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Structure–activity relationship of wedelolactone analogues: Structural requirements for inhibition of Na^+,K^+-ATPase and binding to the central benzodiazepine receptor

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Abstract—Coumestans **2a**–**i**, bearing different patterns of substitution in A- and D-rings, were synthesized and evaluated as inhibitors of kidney Na^+,K^+-ATPase and ligands for the central benzodiazepine (BZP) receptor. The presence of a hydroxyl group in position 2 favours the effect on Na^+,K^+-ATPase but decreases the affinity for the BZP receptor, allowing the design of more selective molecules than the natural wedelolactone. On the other hand, the presence of a catechol in ring D is important for the effect on both molecular targets.

1. Introduction

Wedelolactone (**1**, Fig. 1) is a naturally occurring coumestan from *Eclipta prostrata*, a *Leguminosae* used in folk medicine against snake poison.[1] Some pharmacological effects of wedelolactone are probably important for this use, such as its antihaemorrhagic, antiproteolytic and antiphospholipase activities.[1,2] On the other hand, other effects of wedelolactone are probably unrelated to its antimyotoxic action, such as the inhibition of Na^+,K^+-ATPase and [^{3}H]flunitrazepam binding.[3a] Both effects are potentially relevant and could be useful for the development of new molecules acting on the Na^+,K^+-ATPase or the central benzodiazepine receptor, two molecular targets of clinical importance, as briefly discussed below. In such case, however, it should be important to design molecules with selectivity for only one of these two targets, since there is no rationale for a dual action. We previously described in details the

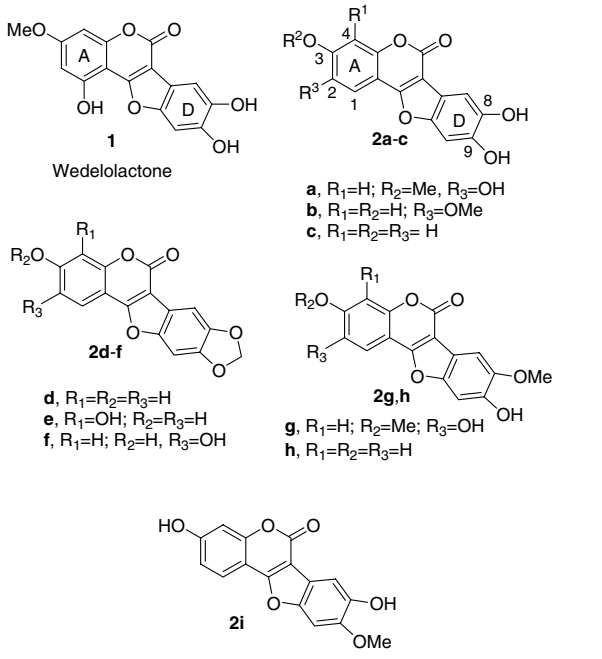

Figure 1. Wedelolactone (**1**) and analogues bearing different patterns of substitution in rings A and D.

Keywords: Wedelolactone; ATPase,Na-K; Benzodiazepine; Coumestan; Isoflavonoid; SAR.
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molecular mechanism of action of 2-methoxy-3,8,9-trihydroxy coumestan (**2b**), a non-steroidal original synthetic derivative from wedelolactone (**1**) (Fig. 1), on both Na^+,K^+-ATPase and (central) benzodiazepine (BZP) site present on the $GABA_A$ receptor complex.[4,5]

Our first molecular target, the Na^+,K^+-ATPase, is a plasma membrane protein responsible for the maintenance of low Na^+, high K^+ cellular concentrations using the energy derived from the hydrolysis of ATP. Besides that, this protein is considered as the molecular target of cardiac glycosides,[6] drugs used in the treatment of heart failure due to their positive inotropic effect and beneficial effects on hemodynamics.[7] The use of cardiac glycosides such as digoxin in the treatment of moderate and severe congestive heart failure, mainly in combined therapy, has been recently reinforced by two multicentric studies[8,9] that confirmed the clinical efficacy of digoxin and its positive effects on morbidity. In addition, a clinical trial sponsored by the NIH[10] demonstrated that the use of digoxin reduces the number of hospitalizations for these patients. However, the adverse effects and narrow therapeutic index of digoxin render its use difficult and support the interest for searching new inotropic compounds acting through the inhibition of Na^+,K^+-ATPase. The interest of non-steroidal or altered steroid-like inhibitors of Na^+,K^+-ATPase has recently emerged in the literature,[11–13] since failure to improve the therapeutic index of digoxin has been attributed to the preservation of the C/D-*cis* junction in the steroid backbone, unique to the cardiac glycosides.[14]

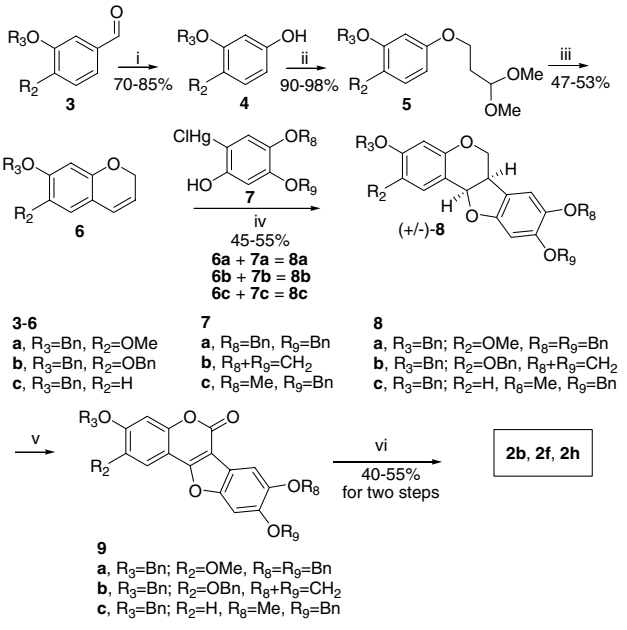
Our second molecular target, the central benzodiazepine receptor, is an allosteric modulatory site present on the $GABA_A$ receptor complex responsible for inhibitory pathways in the central nervous system. Classical benzodiazepines (agonists) potentiate the GABA neuropsychological effects by increasing GABA affinity for its binding sites[15] leading to anxiolytic, sedative/hypnotic, anticonvulsant and myorelaxant effects and amnesia. In spite of being among the most frequently prescribed drugs, benzodiazepines have limitations, particularly in relation to long-term use. As a consequence of an improved definition of the appropriate target (specific sub-type of receptor) the development of anxiolytics without the undesirable effects of classical benzodiazepines is currently a hot topic in medicinal chemistry. On the other hand, BZP receptor inverse agonists are capable of producing convulsions and anxiety but if this molecule bound at a particular sub-type of receptor (α_5) their effects in cognitive function could be clinically useful in Alzheimer's disease and related dementia.[16] The aim of the present work was to perform a structure–activity relationship (SAR) study with nine coumestans (**2a**–**i**) in order to obtain information on the structural requirements for each of the two molecular targets described above, namely Na^+,K^+-ATPase and BZP receptor. Wedelolactone was used as a reference compound. The syntheses of new, non-natural coumestans **2b** and **2f**, as well as 8-methoxycoumestrol (**2h**), a natural product isolated from *Medicago sativa*,[17] are described for the first time, while coumestans **2a**, **2c**, **2d**, **2e**, **2g** and **2i** were synthesized as previously described.[3]

2. Chemistry

Chromenes **6a**–**c** were used as intermediates to prepare coumestans **2b**, **2f** and **2h**. Baeyer–Villiger oxidation of easily available aldehydes **3a**–**c** followed by O-alkylation of the resulting phenols **4a**–**c** with 3-iodo-1,1-dimethoxypropane led to intermediates **5a**–**c** (Scheme 1). Cyclization of these intermediates took place under acid conditions, furnishing chromenes **6a**–**c**. Phenols **4a**–**c** were also used as precursors of organomercurials **7a**–**c**. Oxa-Heck coupling between **6a**–**c** and **7a**–**c** led to the corresponding protected pterocarpans **8a**–**c**. Oxidation of the **8a**–**c** with DDQ in THF furnished the coumestans **9a**–**c**. The benzyl protecting groups in **9a**–**c** were removed by hydrogenolysis (H_2, Pd–C) to give the desired coumestans **2b**, **2f** and **2h**.

3. Pharmacology

All the 10 coumestans tested inhibited the Na^+,K^+-ATPase activity, but with significant differences in potency (Table 1). Comparing the coumestans **2a**, **2b** and **2c**, all bearing a catechol group in the D-ring, with wedelolactone (**1**) we can observe that the naturally occurring coumestan **1** and the synthetic isomer **2a** were the most potent molecules ($IC_{50} \approx 0.7\ \mu M$, Table 1). This result indicates that in A-ring the change of the hydroxyl group from position 1, as in **1**, to position 2, as in **2a**, did not affect the capacity to inhibit the Na^+,K^+-ATPase. On the other hand, when the positions of the hydroxyl and methoxy groups present in **2a** are inverted, as in **2b**, a five times loss of affinity is observed, suggesting that the hydroxyl group in position 2 is important for the interaction with this enzyme. The absence of a



Scheme 1. Synthesis of coumestans **2b**, **2f** and **2h**. Reagents and conditions: (i) MCPBA, CH_2Cl_2; (ii) 3-iodo-1,1-dimethoxypropane, KOH, THF; (iii) HCl (20%), THF; (iv) $PdCl_2$, LiCl, acetone; (v) DDQ, THF; (vi) H_2 (3.5 atm), Pd–C.

Table 1. IC_{50} values for inhibition of rat kidney Na^+,K^+-ATPase activity (NaK) and [^{3}H]flunitrazepam binding to rat cerebral synaptosomes (BZD)

	1	2a	2b	2c	2d	2e	2f	2g	2h	2i
NaK	0.7	0.7	3	10	20	25	3	6	30	25
BZP	2	≫100	16	2	≫30	≫100	≫30	≫30	20	50

IC_{50} values, expressed in μM, were obtained from at least three experiments performed in triplicate. When sufficient data were available, these IC_{50} were calculated by non-linear regression. In the other cases, the values were estimated graphically (see detailed results available in supplementary material). Symbol ≫ indicates that much less than 50% inhibition was obtained at the highest concentration tested (indicated in μM).

hydroxyl group at position 2, as in **2c**, leads to an even higher loss of affinity. The same pattern was observed comparing the potency of **2d**, **2e** and **2f**, all bearing a less polar methylenedioxy group at the D-ring but a different pattern of oxygenation at the A-ring. Coumestan **2f**, bearing a hydroxyl group at the 2-position, is 7–8 times more potent than **2d** and **2e**. When the phenol group at C8 (D-ring) in **2a** was methylated, as in **2g**, the potency also decreased ten times. Finally, the comparison of **2c** with **2h** and **2i**, which do not present a hydroxyl group at C2, shows that the catechol group at the D-ring is the best substituent for Na^+,K^+-ATPase inhibition.

The BZP binding affinity of coumestans was evaluated by their ability to displace [^{3}H]flunitrazepam from its specific binding in rat brain synaptosomes (Table 1). Wedelolactone (**1**) inhibited this binding with a tenuous difference of potency when compared to its inhibitory effect on Na^+,K^+-ATPase (IC_{50} = 2 and 0.7 μM, respectively). However, the structural requirement here seems to be very different from that for Na^+,K^+-ATPase since the presence of a hydroxyl in position 1 or 2 is here unnecessary or even unfavourable, as illustrated by the equipotency of **2c** (lacking a hydroxyl in C1 and C2) and **1** (with a hydroxyl in C1) and by the loss of activity of **2a** as compared to **2b** and **1**.

Besides that, the loss of the catechol group in the D-ring resulted in a decrease of affinity for the benzodiazepine receptor, as observed when comparing **2c** with **2d**, **2h** and **2i**.

4. Conclusion

Our present results indicate that it is chemically possible to separate the inhibition of Na^+,K^+-ATPase from the benzodiazepine-like effect and thus to design more selective compounds against the two molecular targets studied here, namely Na^+,K^+-ATPase and central benzodiazepine receptor. Both the hydroxyl group in position 2 and the catechol in the D-ring are important to modulate the inhibition of rat Na^+,K^+-ATPase. On the other hand, binding to the central BZP receptor is decreased if a hydroxyl is present in C2. As a consequence, comparing to the natural wedelolactone (**1**) which is nearly equipotent for these two molecular targets, it was possible to construct a molecule, like **2a**, with a high selectivity towards the Na^+,K^+-ATPase and another, like **2c**, with a relative selectivity for the benzodiazepine receptor. In both cases this could be interesting for clinical purposes.

5. Experimental

5.1. Chemistry

^{1}H NMR spectra were recorded on a Varian Gemini (200 MHz) instrument using tetramethylsilane (TMS) as standard and $CDCl_3$ as solvent. *J* values are given in Hz.

5.1.1. Baeyer-Villiger oxidation of aldehydes. Synthesis of 4a–c. *m*-Chloroperbenzoic acid (4.5 mmol) was added to a solution of the aldehyde **3a**–**c** (3.0 mmol) in dichloromethane (15 mL), and the mixture was stirred at room temperature for 24 h and then diluted with ethyl acetate. The organic solution was successively washed with saturated aqueous Na_2CO_3 solution and brine. The solvent was evaporated in vacuo to give corresponding formiate. NaOH (6 N) was added to a stirred solution of crude formiate in MeOH (15 mL). After stirring at room temperature for 5 min, was added 10% aq HCl solution. The reaction mixture was diluted with ethyl acetate (50 mL), washed with brine and dried over anhydrous Na_2SO_4. The flash chromatography (7:3 hexane/ethyl acetate) furnished the compounds **4a**–**c** as solids (70–85% yield).

Compound **4a**: ^{1}H NMR δ 7.37 (5H, m); 6.75(1H, d, *J* 8.61 Hz); 6.45 (1H, d, *J* 2.84 Hz); 6.34 (1H, dd, *J* 8.61, 2.84 Hz); 5.09 (2H, s); 3.82 (3H, s).

Compound **4b**: ^{1}H NMR δ 7.36 (10H, m); 6.79 (1H, d, *J* 8.61 Hz); 6.49 (1H, d, *J* 2.84 Hz); 6.29 (1H, dd, *J* 8.61, 2.84 Hz); 5.11(2H, s); 5.06 (2H, s).

Compound **4c**: ^{1}H NMR δ 7.37 (5H, m); 7.09 (1H, t, *J* 8.0 Hz); 6.48 (3H, m); 5.10 (2H, s).

5.1.2. O-Alkylation of phenols. Synthesis of 5a–c. A mixture of KOH (224 mg, 2 mmol) and phenols **4a**–**c** (2.15 mmol) in THF (25 mL) was stirred for 10 min at room temperature. Then 3-iodopropionaldehyde dimethyl acetal (1 mmol) was added slowly and the resulting mixture refluxed for 5 h. The reaction mixture was cooled and the 3-iodopropionaldehyde dimethyl acetal (1 mmol) was added slowly and the resulting mixture refluxed for 12 h. The reaction mixture was cooled, extracted with ethyl acetate, washed with brine, dried over sodium sulfate and concentrated. The flash chromatography (7:3 hexane/ethyl acetate) furnished a yellow oil (90–98% yield).

Compound **5a**: ^{1}H NMR δ 7.37 (5H, m); 6.81 (1H, d, *J* 8.80 Hz); 6.60 (1H, d, *J* 2.60 Hz); 6.43 (1H, dd, *J* 8.80, 2.60 Hz); 5.13 (2H, s); 4.61 (1H, t, *J* 5.8 Hz); 3.95 (2H,

t, *J* 6.40 Hz); 3.84 (3H, s); 3.36 (6H, s); 2.04 (1H, q, *J* 6.00 Hz).

Compound **5b**: ^{1}H NMR δ 7.38 (10H, m); 6.84 (1H, d, *J* 8.79 Hz); 6.57 (1H, d, *J* 2.80 Hz); 6.29 (1H, dd, *J* 8.80, 2.80 Hz); 5.12 (2H, s); 5.07 (2H, s); 4.60 (1H, t, *J* 5.74 Hz); 3.94 (2H, t, *J* 6.35 Hz); 3.35 (6H, s); 2.03 (2H, q, *J* 6.35 Hz).

Compound **5c**: ^{1}H NMR δ 7.4 (5H, m); 7.2 (1H, t, *J* 8.00 Hz); 6.6 (3H, m); 5.00 (2H, s); 4.60 (1H, t, *J* 5.50 Hz); 4.00 (2H, t, *J* 5.50 Hz); 3.4 (6H, s); 2.1 (2H, q, *J* 5.50 Hz). ^{13}C NMR δ 160.00 (C); 159.90 (C); 136.80 (C); 129.80 (CH); 128.40 (CH); 127.80 (CH); 127.30 (CH); 101.9 (CH_2); 101.80 (CH); 101.70 (CH); 69.80 (CH_2); 63.80 (CH_2); 53.10 (CH_3); 32.6 (CH_2).

5.1.3. Synthesis of chromens 6a–c. To a solution of **5a**–**c** (0.98 mmol) in THF (5 mL) was added 10% aq HCl at 0 °C. The reaction mixture was stirred at 0 °C for 1 h. Then the ice bath was removed and the reaction mixture was stirred for 12 h at room temperature. After this time saturated aqueous $NaHCO_3$ solution was added and the mixture was extracted with ethyl acetate, washed with brine, dried over sodium sulfate, concentrated. The flash chromatography (8:2 hexane/ethyl acetate) furnished a yellow oil (47–53%).

Compound **6a**: ^{1}H NMR δ 7.40 (5H, m); 6.59 (1H, s); 6.42 (1H, s); 6.30 (1H, dt, *J* 13.00, 3.20 Hz); 5.61 (1H, dt, *J* 13.00, 4.90); 5.10 (2H,s); 4.78 (2H, dd, *J* 4.90, 3.20); 3.82 (3H, s).

Compound **6c**: ^{1}H NMR δ 7.40 (5H, m); 6.90 (1H, d, *J* 9.80 Hz); 6.50 (2H, dd, *J* 9.80, 3.00 Hz); 6.50 (1H, d, *J* 3.00 Hz); 6.40 (1H, dt, *J* 13.00, 3.2 Hz); 5.60 (1H, dt, *J* 13.00, 4.90 Hz); 5.00 (2H, s); 4.80 (2 H, dd, *J* 4.90, 3.20). ^{13}C NMR δ 159.60 (C); 155.10 (C); 136.70 (C); 128.40 (CH); 127.30 (CH); 127.10 (CH); 131.10 (C); 124.00 (CH); 118.80 (CH); 115.80 (C); 107.70 (CH); 102.50 (CH); 69.90 (CH2); 65. 40 (CH_2).

5.1.4. Oxa-Heck coupling for the synthesis of 8a–c. To a mixture of $PdCl_2$ (87 mg, 0.49 mmol) and LiCl (42 mg, 1.0 mmol) in acetone (5 mL) were added chromens **6a**–**c** (0.46 mmol) in acetone (10 mL). This mixture was stirred for 15 min at 0 °C and then 2-chloromercurio–phenols **7a**–**c** (0.42 mmol) in acetone (10 mL) were added. The suspension obtained was stirred for 12 h at 25 °C. After this time, brine (150 mL) was added to it and the mixture was extracted with ethyl acetate (3× 50 mL), the organic extract dried (Na_2SO_4) and submitted to column chromatography (8:2 hexane/ethyl acetate) to give the compounds **8a**–**c** as solids (45–55% yield).

Compound **8a**: ^{1}H NMR δ 7.40 (5H, m); 6.96 (1H, s); 6.85 (1H, s); 6.54 (1H, s); 6.49 (1H, s); 5.42 (1H, d, *J* 6.41 Hz); 5.13 (2H, s); 5.10 (2H, s); 5.06 (2H, s); 4.14 (1H, dd, *J* 9.61, 3.75 Hz); 3.89 (3H, s); 3.50 (2H, m).

Compound **8b**: ^{1}H NMR δ 7.37 (10H, m); 7.07 (1H, s); 6.70 (1H, s); 6.53 (1H, s); 6.43 (1H, s); 5.90 (1H, d, *J* 5.49 Hz); 5.89 (1H, d, *J* 5.49 Hz); 5.41 (1H, d, *J*6.87 Hz); 5.13 (2H, s); 5.11 (2H, s); 4.18 (1H, dd, *J* 10.8, 4.63 Hz); 3.59 (1H, t, *J* 10.75 Hz); 3.46 (1H, m). ^{13}C NMR δ 153.90 (C); 150.53 (C); 150.23 (C); 147.84 (C); 143.71 (C); 141.82 (C); 137.15 (C); 136.56 (C); 128.31–126.97 (10 CH); 117.67 (C); 116.60 (CH); 111.31 (C); 104.51 (CH); 103.18 (CH); 101.06 (CH_2); 93.53 (CH); 78.30 (CH); 72.01 (CH_2); 70.59 (CH_2); 66.31 (CH_2); 40.00 (CH).

Compound **8c**: ^{1}H NMR δ 7.40 (10H, m); 6.85 (1H, s), 6.70 (1H, dd, *J* 8.60, 2.37 Hz); 6.53 (3H, m); 5.46 (1H, d, *J* 6.23 Hz); 5.11 (2H, s); 5.05 (2H, s); 4.26 (1H, dd, *J* 10.07, 4.21 Hz); 3.86 (3H, s); 3.65 (1H, t, *J* 10.62 Hz); 3.53 (1H, m).

5.1.5. Synthesis of coumestans 2b, 2f and 2h. To a solution of **8a**–**c** (0.1 mmol) in THF (3.5 mL) was added DDQ (41.3 mg, 0.2 mmol). The resulting mixture was stirred at room temperature for 12 h. The coumestans **9a**–**c** precipitated out of solution and they were collected by filtration and washed with cold hexane. The crude products were allowed to react with hydrogen (3 atm) in acetone for 6 h. After this time, the catalyst was filtered (Celite)® and concentrated in vacuum to furnish the compounds **2b**, **2f** and **2h** (40–55% overall yield). Homogeneous spectra were obtained in the three cases. In the case of **2b** and **2h**, the purity was also checked by HPLC (CH_3CN/H_2O; 55:45) and was around 91–96%.

Compound **2b**: ^{1}H NMR δ 10.39 (1H, s); 9.48 (1H, s); 9.42 (1H, s); 7.37 (1H, s); 7.23 (1H, s); 7.17 (1H, s); 6.95 (1H, s); 3.91 (3H, s). Anal. Calcd for $C_{16}H_{10}O_7$: C, 61.15; H, 3.21. Found: C, 62.00; H, 3.11.

Compound **2f**: ^{1}H NMR δ 7.38 (1H, s); 7.36 (1H, s); 7.33 (1H, s); 6.99 (1H, s); 6.16 (2H, s). Anal. Calcd for $C_{16}H_8O_7$: C, 61.55; H, 2.58. Found: C, 61.00; H, 2.48.

Compound **2h**: ^{1}H NMR δ 10.66 (1H, s); 9.62 (1H, s); 7.85 (1H, d, *J* 9.16 Hz); 7.32 (1H, s); 7.25 (1H, s); 6.94 (2H, m).[17]

5.2. Pharmacology

A 30 mM stock solution in DMSO was made for all compounds. Further dilutions were done immediately before use in water or Tris–HCl-buffered Krebs solution for Na^+,K^+-ATPase inhibition and [^{3}H]flunitrazepam binding, respectively.

5.3. Tissue preparation

5.3.1. Na^+,K^+-ATPase. Adult male Wistar rats were killed by decapitation and their kidneys were rapidly excised and stored at −80 °C. Preparations enriched in Na^+,K^+-ATPase were obtained by chaotropic treatment with 2 M KI for 1 h and 0.1% DOC (sodium deoxicholate) overnight, followed by differential centrifugation, as earlier described.[18] The protein concentration was measured according to the method of Lowry et al.[19] using bovine serum albumin as the standard.

5.3.2. Benzodiazepine receptor. Brain hemispheres were obtained from male Wistar rats sacrificed by decapitation. Briefly, tissues were homogenized in a Potter apparatus with a motor-driven Teflon pestle at 4 °C in 15 volumes of ice-cold 0.32 M buffered sucrose (pH 7.4) per gram of organ. After centrifuging at $1000g_{max}$ for 10 min, the supernatant was centrifuged at $48{,}000g_{av}$ for 20 min to obtain the crude synaptosomes that were resuspended in a buffered Krebs solution and stored at −80 °C until use.

5.3.3. Inhibition of Na^+,K^+-ATPase activity. The Na^+,K^+-ATPase activity was determined by the Fiske and Subbarow method with slight modifications, as described earlier.[20] The specific activity of the enzyme corresponds to the difference between the total ATPase activity and the activity measured in the presence of 1 mM ouabain. The reaction was started by addition of the preparation, incubated at 37 °C for 2 h, in a total volume of 0.5 mL. The incubation was performed in the presence of 84 mM NaCl, 3 mM KCl, 3 mM $MgCl_2$, 1.2 mM ATP-Na_2, 2.5 mM EGTA, 10 mM sodium azide and 20 mM maleic acid buffered to pH 7.4 with Tris, in the presence or absence of increasing concentrations of the coumestans.

5.3.4. [^{3}H]Flunitrazepam binding assay. Synaptosomes (200 μg protein) were incubated at 4 °C for 90 min in a buffered Krebs solution (Tris–HCl, pH 7.4, at 4 °C), containing 0.2 nM [^{3}H]flunitrazepam (85 Ci/mmol, New England Nuclear Life Science Products, USA). After incubation, samples were rapidly diluted with 3 mL of ice-cold Krebs buffer and immediately filtered on glass fibre filters (GMF3, Filtrak, Germany) under vacuum. Filters were then washed once more with 3 mL buffer, dried and immersed in a scintillation cocktail (0.1 g/L POPOP and 4.0 g/L PPO in toluene) and the radioactivity retained in the filters was counted in a Packard Tri-Carb 1600 TR liquid scintillation analyzer. Non-specific binding was estimated in the presence of 5 μM unlabelled flunitrazepam. Competition experiments were performed adding increasing concentrations of the coumestans.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2006.07.053.

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