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Flavylium salts as in vitro precursors of potent ligands to brain GABA-A receptors

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ABSTRACT

The synthesis of a series of derivatized flavylium cations was undertaken and the affinity to the benzodiazepine binding site of the GABA-A receptor evaluated. The observed high affinity for some derivatives (sub-μM range) was explained by an in vitro transformation of the flavylium cations into the corresponding *trans*-retrochalcones, components which are proposed to be the active species in this series.

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γ-Aminobutyric acid (GABA) activates chloride ion channels by binding to GABA-A receptors in the brain. The GABA-A receptor is a heteropentameric, trans-membrane protein complex consisting of multiple combinations of distinct receptor subunits.[1,2] It is generally believed that the most abundant GABA-A receptor in the adult mammalian brain consists of an $\alpha_1\beta_2\gamma_2$ subunit combination.[3] The brain GABA-A receptor contains a variety of binding sites for compounds, which modulate the functional effects of GABA to operate on the chloride ion channel.[4,5] The benzodiazepine binding site of the GABA-A receptor recognizes a wide range of structurally different compounds, 1,4-benzodiazepines as well as non-benzodiazepine compounds:[6–8] 1,4-benzodiazepines (e.g., diazepam); β-carbolines (e.g., abercanil and DMCM); triazolopyridazines (e.g., CL 218.872); imidazopyridines (e.g., zolpidem); pyrazoloquinolinones (e.g., CGS 9896); cyclopyrrolones (e.g., zopiclone); imidazopyrimidines (e.g., divalone); pyra-zolopyrimidines (e.g., zaleplon); triazolopyridazines (e.g., L-838.417); imidazobenzodiazepines (e.g., RY 80); and pyridoindoles (e.g., SL 651.498). Amentoflavone was the first non-nitrogen containing compound to be identified showing high affinity to benzodiazepine binding site of the GABA-A receptor in vitro.[9] Later a series of natural as well as synthetic flavone derivatives were identified having low nanomolar affinity to benzodiazepine binding site.[10,11]

In our search for new natural compounds acting on the GABA receptor, we decided to investigate further the action of flavonoids by testing flavylium compounds (Fig. 1) for which some biological properties have already been described such as anti-mutagenic,[12] anti-tumoral,[13] or anti-malarial[14] activities.

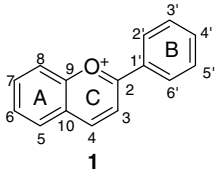

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Figure 1. The flavylium skeleton.

Flavylium derivatives, including anthocyanidins or anthocyanins, are very important plant pigments which are compounds known to be subjected to photochromism. This phenomenon occurs through *cis–trans* photochemical isomerization of the chalcone forms resulting from the reversible pyrylium ring opening by water.[15] Because flavylium compounds are stable powders which are easily prepared, they could be used as convenient in vitro precursors of retrochalcones when dissolved in neutral buffers, resulting in a possible delayed action on the GABA receptor.

In this letter, we describe the synthesis of diversely substituted flavylium derivatives and the binding assays for the benzodiazepine binding site of the GABA-A receptor on rat-brain membranes. A range of various affinities were observed reaching nM values for the most active compounds for which analyses of the chemical transformation correlated with time-dependent binding assays were performed.

Flavylium hexafluorophosphates, substituted on their A-ring, were synthesized in one step by condensing one equivalent of

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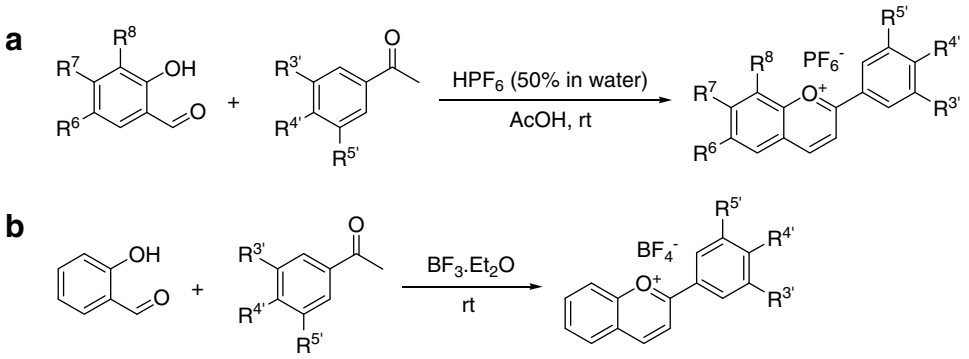


Scheme 1. Condensation step for the synthesis of flavylium salts: for (a) A-ring substituted and (b) A-ring unsubstituted derivatives.

Table 1
Binding data for flavylium cations **1a–q**

Compound	Substitution						Affinity[a] K_i[b](μM)
	R^6	R^7	R^8	$R^{3'}$	$R^{4'}$	$R^{5'}$	
1a	OMe	H	H	H	H	H	0.18
1b	OMe	H	H	H	OMe	H	0.06
1c	OMe	H	H	H	OH	H	0.16
1d	OH	H	H	H	OMe	H	0.32
1e	OH	H	H	H	OH	H	1.50
1f	H	H	H	H	OMe	H	0.48
1g	Br	H	H	H	OMe	H	0.14
1h	OMe	H	H	OMe	OMe	H	n.a. at 10
1i	H	H	H	OMe	OMe	H	n.a. at 10
1j	H	H	H	OMe	OMe	OMe	n.a. at 10
1k	H	OH	H	H	OH	H	0.28
1l	H	OH	H	H	H	H	0.26
1m	H	OMe	H	H	H	H	1.80
1n	H	OMe	H	H	OMe	H	1.20
1o	H	H	OH	H	OH	H	n.a. at 10
1p	H	OH	OH	H	OH	H	n.a. at 10
1q	OMe	OH	H	H	OMe	H	7.80

[a] n.a. denotes no inhibition of specific [^{3}H]-flumazenil binding.
[b] Each K_i value is the mean of three determinations.

benzaldehyde with one equivalent of acetophenone in acetic acid in the presence of hexafluorophosphoric acid.[16,17] In the absence of substituents on the A-ring, we performed a solvent-free condensation in the presence of $BF_3{\cdot}Et_2O$[18,19] (Scheme 1).

The results of the competition binding assays with the benzodiazepine antagonist [^{3}H]-flumazenil on membranes from rat-brain in vitro are listed in Table 1.[20]

A clear structure–activity analysis cannot be drawn from these data obtained on hydroxy- and methoxy-substituted flavylium derivatives. The effect of the compounds was measured on the benzodiazepine binding site of the GABA-A receptors in rat cortical membranes in vitro. The derivatives showed a range of affinities reaching nanomolar values for the most active compounds. Possibly, the 3′-OMe or 8-OH substituted derivatives display very low recognition of the binding site. All these values were determined in standard binding conditions by incubating the probes for 15 min in the buffer before performing the test. Knowing the poor stability of the flavylium salts in aqueous medium prompted us to analyze these reactions in further detail.

The chemical transformations occurring in aqueous medium are well described in the literature[15] and are summarized in Scheme 2.

For 4′-OH substituted derivatives (compounds **1c**, **1e**, **1k**, **1o**, and **1p**), two competing processes are in equilibria: deprotonation of the hydroxyl phenolic group, leading to quinone species versus addition of water at position-2 of the flavylium salt, leading to reversible ring opening as illustrated in Scheme 2, the *trans*-retrochalcone being the ultimate structure formed. The latter mechanism accounts for all the flavylium derivatives.

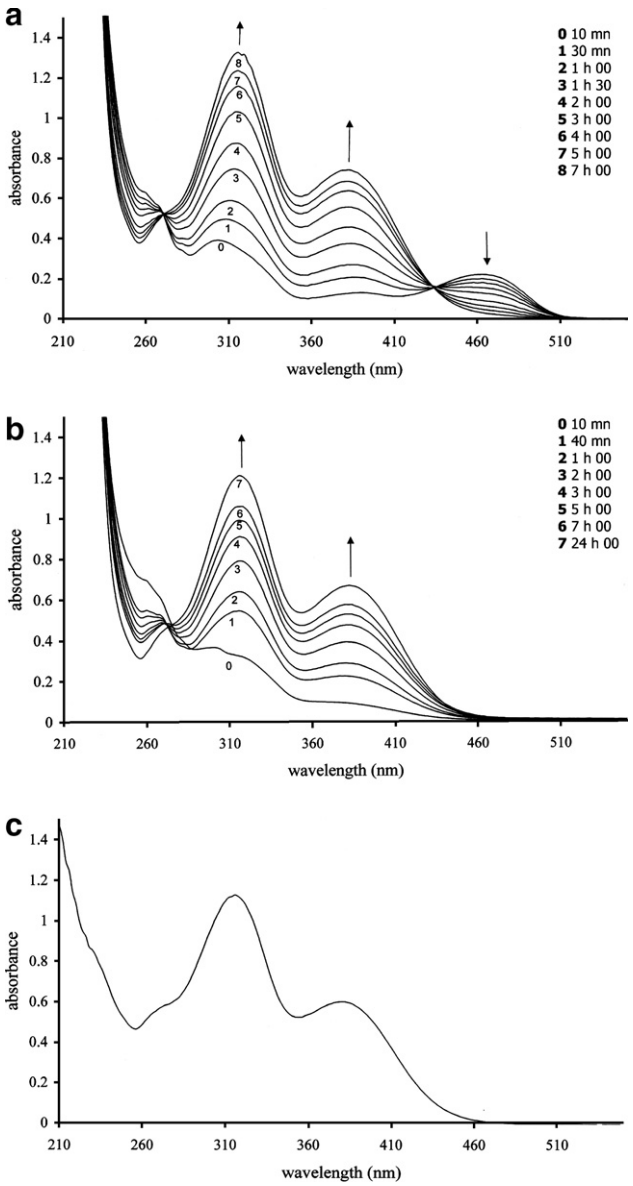
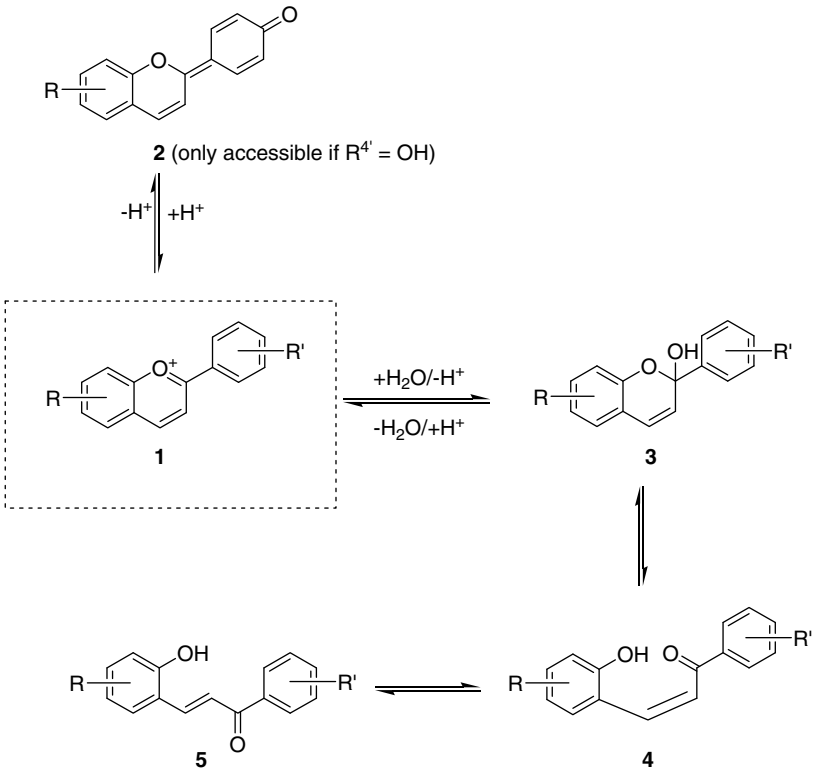


Figure 2. UV-analysis of the evolution of flavylium cation **1b** in aqueous media. (a) In aqueous ethanol. (b) In buffer (Tris–citrate, pH 7.1). (c) UV–visible spectra of *trans*-chalcone **5b** in aqueous ethanol.



Scheme 2. Chemical transformation of flavylium cations in aqueous media.

As the relative concentrations of the different flavylium derivatives in solution (structures **1**–**5**) are time and pH-dependent, we analyzed the transformations by UV spectroscopy. The binding assays use aqueous ethanol as initial incubation medium before dilution in buffer (pH 7.1), therefore we analyzed by UV these transformations on the most potent flavylium cation **1b**, subsequently in aqueous ethanol and in buffer (Fig. 2a and b).

Clearly, the UV analyses in water/alcohol or buffer suggest the transformation of the flavylium salt into a similar compound, presumably the *trans*-retrochalcone. A main difference, however, can be noticed around 460 nm, which is characteristic of the flavylium absorbance. There is a slow and progressive disappearance of this peak in water/alcohol while in buffer, there is an instant loss of the orange flavylium color, likely due to addition of a water molecule to the flavylium chromophore (see Scheme 2) before a slow conversion to the *trans*-retrochalcone **5b**. A quantitative analysis of this transformation performed in a 1:1 CH_3CN/H_2O mixture allowed isolation after 24 h of the *trans*-retrochalcone with 67% yield after work up and chromatography on silica gel.[21] The corresponding UV–visible spectrum in aqueous ethanol is given in Figure 2c.

To corroborate the ligand–receptor interactions with these chemical transformations, we checked the influence of pre-incubation of the sample in aqueous ethanol on the binding affinities. Pre-incubation of flavylium **1b** for 15 min and 24 h, respectively, before keeping the samples for 40 min at 0–4 °C in the binding assay, led to a slight increase in affinity going from 60 to 18 nM. The synthesized *trans*-retrochalcone **5b** showed a K_i value of 28 nM, value which is slightly higher than the K_i value (18 nM) obtained on the corresponding flavylium derivative **1b**. A similar change in affinity was observed for the 6-Br-4′-OMe flavylium **1g** increasing from 136 to 66 nM in the same pre-incubation time range. Even though, each flavylium salt will display individual kinetics of transformation to the corresponding *trans*-retrochalcone, the disappearance of the flavylium salt in buffer is instant and the binding values given in Table 1 correspond therefore to a mixture of chalcone species. These binding values might be slightly changed to lower K_i values by longer incubation times as illustrated for compounds **1b** and **1g**.

This letter describes for the first time that transformation of flavylium salts in vitro leads to compounds showing nanomolar affinity to the benzodiazepine binding site of the brain GABA-A receptor. Flavylium salts are stable and convenient synthetic precursors of retrochalcones, which are suggested to be the biologically active species. Further studies are needed to characterize the interaction of the flavylium derivatives on GABA-A receptors.

Acknowledgments

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17. *Typical procedure for the preparation of flavylium hexafluorophosphates* ***1a–e, 1g–h*** *and* ***1k–q****:* To 1.0 mmol of benzaldehyde (1.0 equiv) and 1.0 mmol of acetophenone (1.0 equiv), solubilized in the minimum of acetic acid, is added an excess of hexafluorophosphoric acid, 50% in H_2O. The mixture becomes immediately dark red and is stirred at room temperature for 48 h. The mixture is then plunged in 20 ml of diethyl ether and a colored solid precipitates. This solid is isolated by filtration and washed several times with diethylether to give the expected flavylium hexafluoro-phosphate in pure form.*6,4′-Dimethoxyflavylium hexafluorophosphate (**1b**):* 89%, orange solid. UV/vis (EtOH/10% 1 N HCl): λ_{max} (ε) = 298 (15,100), 392 (ép.), 464 (33,400) nm (M^{-1} cm^{-1}). RMN ^1H (300 MHz, CD_3CN/1% TFAd_1, 25 °C): δ = 4.01 (s, 3H, OCH_3), 4.02 (s, 3H, OCH_3), 7.29 (m, 2H, AA′ part of an AA′MM′ system, 3′-H and 5′-H), 7.61 (d, 1H, $^4J_{5-7}$ = 2.9 Hz, 5-H), 7.85 (dd, 1H, $^3J_{7-8}$ = 9.5 Hz, $^4J_{7-5}$ = 2.9 Hz, 7-H), 8.19 (dd, 1H, $^3J_{8-7}$ = 9.5 Hz, $^5J_{8-4}$ = 0.7 Hz, 8-H), 8.47 (d, 1H, $^3J_{3-4}$ = 9.1 Hz, 3-H), 8.50 (m, 2H, MM′ part of an AA′MM′ system, 2′-H and 6′-H), 9.10 (dd, 1H, $^3J_{4-3}$ = 9.1 Hz, $^5J_{4-8}$ = 0.7 Hz, 4-H) ppm. RMN ^{13}C (75 MHz, CD_3CN/1% TFAd_1, 25 °C): δ = 56.3, 56.4, 108.4, 116.3, 117.4, 120.5, 121.0 (Cq), 125.5 (Cq), 130.0, 133.1, 151.7 (Cq), 153.6, 160.0 (Cq), 168.0 (Cq), 173.1 (Cq) ppm. MS (ESI positive mode): *m/z* (%) = 267 (100) $[M]^+$. Calcd pour $C_{17}H_{15}O_3$ $[M]^+$ 267.1016, found 267.1047.
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19. *Typical procedure for the preparation of flavylium tetrafluoroborates* ***1f, 1i–j****:* To 122 mg of salicylaldehyde (1 mmol, 1.0 equiv) and 1 mmol of acetophenone (1.0 equiv) is added, at room temperature, 150 µl of $BF_3{\cdot}Et_2O$ (1.4 mmol, 1.4 equiv). The mixture becomes immediately dark red and is stirred at room temperature until obtention of a solid. Then, a small volume of diethylether is added and the mixture is heated to reflux and stirred one hour. The resulting colored solid is isolated by filtration and washed several times with diethylether to give the expected flavylium tetrafluoroborate in pure form.*4′-Methoxyflavylium tetrafluoroborate (**1f**):* 57%, yellow solid. UV/vis (EtOH/10% 1 N HCl): λ_{max} (ε) = 254 (11,700), 282 (11,800), 444 (44,500) nm (M^{-1} cm^{-1}). RMN ^1H (300 MHz, CD_3CN/1% TFAd_1, 25 °C): δ = 4.00 (s, 3H, OCH_3), 7.27 (m, 2H, AA′ part of an AA′MM′ system, 3′-H and 5′-H), 7.90 (m, 1H), 8.23 (m, 3H), 8.49 (d, 1H, $^3J_{3-4}$ = 9.1 Hz, 3-H), 8.54 (m, 2H, MM′ part of an AA′MM′ system, 2′-H and 6′-H), 9.18 (d, 1H, $^3J_{4-3}$ = 9.1 Hz, 4-H) ppm. RMN ^{13}C (75 MHz, CD_3CN/1% TFAd_1, 25 °C): δ = 56.2, 116.4, 117.0, 118.8, 120.7 (Cq), 123.7 (Cq), 129.8, 130.3, 133.9, 138.8, 154.9, 155.7 (Cq), 168.8 (Cq), 175.0 (Cq) ppm. MS (ESI positive mode): *m/z* (%) = 237 (100) $[M]^+$. Calcd for $C_{16}H_{13}O_2$ $[M]^+$ 237.0910, found 237.1068.
20. *Typical procedure for the radioligand binding assays. Tissue preparation:* Cerebral cortex from male Wistar rats (150–200 g) is homogenized for 5–10 s in 20 ml Tris–HCl (30 mM, pH 7.4) using an Ultra-Turrax homogenizer. The suspension is centrifuged at 27,000*g* for 15 min at 0–4 °C followed by three centrifugation (15,000*g*, 10 min, 0–4 °C) resuspension cycles (Tris, HCl 50 mM, pH 7.4). The washed pellet is homogenized in 20 ml of Tris, HCl buffer and incubated on a water bath (37 °C) for 30 min to remove endogenous GABA and then centrifuged for 10 min at 27,000*g* (0–4 °C). The final pellet is resuspended into 10 ml of Tris, HCl buffer (concentration 20 mg original tissue per ml). The preparation is frozen and stored at −20 °C until use within a maximum of 3 months.*Binding assay:* The membrane preparation is thawed and centrifuged at 0–4 °C for 10 min at 27,000*g*. The pellet is resuspended in 50 mM Tris, citrate, pH 7.1 (1 mg original tissue per 0.5 ml buffer) and then used for binding assay. Aliquots of 500 µl membrane preparation are added to 25 µl of test solution and 25 µl of [^{3}H]-flumazenil (^{3}H-Ro 15-1788, 70.8 Ci/mmol, NEN Life Sciences products) (1 nM, final concentration in the assay), mixed and incubated for 40 min at 0–4 °C. Non-specific binding is determined using Clonazepam (1 µM, final concentration). Following incubation, the samples are added to 3 ml of ice-cold Tris, citrate (50 mM, pH 7.1) and poured directly on to Whatman GF/C glass fiber filters under suction and immediately washed with 3 ml ice-cold Tris, citrate buffer. The amounts of tritium on the filters are determined by conventional liquid scintillation counting. All samples are done in triplicate. Stock solutions (10 mM) of flavylium derivatives are made in DMSO. For the determination of IC_{50} values, 5–7 different concentrations of substances are made into 48% of ethanol.
21. *Procedure for the preparation of trans-6,4′-dimethoxyretrochalcone (**5b**):* 0.1 mmol (41 mg) of 6,4′-dimethoxyflavylium hexafluorophosphate **1b** were dissolved in 40 ml of a 1:1 CH_3CN/H_2O mixture. The resulting solution was stirred for 24 h at room temperature, then acetonitrile was evaporated under vacuum. The resulting aqueous phase was extracted three times with dichloromethane and the organic layer was washed with water and brine and then dried over Na_2SO_4. After evaporation of the solvent, the resulting crude product was purified by column chromatography (heptane/ethyl acetate 2:1) to give 0.67 mmol of *trans*-6,4′-dimethoxyretrochalcone **5b** in pure form.*trans-6,4′-Dimethoxyretrochalcone (**5b**):* 67%, yellow oil. UV/vis (EtOH/10% 1 N HCl): λ_{max} = 316, 380 nm (M^{-1} cm^{-1}). RMN ^1H (300 MHz, CD_3OD, 25 °C): δ = 3,78 (s, 3H), 3,88 (s, 3H), 6.80 (d, 1H, ^3J = 8.8 Hz), 6.86 (dd, 1H, ^3J = 8.8 Hz, ^4J = 2.9 Hz), 7.04 (m, 2H, AA′ part of an AA′MM′ system), 7.20 (d, 1H, ^4J = 2.9 Hz), 7.78 (d, 1H, ^3J = 15.7 Hz), 8.06 (m, 2H, MM′ part of an AA′MM′ system), 8.07 (d, 1H, ^3J = 15.7 Hz) ppm. RMN ^{13}C (75 MHz, CD_3OD, 25 °C): δ = 54.6, 54.9, 112.2, 113.6, 116.6, 118.3, 121.1, 122.0 (Cq), 130.6, 130.9 (Cq), 140.1, 151.6 (Cq), 153.0 (Cq), 163.8 (Cq), 190.1 (Cq) ppm.