of bath solution containing GABA EC_{5-10}.[7,8] For concentration−response experiments with compound **1**, a stock solution (10 mM in dimethyl sulfoxide) was diluted with bath solution to concentrations ranging from 3 to 500 μM and then mixed with GABA EC_{5-10}. Modulations of chloride currents through $GABA_A$ receptors composed of α_1, β_2, and γ_{2S} subunits were analyzed by coapplication of GABA EC_{5-10} and **1**. To evaluate the agonistic activity of compound **1**, samples of the same concentrations were applied to the oocyte without containing GABA EC_{5-10}.

Semipreparative HPLC Purification for Off-line Microprobe NMR. The CH_2Cl_2 extract was dissolved in dimethyl sulfoxide and submitted to semipreparative HPLC with MeOH (solvent A) and H_2O (solvent B) with the following gradient: 65% A → 80% over 8 min, flow rate 5 mL/min, to afford a fraction containing **1** (3.8 mg, t_R 6.1 min). Final purification on Sephadex LH-20 (5 × 30 cm) with chloroform/methanol (5:7, v/v) gave **1** (2.7 mg).

(*S*)-4-Methoxy-7-phenyl-7,8-dihydro[1,3]dioxolo[4,5-*g*]isochromen-5-one (1): pale yellow solid; $[\alpha]^{20}_D$ −36.7 (*c* 0.54, $CHCl_3$); UV (MeOH) λ_{max} 234, 275, 308 nm; ^1H and ^{13}C NMR data, see Table 1; HRESIMS *m/z* 321.0725 $[M + Na]^+$, calcd for $C_{17}H_{14}NaO_5$, 321.0733.

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Supporting Information Available: ^1H NMR, ^{13}C NMR, $^1H-^1H$ COSY, NOESY, HSQC, HMBC, and CD spectra of **1** are available free of charge via the Internet at http://pubs.acs.org.

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