Luteolin, a Compound with Adenosine A_1 Receptor-Binding Activity, and Chromone and Dihydronaphthalenone Constituents from *Senna siamea*

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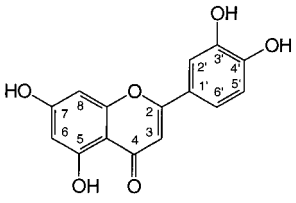
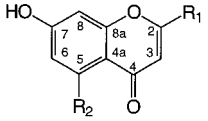
Activity-guided fractionation led to the isolation of luteolin (**1**) from the leaves of *Senna siamea* (syn. *Cassia siamea*). This compound was found to be an antagonist at the adenosine A_1 receptor with a K_i value in the low micromolar range. Four additional nonactive compounds (**2**−**5**) were also isolated, and their structures were elucidated. One compound was identified as cassia chromone (5-acetonyl-7-hydroxy-2-methylchromone) (**2**). Three other compounds are new, and they were identified as 5-acetonyl-7-hydroxy-2-hydroxymethyl-chromone (**3**), 4-(*trans*)-acetyl-3,6,8-trihydroxy-3-methyldihydronaphthalenone (**4**), and 4-(*cis*)-acetyl-3,6,8-trihydroxy-3-methyldihydronaphthalenone (**5**).

Senna siamea (Lam.) Irwin & Barneby (syn. *Cassia siamea* Lam.) (Fabaceae), known as "Kheelek" in Thai, has been reported to contain anthraquinones, alkaloids, flavonoids, chromones, and terpenoids.[1,2] It is used widely in Thailand and the rest of Southeast Asia as a food plant and in herbal medicine. Some pharmacological effects of the extract from this plant have been studied. In 1949, Arunlakshana reported the effect of an alcoholic extract from the leaves of *S. siamea* on the central nervous system, on smooth muscle, and on diuresis.[3] The diuretic effect was observed again by Aswal et al. in 1984.[4] Thongsaard et al., in 1996 reported the anxiolytic effect of barakol, a compound extracted from this plant.[5]

Using an adenosine A_1 receptor binding assay[6] as a screening method, we found that the ethanolic extract of the leaves from this plant showed in vitro binding activity to the receptor. Therefore, a further investigation was performed, resulting in the isolation of the active compound, luteolin (**1**), along with four other compounds (**2**−**5**).

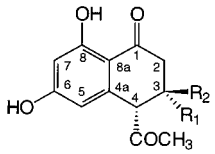


1



2 R_1=CH_3, R_2=CH_2COCH_3

3 R_1=CH_2OH, R_2=CH_2COCH_3



4 R_1=CH_3, R_2=OH

5 R_1=OH, R_2=CH_3

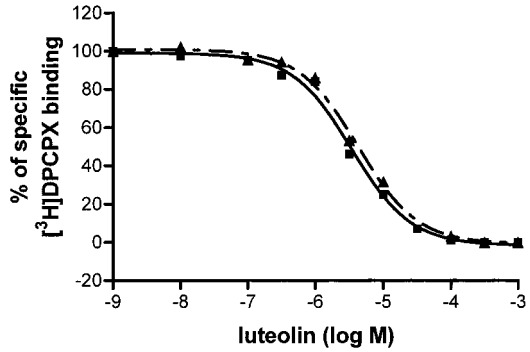


Figure 1. Displacement of [^{3}H]DPCPX binding to adenosine A_1 receptor by luteolin (**1**) in the presence (▲) and absence (■) of 0.5 mM GTP. Data are from one typical experiment performed in duplicate.

Results and Discussion

Ethanolic extract of the leaves of *S. siamea* was fractionated by centrifugal partition chromatography (CPC) using the procedure that has been developed as a general prefractionation step before bioactivity screening.[7] The fractions were screened in an adenosine A_1 receptor-binding assay at a concentration of 0.1 mg/mL. Apart from the fatty-acid-rich fractions, which showed noncompetitive binding activity in this assay,[8] a strong activity was found in another fraction, indicating high affinity to the receptor. The active principle was isolated and identified as the known flavonoid luteolin (**1**) by comparing spectral data with the literature values.[9] The K_i value of this compound was determined as 1.66 ± 0.69 μM, and the K_i value measured in the presence of 0.5 mM guanosine 5′-triphosphate (GTP) was 1.55 ± 0.25 μM (Figure 1). As GTP did not show a significant effect on the binding activity of **1**, it is most likely that **1** acts as an antagonist in the assay.[10] The affinity of this flavone for the adenosine A_1 receptor has not been reported before. This affinity is relatively high in comparison to those of caffeine and theophylline, well-known adenosine A_1 antagonists, which have K_i values of 29 and 8.5 μM, respectively.[11] Ji et al. also reported on the interaction of some other flavonoids with adenosine receptors at micromolar concentrations and discussed the structure−activity relationships.[12] They suggested that the carbonyl group at the C-4 position promoted affinity at adenosine receptors, while hydroxyl groups on both rings A and B of flavones had no effect on adenosine

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A_1 affinity. Some other flavonoids have been shown to be active at adenosine receptors as well.[13,14]

In the present investigation, four additional compounds were obtained. The MS and ^{1}H NMR spectral data of **2** corresponded to 5-acetonyl-7-hydroxy-2-methylchromone (cassia chromone), which has been reported before from this plant.[1,2,15] The proton assignments were in agreement with those of Wagner et al.[1]

In the MS, compound **3** exhibited a molecular ion at 248, which was 16 amu different from compound **2**. The ^{1}H NMR spectra of **3** differed from that of **2** by the absence of one methyl group and the presence of an additional methylene group at 4.44. The downfield chemical shift suggested that the methylene carbon was attached to a hydroxyl group. This methylene hydroxyl signal showed a coupling with H-3 (J = 0.9 Hz). Moreover, from the HMBC spectrum, the long-range correlation of the methylene proton to C-2,C-3 and of H-3 to C-4a,C-2 and the methylene carbon suggested the location of the methylene hydroxyl group at C-2. The rest of the carbon resonances of **3** were assigned by its HMQC and HMBC spectral data. Thus, the structure of **3** was identified as 5-acetonyl-7-hydroxy-2-hydroxymethylchromone, which is a novel natural product.

Compounds **4** and **5** showed similar UV spectra and exhibited an identical molecular weight of 250 in an LC−MS experiment. They were then purified by HPLC. The ^{1}H NMR spectra of both compounds showed the same pattern, consisting of two signals of methyl protons at δ 1.34 and 2.34 for **4** and at δ 1.27 and 2.35 for **5**, a pair of geminal protons at δ 2.47 (J = 1.2,17 Hz) and 3.13 (J = 17 Hz) for **4** and at δ 2.45 (J = 1.2,17 Hz) and 3.13 (J = 17 Hz) for **5**, a methine proton at δ 4.14 for **4** and at δ 4.16 for **5**, and a pair of *meta*-aromatic protons at δ 6.16 (J = 2.2 Hz) and 6.27 (J = 2.2 Hz) for **4** and at δ 6.13 (J = 2.2 Hz) and 6.18 (J = 2.2 Hz) for **5**. The molecular weight and the number of carbons determined by ^{13}C NMR suggested the molecular formula $C_{13}H_{14}O_5$, indicating the presence of seven double-bond equivalents in the molecule. The chemical shifts from the ^{13}C NMR spectra showed the presence of two carbonyl signals resonating at δ 208.3 and 202.3 for **4** and at δ 209.5 and 203.1 for **5**, and six signals in the aromatic region. This information suggested that the structures of these two isomers might consist of a benzene ring, two carbonyls, and an aliphatic ring.

2D NMR experiments (COSY, NOESY, HMQC, and HMBC) showed the various correlations between the signals and allowed the complete assignment of hydrogens and carbons of compounds **4** and **5**. In compound **4**, the long-range correlation of the methine proton H-4 to the aromatic carbons C-4a, C-5, and C-8a and the long-range correlation of this proton to the aliphatic protons H-3 and H-2 suggested that the benzene ring is connected to the aliphatic ring at C-4a and C-8a. Moreover, H-4 showed a long-range correlation to the two carbons from the acetyl group, indicating the location of the acetyl side chain at C-4. The long-range correlation of H-2β to the carbonyl carbon C-1 and the aromatic carbon C-8a established the dihydronaphthalenone structure. The assignment of C-5 and C-7 was confirmed by the fact that in the NOESY experiment, H-5 showed a cross-peak to the acetyl proton and H-4. Also, the assignment of C-6 and C-8 was confirmed by the correlation of H-5 to C-6 in the HMBC spectrum. Based on these results, we identified **4** as 4-acetyl-3,6,8-trihydroxy-3-methyldihydronaphthalenone.

Compound **4** is a diastereoisomer of **5** because it has the same chemical structure as **5** based on 1D and 2D NMR experiments, including HMQC and HMBC data, but has a different stereochemistry based on NOESY experiments. In both compounds, the *W*-couplings of H-2β and H-4 observed in the ^{1}H−^{1}H COSY spectrum suggested equatorial orientations for these protons. In addition, in the H NMR spectrum, doublet of doublet signals of H-2β (J = 17, 1.2 Hz) and the broad singlet signals of H-4 in both compounds were observed. In the NOESY experiment, NOEs between the methyl group at C-3 and the methyl group of the acetyl side chain at C-4 were observed in **4** but not in **5**. Therefore, the methyl group at C-3 and the acetyl group at C-4 of **4** are on the same side of the molecule, which are equatorial and axial orientations, respectively. This led to the relative configuration elucidation of **4** as 4-(*trans*)-acetyl-3,6,8-trihydroxy-3-methyldihydronaphthalenone. Compound **5**, of which the methyl group at C-3 and the acetyl group at C-4 are in an axial orientation, was thus identified as 4-(*cis*)-acetyl-3,6,8-trihydroxy-3-methyldihydronaphthalenone. Both compounds have never been reported before. The absolute configurations of **4** and **5** could not be determined.

The affinities of compounds **2**−**5** for adenosine A_1 receptors were also determined. Despite the presence of the carbonyl groups similar to flavonoids, the affinities of these compounds were very low (K_i > 50 μM).

Experimental Section

General Experimental Procedures. A modular Sanki (Kyoto, Japan) centrifugal partition chromatograph (type LLN) was used. It consisted of a power supply (model SPL), a triple-head constant-flow pump (model LBP-V), and a centrifuge (model NMF). A Panasonic Pen-recorder (model VP 67222A) was connected to a UVIS 200 detector (Linear Instruments, Reno, NV). Fractions were collected by means of a LKB 2211 Superrac fraction collector. In all experiments, six cartridges (total internal volume 125 mL) were used. The pressure was limited to 60 bar. The flow rate was set to 2 mL/min. The fraction size was 8 mL unless stated otherwise.

TLC analyses were performed on 20 × 10 cm Si gel plates F_{254} no. 5554 (Merck, Darmstadt, Germany) and developed in saturated TLC chambers that were preequilibrated for about 30 min. Two TLC solvent systems, that is, $CHCl_3$−MeOH (9:1) and EtOAc−formic acid−acetic acid−water (100:11:11:27) were used. The visual detection was performed under UV 254 nm and UV 366 nm. Then, each TLC plate was sprayed with modified anisaldehyde−sulfuric acid spray reagent. After spraying, the plates were heated with a hot-air blower for 2 min.

An LKB pump type 2150 (Bromma, Sweden), a Waters 710B WISP autosampler, and Waters 990 photodiode array detector were used with a 10-μm Bondapack C_{18} 300 × 7.8 mm (i.d.) preparative column (Waters, Milfort, MA) for the last purification step of **1** and a 5-μm Hypersil C_{18} 250 × 4.6 mm (i.d.) column (Shandon, Cheshire, England) for the isolation of **4** and **5**. LC−MS and LC−MS−MS were acquired using a Finnigan MAT TSQ-700 instrument equipped with a custom-made electrospray interface (ESI). UV spectra (in MeOH) were measured on a Cary 1Bio UV−vis spectrophotometer. IR spectra were recorded with a SP3−200 infrared spectrophotometer. NMR spectra were measured on Bruker DPX-300 and on Bruker DMX-600 spectrometers. Standard pulse sequences were used for COSY, HMQC, HMBC ($^NJ_{C-H}$ = 8.3 Hz), and NOESY (with a mixing time of 1 s).

Chemicals. GTP was obtained from Sigma (St. Louis, MO). [^{3}H]1,3-dipropyl-8-cyclopentylxanthine ([^{3}H]DPCPX) was obtained from NEN (Du Pont Nemours, 's-Hertogenbosch, The Netherlands). N^6-Cyclopentyl adenosine (CPA) was obtained from RBI (Natick, MA). Anisaldehyde was purchased from Acros Organic (Geel, Belgium). All organic solvents (analytical reagent grade) were purchased from J. T. Baker (Deventer, The Netherlands).

Plant Material. Leaves of *S. siamea* were collected from Naresuan University, Phitsanulok, Thailand, in January 1998.

A voucher specimen (19980001) is deposited at the Department of Medicinal Chemistry and Pharmacognosy, Faculty of Pharmaceutical Sciences, Naresuan University, Thailand.

Extraction and Isolation. Dried leaves of *S. siamea* were extracted with ethanol, and the extract was then evaporated under reduced pressure, yielding a brownish residue. Three grams of this extract was separated by CPC with the solvent system heptane−EtOAc−MeOH−water (6:1:6:1). Ascending-mode elution was performed to collect 20 fractions, then the mode of elution was changed to descending to collect another 20 fractions. The run was repeated five times to inject 15 g of extract in total. The fractions were combined based on TLC patterns and tested in the adenosine A_1 receptor-binding assay. Fraction 16 (4 g) was loaded into a CPC (a two-phase system, $CHCl_3$−MeOH−water (7:13:8). Descending-mode elution was performed to collect 20 fractions; afterward the ascending mode was used to collect another 20 fractions. Fractions were once again pooled based on TLC to give nine pools (16/1−16/9). Fraction 16/4 (70 mg) was separated by CPC, solvent system EtOAc−EtOH−water (10:3:10). Thirty fractions of 4 mL each were obtained in ascending mode, and 20 fractions were obtained in descending mode. Using HPLC with MeOH−water−acetic acid (60:40:0.1) as eluent, compound **1** (4 mg) was obtained from fraction 16/4/1. Compounds **4** (2 mg) and **5** (3 mg) were isolated from fraction 16/4/2 by HPLC using MeOH−water−acetic acid (28:72:0.5) as eluent. Compound **2** (30 mg) was obtained from the separation of fraction 16/2 (676 mg) by CPC with the solvent system heptane−EtOAc−MeOH−water (4:6:4:6), using descending mode for fractions 1−20 and ascending mode for fractions 21−40. Compound **3** (10 mg) was obtained from the separation of fraction 16/5 (270 mg) by CPC with the solvent system EtOAc−EtOH−water (7:3:10), using ascending mode for fractions 1−20 and descending mode for fractions 21−40.

5-Acetonyl-7-hydroxy-2-hydroxymethylchromone (3): colorless solid; UV (MeOH) λ_{max} (log ϵ) 206 (4.20), 248 (3.95), 280 (3.79) nm; IR (KBr) ν_{max} 3400, 3100, 2910, 1690, 1650, 1610, 1360, 1150 cm^{-1}; ^1H NMR (CD_3OD, 600 MHz) δ 6.76 (1H, d, J = 2.4 Hz, H-8), 6.62 (1H, d, J = 2.4 Hz, H-6), 6.20 (1H, t, J = 0.9 Hz, H-3), 4.44 (2H, d, J = 0.9 Hz, CH_2OH), 4.18 (2H, s, H-1′), 2.29 (3H, s, H-3′); ^{13}C NMR (CD_3OD, 150 MHz) δ 208.4 (C-2′), 181.2 (C-4), 169.5 (C-2), 163.5 (C-7), 161.1 (C-8a), 139.6 (C-5), 119.7 (C-6), 115.6 (C-4a), 108.7 (C-3), 102.0 (C-8), 61.3 (CH_2OH), 50.0 (C-1′), 30.0 (C-3′); positive ESIMS m/z 249 $[M + H]^+$; EIMS m/z 249 (100), 231 (19), 206 (18).

4-(*trans*)-Acetyl-3,6,8-trihydroxy-3-methyldihydronaphthalenone (4): colorless solid; UV (MeOH) λ_{max} (log ϵ) 215 (4.26), 284 (4.09), 328 (3.79) nm; IR (KBr) ν_{max} 3400, 3150, 1690, 1630, 1160 cm^{-1}; ^1H NMR (CD_3OD, 600 MHz) δ 6.27 (1H, d, J = 2.2 Hz, H-5), 6.16 (1H, d, J = 2.2 Hz, H-7), 4.14 (1H, br s, H-4), 3.13 (1H, d, J = 17 Hz, H-2α), 2.47 (1H, dd, J = 17, 1.2 Hz, H-2β), 2.34 (3H, s, CH_3CO), 1.34 (3H, s, CH_3C-3); ^{13}C NMR (CD_3OD, 150 MHz) δ 208.3 (*C*OC-4), 202.3 (C-1), 166.9 (C-8), 166.3 (C-6), 144.6 (C-4a), 110.6 (C-8a), 109.8 (C-5), 102.5 (C-7), 72.2 (C-3), 64.3 (C-4), 49.1 (C-2), 32.5 (*C*H_3-CO), 27.6 (*C*H_3C-3); positive ESIMS m/z 251 $[M + H]^+$; EIMS m/z 251 (8), 233 (100), 193 (84).

4-(*cis*)-Acetyl-3,6,8-trihydroxy-3-methyl-dihydronaphthalenone (5): colorless solid; UV λ_{max} (log ϵ) 218 (4.26), 282 (4.11), 325 (3.82) nm; IR (KBr) ν_{max} 3400, 3130, 1690, 1620, 1160 cm^{-1}; ^1H NMR (CD_3OD, 600 MHz) δ 6.18 (1H, d, J = 2.2 Hz, H-5), 6.13 (1H, d, J = 2.2 Hz, H-7), 4.16 (1H, br s, H-4), 3.12 (1H, d, J = 17 Hz, H-2α), 2.45 (1H, dd, J = 17, 1.2 Hz, H-2β), 2.35 (3H, s, CH_3CO), 1.27 (3H, s, CH_3C-3); ^{13}C NMR (CD_3OD, 150 MHz) δ 209.5 (*C*OC-4), 203.1 (C-1), 166.9 (C-8), 166.5 (C-6), 144.4 (C-4a), 110.5 (C-8a), 109.4 (C-5), 102.6 (C-7), 72.6 (C-3), 64.1 (C-4), 49.1 (C-2), 33.6 (*C*H_3CO), 29.2 (*C*H_3C-3); positive ESIMS m/z 251 $[M + H]^+$; EIMS m/z 251 (4), 233 (10), 191 (100).

Radioligand Receptor Binding Assay. The adenosine A_1 receptor-binding studies were carried out on membranes of rat cortical brains. Membranes were prepared according to the method of Lohse et al.,[16] except that the membranes were incubated with 2 IU/mL adenosine deaminase at 37 °C before storage, as described by Pirovano et al.[17] Protein concentrations were measured by the bicinchonic acid method.[18]

The adenosine A_1 receptor-binding assays were performed with 0.4 nM [^{3}H]DPCPX as the radioligand (K_d 0.39 nM). The assays were performed as originally described by Lohse et al.[6] The incubation mixture consisted of 100 μL [^{3}H]DPCPX, 100 μL 10^{-5} M N^6-cyclopentyladenosine (CPA) or test compound in different concentrations, 100 μL Tris−HCl 50 mM buffer pH 7.4, and 100 μL rat-brain homogenate containing 30 μg of brain tissue. After an incubation at 25 °C for 60 min, the mixture was put on ice and filtered over glass-fiber filters (GF/B Whatman) under reduced pressure. The filters were washed three times with 2 mL ice-cold 50 mM Tris−HCl buffer pH 7.4. The radioactivity of the washed filters was counted for 4 min by a Hewlett-Packard Tri-Carb 1500 liquid scintillation counter after adding 3.5 mL scintillation liquid. Non-specific binding was determined in the presence of 10^{-5} M CPA. Radioligand binding data were analyzed with the software package Prism (Graph Pad Inc, San Diego, CA). The K_i values of specific [^{3}H] DPCPX binding were determined by log-probit analysis using six to eight different concentrations of CPA as a displacer. Data are means ± SD of three individual determinations, each performed in duplicate.

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