Inhibition of [^{3}H]-LSD Binding to 5-HT$_7$ Receptors by Flavonoids from *Scutellaria lateriflora*

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Received October 30, 2002

The hot water and 70% ethanol extracts of dried mad-dog skullcap (*Scutellaria lateriflora*) both bound to the 5-HT$_7$ receptor, with 87.2 ± 6.2% and 56.7 ± 1.3% inhibition of [^{3}H]-LSD binding to the receptor at 100 μg/mL, respectively. The on-line analysis of a 70% ethanol extract by HPLC-UV/MS resulted in the identification of five flavones (**1**−**5**). Fractionation of the ethanol extract resulted in the isolation of three flavone-glucuronides (**6**−**8**) and a flavanone-glucuronide (**9**), including one new compound, lateriflorin (5,6,-dihydroxy-7-glucuronyloxy-2′-methoxyflavone) (**8**). The structure of **8** was determined by NMR (^{1}H NMR, ^{13}C NMR, and NOESY experiments) and MS analysis. From the results obtained in the testing of the pure compounds, it is evident that the activity on the 5-HT$_7$ receptor is at least partly due to the presence of flavonoids. Scutellarin and ikonnikoside I showed the highest inhibition of [^{3}H]-LSD binding with IC$_{50}$ values of 63.4 and 135.1 μM, respectively.

Mad-dog skullcap (*Scutellaria lateriflora* L., Lamiaceae) is a perennial herb indigenous to North America that grows in wet places from Canada to Florida and westward to British Columbia, Oregon, and New Mexico. It derives its common name from the helmet-shaped upper lid of the seed pods. The aqueous extract of the flowering parts has been traditionally used by Native Americans as a nerve tonic and for its sedative and diuretic properties.[1,2] Most recent research has focused on Baikal skullcap (*Scutellaria baicalensis* Georgi), which has antioxidant and antiinflammatory activities.[3,4] Several flavonoids of *S. baicalensis* have been evaluated for their ability to bind to the benzodiazepine site of the GABA$_A$ receptor. Baicalein, baicalin, and scutellarein are weak ligands of this receptor. The binding capacity of wogonin was contradictory in two studies.[5,6] Baicalein further showed potent inhibition of leukotriene B$_4$ and leukotriene C$_4$ release from human polymorphonuclear leukocytes in an in vitro model for activity against bronchial asthma.[7]

Compared to *S. baicalensis*, little is known of the chemistry of *S. lateriflora*. Mono- and diterpenes[8−10] have been reported as well as the flavonoid baicalin.[11] The aim of this study is to increase the body of information known about the chemistry of *S. lateriflora* and to evaluate the ability of *S. lateriflora* crude extracts and isolated flavonoids to inhibit [^{3}H]-LSD binding to the serotonin-7 receptor (5-HT$_7$ receptor). The pharmacology of the 5-HT$_7$ receptor agonists and antagonists is still relatively unexplored. It has been suggested, however, that 5-HT$_7$ receptor ligands could be useful in the therapy of various ailments such as sleep disorders, depression, migraine, pain, or memory impairment.[12]

The 70% ethanol extract of dried aboveground parts of *S. lateriflora* was used for the screening by HPLC-UV/MS. The UV spectra of the major compounds indicated the presence of flavones. Comparison of the UV and MS spectra with reference standards allowed the identification of baicalein (**1**), baicalin (**2**), scutellarin (**3**), wogonin (**4**), and 5,6,7-trihydroxy-2′-methoxyflavone (**5**) in the extract.

As some of the major peaks in the HPLC-UV trace could not be identified with on-line methods, the ethanol extract was fractionated, and three flavone-glycosides (**6**−**8**) as well as a flavanone-glycoside (**9**) were isolated. Compounds **6**, **7**, and **9** were identified as 7-glucuronyloxy-5,6,2′-trihydroxyflavone (ikonnikoside I), oroxylin A 7-*O*-glucuronide, and dihydrobaicalin, respectively, by comparison of the ^{1}H NMR and ^{13}C NMR data with literature values.[13−15] The UV spectra of compound **8** (λ_{max}: 277 and 325 nm) exhibited bands very similar to those of **6**, indicating a flavone with a similar substitution pattern. The ESI-MS data of **8** (*m*/*z* 477 [M + H]$^+$) gave a difference of 14 u from **6**, which suggested an additional methylene or methoxyl group present in **8**. The presence of a methoxyl group was confirmed by the ^{1}H NMR data by a singlet at δ_H 3.87 (3H). The coupling pattern of the signals in the aromatic region, δ_H 7.87 (1H, dd, J = 7.2, 1.8 Hz), 7.59 (1H, ddd, J = 8.7, 8.4, 1.8 Hz), 7.26 (1H, d, J = 8.7 Hz), 7.17 (1H, dd, J = 8.4, 7.2 Hz), gave evidence for a flavone substituted at position 2′. The two singlets at δ = 6.97 and 6.87, each integrating as one hydrogen, indicated that the position C-3 and one carbon on ring A were not substituted. Ring A was shown by means of the ^{13}C NMR experiment as 5,6,7-trioxygenated with a glucuronic acid moiety attached at position 7. The shift of the methoxyl carbon at δ = 56.1 indicated that one of the adjacent carbons was unsubstituted, as di-*ortho*-substituted aromatic methoxyl groups give signals above δ = 60.0.[16] This fact suggested that the C-2′ was methoxylated. The NOESY cross-peaks between the methoxyl group and H-3 and H-3′ gave further proof that this group was attached to the carbon C-2′. Therefore, compound **8**, a newly isolated natural product, was identified as 5,6-dihydroxy-7-glucuronyloxy-2′-methoxyflavone and named lateriflorin.

All of the flavonoids tested (**1**−**4**, **6**, **9**) were able to bind to the 5-HT$_7$ receptor (see Table 1) and therefore contribute to the binding activity of the extracts. The fact that content and composition of flavonoids are very similar in the water

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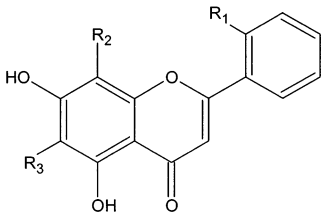
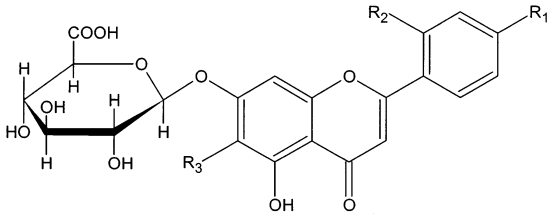
10.1021/np0205102 CCC: $25.00
Published on Web 03/13/2003



1: $R_1 = R_2 = H$, $R_3 = OH$

4: $R_1 = R_3 = H$, $R_2 = OCH_3$

5: $R_1 = OCH_3$, $R_2 = H$, $R_3 = OH$



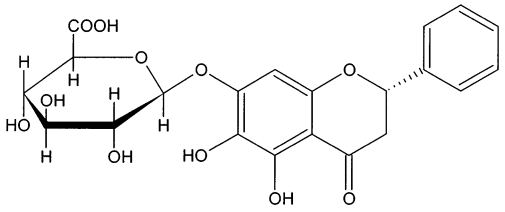
2: $R_1 = R_2 = H$, $R_3 = OH$

3: $R_1 = R_3 = OH$, $R_2 = H$

6: $R_1 = H$, $R_2 = R_3 = OH$

7: $R_1 = R_2 = H$, $R_3 = OCH_3$

8: $R_1 = H$, $R_2 = OCH_3$, $R_3 = OH$



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and the alcohol extract, but the water extract is more active in the assay (the hot water and 70% alcohol extracts showed 87.21 ± 6.20% and 56.65 ± 1.33% inhibition of [^{3}H]-LSD binding to the receptor at 100 μg/mL), suggests that compounds other than the flavonoids, or some of the minor flavonoids present in the extract, might contribute to the activity.

The flavone-glucuronides showed a higher affinity for the receptor than the non-glycosylated compounds. This is very interesting in light of recent findings indicating that baicalin is transformed to its aglycone baicalein by intestinal bacteria.[17] Baicalein is readily absorbed intestinally but is efficiently conjugated back to baicalin in the plasma.[18] It is possible that other flavone-7-*O*-glucuronides are absorbed in a similar way. The extent to which a compound is able to cross the blood−brain barrier, however, remains to be confirmed.

These findings may help to explain the use of *S. lateriflora* as a sedative and nerve tonic. More work should be done to determine whether the flavonoid-glucuronides act as agonists or antagonists of the receptor, and if the compounds act specifically on the 5-HT$_7$ receptor or bind to other serotonin receptors as well.

Table 1. Inhibition of [^{3}H]-LSD Binding to the 5-HT$_7$ Receptor by Flavonoids from *S. lateriflora*[a]

compound	IC_{50} (μM)
1	>350
2	>200
3	63.4 ± 4.8
4	>350
6	135 ± 8
9	179 ± 9
5-HT	2.55×10^{-3}

[a] The competitive binding capability of the sample was calculated in comparison with the inhibition of 1 μM 5-HT (100%).

Experimental Section

General Experimental Procedures. Baicalein and baicalin were purchased from Indofine Chemical Co. (Somerville, NJ). Scutellarin was ordered from Herbstandard, Inc. (Chesterfield, MO). Wogonin was obtained from Chromadex, Inc. (Laguna Hills, CA). 5,6,7-Trihydroxy-2′-methoxyflavone was obtained by acid hydrolysis of 5,6-dihydroxy-7-glucuronyloxy-2′-methoxyflavone. Sodium pyruvate, gentamycin, Pen/Strep, and [^{3}H]-LSD were purchased from Perkin-Elmer Life Sciences (Boston, MA). All other chemicals were ordered from Sigma Chemical Co. (St. Louis, MO). The HPLC system consisted of an Agilent quaternary pump, UV/vis detector (DAD), and automatic sample injector, and LC-MS data were obtained with an Agilent 1100 series LC/MSD trap (Agilent 1100 series, Agilent Technologies, Burlington, MA). For the HPLC-UV/MS analysis, the following conditions were used: column, Zorbax XDB C-18 (5 μm; 150 × 4.6 mm ID); solvent, MeCN (0.05% TFA)−H_2O (0.05% TFA); gradient, 19:81 → 52:48 in 30 min; flow, 0.9 mL/min. ESI-MS: positive ion mode (source: capillary voltage −4000, end plate offset voltage −500, scan range from *m*/*z* 150−600, skim 1 voltage 27.2, capillary exit offset voltage 50.3, trap drive 43.5). UV spectra were measured on a Varian Cary 50 UV/vis spectrophotometer. ^{1}H NMR and ^{13}C NMR data were collected on a GE NMR instrument at 300 and 75 MHz, respectively, or a Bruker Avance instrument at 500 and 125 MHz, respectively. Silica gel (170−400 mesh) was from Fisher Scientific Co. (Pittsburgh, PA), Sephadex LH-20 was from Amersham Pharmacia Biotech (Uppsala, Sweden), and the high load C18 solid phase extraction cartridges were from Alltech Corp. (Deerfield, IL).

Plant Material. *Scutellaria lateriflora* L. was cultivated at the Tom's of Maine Green Mountain Herbs Farm, Rockingham, VT, or was obtained from Blessed Herbs (Oakham, MA). The aboveground parts were extracted with either 70% ethanol (ratio plant material−solvent 1:10) for 24 h or hot water (ratio plant material−solvent 1:20) for 15 min on a platform shaker. A voucher specimen (No. GMH R1046) was deposited at the herbarium of the Farm.

5-HT$_7$ Binding Assay. Membrane Preparation: The human 5-HT$_7$-transfected CHO cells were grown in Ham's F12 media supplemented with 10% FBS, MEM sodium pyruvate (1 mM), gentamycin (50 mg/mL), and penicillin/streptomycin (50 U/mL). Cells were scraped from the culture dishes at full confluence, as described previously.[19] These cells were homogenized and centrifuged twice at 12000*g* for 20 min. The pellets were dissolved in TEM buffer (75 mM Tris, 1 mM EDTA, 12.5 mM of $MgCl_2$, pH 7.4) and were stored at −80 °C. Protein concentrations were determined by the Lowry method using bovine serum albumin as the standard. All preparations were kept on ice.

Serotonin Receptor Binding Assay. Radioligand binding studies were performed in 96-well plates according to the methodology outlined in refs 19 and 20. Briefly, 5 μL of samples in DMSO were mixed into a total amount of 200 μL of incubation buffer containing 40−60 μg/well total protein and 6 nM of [^{3}H]-LSD (protected from light) for h5-HT$_7$. After 1 h incubation at 37 °C, the incubated mixtures were filtered over 934-AH Whatman filters presoaked in 0.5% polyethylenimine (PEI) and washed five times in ice-cold 50 mM TRIS buffer (pH 7.4) using a 96-well Tomtec-Harvester (Orange, CT). The filter was dried, suspended in Wallac microbeta plate scintillation fluid (Turku, Finland), and counted by a Wallac 1450 MICROBETA liquid scintillation counter (Turku, Finland). 5-HT (250 nM) was used to define nonspecific binding, which was less than 10% of total binding. The percent inhibition of [^{3}H]-LSD binding to the 5-HT$_7$ receptor was determined as $[1 - (dpm_{sample} - dpm_{blank})/(dpm_{DMSO} - dpm_{blank})] \times 100$. The

inhibition of [^{3}H]-LSD binding (%) of the sample was calculated in comparison with the inhibition of 1 μM 5-HT (100%). For the most potent compounds, IC_{50} values were determined by evaluation of the percent inhibition of [^{3}H]-LSD binding in a number of serial dilutions. The data represent the average of triplicate determinations.

Extraction and Isolation. A total of 100 g of dried plant material was extracted with 70% ethanol. After drying (5.5 g), the extract was submitted to column chromatography on silica gel using mixtures of CH_2Cl_2−EtOH (1:1, 1:4) and EtOH−H_2O (9:1 and 7:3) as solvent in a gradient manner. Fraction 3 was further separated by gel filtration on Sephadex LH-20 with MeOH−H_2O (1:1) to give fractions 1a−10a. Fraction 10a contained compound **6** (0.9 mg). Compounds **2** (16.6 mg), **9** (8.45 mg), and **6** (6.07 mg) were obtained from fraction 4 after a separation step on Sephadex LH-20 (MeOH−H_2O, 1:1, fractions 1b−15b). Separation of fraction 12b on a C-18 solid phase extraction cartridge using first H_2O and then a mixture of MeCN−H_2O (1:4) yielded 1.20 mg of **2** and 1.83 mg of **8**. Compound **7** (2.27 mg) was obtained from combined fractions 5a and 5b after a separation step using gel filtration on Sephadex LH-20 with MeOH−H_2O (1:1) and a final purification by solid phase extraction on a C-18 cartridge using MeCN−H_2O (2:3) as solvent.

5,6-Dihydroxy-7-glucuronyloxy-2′-methoxyflavone (8): pale yellow needles; mp > 200 °C (dec); UV (MeOH) λ_{max} (log ϵ) 277 (4.30), 325 (4.12) nm; ^{1}H NMR (300 MHz, DMSO-d_6) 12.58 (1H, s, HO−C(5)), 8.68 (1H, br s, HO−C(6)), 7.87 (1H, dd, J = 7.2, 1.8 Hz, H-6′), 7.59 (1H, ddd, J = 8.7, 8.4, 1.8 Hz, H-4′), 7.26 (1H, d, J = 8.7 Hz, H-3′), 7.17 (1H, dd, J = 8.4, 7.2 Hz, H-5′), 6.97 (1H, s, H-3), 6.87 (1H, s, H-8), 5.21 (1H, d, J = 7.6 Hz, H-1″), 3.95 (1H, d, J = 8.3 Hz, H-5″), 3.87 (3H, s, OCH_3); ^{13}C NMR (75 MHz, DMSO-d_6) 170.2 (C-6″), 163.4 (C-2), 158.0 (C-2′), 151.2 (C-7), 149.8 (C-9), 145.9 (C-5), 132.1 (C-4′), 131.3 (C-6), 129.2 (C-6′), 120.8 (C-5′), 119.0 (C-1′), 112.6 (C-3′), 110.2 (C-3), 99.2 (C-1″), 93.7 (C-8), 75.5 (C-5″), 75.2 (C-3″), 72.2 (C-2″), 71.5 (C-4″), 56.1 (OCH_3); the signals for C-10 and C-4 were not clearly distinguished due to the small amount of compound available; ESI-MS m/z (rel int) 476 $[M]^-$ (24), 475 $[M - H]^-$ (100), 299 $[M - H -$ glucuronyl$]^-$ (10); HRMS (MALDI-TOF) m/z 477.0490 (calcd for $C_{22}H_{21}O_{12}$, $[M + H]^+$ 477.1033).

Acid Hydrolysis of 5,6-Dihydroxy-7-glucuronyloxy-2′-methoxyflavone (8). Compound **8** (1.0 mg) was hydrolyzed for 3 h under reflux conditions in 2 mL of 2 N HCl. The solution was cooled, and 2 mL was directly injected into the HPLC. The hydrolysis product was identified as 5,6,7-trihydroxy-2′-methoxyflavone (**5**) on the basis of the UV- and MS-spectral data. An HPLC-UV/MS analysis, using the conditions mentioned above, allowed the identification of **5** in the skullcap extract as well.

Acknowledgment. The authors wish to acknowledge Dr. David R. Sibley, Chief of the Molecular Neuropharmacology Section in the NIH National Institute of Neurological Disorders and Stroke, for providing the CHO cells. We would like to thank Dr. Tony Durst for running the NMR experiments and Dr. Devenand Pinto for the high-resolution mass spectral data. The work of J.E.B. has been supported by the UIC/NIH Center for Botanical and Dietary Supplements Research, NIH Grant P50 AT00155.

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NP0205102