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Synthesis and Biological Activity of 4-Alkoxy Chalcones: Potential Hydrophobic Modulators of P-Glycoprotein-Mediated Multidrug Resistance

Frédéric Bois,[a] Ahcène Boumendjel,[a,*] Anne-Marie Mariotte,[a] Gwenaëlle Conseil[b] and Attilio Di Petro[b]

[a]*Laboratoire de Pharmacognosie-UFR de Pharmacie de Grenoble,Université Joseph Fourier, 38706 La Tronche, France*
[b]*Institut de Biologie et Chimie des Protéines, UPR 412-CNRS, 7 Passage du Vercors, 69367 Lyon Cedex 07, France*

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Abstract—A series of 4-alkoxy-2′,4′,6′-trihydroxychalcones have been synthesized and evaluated for their ability to inhibit P-glycoprotein-mediated multidrug resistance (MDR) by direct binding to a purified protein domain containing an ATP-binding site and a modulator-interacting region. The introduction of hydrophobic alkoxy goups at position 4 led to much more active compounds as compared to the parent chalcone. The binding affinity increased as a function of the chain length, up to the octyloxy derivative for which a K_D of 20 nM was obtained.

Introduction

Resistance to chemotherapy remains a serious impediment toward the use of cytotoxic drugs in the treatment of cancer. Resistance to a wide variety of drugs with unrelated chemical structures and different mechanisms of action is commonly known as multidrug resistance (MDR). Overexpression of P-glycoprotein, a 170-kDa transmembrane transporter protein belonging to the large superfamily of ABC transporters, also known as traffic ATPases, can render cells resistant to a variety of chemotherapeutic drugs.[1–3] It uses the energy driven from ATP hydrolysis to transport cytotoxic drugs from inside to outside of cancer cells, preventing their killing effects.[4] Because of the deleterious effect of P-glycoprotein toward chemotherapeutic efficiency, compounds that modify its function are of potential clinical value. Within the last decade, several inhibitors of P-glycoprotein-mediated drug efflux have been identified. These so-called MDR modulators lead to resensitization of multidrug-resistant tumor cells to chemotherapeutic agents: they include calcium channel blockers such as verapamil or trifluoperazine, and immunosuppressors such as cyclosporin A which usually act by competing with cytotoxic drugs for binding to P-glycoprotein.[5–11]

Flavonoids have been reported to modulate drug efflux in MDR cancer cells.[12,13] For example, quercetin **I** (Fig. 1) has been shown to restore sensitivity to adriamycin in multidrug resistance cells[14] by inhibiting P-glycoprotein ATPase activity.[15] By using a purified recombinant protein corresponding to a cytosolic nucleotide-binding domain of the transporter,[16–19] we have tested a number of flavonoids and chalcones and shown that their binding site was cytosolic and partly overlapped the ATP-binding site and the modulator-interacting region. The binding affinity appeared to be dependent both on the class of flavonoids and on their substituents.[19] Structure–activity relationship studies conducted on chalcones **II** (Fig. 1) showed that modification of the B-ring moiety by addition of a halogen atom at position 4 increased the activity with the following efficiency sequence: I > Br > Cl > F > H.[20] This increase in binding affinity appeared to be correlated to the increase in hydrophobicity,[21] rather than to electron density and related strength as hydrogen-bond acceptor. In order to further establish the importance of B ring hydrophobicity for binding affinity toward P-glycoprotein, we synthesized a series of increasingly hydrophobic 4-alkoxy chalcones, with the general formula **III** (Fig. 1) and differring from each other by C_2H_4 constant units. With these compounds, the contribution of the substituent

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* Corresponding author. Tel.: +33-4-7651-8688; fax: +33-4-7663-7165; e-mail: Ahcene.Boumendjel@ujf-grenoble.fr

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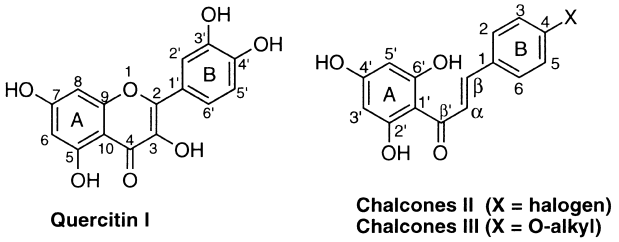


Figure 1.

lipophilicity may be discerned and correlated to the binding affinity for P-glycoprotein; in addition, the optimal substituent length can be identified.[22]

Results and Discussion

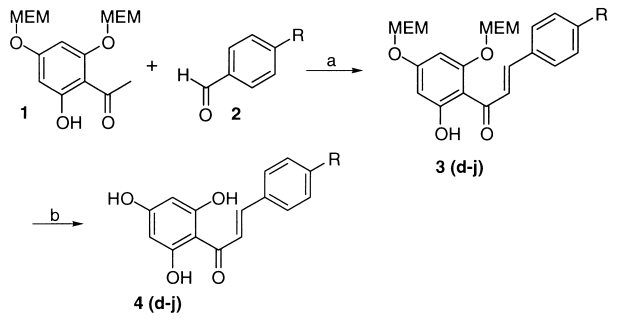
Chemistry

The synthesis of chalcones **4(d–j)** was accomplished by a Claisen–Schmidt condensation[23] between the appropriate 4′-alkoxybenzaldehyde and acetophenone, as shown in Scheme 1. To increase the yield, hydroxy groups in the starting acetophenone were masked as MEM ethers before condensation, and finally deprotected by acidic hydrolysis. Chalcones **4a** (R = H), **4b** (R = OH) and **4c** (R = OMe) are naturally occurring products and their syntheses are not reported here.

Biology

The binding affinity was evaluated by using the C-terminal nucleotide-binding domain (NBD2) of mouse P-glycoprotein.[19,20] The quenching of protein intrinsic fluorescence, due to the single tryptophan residue at position 1106 of P-glycoprotein, was measured as previously described.[16,18–20] The low dissociation constant K_D, in the nano/micromolar range, and the high maximal fluorescence-quenching ΔF_{max}, $>50\%$, were determined by using the Graphit program (Erithacus software).

In this study, we report the synthesis of seven chalcones **4(d–j)**. The compounds were dissolved in dimethylsulfoxide and then diluted in aqueous medium to be assayed for their ability to bind, in vitro, to the P-glycoprotein purified domain NBD2 (Table 1). Overall, the



Scheme 1. Reagents: (a) KOH, MeOH, reflux; (b) HCl, Et_2O/MeOH, reflux. R = O-alkyl; MEM, -$CH_2OCH_2CH_2OCH_3$.

Table 1. Structure of chalcones and binding to the NBD2 cytosolic domain of P-glycoprotein

Chalcone **4** R=	K_D (μM)	ΔF_{max} (%)
4a H	4.6 ± 0.3	87.8 ± 1.5
4b OH	4.8 ± 0.5	86.3 ± 0.5
4c O–CH_3	2.3 ± 0.2	85.5 ± 1.3
4d O–*n*-C_2H_5	2.1 ± 0.2	84.5 ± 1.5
4e O–*n*-C_4H_9	1.0 ± 0.08	84.2 ± 1.6
4f O–*n*-C_6H_{13}	0.27 ± 0.05	74.6 ± 2.5
4g O–cyclohexyl	0.53 ± 0.07	73.2 ± 2.9
4h O–*n*-C_8H_{17}	0.02 ± 0.04	54.5 ± 5.3
4i O–*n*-$C_{10}H_{21}$	0.06 ± 0.04	50.2 ± 4.0
4j O–*n*-$C_{14}H_{29}$	14.2 ± 2.5	89.4 ± 6.9

data clearly show that the binding affinity was directly correlated to the chain length when it contained up to eight carbon atoms. The most active compound, 4-octyloxy chalcone **4h**, exhibited a K_D value of 20 nM, indicating a 200-fold increased affinity as compared to the parent chalcone. However, further increase in the chain length began to alter the binding for the decyloxy derivative. It even exhibited a dramatic effect for the tetradecyloxy chalcone, since the apparent affinity was lower than the parent chalcone.

The key roles played by the A-ring and α,β-ketone system in the affinity were most likely due to mimicking of the adenine moiety of ATP, as demonstrated by co-crystallization of cyclin-dependent kinase 2[24] and Hck tyrosine kinase[25] with flavonoids or derivatives. On the other hand, it has been shown that recombinant cytosolic domains of P-glycoprotein contain a steroid-interacting hydrophobic region in addition to the ATP-binding site.[18,19] Based on these data, we can imagine that the present 4-alkoxy chalcones overlap the two binding sites of the cytosolic domain where the B-ring substituted by the hydrophobic alkoxy group could bind to the steroid-interacting region. The increase in hydrophobicity up to the octyloxy derivative would thus increase the strength of the interaction with this region, but excess hydrophobicity in the tetradecyloxy **4j** chalcone might produce a shift and new positioning of the compound, toward another hydrophobic area, preventing any interaction at the ATP site. Therefore, tetradecyloxy chalcone would not exhibit, contrarily to the octyloxy derivative, a bifunctional interaction. Accordingly, C_5 dimethylallyl substitution of ring A in flavonoids was recently shown (i) to increase interaction within the cytosolic domain of the parasite P-glycoprotein-like multidrug transporter, (ii) to inhibit the drug-efflux activity of the transporter and (iii) to chemosensitize the parasite growth to the presence of daunomycin.[26]

Conclusions

In summary, the potency of type **4** chalcones was dependent on chain length, and therefore on lipophilicity. The high binding affinity observed may be due to simultaneous overlapping of ATP-binding site and steroid-interacting region present in the purified NBD2

domain. Efforts to examine the effects produced on binding to P-glycoprotein by chalcone analogues where the B-ring is replaced by a steroid are being made and will be reported in the near future.

Experimental

General chemistry methods

^{1}H and ^{13}C NMR spectra were recorded on a Bruker AC-200 instrument (200 MHz for ^{1}H, 50 MHz for ^{13}C). Chemical shifts are reported as δ values (ppm) relative to Me_4Si as an internal standard. EI mass spectra were obtained at 70 eV using a Fisons Trio 1000 instrument. The ionization current and the chamber temperature were 150 mA and 200°C, respectively. Elemental analyses were performed by the Analytical Department of CNRS-Vernaison, France. Thin-layer chromatography (TLC) was carried out using Merck silica gel F-254 plates (thickness 0.25 mm). Flash chromatography was carried out using Merck silica gel 60, 200–400 mesh or LiChroprep® DIOL (40–63 mm). All solvents were distilled prior to use. Diethylether was purchased as anhydrous and used as received.

4-Alkoxy-2′-hydroxy-4′,6′-di-*O*-MEM-chalcones and chalcones (3): General method. To a solution of 2′-hydroxy-4′,6′-di-*O*-MEM-acetophenone in methanol (7 mL/mmol) was first added *p*-alkoxybenzaldehyde (1.5 equiv) and then an aqueous solution of KOH (50%, 1 mL/mmol of di-*O*-MEM-acetophenone), and the mixture was heated at 70°C. The reaction was monitored by TLC until completion (1.5–2 h) and water (20 mL) was then added; methanol was evaporated and the solution was submitted to an extraction with CH_2Cl_2. The organic layer was dried and evaporated to dryness, then purified by column chromatography (silica gel, AcOEt: hexane 1:1) to afford a yellow powder of chalcones **3**.

4-Ethoxy-2′-hydroxy-4′,6′-di-*O*-MEM-chalcone (3d). ^{1}H NMR (acetone-d_6): δ 13.89 (s, 1H); 7.94 (d, J = 15.6 Hz, 1H); 7.75 (d, J = 15.6 Hz, 1H); 7.67 (dd, J_1 = 1.9 Hz, J_2 = 8.2 Hz, 2H); 6.98 (dd, J_1 = 1.9 Hz, J_2 = 8.2 Hz, 2H); 6.32 (d, J = 2.3 Hz, 1H); 6.27 (d, J = 2.3 Hz, 1H); 5.38 (s, 2H); 5.28 (s, 2H); 4.05 (t, J = 6.4 Hz, 2H); 3.87 (m, 2H); 3.87 (s, 3H); 3.80 (m, 2H); 3.51 (m, 4H); 3.38 (s, 3H); 3.36 (s, 3H). MS (EI) *m*/*e* 476 $[M]^+$. Anal. calcd for $C_{25}H_{32}O_9$: C, 63.01; H, 6.77. Found: C, 63.08; H, 6.79.

4-Butyloxy-2′-hydroxy-4′,6′-di-*O*-MEM-chalcone (3e). ^{1}H NMR (acetone-d_6): δ 7.95 (d, J = 15.5 Hz, 1H); 7.76 (d, J = 15.5 Hz, 1H); 7.67 (dd, J_1 = 2 Hz, J_2 = 7.8 Hz, 2H); 6.98 (dd, J_1 = 2 Hz, J_2 = 7.8 Hz, 2H); 6.36 (d, J = 2.3 Hz, 1H); 6.25 (d, J = 2.3 Hz, 1H); 5.48 (s, 2H); 5.32 (s, 2H); 4.07 (t, J = 6.3 Hz, 2H); 3.88 (m, 2H); 3.79 (m, 2H); 3.53 (m, 4H); 3.28 (s, 3H); 3.27 (s, 3H); 1.75 (m, 2H); 1.49 (m, 2H); 0.96 (t, J = 7.27 Hz, 3H). MS (EI) *m*/*e* 504 $[M]^+$. Anal. calcd for $C_{27}H_{36}O_9$: C, 64.27; H, 7.19. Found: C, 64.38; H, 7.22.

4-Hexyloxy-2′-hydroxy-4′,6′-di-*O*-MEM-chalcone (3f). ^{1}H NMR (acetone-d_6): δ 7.95 (d, J = 15.6 Hz, 1H); 7.86 (d, J = 15.6 Hz, 1H); 7.67 (dd, J_1 = 2 Hz, J_2 = 7.8 Hz, 2H); 6.98 (dd, J_1 = 2 Hz, J_2 = 7.8 Hz, 2H); 6.78 (d, J = 2.3 Hz, 1H); 6.72 (d, J = 2.3 Hz, 1H); 5.83 (s, 2H); 5.73 (s, 2H); 4.07 (t, J = 6.3 Hz, 2H); 3.88 (m, 2H); 3.79 (m, 2H); 3.53 (m, 4H); 3.28 (s, 3H); 3.27 (s, 3H); 1.75 (m, 2H); 1.49 (m, 4H); 0.96 (t, J = 7.3 Hz, 3H). MS (EI) *m*/*e* 532 $[M]^+$. Anal. calcd for $C_{29}H_{40}O_9$: C, 65.39; H, 7.57. Found: C, 65.42; H, 7.63.

4-Cyclohexyloxy-2′-hydroxy-4′,6′-di-*O*-MEM-chalcone (3g). ^{1}H NMR (acetone-d_6): δ 13.92 (s, 1H); 7.94 (d, J = 15.6 Hz, 1H); 7.76 (d, J = 15.6 Hz, 1H); 7.67 (d, J = 8.8 Hz, 2H); 6.99 (d, J = 8.8 Hz, 2H); 6.36 (d, J = 2.3 Hz, 1H); 6.25 (d, J = 2.3 Hz, 1H); 5.48 (s, 2H); 5.32 (s, 2H); 4.45 (m, 1H); 3.88 (m, 2H); 3.78 (m, 2H); 3.54 (m, 4H); 3.28 (s, 3H); 3.27 (s, 3H); 1.98 (m, 2H); 1.78 (m, 2H); 1.45 (m, 6H). MS (EI) *m*/*e* 530 $[M]^+$. Anal. calcd for $C_{29}H_{38}O_9$: C, 65.64; H, 7.22; Found: C, 65.69; H, 7.26.

4-Octyloxy-2′-hydroxy-4′,6′-di-*O*-MEM-chalcone (3h). ^{1}H NMR (acetone-d_6): δ 13.07 (s, 1H); 7.94 (d, J = 15.6 Hz, 1H); 7.75 (d, J = 15.6 Hz, 1H); 7.67 (dd, J_1 = 1.93 Hz, J_2 = 8.2 Hz, 2H); 6.98 (dd, J_1 = 1.93 Hz, J_2 = 8.2 Hz, 2H); 6.34 (d, J_1 = 2.3 Hz, 1H); 6.24 (d, J_1 = 2.3 Hz, 1H); 5.47 (s, 2H); 5.31 (s, 2H); 4.05 (t, J = 6.4 Hz, 2H); 3.87 (m, 2H); 3.80 (m, 2H); 3.51 (m, 4H); 3.27 (s, 3H); 3.26 (s, 3H); 1.74 (m, 2H); 1.30 (m, 10H); 0.88 (t, J = 6.4 Hz, 3H). MS (EI) *m*/*e* 560 $[M]^+$. Anal. calcd for $C_{31}H_{44}O_9$: C, 66.41; H, 7.91. Found: C, 66.46; H, 8.01.

4-Decyloxy-2′-hydroxy-4′,6′-di-*O*-MEM-chalcone (3i). ^{1}H NMR (acetone-d_6): δ 7.95 (d, J = 15.6 Hz, 1H); 7.77 (d, J = 15.6 Hz, 1H); 7.67 (d, J = 8.8 Hz, 2H); 7.0 (d, J = 8.8 Hz, 2H); 6.36 (d, J = 2.3 Hz, 1H); 6.25 (d, J = 2.3 Hz, 1H); 5.48 (s, 2H); 5.33 (s, 2H); 4.07 (t, J = 6.4 Hz, 2H); 3.89 (m, 2H); 3.78 (m, 2H); 3.62 (m, 4H); 3.29 (s, 3H); 3.27 (s, 3H); 1.32 (m, 16H); 0.87 (t, J = 6.1 Hz, 3H). MS (EI) *m*/*e* 588 $[M]^+$. Anal. calcd for $C_{33}H_{48}O_9$: C, 67.32; H, 8.22. Found: C, 67.37; H, 8.25.

4-Tetradecyloxy-2′-hydroxy-4′,6′-di-*O*-MEM-chalcone (3j). ^{1}H NMR (acetone-d_6): δ 7.94 (d, J = 15.5 Hz, 1H); 7.74 (d, J = 15.5 Hz, 1H); 7.67 (d, J = 8.8 Hz, 2H); 6.99 (d, J = 8.8 Hz, 2H); 6.35 (d, J = 2.2Hz, 1H); 6.24 (d, J = 2.3 Hz, 1H); 5.48 (s, 2H); 5.30 (s, 2H); 4.07 (t, J = 6.4 Hz, 2H); 3.88 (m, 2H); 3.78 (m, 2H); 3.62 (m, 4H); 3.29 (s, 3H); 3.27 (s, 3H); 1.32 (m, 24H); 0.87 (t, J = 6.1 Hz, 3H). MS (EI) *m*/*e* 644 [M]+. Anal. calcd for $C_{37}H_{56}O_9$: C, 68.91, H, 8.75. Found: C, 69.01; H, 8.78.

4-Alkoxy-2′,4′,6′-trihydroxy-chalcones and chalcones (4): General method. To a stirred solution of MEM-protected chalcone **3** in methanol (30 mL/mmol) was added HCl (1% in ether, 10 mL/mmol). The solution was heated at 60°C and monitored by TLC; after completion (2–3 h), the reaction was cooled to room temperature and water was added. The solution was diluted with CH_2Cl_2 (100 mL) and the organic layer was separated, washed twice with water, dried and concentrated. The solid obtained was washed three times with hexane and the solid was collected by filtration to afford a crude

material which was purified by column chromatography (LiChroprep® DIOL–$CHCl_3$).

4-Ethoxy-2′,4′,6′-trihydroxychalcone (4d). ^{1}H NMR (acetone-d_6): δ 8.12 (d, J = 15.5 Hz, 1H); 7.74 (d, J = 15.5 Hz, 1H); 7.6 (dd, J_1 = 2 Hz, J_2 = 6.7 Hz, 2H); 6.96 (dd, J_1 = 2 Hz, J_2 = 6.7 Hz, 2H); 5.95 (s, 2H); 4.12 (q, J = 6.9 Hz, 2H); 1.38 (t, J = 6.9 Hz, 3H). MS (EI) m/e 300 $[M]^+$. Anal. calcd for $C_{17}H_{16}O_5$: C, 67.99; H, 5.37. Found: C, 68.03; H, 5.42.

4-Butyloxy-2′,4′,6′-trihydroxychalcone (4e). ^{1}H NMR (acetone-d_6): δ 11.62 (s, 2H); 8.80 (s, 1H); 7.95 (d, J = 15.5 Hz, 1H); 7.41 (d, J = 15.5 Hz, 1H); 7.28 (dd, J_1 = 1.9 Hz, J_2 = 6.7 Hz, 2H); 6.65 (dd, J_1 = 1.9 Hz, J_2 = 6.7 Hz, 2H); 5.62 (s, 2H); 3.72 (t, J = 6.4 Hz, 2H); 1.42 (m, 2H), 1.17 (m, 2H); 0.63 (t, J = 7.2 Hz, 3H). MS (EI) m/e 328 $[M]^+$. Anal. calcd for $C_{19}H_{20}O_5$: C, 69.50; H, 6.14. Found: C, 69.54; H, 6.18.

4-Hexyloxy-2′,4′,6′-trihydroxychalcone (4f). ^{1}H NMR (acetone-d_6): δ 12.00 (s, 2H); 9.25 (s, 1H); 8.20 (d, J = 15.5 Hz, 1H); 7.70 (d, J = 15.5 Hz, 1H); 7.62 (d, J = 6.5 Hz, 2H); 6.96 (d, J = 6.5 Hz, 2H); 6.0 (s, 2H); 4.10 (t, J = 6.3 Hz, 2H); 1.78 (m, 2H); 1.40 (m, 6H); 0.85 (t, J = 7 Hz, 3H). MS (EI) m/e 357 $[M+1]^+$. Anal. calcd for $C_{21}H_{24}O_5$: C, 70.77; H, 6.79. Found: C, 70.81; H, 6.83.

4-Cyclohexyloxy-2′,4′,6′-trihydroxychalcone (4g). ^{1}H NMR (acetone-d_6): δ 11.9 (s, 1H); 9.18 (s, 1H); 8.12 (d, J = 15.5 Hz, 1H); 7.75 (d, J = 15.5 Hz, 1H); 7.60 (dd, J_1 = 6.8 Hz, J_2 = 1.9 Hz, 2H); 6.98 (dd, J_1 = 6.8 Hz, J_2 = 1.9 Hz, 2H); 5.96 (s, 2H); 4.42 (m, 1H); 1.98 (m, 2H); 1.77 (m, 2H); 1.44 (m, 6H). MS (EI) m/e 354 $[M]^+$. Anal. calcd for $C_{21}H_{22}O_5$: C, 71.77; H, 6.26. Found: C, 71.81; H, 6.28.

4-Octyloxy-2′,4′,6′-trihydroxychalcone (4h). ^{1}H NMR (acetone-d_6): δ 11.94 (s, 2H); 9.16 (s, 1H); 8.12 (d, J = 15.5 Hz, 1H); 7.74 (d, J = 15.5 Hz, 1H); 7.95 (dd, J_1 = 2.6 Hz, J_2 = 6.8 Hz, 2H); 6.96 (dd, J_1 = 2.6 Hz, J_2 = 6.8 Hz, 2H); 5.94 (s, 2H); 4.04 (t, J = 6.4 Hz, 2H); 1.74 (m, 2H); 1.35 (m, 10H); 0.86 (t, J = 6.4 Hz, 3H). MS (EI) m/e 384 $[M]^+$. Anal. calcd for $C_{23}H_{28}O_5$: C, 71.85; H, 7.34. Found: C, 71.88; H, 7.36.

4-Decyl-2′,4′,6′-trihydroxychalcone (4i). ^{1}H NMR (MeOH-d_4): δ 8.09 (d, J = 15.5 Hz, 1H); 7.70 (d, J = 15.5 Hz, 1H); 7.54 (d, J = 7 Hz, 2H); 6.92 (d, J = 7 Hz, 2H); 5.89 (s, 2H); 4.02 (t, J = 6.6 Hz, 2H); 1.76 (m, 2H); 1.32 (m, 14H); 0.86 (t, J = 6.5 Hz, 3H). MS (EI) m/e 412 $[M]^+$. Anal. calcd for $C_{25}H_{32}O_5$: C, 72.79; H, 7.82. Found: C, 72.81; H, 7.86.

4-Tetradecyl-2′,4′,6′-trihydroxychalcone (4j). ^{1}H NMR (MeOH-d_4): δ 8.10 (d, J = 15.5 Hz, 1H); 7.72 (d, J = 15.5 Hz, 1H); 7.50 (d, J = 7.2 Hz, 2H); 6.95 (d, J = 7.2 Hz, 2H); 5.90 (s, 2H); 4.05 (t, J = 6.5 Hz, 2H); 1.76 (m, 2H); 1.32 (m, 22H); 0.85 (t, J = 6.4 Hz, 3H). MS (EI) m/e 468 $[M]^+$. Anal. calcd for $C_{25}H_{32}O_5$: C, 74.32; H, 8.60. Found: C, 74.39; H, 8.63.

Biological assays

The recombinant C-terminal cytosolic domain of mouse P-glycoprotein was overexpressed in bacteria and purified by affinity chromatography as described earlier.[12] Fluorescence experiments were performed at 25 ± 0.1°C, using an SLM-Aminco 8000C spectrofluorimeter with spectral bandwidths of 2 nm and 4 nm, respectively, for excitation and emission. The tryptophan-specific intrinsic fluorescence of 0.5 μM recombinant protein in 1.2–2.0 mL of 20 mM potassium phosphate buffer at pH 6.8, containing 0.5 M NaCl, 20% glycerol and 0.01% 6-*O*-(*N*-heptylcarbamoyl)-methyl-α-D-glucopyranoside, was scanned from 310 to 360 nm upon excitation at 295 nm. Contribution for Raman effect of buffer was subtracted, and the fluorescence spectra were integrated. The interaction with chalcones was monitored by the increasing quenching of emission fluorescence produced by successive additions of aliquots from 0.1–0.5 mM dimethylsulfoxide solutions of the compound, up to a 20 μM final concentration. The measurements were corrected for inner-filter effect of chalcones, as determined in parallel experiments with *N*-acetyltryptophanamide. Curve fitting of ligand binding related to fluorescence decrease was performed with Grafit (Erithacus software) as previously described.[18–20]

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