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A Benzopyrane Derivative as a P-Glycoprotein Stimulator: A Potential Agent to Decrease β-Amyloid Accumulation in Alzheimer's Disease

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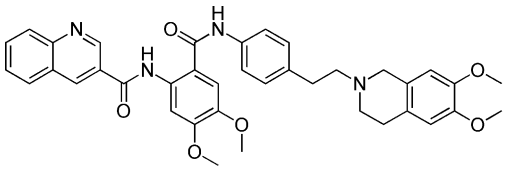
Neurodegenerative disorders such as Alzheimer's disease (AD) are characterized by slow and progressive loss of one or more functions of the central nervous system (CNS). Since many factors, often little-known, are involved in the pathogenesis of these diseases, and their diagnosis is related to the clinical symptoms, AD is usually diagnosed in an advanced stage precluding the possibility of an effective disease-modifying agent rather than a therapy that treats only the symptoms. Recently, P-glycoprotein (P-gp) has been suggested as a new biomarker for the diagnosis of AD in the early stages, using imaging techniques such as positron emission tomography (PET) and Single-photon emission computed tomography (SPECT).[1,2] P-gp belongs to the ATP binding cassette (ABC) transporter family, which are localized at the cell membranes of liver, kidney and placenta cells, and on the villus tip of enterocytes in the gut, and they are important constituents of the blood–brain barrier (BBB), blood–cerebro spinal fluid (B-CSF), and the blood–testis barrier (BTB).[3,4] These pumps use ATP hydrolysis energy to efflux toxins, xenobiotics and antineoplastic agents out of the cells.

Breast cancer resistance protein (BCRP) and multidrug resistance-associated proteins (MRPs) also belong to this family of transporters. These efflux proteins, differently localized with respect to P-gp, are critical components of several biological barriers and are able to efflux different substrates.[5,6] MRPs bear an additional and specific five transmembrane domain compared with P-gp, and as such, they can efflux organic ions with high-molecular weights. In contrast, BCRP is a P-gp monomer, and so is considered a "half transporter"; it effluxes the same substrates as P-gp.

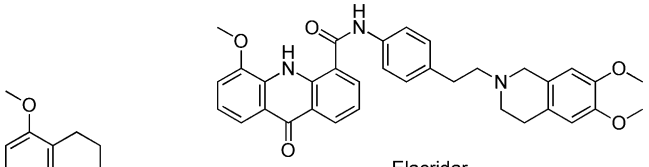
P-gp is a protein involved in the active transport of molecules across biological membranes,[4] and it has been shown that alterations of its expression and/or activity are related to the onset of neurodegenerative disorders.[1,2] Indeed, it has been reported that up-regulation of this efflux pump is responsible for a decrease in β-amyloid intracellular accumulation, which is an important hallmark in AD.[7] Therefore, targeting β-amyloid clearance by P-gp stimulation could be an useful strategy to prevent AD progression.

For this purpose, P-gp ligands are useful for the diagnosis of neurodegenerative diseases by PET or SPECT techniques. In particular, P-gp substrates are needed to detect the activity whereas P-gp inhibitors to measure the expression of the efflux protein. Furthermore, P-gp stimulators could be employed for the treatment of disorders where decreased P-gp expression and/or activity have been proven to play a role in the progression or etiology of the disease.

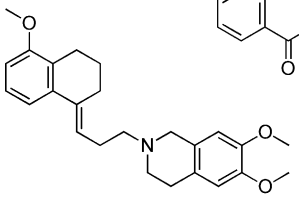
The most recently reported P-gp inhibitors are tariquidar, elacridar, and our ligand MC18.[8–10] However, these molecules have limitations: 1) elacridar shows similar activity towards P-gp and BCRP, therefore it is not a P-gp-selective inhibitor;[11] 2) tariquidar does not exhibit a specific profile towards P-gp because it acts as an inhibitor or substrate in a dose-dependent manner;[12] 3) MC18 lack specificity towards P-gp over MRP1 proteins, exhibiting comparable activities against both ($EC_{50} = 1.50$ μM and 2.80 μM vs P-gp and MRP1, respectively).[13]



Tariquidar



Elacridar



MC18

Previous structure–activity relationship (SAR) studies[10,13,14] allowed us to consider the basic fragment as a pivotal feature for modulating P-gp activity, while the alkyl tetralin nucleus could be modified to obtain selective ligands towards other ABC transporters.

Starting from MC18 as the lead compound, P-gp ligands were designed replacing the alkyl tetralin nucleus with α- or β-benzopyrane nucleus linked to the same basic moiety (6,7-dimethoxytetrahydroisoquinoline) in order to develop and characterize more potent and selective molecules.

The introduction of a benzopyrane system was aimed at decreasing the lipophilicity (cLog*P*) of the new derivatives with

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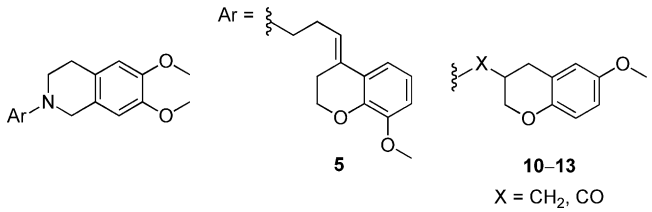
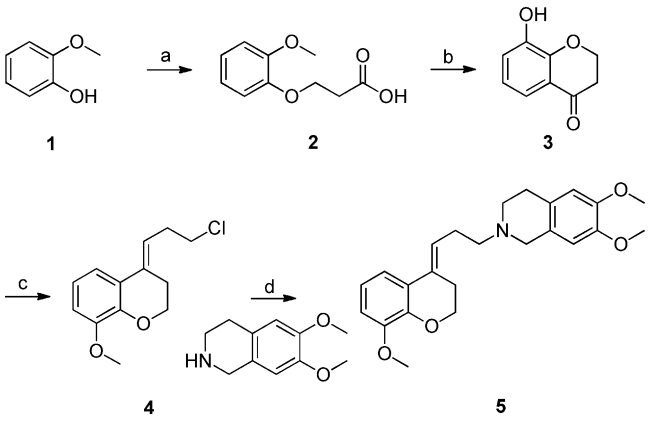


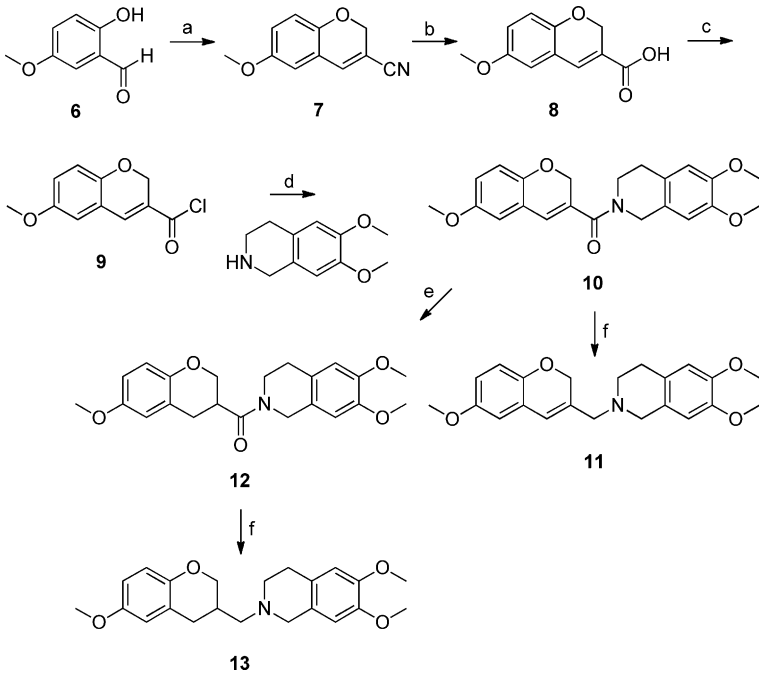
respect to the parent compound MC18. Indeed, cLog*P* values of the benzopyrane derivatives range from 2.87 to 3.73, compared with a value of 4.25 for the lead compound. Moreover, the role of the basicity of the derivatives in modulating their activity against P-gp was also investigated by synthesizing amide derivatives.

Compound **5** was synthesized as shown in Scheme 1. In brief, treatment of guaiacol (**1**) with NaH and 3-bromopropionic acid in DMF gave phenoxypropionic acid **2**.[15] This intermediate cyclized in the presence of polyphosphoric acid to chromanone **3**.[16–18] (*E*)-Chloropropyliden chromene **4** was obtained by reaction of **3** with the Grignard reagent, cyclopropylmagnesium bromide, generated in situ. 6,7-Dimethoxytetrahydroisoquinoline (2 equiv) in the presence of sodium carbonate was condensed with intermediate **4** to give the amine derivative **5**.



Scheme 1. Synthesis of compound **5**. *Reagents and conditions*: a) $BrCH_2CH_2COOH$ (1.2 equiv), NaH (2.6 equiv), DMF, 15 °C, 30 min, 52%; b) Polyphosphoric acid (3.3 equiv), 140 °C, 1 h, 47%; c) 1. Mg, anhydrous THF, cyclopropyl bromide (1.2 equiv), reflux, 12 h; 2. CH_2COOH/HCl, RT, 2 h, 40%; d) Na_2CO_3 (2 equiv), DMF, reflux, 18 h, 65%.

Compounds **10**–**13** were prepared following the reaction sequence outlined in Scheme 2. Nitrile **7** was obtained by coupling in of 2-hydroxy-5-methoxybenzaldehyde with acrylonitrile in the presence of 1,4-diazabicyclo[2.2.2]octane (DABCO) as a catalyst.[19] Alkaline nitrile hydrolysis was carried out in refluxing aqueous sodium hydroxide. The mixture was acidified with concentrated hydrochloric acid, and carboxylic acid **8** was collected by filtration.[19] Reaction of **8** with thionyl chloride gave intermediate **9**, which was used without isolation and condensed with tetrahydroisoquinoline (2 equiv) to give amide **10**. Reduction of compound **10** with lithium aluminum hydride gave amine **11**, while reduction using standard hydrogenation conditions afforded amide **12**. Finally, amine **13** was obtained by reduction of the corresponding amide **12** under the same conditions used to generate **11**.



Scheme 2. Synthesis of compounds **10**–**13**. *Reagents and conditions*: a) CH_2CHCN (5 equiv), DABCO (0.25 equiv), reflux, 2 h, 65%; b) 1. NaOH (10%), reflux, 6 h; 2. HCl (37%), 95%; c) $SOCl_2$ (2 equiv), Et_3N, reflux, 1 h; d) Na_2CO_3, DMF, reflux, 18 h, 63%; e) Pd/C (10%), H_2, EtOH, RT, 12 h, 70%; f) $LiAlH_4$ (2 equiv), anhydrous THF, reflux, 2 h, 56%.

The biological evaluation of compounds **5**, **10**–**13** was carried out in tumor cell lines overexpressing P-pg (MDCK-MDR1) or MRP1 (MDCK-MRP1). In this assay, calcein-AM, a profluorescent probe, is used as the P-gp or MRP1 substrate. In the presence of a pump inhibitor, calcein-AM is able to permeate the cell membrane, and it is hydrolyzed by cytosolic esterases to calcein, a fluorescent dye that accumulates in cells. Calcein accumulation reflects the P-gp or MRP1 inhibition activity of tested compounds.[20] In Table 1, EC_{50} values are reported for test compounds, and the corresponding dose–response curves for MC18 and compounds **5** and **13** are depicted in Figure 1.

Replacement of the tetralin nucleus, present in MC18, with a benzopyrane fragment (compound **5**) had little effect on the potency of the derivative towards P-gp (EC_{50} = 1.30 μM vs 1.50 μM for MC18) but did improve the selectivity for P-gp over MRP1 (EC_{50} against MRP1 was > 100 μM for compound **5** and 2.80 μM for MC18).

Analogously, β-benzopyrane derivative **13** showed high P-gp modulating activity (EC_{50} = 2.90 μM) and low activity towards MRP1 (EC_{50} > 100 μM). Moreover, the presence of a double bond (compound **11**) decreased the P-gp activity (EC_{50} = 14 μM) compared with compound **13**. Amide derivatives **10** and **12** were found to be poorly active against P-gp (EC_{50} = 11 μM and 26 μM, respectively). While, compounds **10**, **11** and **12** were found to be inactive against MRP1 (EC_{50} = > 100 μM,

Table 1. Biological evaluation of compounds **5**, **10**–**13** towards P-gp and MRP1 pumps.

Compd	cLog *P*[a]	$EC_{50} \pm SEM$[b] [μM]	
		P-gp	MRP1
5	3.73	1.30 ± 0.26	>100
10	2.87	11 ± 0.20	>100
11	3.27	1.4 ± 0.30	81.6 ± 5.2
12	2.69	26 ± 1.5	>100
13	3.09	2.90 ± 0.40	>100
MC18[c]	4.25	1.50 ± 0.20	2.80 ± 0.50

[a] Values were calculated using the Osiris Property explorer program (version Actelion from Organic Chemistry Portal). [b] Data represent the mean value of three independent determinations performed in duplicate. [c] Data taken from Ref. [13].

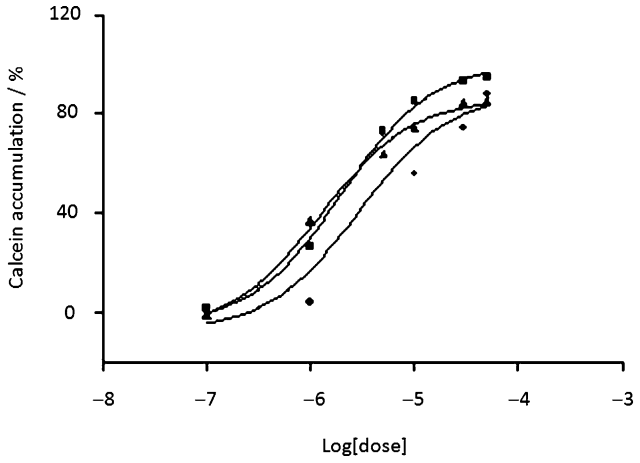


Figure 1. Representative dose–response curves for calcein accumulation in MDCK-MDR1 cells for compounds **5** (▲), **13** (◆), and MC18 (■). Data are the mean of three independent determinations performed in triplicate.

81.6 μM, and >100 μM, respectively). These findings confirm that modifications to the nonbasic fragment of MC18 address the issue of selectivity for P-gp over MRP1. Indeed, benzopyrane derivatives **5** and **13** were found to be potent P-gp-modulating agents, with improved selectivity for P-gp over MRP1 than the parent compound MC18. Moreover, amide derivative **12** was found to be less active than the corresponding amine **13**, while similarly poor P-gp activity was exhibited by amide **10** and the corresponding amine **11**. Finally, a correlation between cLog *P* and EC_{50} values was not found.

Characterization of the type of interaction was also investigated for the best compounds (**5** and **13**) in terms of P-gp activity and selectivity. Derivative **5** displays MC18-like P-gp interacting activity (inhibition), while derivative **13** displays a modulatory activity, as observed in apparent permeability determination and ATP cell depletion assays (data not shown).

Recently, we validated an ex vivo method to investigate the mechanism of P-gp interaction of these compounds: the everted gut sac assay. This model has been shown to be useful for the classification of P-gp interacting molecules as substrates, modulators, inhibitors, and stimulators.[21]

P-gp stimulators are able to up-regulate P-gp activity, causing increased cellular efflux of xenobiotics and a corresponding decrease in their accumulation, which could culminate in an overall decrease in cytotoxicity or, as for our aims, in a detoxification effect of the CNS from β-amyloid peptide.

For this purpose, by using the ex vivo model, the fluxes of rhodamine, a fluorescent P-gp substrate, in the absence and presence of compound **13** were preliminarily evaluated to establish the nature of its interaction with P-gp (Figure 2). In this assay rhodamine is effluxed from the serosal to the mucosal compartment, both by passive and active P-gp-mediated transport. The experimental details have been previously reported elsewhere.[22]

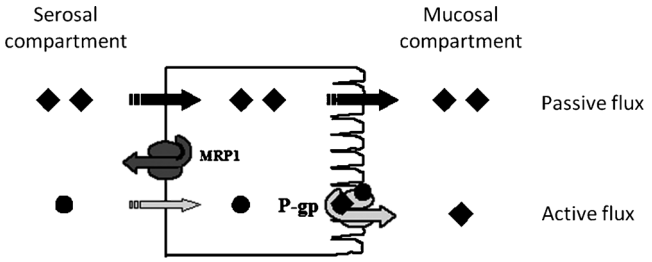


Figure 2. A schematic representation of rhodamine (◆) efflux in the absence and presence of an inducer (●) in an everted gut sac assay.

Kinetic constant (*k*) of rhodamine efflux in the presence of compound **13** slightly increases with respect to the corresponding value measured with rhodamine alone ($k = 8.3 \times 10^{-3}\ min^{-1}$ and $k = 5.9 \times 10^{-3}\ min^{-1}$, respectively). Conversely, the corresponding half-life ($t_{1/2}$) in the presence of compound **13** decreased from 116.8 min to 83.6 min (Figure 3). In light of these preliminary results obtained by using rhodamine as a probe, compound **13** might display P-gp-stimulating activity.

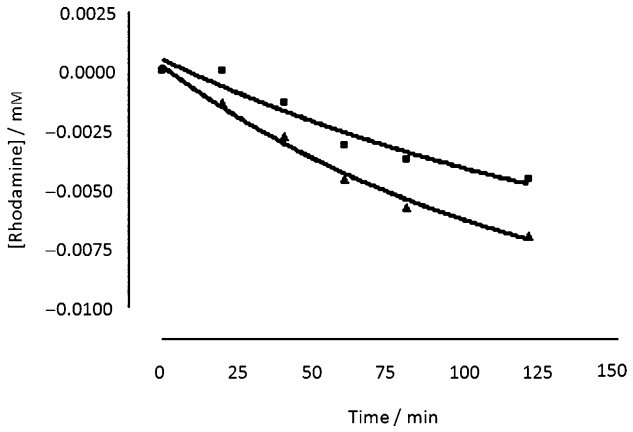


Figure 3. Representative curves of efflux kinetic experiments for rhodamine (100 μM) in the presence (▲) or absence (■) of compound **13** (50 μM). Rhodamine alone gave $k = 5.9 \times 10^{-3}\ min^{-1}$ and $t_{\frac{1}{2}} = 116.8$ min; rhodamine in the presence of **13** gave $k = 8.3 \times 10^{-3}\ min^{-1}$ and $t_{\frac{1}{2}} = 83.6$ min. Data were analyzed using Graph Pad 5.0 (GraphPad Software, San Diego, CA) for Windows. *P* values were calculated using a two-tailed correlation and found to be statistically significant ($P < 0.005$). Standard error of the mean (SEM) values are ±20% for each compound.

Further experiments are in progress to confirm **13** as a P-gp stimulator able to cause β-amyloid efflux.

The apparent discrepancy between the modulating activity shown by compound **13** in the calcein-AM assay and the stimulator profile displayed in our everted gut sac assay can be explained by considering that the ex vivo model is a more complex system where other pumps are involved in the final effect, whereas in the in vitro assay only P-gp interactions are measured. Therefore, the in vitro method is useful to determine the potency of the tested compounds, while the ex vivo model is more predictive of the final physiological effect.

The present study provides evidenced that β-benzopyrane derivative **13** is a potent P-gp ligand with increased selectivity for P-gp over the MRP1 pump when compared with the lead compound MC18. However, initial results indicate that **13** is a P-gp inducing agent, whereas MC18 is a potent P-gp inhibitor. This is an important finding because a potential application of P-gp stimulators is the treatment of AD, since in vitro studies have shown that β-amyloid is a P-gp substrate, and consequently that P-gp is involved in the clearance of β-amyloid from both healthy (aging) and AD cells. Therefore, restoration of P-gp activity by stimulators in situations where P-gp activity or expression is decreased, such as Parkinson's and Alzheimer's diseases, could be a new perspective in the therapy of these disorders.

Experimental Section

Compound **3** was prepared as reported in Ref. [18], and compounds **7–9** were synthesized as reported in Ref. [19]. All characterization data were in accordance with those previously reported.[18,19] ^{1}H NMR spectra were recorded in $CDCl_3$ at 300 MHz on a Varian Mercury-VX Spectrometer. All chemical shift values (δ) are reported in ppm. MS data were collected on an HP6890-5973 MSD GC–MS instrument

(*E*)-4-(3-Chloropropylidene)-3,4-dihydro-8-methoxy-2*H*-chromene (4): Purification by column chromatography (petroleum ether/$CHCl_3$, 3:7) gave a colorless oil (15%); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.2–6.75 (m, 3H), 6.08 (t, J = 2.0 Hz, 1H), 4.30 (t, J = 6.0 Hz, 2H), 3.88 (s, 3H), 3.60 (t, J = 7.0 Hz, 2H), 2.70–2.67 ppm (m, 4H).

1,2,3,4-Tetrahydro-2-((*E*)-3-(2,3-dihydro-8-methoxychromen-4-ylidene)propyl)-6,7-dimethoxyisoquinoline (5): Purification by column chromatography (CH_2Cl_2/MeOH, 19:1) gave a yellow solid (21%); mp: 223–224 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 6.85–6.50 (m, 5H), 6.05 (t, J = 7.0 Hz, 1H), 4.30 (t, J = 5.0 Hz, 2H), 3.90–3.62 (m, 11H), 2.90–2.72 (m, 4H), 2.72–2.60 (m, 4H), 2.55–2.45 ppm (m, 2H); GC–MS: *m/z* (%): 395 [M^+] (6.8), 206 (100); Anal. calcd for $C_{24}H_{29}NO_4$·HCl·H_2O: C 64.06, H 7.17, N 3.11; found: C 64.69, H 6.83, N 3.31.

(3,4-Dihydro-6,7-dimethoxyisoquinolin-2(1*H*)-yl)(6-methoxy-2*H*-chromen-3-yl)methanone (10): Purification by column chromatography (petroleum ether/EtOAc, 1:1) gave a yellow waxy solid (32%); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 6.82–6.62 (m, 5H), 6.60 (s, 1H), 4.86 (s, 2H), 3.98–4.10 (m, 2H), 4.75 (s, 2H), 3.96 (s, 3H), 3.90 (s, 3H), 3.88 (s, 3H), 2.91–2.80 ppm (m, 2H); GC–MS: *m/z* (%): 381[M^+] (78.6), 192 (100).

1,2,3,4-Tetrahydro-6,7-dimethoxy-2-((6-methoxy-2*H*-chromen-3-yl)methyl)isoquinoline (11): Purification by column chromatography ($CHCl_3$/EtOAc, 9:1) gave a yellow solid (34%); mp: 238 °C (dec); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 6.80–6.50 (m, 5H), 6.38 (s, 1H), 4.79 (s, 2H), 3.95–3.87 (m, 9H), 3.59 (s, 2H), 3.22 (s, 2H), 2.92–2.71 ppm (m, 4H); GC–MS: *m/z* (%): 367 [M^+] (1.3), 174 (100); Anal. calcd for $C_{22}H_{25}O_4N$·HCl·0.5H_2O: C 63.99, H 6.59, N 3.47; found: C 63.38, H 6.30, N 3.33.

(3,4-Dihydro-6,7-dimethoxyisoquinolin-2(1*H*)-yl)(3,4-dihydro-6-methoxy-2*H*-chromen-3-yl)methanone (12): Purification by column chromatography ($CHCl_3$/EtOAc, 19:1) gave a white waxy solid (38%); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 6.81–6.58 (m, 5H), 4.7 (s, 2H), 4.41–4.33 (m, 1H, OCH), 4.21–4.18 (m, 1H), 3.91–3.72 (m, 11H), 3.31–3.29 (m, 2H), 2.90–2.78 ppm (m, 3H); GC–MS: *m/z* (%): 383 [M^+] (100), 192 (53.3).

1,2,3,4-Tetrahydro-2-((3,4-dihydro-6-methoxy-2*H*-chromen-3-yl)-methyl)-6,7-dimethoxyisoquinoline (13): Purification by column chromatography ($CHCl_3$) gave a white solid (84%); mp: 222–224 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ = 6.90–6.58 (m, 5H), 3.92–3.88 (m, 1H) 4.32–4.22 (m, 1H), 3.84–3.72 (m, 9H), 3.61 (s, 2H), 2.34–3.05 ppm (m, 9H); GC–MS: *m/z* (%): 369 [M^+] (12.7), 206 (100); Anal. calcd for $C_{22}H_{27}O_4N$·HCl: C 65.10, H 6.95, N 3.45; found: C 65.00, H 6.96, N 3.49.

Acknowledgements

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Keywords: alzheimer's disease · benzopyranes · everted gut sac assay · multidrug resistance-associated proteins · P-glycoprotein stimulators

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