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6-Bromoflavone, a High Affinity Ligand for the Central Benzodiazepine Receptors Is a Member of a Family of Active Flavonoids

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6-Bromoflavone, obtained by bromination of flavanone, binds to central benzodiazepine receptors with a Ki = 70 nM and has a clear anxiolytic activity in mice, at 0.5 mg/kg, i.p. A survey of the structure/affinity relationship for those receptors in a series of natural and synthetic flavonoids is presented.

Previous work from our laboratories has shown that some naturally occurring flavonoids, like chrysin (5,7-dihydroxyflavone) and apigenin (5,7,4′-trihydroxyflavone), are ligands for the BDZ-Rs which exhibit anxiolytic activity with relatively minor sedative or myorelaxant effects (1–3).

Since electronegative chemical groups were shown to be essential for the activity of the classical BDZ anxiolytics (4), we thought we might be able to increase the affinity of chrysin for the BDZ-Rs by introducing halogen atoms in its molecule. Unfortunately the affinities of the products were the same or less than that of the lead compound (see Table 1: compounds: 23–26).

However, bromination of flavanone had a positive result since it yielded a flavone derivative with greatly enhanced binding affinity and anxiolytic properties which are described here.

The affinities to BDZ-Rs of a series of natural and synthetic flavonoids are collected in Table 1 and analyzed in terms of their structure.

MATERIALS AND METHODS

Flavanone was obtained from Extrasynthese, Genay, France. Melting points are uncorrected. EIMS were measured in a Shimadzu QP-1000 quadrupole mass spectrometer. NMR spectra were recorded in a Bruker AMX 400 spectrometer. UV spectra were determined in a Shimadzu UV-160A recording spectrophotometer. Adsorption chromatography was performed in columns (2.5 cm×40 cm) of silica gel, 10–40 μ, type H (Sigma, USA), eluted stepwise with 200 ml of toluene, followed by equal volumes of toluene containing 1, 2, 3, 4 and 5% acetone (v/v). HPLC fractionations were performed using C18 reversed phase Vydac columns (The Separation Group, Hesperia, CAL, USA). Elutions were carried out with a lineal gradient of 30 to 80% ACN in water, in 45 min, at a flow rate of 5 mL/min. The following flavonoids were kindly provided by Dr. A. Pomilio: **9, 11, 12, 13, 14, 18, 19** and **20.**

The synthesis of the brominated flavones was performed as follows: to a solution of flavanone (2.7 mmol) and pyridine (0.15 mmol) in CCl_4 (9.6 ml), at 0°C, a solution of bromine (6.2 nmol) in CCl_4 (3.72 ml) was added dropwise. The mixture was stirred for 1 h at 30°C followed by 45 min. at 65°C. After cooling, the reaction mixture was washed with two 10 mL portions of a saturated aqueous solution of $Na_2S_2O_5$ and then with water, dried over Na_2SO_4 and concentrated to dryness in a rotary evaporator.

The crude product dissolved in toluene was chromatographed in a column of silica gel, as described before. The fractions

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Abbreviations: BDZ, benzodiazepine; BDZ-R, benzodiazepine receptor; FNZ, flunitrazepam; [^{3}H]FNZ, tritium-labeled flunitrazepam; GABA, gamma-aminobutyric acid; AMPA, α-amino-3-hydroxy-5-methyl isoxazole-4-propionic acid; EIMS, electron impact mass spectrometry; ACN, acetonitrile, HPLC, high performance liquid chromatography; VEH, vehicle.

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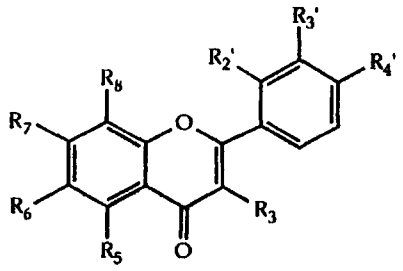
TABLE 1
Binding Affinity of Different Flavonoids for the BDZ-R



Flavone

	R_3	R_5	R_6	R_7	R_8	$R_{2'}$	$R_{3'}$	$R_{4'}$	K_i[a,b] (μM)
1	H_2	H	H	H	H	H	H	H	>40
2	H	H	H	H	H	H	H	H	1.0 ± 0.6
3	Br	H	H	H	H	H	H	H	>75
4	H	H	Br	H	H	H	H	H	0.070 ± 0.009
5	Br	H	Br	H	H	H	H	H	>75
6					Tribromoflavone				>75
7	H	OH	H	OH	H	H	H	H	3[c]
8	H	OH	H	OH	H	H	H	OH	4[d]
9	OH	OH	H	OH	H	H	H	H	>100
10	OH	OH	H	OH	H	H	H	OH	93[e]
11	H	OH	H	OH	H	H	OH	OH	>100[e]
12	OH	H	H	OH	H	H	OH	OH	>100[e]
13	OH	OH	H	OH	H	H	OH	OH	>100[e]
14	OH	OH	H	OH	H	OH	H	OH	>100[e]
15	H	OH	OCH_3	OCH_3	H	H	OH	OH	>100[f]
16	H	OCH_3	H	OCH_3	H	H	H	H	>100[g]
17	H	OH	H	OCH_3	H	H	H	H	50[g]
18	H	OH	H	OH	H	H	H	OCH_3	>100
19	H	OCH_3	OH	OCH_3	H	H	H	H	>100
20	H	OCH_3	OPh	OCH_3	H	H	H	H	>100
21	H	OH	OCH_3	OCH_3	H	H	H	OH	23[h]
22	H	OH	OCH_3	OH	H	H	H	OH	1[h]
23	H	OH	Br	OH	Br	H	H	H	0.7[g]
24	H	OH	I	OH	I	H	H	H	1[g]
25	H	OH	H	OH	H	F	H	H	8[g]
26	H	OH	H	OH	H	Cl	H	H	8[g]
27	H	H	NO_2	H	H	H	NO_2	H	0.012 ± 0.001[i]

[a] Binding affinities to bovine cortical membranes were determined as described under Materials and Methods.

[b] K_i ± SEM values are means of 3–5 independent determinations. For values ranging from 0.7 to 8, the SEM varied between 10 to 20% of the absolute values listed. The low affinity values are the result of duplicate measurements.

[c] Reference 2.

[d] Reference 3.

[e] Reference 10.

[f] Reference 11.

[g] Cassels B. K., Paladini A. C. and Medina J. H., unpublished results.

[h] Reference 12.

[i] Reference 15.

obtained were pooled according to the results of their analysis by TLC on silica gel on polyester sheets, with 254 nm fluorescent indicator (Sigma, USA).

These pools were purified by HPLC as described. All the material in the major peaks in the chromatograms was recovered by evaporation of the solvent, recrystalized from ethanol-water and used for identification and assay.

Binding assays to central BDZ-Rs. Extensively washed synaptosomal membranes obtained from bovine cerebral cortices were used (5). The binding of [^{3}H]FNZ (81.8 Ci/nmol; NEN) was carried out as described previously (6).

Animals. Male Swiss mice from our breeding stock, weighing 28–35 g were used. The animals were placed in groups of 10 with free access to water and food and maintained on a 12 h/12h day/night cycle.

Pharmacological tests. In all the tests the mice were injected i.p. with vehicle or a solution of the drugs, 15 min before the assay.

The following tests were used: **spontaneous ambulatory activity counts** in an Opto-Varimex apparatus to measure *locomotor activity;* **the elevated plus maze** to measure *anxiolityc effects;* the **holeboard** to assay for *sedative behavior* and **the horizontal-wire** to detect *myorelaxation.* All these tests have been described previously (3).

RESULTS AND DISCUSSION

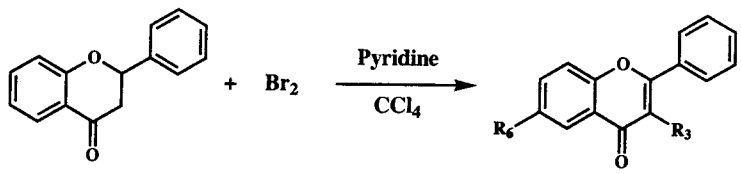
Non specific bromination of flavanone **(1)** yielded flavone **(2)** and four brominated flavones (Fig. 1) These have been separated by silica gel chromatography and HPLC. NMR analysis permitted the identification of 3-bromoflavone **(3),** 6-bromoflavone **(4)** and 3,6-dibromoflavone **(5).** The HPLC retention times (in min) for these compounds and of an unidentified tri-brominated flavone derivative **(6)** are: 16.5 **(2),** 20.0 **(3),** 23.7 **(4),** 26.4 **(5),** 30.7 **(6).**

Identification of the bromoderivatives was achieved on the basis of ^{1}H and ^{13}C NMR 1-dimensional experiments accompanied by COSY-45 (^{1}H-^{1}H-coupling), HC-COBI-DEC (^{1}H-^{13}C direct 1-bond coupling), **(7),** and HMBC (^{1}H-^{13}C long-range, 2- and 3- bond coupling), 2- dimensional experiments (8):

3-Bromoflavone **(3).** mp: 122–123 °C. UV λ_{max} 247, 307 nm. EIMS M^+ 300 and 302 ($C_{15}H_9O_2Br$). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.30 (1H, dd, J = 8, 2 Hz, H-5), 7.87 (2H, dd, J = 8, 2 Hz, H-2′/H-6′), 7.54–7.58 (3H, m, H-3′/H-4′/H-5′), 7.50 (1H, dd, J = 8, 2 Hz, H-8), 7.47 (1H, dt, J = 2, 8 Hz, H-6), 7.37 (1H, dt, J = 2, 8 Hz, H-7). ^{13}C NMR (100 MHz) 162.3 (s, C-2), 109.5 (s, C-3), 173.3 (s, C-4), 122.0 (s, C-4a), 126.0 (d, C-5), 126.0 (d, C-6), 134.4 (d, C-7), 118.8 (d, C-8), 155.9 (s, C-8a), 133.1 (s, C-1′), 129.6 (C-2′/C-6′), 128.8 (C-3′/C-5′), 131.4 C-4′).

6-Bromoflavone **(4).** mp: 189–190 °C. UV λ_{max} 256, 299 nm. EIMS M^+ 300 and 302 ($C_{15}H_9O_2Br$). ^{1}H NMR (DMSO-d_6, 400 MHz) δ 8.11–8.13 (3H, m, H-5, H-2′, H-6′), 8.00 (1H, dd, J = 8, 2 Hz, H-7), 7.47 (1H, d, J = 8 Hz, H-8), 7.57–7.63 (3H, m, H-3′, H-4′, H-5′), 7.11 (1H, s, H-3). ^{13}C NMR (100 MHz) 162.9 (s, C-2), 106.9 (d, C-3), 175.9 (s, C-4), 124.9 (s, C-4a), 126.9 (d, C-5), 117.9 (s, C-6), 136.9 (d, C-7), 121.3 (d, C-8), 154.7 (s, C-8a), 130.8 (s, C-1′), 126.5 (C-2′/C-6′), 129.1 (C-3′/C-5′), 132.0 (C-4′).

3,6-Dibromoflavone **(5).** mp: 182–183 °C. UV λ_{max} 249, 299 nm. EIMS M^+ 380 and 382 ($C_{15}H_8O_2Br_2$). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.42 (1H, d, J = 2 Hz, H-5), 7.85 (2H, dd, J = 8, 2 Hz, H-2′/H-6′), 7.80 (1H, dd, J = 8, 2 Hz, H-7), 7.53–7.60 (3H, m, H-3′/H-4′/H-5′), 7.41 (1H, d, J = 8 Hz, H-8). ^{13}C NMR (100 MHz) 162.5 (s, C-2), 109.5 (s, C-3), 172.2 (s, C-4), 123.3 (s, C-4a), 129.3 (d, C-5), 119.4 (d, C-6), 137.4 (d, C-7), 120.1 (d, C-8), 154.7 (s, C-8a), 132.8 (s, C-1′), 129.6 (C-2′/C-6′), 128.7 (C-3′/C-5′), 131.6 (C-4′).



flavanone **1**

2 R_3= H R_6= H
3 R_3= Br R_6= H
4 R_3= H R_6= Br
5 R_3= Br R_6= Br
6 tribromoflavone

FIG. 1. Synthesis of brominated flavones.

Tribromoflavone (**6**). EIMS M^+ 460 and 462 ($C_{15}H_8O_2Br_3$). The amount of 6 recovered was so small that identification was not feasible.

Within the set of brominated flavones only **4** showed high affinity for the BDZ-R (Table 1). As a consequence **4** was further examined biochemically and for pharmacological activity.

Saturation curves of [^{3}H]FNZ revealed that **4** is a competitive ligand for the BDZ-Rs (Fig. 2) Displacement curves for [^{3}H]FNZ in the presence or absence of 10 μM GABA showed that **4** behaves as a full agonist (GABA ratio = 1.6–2.0; N = 4(9)).

Compound **4** (20μM) was not able to displace the binding of [^{3}H]N-methylscopolamine, [^{3}H]prazosin, [^{3}H]muscimol or [^{3}H]AMPA to muscarinic-cholinergic, α-adrenergic, $GABA_A$ or glutamate AMPA receptors, respectively.

The behavior of **4** in the pharmacological tests was as follows:

— it did not alter the spontaneous ambulatory locomotor activity of mice injected with doses up to 3 mg/kg, i.p. (data analyzed by Newman-Keuls after ANOVA);

— a significant anxiolytic activity was detected on the elevated plus maze in mice injected with 0.5 mg/Kg; i.p., as shown in Fig. 3;

— in the holeboard test the results in Fig. 4 are indicative of sedative properties at 3 mg/kg, i.p.;

— the decrease in the percentage of animals grasping the horizontal wire was significant only at doses of 10 mg/kg, ($p<0.0001$), (X^2 test).

Based on the abovementioned biochemical and pharmacological characterizations, we can conclude that **4** recognizes central BDZ-Rs specifically and in a competitive manner with a Ki of 70 nM and possesses anxiolytic properties at doses quite similar to those observed previously with diazepam (2). However, the anxiolytic action of **4** at doses less than 1 mg/kg is not "contaminated" with sedative effects. The present findings indicate that **4** behaves as a full agonist of the central BDZ-Rs.

The affinities for the BDZ-Rs of flavanone and a range of natural and synthetic derivatives of flavone are now available and have been collected in Table 1.

Flavone itself (**2**) shows a significant affinity (Ki = 1μM) but the introduction of a bromine atom at C6 (**4**) raises this parameter 14 times, as already described, while bromination at C3 sharply decreases the affinity of the parent compound **(3,5).**

Hydroxyl groups at C5 and C7 (7) or C5, C7 and C4′ (**8**) do not substantially modify the affinity

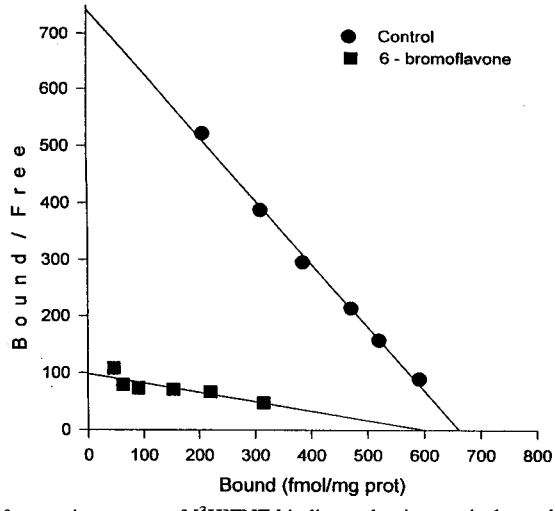


FIG. 2. Scatchard plots of saturation curves of [^{3}H]FNZ binding to bovine cortical membranes in the presence (■) and in the absence (●) of **4** (100 nM). This is a representative experiment of three independent determinations.

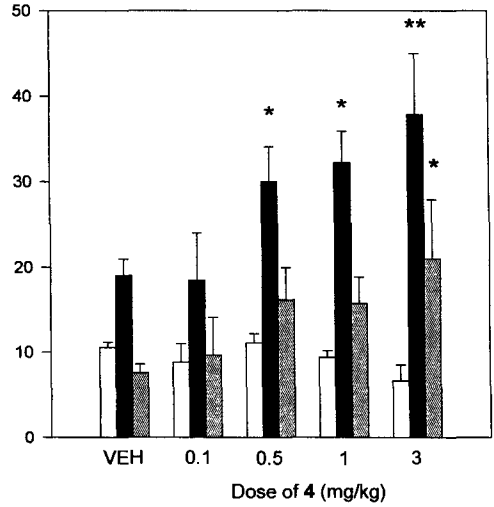


FIG. 3. Performance of mice during a 5 min test in the elevated plus-maze test, 15 min after i.p. injection with VEH or **4** (0.1–3 mg/Kg). Results are expressed as mean ± SEM of the number of total arms entries (open bars), percentage of open arms entries (closed bars) and percentage of time spent in the open arms (hatched bars). *p<0.05, **p<0.01, Dunnett's multiple comparison test. The number of experimental mice per group ranged between 15 and 30.

of flavone for central BDZ-Rs. Hydroxylation at C3 **(9, 10, 12–14),** however, appears to be associated with much reduced affinity, and the same seems to be true for the presence of a catechol group at C3′ and C4′ **(11–13, 15).** The effect of hydroxylation a C2′ **(14)** is not sufficiently clear at this time. *O*-Methylation of hydroxil groups at C5, C7 or C4′ **(16, 18, 19–21)** appears to be generally unfavorable.

Introduction of a methoxyl group at C6 **(15, 21, 22)** does not seem to affect the affinity of the parent apigenin **(8)** or its 7-*O*-methyl ether derivative **(21)** to any great extent, and the same is true for bromination or iodination of chrysin **(7)** at C6 and C8 **(23, 24),** unlike the abovementioned

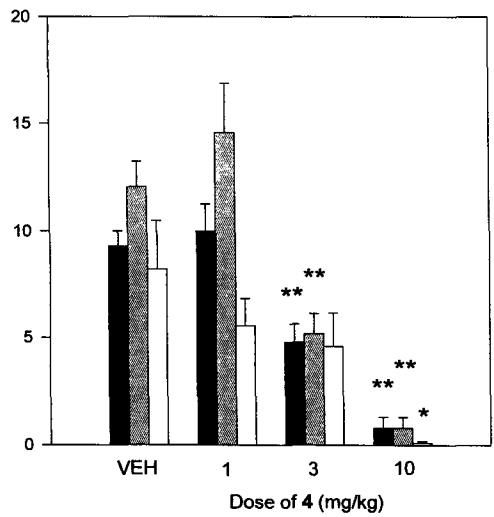


FIG. 4. Mean ± SEM of the number of head-dips (closed bars), time spent head-dipping (hatched bars), and number of rearings (open bars) for mice during a 5 min test in the holeboard 15 min after i.p. injection of VEH or **4** (1–10 mg/Kg). *p<0.01, **p<0.001 significantly different from controls (Newman-Keuls after ANOVA). The number of animals per group ranged between 7–13.

bromination of unsubstituted flavone at C6 **(4).** On the other hand, a chlorine or fluorine atom at C2′ **(25, 26)** seems to reduce affinity somewhat. However, the addition of nitro groups at positions 6 and 3′ of the flavone nucleus (27) induces a striking increase in affinity.

With respect to the pharmacological properties of these compounds the following correlations were detected:

— in general, a very low affinity for the BDZ-R is associated with no anxiolytic activity as found with **10** and **15;**

— compounds **7** (1, 2), **8** (3) and **23** exhibited medium-low affinity for the BDZ-Rs, and had moderate anxiolytic properties with partial agonists behavior;

— when the affinity reaches high levels, as shown in Table 1 for **4** and **27** (15), the compounds behave either as full or partial agonists, respectively.

Häberlein *et al* (13) have published a quantum chemical and spectroscopic approach to the study of structure-activity relationships in this class of compounds. Their conclusions are based on a small group of inactive flavanones plus two flavone derivatives with very low affinity for the BDZ-Rs and two flavones claimed to have low to medium affinity. One of these latter compounds, 5,7 dimethoxyflavone, should be identical to **16** in Table 1 of the present work. The structure of **16** is supported by our NMR data (14). Unfortunately **16** is inactive in our assays (see Table 1). Until this discrepancy can be clarified we think that the conclusions of Häberlein *et al* (13) should be considered preliminary.

In conclusion, the present results strongly support our hypothesis that natural and/or synthetic flavonoids represent a new family of BDZ-Rs ligands with interesting pharmacological profiles.

ACKNOWLEDGMENTS

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14. ^{1}H-NMR ($CDCl_3$, 300 MHz) of compound **15** (Table 1): δ 7.86 (2H, m, H-2′, H-6′), 7.48 (3H, m, H-3′, H-4′, H-5′), 6.67 (1H, s, H-3), 6.56 (1H, d, J = 2.2 Hz, H-8), 6.37 (1H, d, J = 2.2 Hz, H-6), 3.95, 3.90 (2×3H, 2×s, 2×OMc).
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