Structure−Activity Relationships and Molecular Modeling Analysis of Flavonoids Binding to the Benzodiazepine Site of the Rat Brain $GABA_A$ Receptor Complex

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The affinities for the benzodiazepine binding site of the $GABA_A$ receptor of 21 flavonoids have been studied using [^{3}H]flumazenil binding to rat cortical membranes in vitro. We show that flavonoids with high affinity for the benzodiazepine receptor in vitro spanning the whole efficacy range from agonists (**1q**) to inverse agonists (**1l**) can be synthesized. The receptor binding properties of the flavonoids studied can successfully be rationalized in terms of a comprehensive pharmacophore model recently developed by Cook and co-workers (*Drug Des. Dev.* **1995**, *12*, 193−248), supporting the validity of this model. However, in contrast to the requirement by the model that an interaction with the hydrogen bond-accepting site A2 is necessary for compounds to display inverse agonistic activity, 6-methyl-3′-nitroflavone (**1l**), which cannot engage in such an interaction, nevertheless displays inverse agonism. The analysis of the binding affinities of 3′- and 4′-substituted flavones in terms of the pharmacophore model has yielded new information for the further development of the pharmacophore model.

Introduction

The major inhibitory neurotransmitter in the mammalian central nervous system (CNS), γ-aminobutyric acid (GABA), exerts its physiological effects by binding to three different receptor types in the neuronal membrane: the $GABA_A$, $GABA_B$, and $GABA_C$ receptors. The $GABA_B$ receptor belongs to the G-protein-coupled receptor superfamily,[2] while the $GABA_A$[3] and $GABA_C$[4] receptors are ligand-gated chloride ion channel complexes. The $GABA_A$ receptor is thought to form a pentameric structure assembled from a gene family consisting of six α, four β, three γ, one δ, one ϵ, one π, and three ρ subunits, each encoded by different genes.[5] In addition to GABA binding sites, the $GABA_A$ receptor chloride channel complex possesses binding sites for compounds that allosterically modify the chloride channel gating of GABA, such as benzodiazepines (BZDs), β-carbolines, barbiturates, and certain steroids.[6,7] BZDs pharmacological effects (anxiolytic, anticonvulsant, muscle relaxant, and sedative-hypnotic) make them the most important $GABA_A$ receptor-modulating drugs in clinical use.[8] The interaction of BZD agonists with the $GABA_A$ receptor complex increases the GABA-induced chloride channel opening frequency, which results in membrane hyperpolarization, thus reducing neuronal excitability. The BZD binding sites in the brain were identified and described by radioligand receptor binding assays using [^{3}H]BZDs as ligands, and originally it was found that only 1,4-BZD derivatives bind to these receptors. It has since been shown that many groups of compounds bind to the BZD receptor (BzR) with high affinity, e.g., triazolopyridazines, cyclopyrrolones, quinolines, and β-carbolines.[9] Additionally, it has been found that some naturally occurring flavones and synthetic derivatives bind with high affinity to the BzR.[10,11] More than 4000 chemically unique flavonoids have been isolated from plants, making the flavonoids one of the most important classes of metabolites.[12]

A comprehensive pharmacophore model for the BzR based on structure−activity relationship studies for 136 different ligands from 10 structurally different classes of compounds has recently been developed by Cook and co-workers.[1] In this model the authors assume that BzR agonists, antagonists, and inverse agonists share the same binding pocket. The pharmacophore model has successfully been employed in the design of novel BzR ligands[13,14] and has recently been used in the structure−activity analyses of novel β-carboline ligands[15] such as *N*-(indol-3-ylglyoxylo)benzylamines[16] and *N*′-(phenylindol-3-yl)glyoxylohydrazides.[17] Although the pharmacophore model is based on ligands from a large number of different classes of compounds, flavonoids were not included in the development of the model. We have used radioligand receptor binding techniques to investigate the affinities for the benzodiazepine receptor of 21 flavonoid derivatives (9 commercially available, 5 reported in the literature, and 7 developed in our laboratory), providing an opportunity to test the validity and usefulness of the model.

Results and Discussion

Chemistry. The structures of the flavonoids investigated in this study are shown in Chart 1. 6-Methyl-2′-nitroflavone (**1j**), 3′,6-dimethylflavone (**1k**), 6-methyl-

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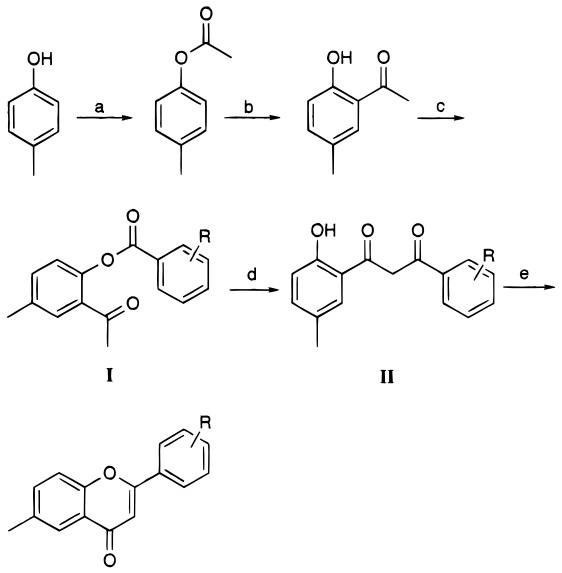
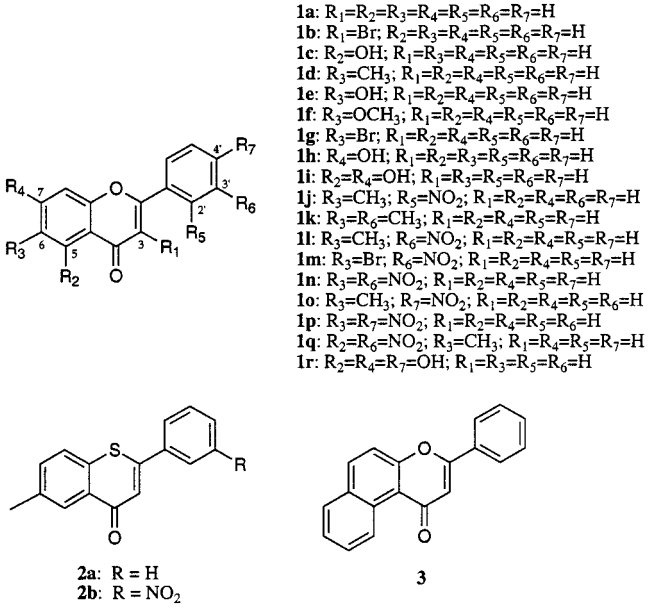
Scheme 1[a]



[a] (a) Ac_2O, pyridine; (b) $AlCl_3$; (c) *o*-, *m*-, *p*-nitrobenzoyl chloride or *m*-toluoyl chloride, pyridine; (d) KOH, pyridine; (e) H_2SO_4.

Chart 1

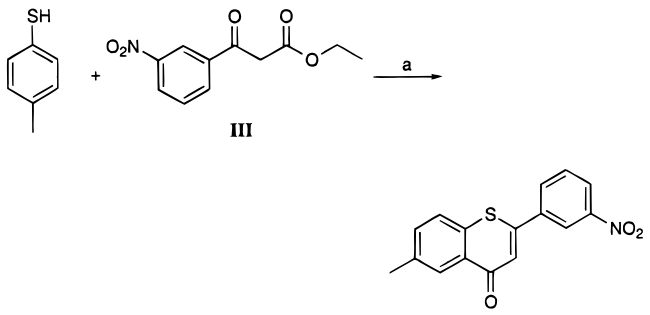


3′-nitroflavone (**1l**), and 6-methyl-4′-nitroflavone (**1o**) were synthesized according to procedures that previously have been described for other flavones.[18] The synthesis involved an acetylation, a Fries rearrangement, a modified Schotten−Baumann reaction, and a Baker−Venkataraman rearrangement followed by a cyclization with acid (see Scheme 1).

6-Methylthioflavone (**2a**) was prepared as previously published,[19] and the same procedure was adapted for the synthesis of 6-methyl-3′-nitrothioflavone (**2b**) according to Scheme 2. 6-Methyl-3′,5-dinitroflavone (**1q**) was synthesized by nonspecific nitration of 6-methylflavone (**1d**), according to Scheme 3.

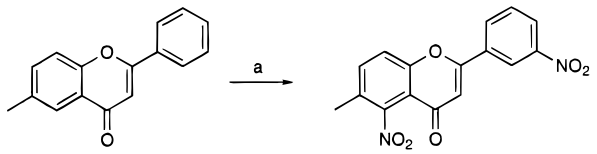
Receptor Binding. As shown in Table 1 the tested flavonoids inhibit [^{3}H]flumazenil binding with a wide range of affinities, from low nanomolar (**1q** and **1l**) to high micromolar (**1b** and **1p**) concentrations. Compound **1q** shows the highest affinity with a K_i value of 1.9 nM

Scheme 2[a]



[a] (a) H_2PO_4, P_2O_5.

Scheme 3[a]



[a] (a) HNO_3, P_2O_5.

Table 1. K_i Values and GABA Ratio of Flavones Tested on [^{3}H]Flumazenil[a] Binding in Vitro to Rat Cortical Membranes

no.	name	K_i value (nM)	GABA ratio
1a	flavone	4200 ± 30	1.05 ± 0.03
1b	3-bromoflavone[a,28]	>75000	NR
1c	5-hydroxyflavone	1900 ± 34	1.03 ± 0.07
1d	6-methylflavone	125 ± 8	0.98 ± 0.08
1e	6-hydroxyflavone	400 ± 60	1.06 ± 0.10
1f	6-methoxyflavone	570 ± 30	1.51 ± 0.16
1g	6-bromoflavone[a,28]	70 ± 9	1.80
1h	7-hydroxyflavone	4200 ± 270	1.14 ± 0.08
1i	5,7-dihydroxyflavone	920 ± 80	0.90 ± 0.11
1j	6-methyl-2′-nitroflavone	1000 ± 40	1.49 ± 0.32
1k	3′,6-dimethylflavone	29 ± 2	1.63 ± 0.27
1l	6-methyl-3′-nitroflavone	5.6 ± 0.7	0.72 ± 0.05
1m	6-bromo-3′-nitroflavone[a,28]	16	1.4
1n	3′,6-dinitroflavone[a,29]	26 ± 3	1.3
1o	6-methyl-4′-nitroflavone	7300 ± 2300	ND
1p	4′,6-dinitroflavone[a,30]	17000 ± 5000	NR
1q	6-methyl-3′,5-dinitroflavone	1.9 ± 0.3	1.41 ± 0.11
1r	4′,5,7-trihydroxyflavone	770 ± 40	1.03 ± 0.12
2a	6-methylthioflavone	310 ± 37	1.35 ± 0.18
2b	6-methyl-3′-nitrothioflavone	33 ± 2	1.51 ± 0.19
3	5,6-benzoflavone	1480 ± 15	1.28 ± 0.05
	diazepam	8.5 ± 1.6	2.13 ± 0.35
	flumazenil	1.2 ± 0.4	1.05 ± 0.11
	DMCM	7.6 ± 1.3	0.57 ± 0.14

[a] Compounds referred from literature were tested on [^{3}H]flunitrazepam binding. All values are the mean ± SEM of three to five separate experiments. NR, not reported; ND, not determined.

on [^{3}H]flumazenil binding to rat cortical membranes; this affinity is of similar potency as that of flumazenil (K_i = 1.2 nM). Compound **1q** shows a competitive inhibition (Figure 1) of [^{3}H]flumazenil binding (with an increase in the K_D value while the number of binding sites was unaffected). Several other flavone derivatives investigated in this study showed competitive inhibition of [^{3}H]flumazenil binding (data not shown).

To evaluate the pharmacological profile of compounds interacting with the BzR by receptor binding assays, the GABA ratio (see Experimental Section) can be useful to indicate the efficacy of BzR ligands.[20] GABA ratios > 1 indicate that compounds have agonistic profiles, e.g. diazepam, while GABA ratios < 1 indicate compounds with inverse agonistic profiles, e.g. DMCM, and GABA ratios ∼ 1 indicate that compounds have antagonistic

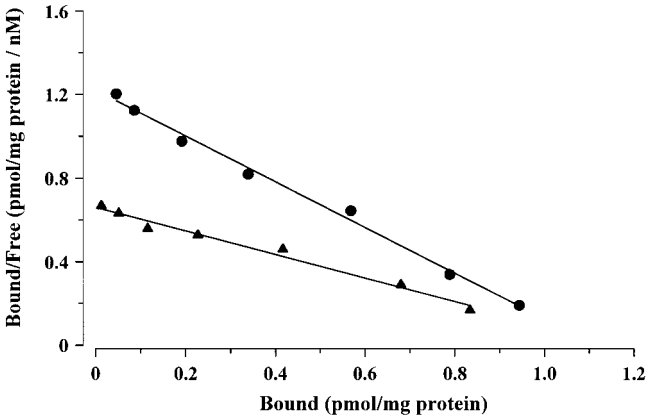


Figure 1. Representative Scatchard plots of [^{3}H]flumazenil binding to rat cortical $GABA_A$ receptors in vitro in the absence (●) and presence (▲) of **1q** (6-methyl-3′,5-dinitroflavone) (2.5 nM): (●) K_D = 0.9 nM, B_{max} = 1.1 pmol/mg of protein; (▲) K_D = 1.8 nM, B_{max} = 1.2 pmol/mg of protein.

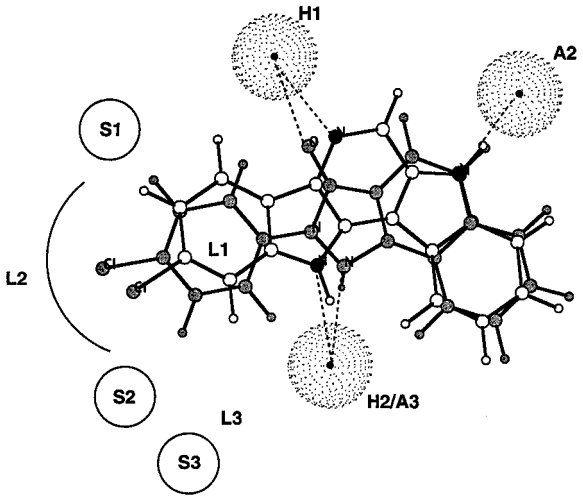
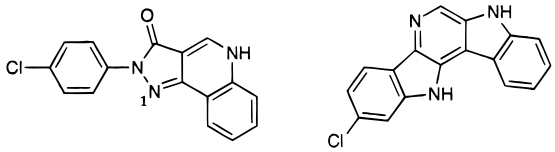


Figure 2. Pharmacophore model developed by Cook and co-workers.[1] CGS-9896 (**4**; filled atoms) and dihydropyrido[3,4-*b*:5,4-*b*′]diindole (**5**; unfilled carbon atoms) (see also Chart 2) are shown in the model.

profiles, e.g. flumazenil (see Table 1). As shown in Table 1, **1q** has a GABA ratio of 1.4 suggesting that this compound is a partial agonist as compared to diazepam (GABA ratio 2.1 in the assay conditions used here), which is a full agonist. The GABA ratios of **1f**, **1g**, **1j**, **1k**, **1m**, **1n**, **2a**, **2b**, and **3** suggest that these compounds have a partial agonistic profile, while for compounds **1a**, **1c**, **1d**, **1e**, **1h**, **1i**, and **1r** an antagonistic profile would be predicted. Compound **1l** (K_i = 5.6 nM) has a GABA ratio of 0.7 indicating an inverse agonistic activity. Thus it seems that, as for other classes of BzR ligands,[9] flavone derivatives spanning the whole efficacy range can be developed.

Pharmacophore Model. The key elements of the pharmacophore model developed by Cook and co-workers[1] are shown in Figure 2. H1 and A2 are hydrogen bond donor and acceptor sites, respectively, whereas H2/A3 is a bifunctional hydrogen bond donor/acceptor site. L1, L2, and L3 are three lipophilic pockets, and S1, S2, and S3 denote regions of steric repulsive ligand−receptor interactions (receptor-essential volumes). According to the analysis of Zhang et al.,[1] occupation of areas L2 and/or L3 and interactions at H1, H2, and L1 are important for agonist activity, whereas interactions

Chart 2



4 **5**

at A2, H1, and L1 are required for potent inverse agonism. Furthermore, it has been concluded that a planar or close-to-planar geometry is required for ligands with potent activity.[1]

Conformational Analysis of Flavones. Conformational analysis by using the MM3(92) and MM2(91) programs (see Experimental Section) indicates that all flavones which do not contain a 3- or 2′-substituent are calculated to be almost planar molecules. In the global energy minimum, these flavones display only a moderate twist (ca. 20°) of the 2-phenyl ring with respect to the bicyclic ring system. In addition, the energy required for coplanarity of the two ring systems is calculated to be small, less than 0.2 kcal/mol. In contrast, due to strong steric repulsions between the bromo substituent and the *o*-hydrogens of the 2-phenyl ring in 3-bromoflavone (**1b**), the phenyl ring in this compound is calculated to be 63° twisted and the energy required for planarity is calculated to be high, 6.4 kcal/mol. Thus, the inactivity of **1b** (K_i > 75 000 nM, Table 1) may be attributed to the high energy for the planar conformation in accordance with the conclusion drawn by Zhang et al.[1] that a planar or close-to-planar geometry is required for ligand binding to the benzodiazepine receptor.

The 2-phenyl ring of the 2′-NO_2-substituted flavone **1j** is calculated to have a twist of 45°. However, the energy required for coplanarity of the 2-phenyl ring and the bicyclic ring system is quite small, 1.5 kcal/mol. The coplanar 2-phenyl group rotamer with the NO_2 group pointing toward the ether oxygen is calculated to be 2.9 kcal/mol lower in energy than the alternative one. It should be noted that in the planar conformation of **1j** the 2′-NO_2 group is calculated to be essentially orthogonal to the 2-phenyl ring.

Due to the larger size of the sulfur atom compared to the oxygen atom, the thioflavones **2a** and **2b** display a larger twist (30−32°) of the 2-phenyl ring than the corresponding flavones **1d** and **1l**. The conformational energy required for a planar geometry of **2a** and **2b** is calculated to be 1.2−1.4 kcal/mol.

In conclusion, all flavones studied in the present work, except **1b**, may attain coplanarity or close-to-coplanarity of the 2-phenyl ring and the bicyclic ring system with only a small increase of the conformational energy.

Fitting of the Flavones to the Pharmacophore Model. To fit the flavones studied in the present work to the pharmacophore model, the high-affinity partial agonist CGS-9896 (**4**) (see Chart 2) was employed as a template. The conformation of the flavones with coplanar ring systems was used in the molecular superimpositions. The fitting points used are (i) the centroids of the fused benzene ring in **4** and of the 2-phenyl ring in the flavones, (ii) the carbonyl oxygen, and (iii) the N1 atom in **4** and the oxygen or sulfur atom in position 1 in the flavones. The fitting of the flavone derivative **1d** to the pharmacophore model is shown in Figure 3.

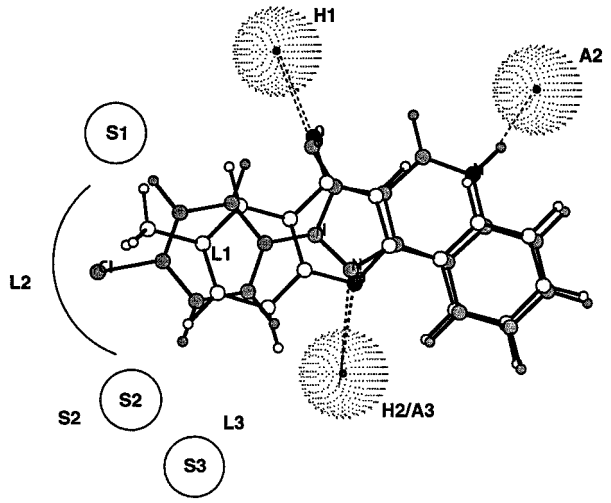


Figure 3. 6-Methylflavone (**1d**; unfilled carbon atoms) fitted to the pharmacophore model by superimposition with CGS-9896 (**4**; filled atoms).

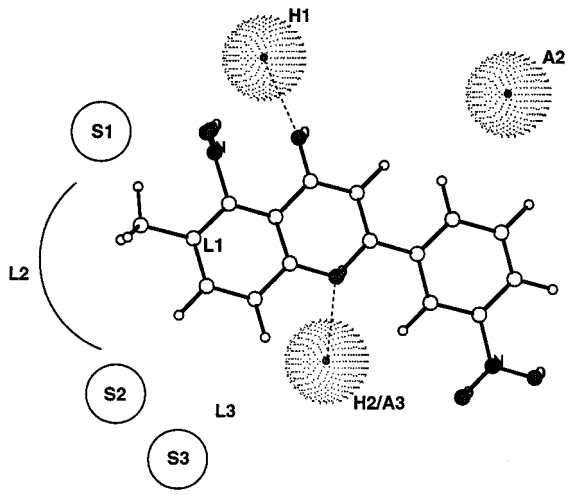


Figure 4. Proposed binding mode of 6-methyl-3′,5-dinitroflavone (**1q**) in the pharmacophore model.

Interactions of the Flavones with the Sites of the Pharmacophore Model. As shown in Figure 3, the flavone carbonyl group and the carbonyl group in **4** (see Chart 2) interact with the hydrogen bond-donating site H1 in essentially the same manner. Furthermore, the benzene rings chosen for fitting superimpose almost perfectly and the position of the ether oxygen in the flavones is very close to that of N1 in **4**, making very similar hydrogen bond interactions with site H2 possible. The fused phenyl ring in the flavones occupies the lipophilic region L1 as does the corresponding benzene ring in **4**. The 6-methyl substituent in **1d** fills out the lipophilic site L2, but not to the same extent as the chlorine atom in **4** (Figure 3).

The 6-substituted flavones clearly fulfill the interaction criteria for agonist activity described above. The data in Table 1 show that the affinity of the flavone ligand increases significantly when a 6-substituent, in particular a less polar one as in **1d** and **1g**, is present. In addition, the larger methoxy (**1f**) and bromo (**1g**) substituents efficiently fill out the lipophilic pocket L2 which, in agreement with the proposed pharmacological effects of such an interaction,[1] enhances the agonist properties of the compounds (Table 1). In contrast to this, a substituent in the 7-position, as in **1h**, does not give a positive contribution to the affinity, presumably due to repulsive interactions with the receptor essential volume at the S2 site.

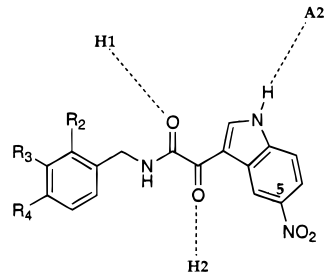
Interestingly, interaction with the hydrogen bond-accepting site A2 is not possible for the flavones studied in the present work. According to the pharmacophore model such an interaction is required for potent inverse agonism.[1] Despite this, the 6-methyl-3′-nitro derivative **1l** displays inverse agonist activity (GABA ratio 0.7, see Table 1).

Replacement of the ether oxygen in **1d** and **1l** by sulfur giving **2a** and **2b** results in a modest affinity decrease by a factor of 3−6. This is most probably due to the weaker hydrogen bond-accepting properties of sulfur compared to oxygen and is consistent with the result from a 3D-QSAR study that a less negative electrostatic potential corresponding to a weaker hydrogen bond at the hydrogen bond-donating H2 site decreases the affinity.[21,22]

Compound **1o** with a 4′-NO_2 substituent in the 2-phenyl ring displays a drastically decreased affinity compared to its parent compound **1d**. Similarly, the 4′-NO_2 group in **1p** is responsible for the very low affinity of this compound. On comparing the affinities of **1n** and **1p** it is clear that the 6-NO_2 group in the 6,4′-di-NO_2 compound **1p** is compatible with a reasonably high affinity. The area in the vicinity of the 4′-position in the flavones (see Figure 3) does not seem to have been mapped out in the pharmacophore model developed by Cook and co-workers.[1] According to the alignment rules of the pharmacophore model, the 4′-position in the flavones corresponds to the 6-position in β-carbolines, and a methoxy substituent in this position in the β-carbolines (DMCM) is compatible with high affinity for the receptor (Table 1). In addition, from a comparison of the affinities of **1j** and **1r** it is clear that the 4′-hydroxy group in **1r** does not result in a decrease of the affinity. Molecular electrostatic potentials calculated by the AM1 method show very little change of the electrostatic potentials in the vicinity of carbonyl and ether oxygens due to the presence of a 4′-NO_2 substituent. Thus, it may be concluded that the very low affinities of the 4′-NO_2 flavones **1o** and **1p** are most probably due to electrostatic repulsive interactions between the strongly electron-rich 4′-NO_2 substituent and the receptor.

The 2′- and 3′-NO_2 groups in compounds **1j**, **1l**, **1m**, **1n**, **1q**, and **2b** may be placed in two alternative positions in the superimposition shown in Figure 3. This substituent may be positioned "upwards" toward the A2 site or "downwards" toward the H2/A3 site. As described above, a 2′-NO_2 group strongly favors a "downwards" orientation of the substituent, whereas the two phenyl group rotamers have essentially identical energies for the 3′-NO_2-substituted compounds. However, considering that the A2 site is a hydrogen bond-accepting site and, thus, is negatively charged or at least electron-rich, a positioning of the NO_2 groups toward this site in the pharmacophore model should lead to prohibitive electrostatic repulsions. Thus, 2′- and 3′-NO_2 groups should be directed "downwards" as exemplified by the 3′-NO_2 group in compound **1q** displayed in Figure 4. Da Settimo et al.[16,17] recently reported structure−activity relationships for a series of *N*-(indol-3-ylglyoxylyl)benzylamines.

Chart 3



6

The compounds were analyzed in terms of the pharmacophore model developed by Cook and co-workers[1]. Comparing the molecular alignments in ref 16 with the alignment displayed in Figure 3, it is clear that the position of the 5-NO_2 group in **6** (Chart 3) corresponds to the 3′-NO_2 group in the flavones **1l**, **1m**, **1n**, **1q**, and **2b** when the 3′-NO_2 group is positioned as in Figure 4. As shown in Table 1, a 3′-NO_2 group in the flavones leads to a significant increase of the affinity (cf. **1d** vs **1l**, **1g** vs **1m**, and **2a** vs **2b**). Similarly, a 5-NO_2 in the *N*-(indol-3-ylglyoxylyl)benzylamine series increases the affinity for the receptor.[16] This strongly supports the proposed bioactive conformation of the 3′-NO_2-phenyl group in the flavone series shown in Figure 4. Other types of substituents in this position in the pharmacophore model such as a 3′-methyl group in the flavone series (**1k**) and a 5-chloro group in the *N*-(indol-3-ylglyoxylyl)benzylamine series[16] also result in an affinity increase, albeit somewhat smaller than shown by a NO_2 substituent. Thus, this substituent position is clearly of great interest for further exploration.

Adding a 5-nitro group to **1l** giving **1q** increases the affinity by a factor of 3 and gives the most potent compound in the present series. According to MM3(92) supported by ab initio HF/6-31G* calculations, the 5-nitro group in **1q** is, as shown in Figure 4, calculated to be essentially orthogonal to the bicylic part of the flavone skeleton. This is due to steric repulsions between the oxygens of the nitro group and the flanking methyl and carbonyl groups. As illustrated in Figure 4, the 5-NO_2 group in **1q** fits nicely between the steric repulsive region S1 and the hydrogen donor site H1 in the pharmacophore model. A rationalization of the positive effect of the 5-NO_2 group on the affinity is shown in Figure 5. Molecular electrostatic potentials calculated by ab initio HF/6-31G* calculations show that the 5-NO_2 group increases the negative electrostatic potential at a position corresponding to a hydrogen-bonding hydrogen at site H1. According to a 3D-QSAR analysis by Allen et al.,[22] this is predicted to increase the affinity of the ligand. On comparing the affinities of compounds **1a** and **1h** with those of **1c** and **1i**, respectively, it is clear that a 5-hydroxy substituent gives a similar increase in the affinity. Calculations of the electrostatic potentials as above also in this case display an increased electrostatic potential at site H1 when the hydroxy group acts as a hydrogen bond acceptor in the direction toward H1.

The 2′-NO_2 group decreases the affinity of **1j** compared to that of its parent compound **1d** by a factor of 8. As discussed above, the bioactive conformation of a 2′-NO_2 flavone derivative most probably has its nitro group directed toward the H2/A3 site in the pharmacophore model. To attain coplanarity of the ring systems in **1j** the NO_2 group is calculated to be essentially orthogonal to the 2-phenyl ring. This orthogonality of the 2′-NO_2 group may be detrimental to high-affinity binding. Another reason for its low affinity may be a steric conflict between the 2′-NO_2 group and the H2/A3 site. Such a conflict may result in rotation about the 1′−2 bond and consequently to a noncoplanarity of the flavone ring systems.

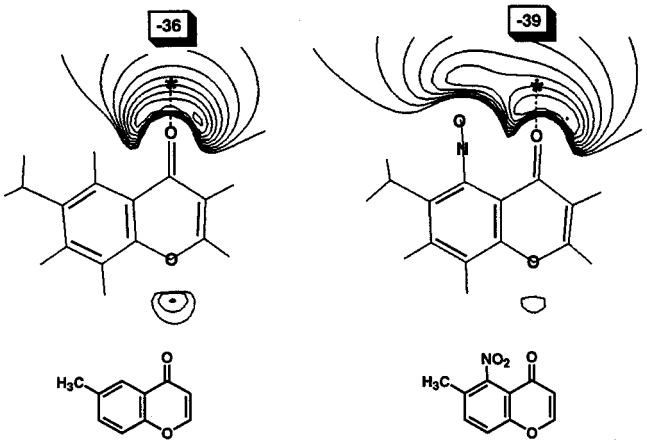


Figure 5. Molecular electrostatic potentials (kcal/mol) calculated by ab initio HF/6-31G* calculations. The electrostatic potential in the plane of the carbonyl group and at a distance 2.0 Å from the carbonyl oxygen in the direction toward the H1 site is denoted by an asterisk.

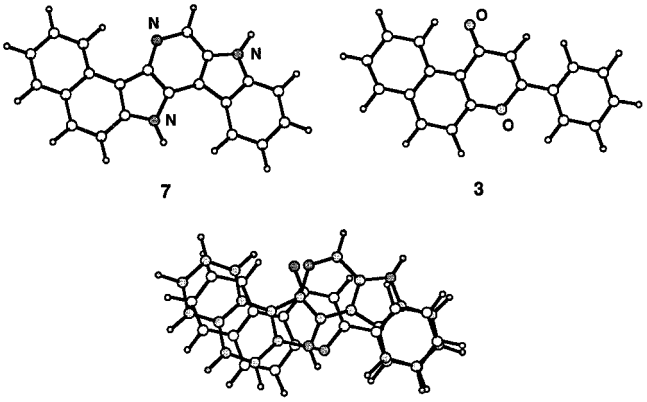


Figure 6. Superimposition of 5,6-benzoflavone (**3**) and the inactive dihydropyrido[3,4-*b*:5,4-*b*′]diindole (**7**).

The low affinity of compound **3** compared to the 6-methyl derivative **1d** may be rationalized by the presence of steric repulsive interactions with the receptor in region S1 (Figure 2) due to the fused benzene ring in **3**. Such steric repulsions are also displayed by the inactive dihydropyrido[3,4-*b*:5,4-*b*′]diindole (**7**) shown in Figure 6.[1] Figure 6 also displays a superimposition of **3** and **7** demonstrating the similarities in the positions of the corresponding fused benzene rings in the two compounds.

Conclusions

We have shown that flavonoids with high affinity (e.g. **1q** and **1l**) for the BzR can be synthesized, spanning the whole efficacy range of BzR ligands from agonistic (**1q**) over antagonistic (**1d**) to inverse agonistic (**1l**)

properties. The receptor binding properties of the flavonoids studied in this work can successfully be rationalized in terms of the pharmacophore model developed by Cook and co-workers[1] supporting the validity of this model. However, in contrast to the requirement by the model of an interaction with the hydrogen bond-accepting site A2 in order to obtain potent inverse agonism, 6-methyl-3′-nitroflavone (**1l**), which cannot engage in such an interaction, is an inverse agonist. Analysis of the affinities of 3′- and 4′-substituted flavones in terms of the pharmacophore model has yielded new information on the corresponding areas of the pharmacophore model. This information may be employed in the further development of the model.

Experimental Section

Chemistry. ^{1}H and ^{13}C NMR were recorded at room temperature with a Bruker DRX400 or a Bruker ARX500 spectrometer. The spectra were recorded in $CDCl_3$, and the solvent signals (7.26 and 77.0 ppm, respectively) were used as reference; data are reported in δ values and J in Hz. The raw data were transformed and the spectra were evaluated with the standard Bruker UXNMR software (rev. 941001). Analytical thin layer chromatography (TLC) was performed on Kiselgel 60 F_{254} plates (Merck), while preparative TLC was performed on precoated PLC plates, silica gel 60F-254, 2 mm. Column chromatography was performed on SiO_2 (Matrex LC-gel: 60A, 35-70 MY; Grace), using a mixture of ethyl acetate and heptane as an eluent. 6-Methylthioflavone (**2a**) was prepared according to ref 19. 2-Acetyl-4-methylphenol was prepared from *p*-cresol by esterification followed by a Fries rearrangement according to ref 18. Compounds **1a**, **1c**, **1d**, **1e**, **1f**, **1h**, **1i**, **1r**, and **3** (>90% pure) were purchased from Extrasynthese S.A. (Lyon, France).

2-Acetyl-1-(2-nitrobenzoyloxy)-4-methylbenzene (Scheme 1; I leading to 1j). 2-Nitrobenzoyl chloride (9.3 g, 50 mmol) was added dropwise to a stirred solution of 2-acetyl-4-methylphenol (5 g, 33 mmol) in 7 mL of pyridine over a period of 10 min. After warming to 50 °C and stirring for 20 min the solution was poured into a mixture of 100 g of ice and 200 mL of 1 M hydrochloric acid. The crude product was collected by filtration, washed with water, and recrystallized from acetone to give white crystals (yield 71%): mp 139−140 °C; ^{1}H NMR ($CDCl_3$) 2.45 (s, 3H), 2.58 (s, 3H), 7.29 (d, 1H, J = 8.0), 7.45 (d, 1H, J = 8.2), 7.67 (brs, 1H), 7.71 (dd, 1H, J = 8.2, 7.5), 7.82 (dd, 1H, J = 8), 8.09 (d, 1H, J = 8.1), 8.12 (d, 1H, J = 7.6); ^{13}C NMR ($CDCl_3$) 21.4, 29.6, 123.8, 124.5, 128.4, 130.5, 131.4, 132.2, 134.2, 134.9, 137.1, 146.7, 147.7, 164.7, 198.1; MS *m*/*z* (% rel int) 299.0798 (M^+, 5, $C_{16}H_{13}NO_5$ requires 299.0794), 150 (100%).

1-(2-Nitrophenyl)-3-[2-(1-hydroxy-4-methylphenyl)]propane-1,3-dione (Scheme 1; II leading to 1j). 2-Acetyl-1-(2-nitrobenzoyloxy)-4-methylbenzene (6 g, 20.1 mmol) was dissolved in 25 mL of pyridine and heated to 50 °C. Finely powdered potassium hydroxide (1.6 g, 28.1 mmol) was added in small portions over a period of 20 min, and stirring was continued for an additional 15 min at 50 °C. After cooling the mixture to room temperature and addition of 30 mL of 10% acetic acid in water, the precipitate formed was collected by filtration and recrystallized from acetone to give yellow crystals (yield 89%), mp 133−135 °C. In chloroform, the product is present as a mixture of the keto (K) and enol (E) forms, 1 to 4: ^{1}H NMR ($CDCl_3$) 2.31 (s, 3H, (E)), 2.33 (s, 3H, (K)), 4.57 (s, 2H, (K)), 6.55 (s, 1H, (E)), 6.90 (d, 1H, J = 8.7, (K)), 6.92 (d, 1H, J = 8.5, (E)), 7.31 (dd, 1H, J = 8.5, 1.9 (E)), 7.32 (m, 1H, (K)), 7.45 (d, 1H, J = 1.4 (E)), 7.55 (d, 1H, J = 1.3 (K)), 7.61 (dd, 1H, J = 7.7, 1.3 (K)), 7.64 (ddd, 1H, J = 8.2, 7.9, 1.3 (K)), 7.65 (ddd, 1H, J = 8.1, 7.8, 1.2 (E)), 7.67 (dd, 1H, J = 8.0, 1.1 (E)), 7.69 (ddd, 1H, J = 8.1, 8.0, 0.8 (K)), 7.76 (ddd, 1H, J = 7.9, 7.7, 0.9 (K)), 7.96 (dd, 1H, J = 7.8, 0.8 (E)), 8.18 (dd, 1H, J = 8.2, 0.9 (K)), 11.60 (s, 1H, (K)), 11.69 (s, 1H, (E)); ^{13}C NMR ($CDCl_3$) 20.9, 21.0, 53.4, 96.7, 118.5, 118.8, 119.1, 119.3, 124.8, 125.0, 128.8, 128.9, 128.9, 129.2, 130.5, 130.5, 130.8, 131.4, 132.0, 133.3, 135.2, 137.6, 138.1, 139.0, 148.8, 161.1, 176.3, 196.2, 196.5, 199.7; MS *m*/*z* (% rel int) 299.0796 (M^+, 12, $C_{16}H_{13}NO_5$ requires 299.0794), 165 (16%), 150 (22%), 135 (100%), 109 (11%), 77 (16%).

6-Methyl-2′-nitroflavone (1j). Concentrated sulfuric acid (95−97%) (1.32 g, 13.5 mmol) was added to a suspension of the diketone **II** (4.0 g, 13.4 mmol) in 20 mL of glacial acetic acid and refluxed for 2 days, whereafter the mixture was poured onto 160 g of ice. After 30 min the white crystals formed were collected by filtration, washed with 200 mL of water and recrystallized from acetone to give white crystals (yield 100%): mp 178−181 °C; ^{1}H NMR ($CDCl_3$) 2.48 (s, 3H), 6.59 (s, 1H), 7.31 (d, 1H, J = 8.6), 7.70 (ddd, 1H, J = 7.3, 1.8, 0.5), 7.73 (ddd, 1H, J = 8.1, 7.8, 1.8), 7.78 (ddd, 1H, J = 7.8, 7.3, 1.5), 8.04 (s, 1H), 8.09 (d, 1H, J = 8.1); ^{13}C NMR ($CDCl_3$) 21.4, 111.6,118.2, 123.9, 125.4, 125.5, 128.4, 131.6, 132.2, 133.8, 135.8, 136.1, 148.6, 155.1, 162.4, 178.6; MS *m*/*z* (% rel int) 281.0685 (M^+, 33, $C_{16}H_{11}NO_4$ requires 281.0688), 253 (12%), 225 (11%), 224 (9%), 196 (8%), 178 (11%), 152 (6%), 134 (100%), 106 (23%).

2-Acetyl-1-(4-methylbenzoyloxy)-4-methylbenzene (Scheme 1; I leading to 1k). The compound was prepared according to the procedure described for **I** leading to **1j**. The crude product was recrystallized from methanol to give white crystals and purified by flash chromatography (heptane:ethyl acetate, 40:1). The yield was 90%: mp 70−73 °C; ^{1}H NMR ($CDCl_3$) 2.44 (s, 3H), 2.46 (s, 3H), 2.54 (s, 3H), 7.12 (d, 1H, J = 8.2), 7.39 (ddd, 1H, J = 8.2, 2.3, 0.7), 7.42 (dd, 1H, J = 7.9, 7.6), 7.47 (d, 1H, J = 8.3), 7.67 (d, 1H, J = 2.0), 8.02 (d, 1H, J = 8.3), 8.04 (s, 1H); ^{13}C NMR ($CDCl_3$) 21.3, 21.8, 30.3, 124.0, 127.9, 129.0, 129.6, 131.0, 131.2, 131.4, 134.5, 135.0, 136.4, 139.0, 147.7, 165.9, 198.2; MS *m*/*z* (% rel int) 268.1091 (M^+, 26, $C_{17}H_{16}O_3$ requires 268.1099), 150 (26%), 135 (29%), 119 (100%), 91 (22%).

1-(3-Methylphenyl)-3-[2-(1-hydroxy-4-methylphenyl)]-propane-1,3-dione (Scheme 1; II leading to 1k). The compound was prepared according to the procedure described for **II** leading to **1j**. The crude product ware crystallized from ethanol to give yellow crystals. The mother liquor was purified with flash chromatography (heptane:ethyl acetate, 40:1). The yield was 73%, mp 91−93 °C. Only the enol form was obtained: ^{1}H NMR ($CDCl_3$) 2.37 (s, 3H), 2.47 (s, 3H), 6.83 (s, 1H), 6.93 (d, 1H, J = 8.5), 7.30 (dd, 1H, J = 8.5, 2.1), 7.38−7.41 (m, 2H), 7.57 (d, 1H, J = 1.5), 7.76 (d, 1H, J = 7.8), 7.77 (s, 1H), 11.95 (s, 1H); ^{13}C NMR ($CDCl_3$) 21.1, 21.9, 92.7, 119.0, 119.0, 124.5, 127.8, 128.6, 128.7, 129.1, 133.6, 134.1, 137.3, 139.0, 160.8, 178.2, 196.0; MS *m*/*z* (% rel int) 268.1091 (M^+, 67, $C_{17}H_{16}O_3$ requires 268.1099), 135 (27%), 119 (100%), 91 (26%).

3′,6-Dimethylflavone (1k). The compound was prepared according to the procedure described for **1j**. The crude product was recrystallized from acetone to give white crystals (yield 98%): mp 135−137 °C; ^{1}H NMR ($CDCl_3$) 2.47 (s, 3H), 2.48 (s, 3H), 6.81 (s, 1H), 7.36 (d, 1H, J = 7.3), 7.42 (dd, 1H, J = 8), 7.49 (d, 1H, J = 8.4), 7.53 (dd, 1H, J = 8.6, 2.0), 7.74 (d, 2H, J = 8.1), 8.03 (s, 1H); ^{13}C NMR ($CDCl_3$) 21.4, 22.0, 107.9, 118.3, 123.9, 124.1, 125.5, 127.3, 129.4, 132.3, 132.8, 135.4, 135.6, 139.2, 155.0, 163.9, 179.1; MS *m*/*z* (% rel int) 250.0996 (M^+, 100, $C_{17}H_{14}O_2$ requires 250.0994), 222 (17%), 134 (47%).

2-Acetyl-1-(3-nitrobenzoyloxy)-4-methylbenzene (Scheme 1; I leading to 1l). The compound was prepared according to the procedure described for **I** leading to **1j**. The crude product was recrystallized from acetone/H_2O to give white crystals (yield 93%): mp 108−110 °C; ^{1}H NMR ($CDCl_3$) 2.47 (s, 3H), 2.56 (s, 3H), 7.15 (d, 1H, J = 8.2), 7.44 (dd, 1H, J = 8.2, 2.2), 7.71 (d, 1H, J = 1.8), 7.75 (dd, 1H, J = 8), 7.51 (ddd, 1H, J = 8.3, 2.3, 1.6), 8.54 (ddd, 1H, J = 7.8, 1.6, 1.5), 9.05 (dd, 1H, J = 2); ^{13}C NMR ($CDCl_3$) 21.4, 29.6, 124.0, 125.7, 128.4, 130.3, 130.3, 131.6, 131.8, 134.8, 136.4, 137.0, 147.1, 148.8, 164.0, 197.8; MS *m*/*z* (% rel int) 299.0786 (M^+, 38, $C_{16}H_{13}NO_5$ requires 299.0794), 150 (100%), 104 (16%).

1-(3-Nitrophenyl)-3-[2-(1-hydroxy-4-methylphenyl)]propane-1,3-dione (Scheme 1; II leading to 1l). The compound was prepared according to the procedure described for **II** leading to **1j**. The crude product was recrystallized from acetone to give yellow crystals (yield 96%), mp 165−167 °C. Only the keto form was obtained: ^{1}H NMR ($CDCl_3$) 2.39 (s, 3H), 6.91 (s, 1H), 6.95 (d, 1H, J = 8.5), 7.34 (dd, 1H, J = 8.5, 2.1), 7.59 (d, 1H, J = 1.5), 7.72 (dd, 1H, J = 8), 8.30 (d, 1H, J = 7.9), 8.41 (ddd, 1H, J = 8.2, 2.3, 1.0), 8.78 (dd, 1H, J = 2), 11.76 (S, 1H); ^{13}C NMR ($CDCl_3$) 21.0, 93.7, 118.7, 119.2, 122.1, 126.9, 128.7, 129.0, 130.4, 132.8, 136.0, 138.2, 149.0, 161.1, 174.1, 196.7; MS *m*/*z* (% rel int) 299.0787 (M^+, 100, $C_{16}H_{13}NO_5$ requires 299.0794), 282 (18%), 177 (9%), 150 (65%), 134 (61%), 104 (12%).

6-Methyl-3′-nitroflavone (1l). The compound was prepared according to the procedure described for **1j**. The crude product was recrystallized from acetone to give white crystals (yield 42%): mp 221−223 °C; ^{1}H NMR ($CDCl_3$) 2.51 (s, 1H), 6.90 (s, 1H), 7.55 (d, 1H, J = 8.5), 7.59 (dd, 1H, J = 8.6, 2.1) 7.76 (dd, 1H, J = 8), 8.05 (m, 1H), 8.23 (ddd, 1H, J = 7.9, 1.7, 1.0), 8.41 (ddd, J = 8.2, 2.2, 1.0), 8.83 (dd, 1H, J = 2.1, 1.9); ^{13}C NMR ($CDCl_3$) 21.4, 109.1, 109.1, 118.4, 121.7, 124.0, 125.6, 125.6, 126.3, 130.7, 123.2, 134.2, 136.0, 136.3, 160.8, 178.6; MS *m*/*z* (% rel int) 281.0692 (M^+, 100, $C_{16}H_{11}NO_4$ requires 281.0688), 253 (13%), 325 (8%), 134 (33%), 106 (8%).

2-Acetyl-1-(4-nitrobenzoyloxy)-4-methylbenzene (Scheme 1; I leading to 1o). The compound was prepared according to the procedure described for **I** leading to **1j**. The crude product was recrystallized from acetone/H_2O to give white crystals (yield 56%): mp 109−111 °C; ^{1}H NMR ($CDCl_3$) 2.46 (s, 3H), 2.56 (s, 3H), 7.15 (d, 1H, J = 8.2), 7.44 (dd, 1H, J = 8.2, 2.2), 7.71 (d, 1H, J = 2.0), 8.37 (d, 2H, J = 8.0), 8.39 (d, 2H, J = 8.0); ^{13}C NMR ($CDCl_3$) 21.4, 29.6, 124.0, 124.2, 130.3, 131.6, 131.8, 134.8, 135.4, 137.0, 147.1, 151.3, 164.2, 197.8; MS *m*/*z* (% rel int) 299.0795 (M^+, 15, $C_{16}H_{13}NO_5$ requires 299.0794), 150 (100%), 120 (9%), 104 (33%), 76 (20%).

1-(4-Nitrophenyl)-3-[2-(1-hydroxy-4-methylphenyl)]propane-1,3-dione (Scheme 1; II leading to 1o). The compound was prepared according to the procedure described for **II** leading to **1j**. The crude product was recrystallized from acetone to give orange crystals (yield 53%), mp 193−195 °C. Only the enol form was obtained: ^{1}H NMR ($CDCl_3$) 2.37 (s, 3H), 6.91 (s, 1H), 6.96 (d, 1H, J = 8.5), 7.35 (dd, 1H, J = 8.5, 2.2), 7.57 (d, 1H, J = 1.3), 8.13 (d, 2H, J = 9.1), 8.36 (d, 2H, J = 9.1), 11.77 (s, 1H); ^{13}C NMR ($CDCl_3$) 21.0, 94.6, 118.8, 119.2, 124.4, 128.1, 128.7, 128.9, 138.2, 139.9, 161.1, 173.9, 196.7; MS *m*/*z* (% rel int) 299.0794 (M^+, 85, $C_{16}H_{13}NO_5$ requires 299.0794), 282 (16%), 177 (8%), 150 (100%), 134 (82%), 104 (20%).

6-Methyl-4′-nitroflavone (1o). The compound was prepared according to the procedure described for **1j**. The crude product was recrystallized from acetone to give white crystals (yield 83%): mp 276−278 °C; ^{1}H NMR ($CDCl_3$) 2.43 (s, 1H), 7.21 (s, 1H), 7.68 (dd, 1H, J = 8.9, 2.1), 7.72 (d, 1H, J = 8.6), 7.85 (m, 1H), 8.37 (s, 4H); ^{13}C NMR ($CDCl_3$) 21.4, 109.9, 119.4, 125.0, 125.0, 128.6, 136.4, 136.6, 138.1, 149.9, 154.9, 160.9, 177.1; MS *m*/*z* (% rel int) 281.0685 (M^+, 100, $C_{16}H_{11}NO_4$ requires 281.0688), 253 (12%), 235 (8%), 134 (34%).

6-Methyl-3′,5-dinitroflavone (1q) (Scheme 3). 100 mg of 6-methylflavone dissolved in 3 mL of dry methylene chloride was added to a mixture of 5 mL of 65% nitric acid with an excess of phosphorus pentaoxide in an ice-cooled 50-mL round-bottomed flask. The mixture was allowed to reach room temperature and was quenched with 6 M KOH after 15 min. After neutralization and extraction with methylene chloride, **1q** was isolated from the crude product mixture by preparative TLC (mobile phase toluene:methyl *tert*-butyl ether, 5:1): ^{1}H NMR (DMSO) 2.06 (s, 3H), 5.74 (s, 3H), 7.88 (dd, 1H, J = 8), 7.94 (d, 1H, J = 8.8), 8.08 (d, 1H, J = 8.7), 8.44 (ddd, 1H, J = 8.2, 2.3, 0.9), 8.56 (ddd, 1H, J = 7.9, 1.8, 1.0), 8.86 (dd, 1H, J = 2); ^{13}C NMR ($CDCl_3$) 16.2, 112.3, 114.9, 121.4, 126.0, 126.8, 127.8, 132.7, 133.8, 135.1, 138.3, 146.6, 148.2, 154.8, 162.9, 174.6; MS *m*/*z* (% rel int) 326.0547 (M^+, 80, $C_{16}H_{10}N_2O_6$ requires 326.0539), 309 (45%), 263 (27%), 234 (16%), 149 (14%), 133 (25%), 84 (100%), 66 (100%).

6-Methyl-3′-nitrothioflavone (2b). 1 g of phosphorus pentoxide (7.05 mmol) and 0.5 mL of 100% phosphoric acid were mixed in a 25-mL round-bottomed flask. The mixture was heated under nitrogen to 80−90 °C for 15 min, whereafter thiocresol (68 mg, 0.55 mmol) and ethyl *m*-nitrobenzoylacetate (135 mg, 0.57 mmol) were added. After 90 min the reaction mixture was poured onto ice, and the water phase was extracted with methylene chloride. The compound was purified by flash chromatography (heptane:ethyl acetate, 9:1) to give white crystals (yield 24%): mp 192−194 °C; ^{1}H NMR ($CDCl_3$) 2.53 (s, 3H), 7.27 (s, 1H), 7.52 (ddd, 1H, J = 8.2, 2.0, 0.4), 7.60 (d, 1H, J = 8.2), 7.73 (dd, 1H, J = 8), 8.02 (ddd, 1H, J = 7.8, 1.8, 1.0), 8.37 (m, 1H), 8.38 (m, 1H), 8.57 (dd, 1H, J = 2); ^{13}C NMR ($CDCl_3$) 21.8, 122.5, 124.9, 125.6, 126.9, 128.9, 130.9, 131.0, 133.1, 133.1, 133.9, 134.3, 138.8, 139.2, 150.2, 181.0; MS *m*/*z* (% rel int) 297.0462 (M^+, 100, $C_{16}H_{11}NO_3S$ requires 297.0460), 269 (27%), 223 (13%), 208 (16%), 150 (15%), 121 (13%).

Binding Studies. [^{3}H]Flumazenil (specific activity 87.5 Ci/mol) was obtained from DuPont, New England Nuclear. Protein concentration was determined using the DC protein assay from Bio-Rad Laboratories. All compounds tested were dissolved in DMSO in a stock solution of 100 μM and diluted into 50% ethanol prior to the binding assay.

Rat Brain Membrane Preparation for Binding Studies. The radioreceptor binding assay was carried out as previously described.[23] In brief, male Wistar rats (weighing about 200 g) were decapitated and the brains excised. The cerebral cortex (∼260 mg) was removed and homogenized in 10 mL of Tris-citrate buffer (50 mM, pH 7.1) using an Ultra-Turrax homogenizer, and the homogenate was centrifuged (20000*g* at 0−4 °C for 10 min). The pellet was resuspended in 15 mL of Tris-citrate buffer (50 mM, pH 7.1). This washing procedure of the membranes was repeated twice, and the final pellet was resuspended in Tris-citrate buffer (50 mM, pH 7.1) to give a final concentration of 2 mg of original tissue/mL and used for [^{3}H]flumazenil binding assays.

[^{3}H]Flumazenil Binding Assay. Each assay consists of 0.5 mL of rat cortical membrane suspensions (2 mg/mL), 25 μL containing test compound, and 25 μL of [^{3}H]flumazenil (final concentration 0.3−0.5 nM). For saturation binding experiments, 6−8 different concentrations of [^{3}H]flumazenil (final concentration 0.05−10 nM) were used. Nonspecific binding was obtained by adding diazepam (final concentration 10 μM) to separate samples. All samples was incubated on an ice bath at 0−4 °C for 40 min, followed by filtration using a semiautomatic cell harvester (Skatron Instruments) to separate bound from free radioactive ligand. Filters were washed with 7 mL of ice-cold Tris-citrate buffer, and the radioactivity on the filters was determined by conventional liquid scintillation counting. Nonspecific binding represented less than 10% of the total binding. All assays were done in duplicate.

The GABA ratio was defined by dividing the K_i value of the test compound without adding GABA to the binding assay by the K_i value of the test compound in the presence of GABA (100 μM) in the binding assay. The K_i value was calculated by the equation: $K_i = IC_{50}/(1 + [^3H]\text{flumazenil}]/K_D)$. The K_D value for [^{3}H]flumazenil binding under the assay conditions used in this study is 1.1 ± 0.2 nM (mean ± SEM of five separate experiments).

Computational Methods. Geometry optimizations and conformational analyses of the flavones were performed by using the molecular mechanics program MM3(92) developed by Allinger and co-workers.[24,25] In addition to the standard MM3(92) parameter set, the following three sets of torsional parameters were used: (O=)C−Csp2−Csp2−O V1 = V3 = 0.0, V2 = 10.0; (O=)C−Csp2−Csp2−N(O_2) V1 = V3 = 0.0, V2 = 10.0; Csp3−Csp2−Csp2−N(O_2) V1 = V3 = 0.0, V2 = 10.0. These parameters are required for the calculations to run. However, the Csp2−Csp2 bonds belong to π-systems which stay essentially planar in all calculations. Thus, the precise

values of the torsional constants do not significantly affect the results. Since π-atom pair parameters for (O═)C···S are not included in the MM3(92) parameter set, geometry optimizations and conformational analyses of the thioflavones **2a** and **2b** were performed by using the MM2(91) program.[26]

For the calculations of molecular electrostatic potentials, the HF/6-31G* basis set was employed with geometries calculated by the same set. The calculations were performed by using the SPARTAN program version 5.0.3.[27]

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