Alkaloids from *Eschscholzia californica* and Their Capacity to Inhibit Binding of [^{3}H]8-Hydroxy-2-(di-*N*-propylamino)tetralin to 5-HT$_{1A}$ Receptors in Vitro[#]

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A 70% ethanol extract of California poppy (*Eschscholzia californica*) was able to bind to 5-HT$_{1A}$ and 5-HT$_7$ receptors at 100 μg/mL. The subsequent isolation procedure yielded the known alkaloids californidine (**1**), escholtzine (**2**), *N*-methyllaurotetanine (**3**), caryachine (**4**), and *O*-methylcaryachine (**5**), along with a new pavine alkaloid, 6*S*,12*S*-neocaryachine-7-*O*-methyl ether *N*-metho salt (**7**). The structure of **7** was determined by spectroscopic data interpretation, while the absolute stereochemistry was determined by means of circular dichroism. From the results obtained from the radioligand-binding assay of the pure compounds, including the commercially available protopine (**6**), it was evident that the activity on the 5-HT$_{1A}$ receptor was at least partly due to the presence of the aporphine alkaloid **3**, which showed the highest inhibition of [^{3}H]8-hydroxy-2-(di-*N*-propylamino)tetralin ([^{3}H]8-OH-DPAT) binding with an EC$_{50}$ value of 155 nM and a K_i of 85 nM.

California poppy (*Eschscholzia californica* Cham., Papaveraceae) is an annual plant found throughout California, usually below 5000 feet, and in parts of Oregon and Washington. The densest populations occur west and south of the Sierra Nevadas to the Mojave Desert. The main medicinal uses of the plant have been as a sedative and anxiolytic; these activities have been confirmed in several studies.[1−3] The mechanisms of action for the sedative and anxiolytic activities have not been clearly established, although Rolland and co-workers[3] suggested an involvement of the benzodiazepine receptors. In addition, some of the activities may be due to an inhibition of the degradation of catecholamines by inhibiting the enzymes dopamine-β-hydroxylase and monoamine oxidase B.[4] In general, the alkaloids present in California poppy are thought to be responsible for the sedative and anxiolytic action, although no studies have been carried out with isolated compounds to confirm this. Overviews on California poppy alkaloids have been published by Fabre et al.[5] and Slavík and Slavíková.[6] Other potentially active compounds are the flavonoids, which occur mainly as glycosides of quercetin and isorhamnetin.[7]

The aim of this study was to test the binding capacity of an aqueous alcohol extract of *Eschscholzia californica* on some of the most important receptors involved in sedation and anxiolysis, and to relate the activity to compounds present in the extract. The GABA−receptor complex was not included in the test series, as the amount of GABA present in the extract may have led to a false positive outcome.

The results of the receptor screening are listed in Table 1. Based on the 50% inhibition criteria set for an active extract, the focus was set on the serotonin receptors. A literature survey showed that some alkaloids are potent ligands for the 5-HT$_{1A}$ receptor,[8−10] while for flavonoid glycosides, no data were found to suggest a possible interaction with this receptor. To confirm that the alkaloids were indeed the ligands for the 5-HT$_{1A}$ receptor, the aqueous alcohol extract was fractionated and six alkaloids were isolated. The main alkaloids were identified as californidine (**1**) and escholtzine (**2**), while the minor alkaloids included *N*-methyllaurotetanine (**3**), caryachine (**4**), and *O*-methylcaryachine (**5**), on the basis of comparison of UV, MS, and MS^2 data and HPLC retention times with published data.[5]

Table 1. Receptor Screening with a 70% Aqueous EtOH Extract of *E. californica* (9 μg/mL)[a]

receptor	inhibition (% ± STDEV)	positive control (EC$_{50}$ in nM)
adenosine A$_1$	21 ± 5	1200
adrenergic α_{2A}	30 ± 7	8.5
adrenergic β1	32 ± 3	1.8
cannabinoid CB$_1$	−4 ± 4	37.6
cholecystokinin CCK$_2$	15 ± 10	0.093
histamine H$_1$	9 ± 1	2.6
serotonin (5-HT$_{1A}$)	64 ± 4	7.5
serotonin (5-HT$_7$)	76 ± 1	0.60
tachykinin NK$_1$	5 ± 2	24.4
tachykinin NK$_2$	4 ± 1	78.1

[a] Positive controls were *R*-(−)-PIA (adenosine A$_1$), yohimbine (adrenergic α_{2A}), *S*-(−)-propanolol (adrenergic β_1), *R*-(+)-WIN-55,212−2 (cannabinoid CB$_1$), sincalide (cholecystokinin CCK$_2$), mepyramine (histamine H$_1$), metergoline (5-HT$_{1A}$), methiothepin (5-HT$_7$), L-703,606 (tachykinin NK$_1$), and MEN-10376 (tachykinin NK$_2$), respectively.

The remaining alkaloid had the same molecular weight as protopine (**6**), an alkaloid that has been isolated previously from the California poppy. However, its HPLC retention time differed from a commercially available protopine standard. The HRFABMS data of *m*/*z* 354.1722 established the molecular formula as $C_{21}H_{24}NO_4$. The ^{1}H NMR spectrum showed four aromatic protons, with one AB system (δ 6.89 and 6.82, both d, J = 8.4 Hz) and two singlets at δ 6.76 and 6.47. Other signals were attributed to an AB system of a methylenedioxy group (δ 5.95 and 5.92, both s), two methoxyl groups at δ 4.03 and 3.86, two *N*-methyl groups at δ 3.58 and 3.56, and the signals for two AMX spin systems with one system appearing at δ 5.50 (d, J = 4.8 Hz), 3.78 (dd, J = 17.4, 4.8 Hz), and 3.07 (d, J = 17.4 Hz) and the other appearing at δ 5.04 (d, J = 4.8 Hz), 3.55 (dd, J = 17.4, 4.8 Hz), and 3.07 (d, J = 17.4 Hz). On the basis of the presence of two aliphatic AMX

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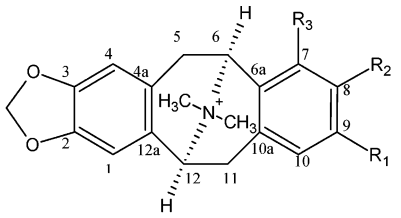
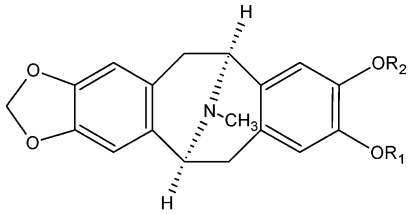
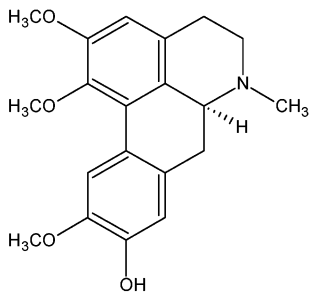


1 $R_1 + R_2 = CH_2OCH_2$, $R_3 = H$
7 $R_1 = H$, $R_2 = R_3 = OCH_3$



2 $R_1 + R_2 = CH_2$
4 $R_1 = CH_3$, $R_2 = H$
5 $R_1 = R_2 = CH_3$



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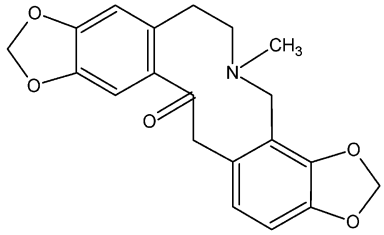

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systems, and the shift of the *N*-methyl protons, the base structure of the molecule was established as that of a quaternary pavine-type alkaloid. The coupling pattern on the aromatic rings indicated that the protons had to be in a *para* position to each other on one ring and in *ortho* position on the other. According to Miura and co-workers,[11] a ^{13}C NMR shift above 60 ppm gives evidence of an *ortho*-disubstituted methoxyl group, which was the case for one of the two methoxyl groups in the alkaloid (δ 62.0 and 56.1). Therefore, the methoxyl groups had to be in positions C-7 and C-8 (or C-9 and C-10), while the methylenedioxy group had to be attached to C-2 and C-3. Finally, three-bond HMBC correlations from H-10 (δ 6.82) to C-11 (δ 33.6), C-6a (δ 125.3), and C-8 (δ 151.8) established the structure as neocaryachine-7-*O*-methyl ether *N*-metho salt (**7**).

The absolute stereochemistry of **7** was determined by means of circular dichroism. The spectrum obtained was compared with the CD data published for the pavine alkaloids munitagenine[12] and argemonine.[13,14] The signs of the two CD bands associated with the α absorption band system were observed from negative to positive from longer to shorter wavelengths; hence the chirality of the transition moments was anticlockwise for an overall negative circular dichroism, consistent with a 6*S*,12*S* stereochemistry. An enantiomeric configuration would have positive and then negative Cotton effects observed from a longer to a shorter wavelength.

Table 2. Inhibition of [^{3}H]8-OH-DPAT Binding to the 5-HT_{1A} Receptor by Alkaloids from *E. californica*[a]

compound	EC_{50} (μM) ± SD	K_i (μM) ± SD
1	>100	n.a.
2	11 ± 4	6 ± 3
3	0.16 ± 0.01	0.085 ± 0.001
4	9 ± 7	5 ± 4
6	>100	n.a.
quercetin	>100	n.a.

[a] The competitive binding capability of the sample was calculated in comparison with the inhibition of 10 μM 5-HT (100%).

For the purpose of this study, binding studies with pure compounds were restricted to the 5-HT_{1A} receptor. It is generally accepted that the 5-HT_{1A} subtype is involved in psychiatric disorders such as anxiety and depression. The capacity of individual alkaloids to inhibit [^{3}H]8-OH-DPAT binding to the 5-HT_{1A} receptor is shown in Table 2. Not surprisingly, the aporphine alkaloid *N*-methyllaurotetanine was most active, with a K_i value of 85 nM. Some aporphine alkaloids are known to be potent, highly specific 5-HT_{1A} agonists and antagonists.[9,10] The activity of **3** was comparable to values for other aporphine alkaloids published in the literature (although there are some more potent synthetic derivatives) and is considered to be responsible to a considerable extent for the strong activity of the 70% aqueous alcohol extract. Some authors have reported that small structural changes in the basic aporphine structure may lead to a drastic change in pharmacological profiles.[9,10] In particular, the absolute stereochemistry at the chiral center and the substitution pattern on the aromatic moiety determine whether the alkaloid exerts serotonergic activities and whether it acts as an agonist or an antagonist.

Minor aporphine alkaloids such as glaucine or isocorydine, previously isolated from California poppy, have not been evaluated in this study, but may contribute to the strong interaction of the 70% aqueous alcohol extract with the 5-HT_{1A} receptor. Further studies are needed to confirm this hypothesis. The other alkaloids showed only weak inhibition of radioligand-binding to the receptor, with the quaternary pavine **1** and protopine (**6**) as the least active at 100 μM. Quercetin, which was the only flavonoid tested on the 5-HT_{1A} receptor, was devoid of any activity.

Despite the longstanding use of California poppy as a medicinal plant, there are some concerns about its safety, mainly due to the presence of the alkaloids. Many alkaloids are used as therapeutic agents with strong pharmacological activities, but often with a relatively narrow therapeutic range. One safety concern is the use of an herbal product concomitantly with a prescription drug, thus leading to the possibility of herb−drug interactions. Many herb−drug interactions occur through the modulation of cytochrome P450 (CYP) activities, in particular CYP3A4, through which many prescription drugs are metabolized.[15] Therefore, the 70% aqueous alcohol extract (containing 1.82% alkaloids, calculated as the sum of **1**−**3**) of *E. californica* was submitted to an in vitro CYP3A4 inhibition assay. This extract showed some enzyme inhibitory activity with an IC_{50} value of 128.6 μg/mL (±61.8 μg/mL), prompting us to look at the CYP3A4 inhibitory activities of some of the major alkaloids and flavonoids present in the extract. Escholtzine (**2**) (IC_{50} = 13.4 ± 4.7 μM) proved to be a strong inhibitor of CYP3A4 in vitro, while the other compounds tested (**1**, **6**, rutin, and isorhamnetin-3-*O*-rutinoside) were less active (IC_{50} > 80 μM). The results provide further evidence that compounds with a methylenedioxyphenyl moiety, such as escholtzine, can strongly inhibit CYP3A4. However it is noteworthy that **1**, a quaternary alkaloid, which differs only by the presence of an additional methyl group on the nitrogen, was significantly less active than **2**. Although such in vitro tests do not represent the conditions

found in humans, caution should be used when *E. californica* preparations are taken concomitantly with drugs metabolized by CYP3A4.

Experimental Section

General Experimental Procedures. Protopine HCl (**6**) and isorhamnetin-3-*O*-rutinoside were purchased from Extrasynthèse SA (Genay, France). HPLC grade acetonitrile was from Fisher Scientific (Pittsburgh, PA) or from Pharmaco (Brookfield, CT). Dibenzylfluorescein was purchased from GENTEST (Woburn, MA). [^{3}H]5-HT and [^{3}H]8-OH-DPAT were obtained from NEN Life Science Products (Boston, MA). All other chemicals were ordered from Sigma Chemical Co. (St. Louis, MO).

A Sanki CPC Model LLN instrument was used for centrifugal partition chromatography. The preparative HPLC system consisted of a Waters 600 quaternary pump, a Waters 486 UV/vis detector (Waters, Milford, MA), and a Rheodyne 2575i manual injector. Columns: YMC ODS-A (5 μm; 150 × 30 mm i.d., Waters, Milford, MA) or Zorbax Eclipse XDB C18 (5 μm; 150 × 21 mm i.d., Agilent Technologies, Burlington, MA). The flow rate was 20 mL/min for the YMC column and 12 mL/min for the Zorbax column. ^{1}H NMR and ^{13}C NMR data were collected on a Bruker Avance DRX instrument at 600 and 150 MHz, respectively. The MS and MS^2 data were obtained with an Agilent 1100 series LC/MSD trap. High-resolution FABMS data were obtained with a JEOL MS route instrument in positive ion mode. Ninety-six-well microtiter plates (model #CSOO-3632) were purchased from Corning Costar (Corning, NY). A Millipore Cytofluor 2350 fluorescence measurement system (Millipore, Bedford, MA) was used for fluorescence measurements. Radioactivity measurements were carried out with a Wallac 1450 Microbeta liquid scintillation counter (Perkin-Elmer Life Sciences, Boston, MA). The EC_{50}, K_d, and K_i values were determined with GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA, www.graphpad.com).

Plant Material. *Eschscholzia californica* Cham. herb (lot # L7976WOC) was obtained from Blessed Herbs (Oakham, MA). A voucher specimen (No. TOM02003) was deposited at the Tom's of Maine herbarium.

Extraction and Isolation. The aboveground parts (1.2 kg) were extracted with 70% aqueous EtOH (ratio plant material−solvent 1:10) for 24 h on a platform shaker. For the isolation procedure, a protocol by Bugatti et al.[16] was followed initially, although it proved to be difficult to remove the ion-pairing agent (sodium lauryl sulfate, SLS) in the final purification steps. The aqueous alcohol extract (240 g in 1.5 L) was brought to approximately pH 1 with sulfuric acid and treated with 40 g of SLS. The solution was partitioned twice with 600 mL of chloroform to yield approximately 10.5 g of alkaloid-rich fraction.

This fraction was submitted to CPC in ascending mode using the upper layer of a solvent system containing hexane−ethyl acetate−MeOH−water (2:3:5:3) to afford nine fractions (A1−A9). Combined fractions A3 to A5 contained mainly alkaloids **1** and **2**. These fractions were evaporated to dryness and treated with perchloric acid in MeOH to precipitate 220 mg of **1** in the form of a perchlorate. Combined fractions A1 and A2 were subjected to CPC in descending mode, using the lower phase of a mixture of toluene−chloroform−MeOH−water (6:2:5:1) as solvent to yield 12 fractions (B1−B12). Fraction B3 was treated with perchloric acid to give 38 mg of **2** in the form of a perchlorate. Compounds **3** (13 mg), **4** (13 mg), **5** (5.0 mg), and **7** (1.0 mg), contained in fractions B5, B6, and B8−B11, were obtained after a purification step by preparative HPLC, using the same conditions as for the purification of **2**.

6*S*,12*S*-Neocaryachine-7-*O*-methyl ether *N*-metho salt (7): amorphous solid; $[\alpha]^{23}_D$ −170 (*c* 0.14, MeOH); CD (*c* 5.08 × 10^{-4} M, MeOH) $[\theta]_{308.0}$ −1195, $[\theta]_{276.5}$ −3739, $[\theta]_{256.5}$ −1277, $[\theta]_{248.0}$ −2464, $[\theta]_{241.5}$ +23, $[\theta]_{237.5}$ +1890, $[\theta]_{232.0}$ −33, $[\theta]_{209.5}$ −94332, $[\theta]_{202.5}$ −153, $[\theta]_{198.0}$ +30561; ^{1}H NMR ($CDCl_3$, 600 MHz) δ 6.89 (1H, d, J = 8.4 Hz, H-9), 6.82 (1H, d, J = 8.4 Hz, H-10), 6.76 (1H, s, H-1), 6.47 (1H, s, H-4), 5.95 and 5.92 (both 1H, s, −OCH_2O−), 5.50 (1H, d, J = 4.8 Hz, H-12), 5.04 (1H, d, J = 4.8 Hz, H-6), 4.03 (3H, s, OCH_3-7) 3.86 (3H, s, OCH_3-8), 3.78 (1H, dd, J = 17.4, 4.8 Hz, H-11a), 3.58 (3H, s, N-CH_3), 3.56 (3H, s, N-CH_3), 3.55 (1H, dd, J = 17.4, 4.8 Hz, H-5a), 3.07 (2H, each d, J = 17.4 Hz, H-5b, H-11b); ^{13}C NMR ($CDCl_3$, 150 MHz) δ 151.8 (C-8), 149.8 (C-2), 148.5 (C-3), 145.7 (C-7), 125.3 (C-6a), 124.8 (C-12a), 124.4 (C-10), 120.4 (C-4a), 119.4 (C-10a), 113.9 (C-9), 108.3 (C-4), 107.6 (C-1), 101.8 (−OCH_2O−), 65.4 (C-12), 62.6 (C-6), 62.0 (OCH_3-7), 56.1 (OCH_3-8), 51.0 (N-CH_3), 51.0 (N-CH_3), 33.6 (C-11), 32.5 (C-5); HRFABMS *m/z* 354.1722 (calcd for $C_{21}H_{24}NO_4$, $[M]^+$ 354.1705).

Receptor Screening. The radioligand-binding assays were performed with a 70% aqueous EtOH extract according to the protocols outlined by MDS Pharma (Bothell, WA). All the studies were carried out at a concentration of 9 μg/mL extract dissolved in DMSO and diluted with assay buffer to a final concentration of 1% DMSO. Each assay was performed in duplicate. The adenosine A_1 binding assay was carried out using human recombinant CHO cells as receptor source and 1 nM of [^{3}H]DPCPX as ligand, according to the protocol of Libert and coworkers.[17] For the α_{2A}-adrenergic assay, human recombinant Sf9 cells were used as source and the ligand was 1 nM [^{3}H]MK-912.[18] The source of β_1-adrenergic receptors was human recombinant Rex16 cells, and the ligand was [^{125}I]cyanopindolol at a 0.03 nM concentration. The assay was carried out according to Feve et al.[19] Human recombinant HEK-293 cells were used as a source for the cannabinoid CB1 receptor, and 8 nM [^{3}H]WIN-55,212-2 was used as the ligand.[20] The cholecystokinin CCK_2 binding assay was carried out with human FGS-7 Jurkat cells as receptor source and 0.072 nM [^{125}I]CCK-8 as ligand.[21,22] For the histamine H_1 assay, human recombinant CHO-K1 cells were used as source and the ligand was 1.2 nM [^{3}H]pyrilamine.[23] The source of 5-HT_{1A}, 5-HT_7, and tachykinin NK_1 and NK_2 receptors was human recombinant CHO cells, and the ligands were 1.5 nM [^{3}H]8-OH-DPAT, 5.5 nM [^{3}H]lysergic acid, 0.25 nM [^{3}H]SR-140333, and 0.5 nM [^{3}H]-SR-48968, respectively.[24−27]

Binding Assay with the 5-HT_{1A} Receptor.[28] The 5-HT_{1A} assays were performed with human recombinant CHO cell membranes (Perkin-Elmer Life Sciences, Boston, MA). Membranes were incubated in buffer (75 mM Tris-HCl, 1.25 mM $MgCl_2$, 1 mM EDTA, 0.1% L-ascorbic acid, 10 μM paragyline, pH 7.4) with [^{3}H]8-OH-DPAT (1.6 nM) at 37 °C for 60 min. After the incubation, the mixture was filtered over 934-H Whatman filters (presoaked in 0.5% polyethyleneimine) and washed twice in ice-cold 50 mM Tris buffer (pH 7.4) using a 96-well Tomtec-Harvester (Tomtec, Orange, CT). Each filter was dried, suspended in Wallac Microbeta plate scintillation fluid, and counted with a Wallac 1450 Microbeta liquid scintillation counter (Perkin-Elmer Life Sciences, Boston, MA). 5-Hydroxytryptamine (10 μM) was used to define nonspecific binding, which accounted for less than 10% of total binding. The percent inhibition of [^{3}H]8-OH-DPAT binding to the 5-HT_{1A} receptor was determined as $[1 - (dpm_{sample} - dpm_{nonspec\ bound})/(dpm_{DMSO} - dpm_{nonspec\ bound})] \times 100$. For the most potent compounds, EC_{50} values were determined by evaluation of the percent inhibition of [^{3}H]8-OH-DPAT binding in a number of serial dilutions. The data represent the average of triplicate determinations. The K_d and K_i values (for [^{3}H]8-OH-DPAT, K_d: 1.9 nM) were calculated using the equations for a single-substrate single-inhibitor model and the GraphPad software.

Cytochrome P450 3A4 (CYP3A4) Enzyme Inhibition Assay. The 70% aqueous EtOH extract was tested for its ability to inhibit cytochrome P450 3A4 in vitro using the fluorometric microtiter plate assay according to the method outlined by Budzinski et al.[29] Test samples were prepared by dissolving the extract at a concentration of 4 mg/mL in 50% aqueous EtOH. Volumes of 4 μL sample solution + 6 μL distilled deionized water were added per test well to keep EtOH at 1% of total well volume (200 μL). Assays were performed in clear-bottom, opaque-welled microtiter plates. Control wells consisted of solvent blank (10 μL of 20% aqueous EtOH), NADPH solution, and live enzyme solution. Control blank wells consisted of solvent blank, NADPH solution, and denatured enzyme solution. Test wells consisted of sample solution, NADPH solution, and live enzyme solution. NADPH was mixed with 0.5 M potassium phosphate buffer to yield a 0.9 mg/mL solution. Enzyme stock solutions were made by prewarming the water/buffer mixture to 37 °C for 10 min, adding the substrate (dibenzylfluorescein, 0.2 mmol/L), vortexing the mixture for 5 s, and adding enzyme. Test-blank wells consisted of sample solution, NADPH solution, and dead enzyme solution. Denatured enzyme was made by boiling live enzyme (CYP3A4, GENTEST, Woburn, MA) for 10 min.

Solutions were added to the appropriate designated wells in the following order: 10 μL of solvent blank or sample solution, 100 μL of NADPH solution, and 90 μL of enzyme solution. Then, the plate was incubated for 1 h at 37 °C. After incubation, 100 μL of stop solution consisting of 2.0 N sodium hydroxide was added, and plates were agitated for 10 s on a Titer Plate Shaker (Lab-Line Instruments, Melrose Park, IL). The fluorescence measurement system was set to a 530 nm

(30 nm bandwidth) excitation filter, and a 590 nm (35 nm bandwidth) emission filter was used to analyze each plate.

Supporting Information Available: The NMR spectra (^{1}H NMR, ^{13}C NMR, COSY, and HMBC) of **7** and the LC-MS analysis profile and spectra of the 70% aqueous ETOH extract of *E. californica*. This material is available free of charge via the Internet at http://pubs.acs.org.

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