Highly Specific and Broadly Potent Inhibitors of Mammalian Secreted Phospholipases A2

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We report a series of inhibitors of secreted phospholipases A2 (sPLA2s) based on substituted indoles, 6,7-benzoindoles, and indolizines derived from LY315920, a well-known indole-based sPLA2 inhibitor. Using the human group X sPLA2 crystal structure, we prepared a highly potent and selective indole-based inhibitor of this enzyme. Also, we report human and mouse group IIA and IIE specific inhibitors and a substituted 6,7-benzoindole that inhibits nearly all human and mouse sPLA2s in the low nanomolar range.

Introduction

Secreted phospholipases A2 (sPLA2s) are a family of disulfide-rich, Ca2+-dependent enzymes that hydrolyze the sn-2 position of glycerolipids to release a fatty acid and a lysophospholipid. The mouse genome encodes 10 sPLA2s (groups IB, IIA, IIC, IID, IIE, IIF, III, V, X, XIIA), whereas the human genome encodes all of these except the group IIC enzyme, which occurs as a pseudogene. More than a decade ago there was interest in human group IIA sPLA2 (hGIIA) as an anti-inflammatory drug target because it was found at high concentrations in synovial fluid from arthritis patients, although a clinical trial with an inhibitor against hGIIA failed to show efficacy in the treatment of rheumatoid arthritis. Interest in inhibitors of sPLA2X has remained because of the possible involvement of these enzymes in inflammation. For example, studies with mGX- and mGV-deficient mice show that these sPLA2s contribute to airway inflammation in a mouse model of allergic asthma. Studies with macrophages from mGV-deficient mice show a partial reduction in eicosanoid production in response to agonists.

Substituted indoles and indolizines first reported by workers at Lilly and Shionogi are the most potent sPLA2 inhibitors and the ones with drug potential in terms of pharmacokinetic profiles. Compounds in this group include the indolizine Indoxam and the substituted indoles Me-Indoxam and 1 (LY315920; Figure 1). The development of these compounds is an early example of structure-guided improvement of binding potency starting from a lead compound obtained through high-throughput screening and making use of the X-ray structure of hGIIA. With the availability of the full set of mouse and human recombinant sPLA2s, it has been recently possible to explore the specificity of these compounds against all mammalian family members. For example, Me-Indoxam inhibits hGIIE, mGIIE, hGIIE, mGIIE, hGV, and mGV sPLA2s with low nanomolar potency, is less potent on hGIB, mGII, hGIX, and mGIX, and inhibits hGIID, mGIID, hGXIIA, and mGXIIA only at micromolar concentrations. Compound 1 potently inhibits hGIIA, mGIIA, hGIIE, mGIIE, hGV, and mGX enzymes and is less potent on the other mammalian sPLA2s.

In the current study we have taken a structure-guided approach using the X-ray structure of hGX to obtain inhibitors in the class shown in Figure 1 that are highly specific for hGX. Along the way we also obtained a highly specific inhibitor that binds only to hGIIA, mGIIA, hGIIE, and mGIIE as well as a broadly potent inhibitor that shows strong inhibition against human and mouse GIB, GIIA, GIID, GIIE, GIIF, GV and GX sPLA2s. These compounds may be useful in the study of the role of various mammalian sPLA2s in cellular and whole animal responses.

Chemistry

Reported compounds were prepared using slightly modified routes. The substituted indole and 6,7-benzoindole inhibitors were prepared using analogous routes starting from 2-carbomethoxy-4-methoxy-indole 4a and 2-carbomethoxy-4-methoxy-6,7-benzoindole 4b, respectively. However, because 4b could not be purchased commercially, it was prepared from commercially available 3-methoxy-2-naphthalenemethanol 2a (Scheme 1). 3-Methoxy-2-naphthalenemethanol 2a was oxidized with PCC to form the aldehyde 2b. The aldehyde was treated with methyl azidoacetate and sodium methoxide to form the azidocinnamate 3. Ring closure of 3 was achieved via the Hemetsberger reaction to give 2-carbomethoxy-4-methoxy-6,7-benzoindole 4b.

Indole-based inhibitors 11c, 11d, 12a, and 12b were prepared by N-1 benzylation of commercially available 4a using sodium hydride as the base to yield 5a (Scheme 2). The methyl ester was saponified to form the 2-carboxylic acid indole 6a. The 2-acetyl indole 7a was formed by treatment of 6a with methylolithium. Reduction of the ketone was carried out with NaBH4 to yield 8a. Deoxygenation of 8a was achieved using a mixture of NaBO2 and trifluoroacetic acid to give 9a. The 2-isobutyl indole intermediate 9b was prepared in a similar fashion as 9a except isobutyllithium was used in place of methylolithium to form 7b with subsequent transformations to give 9b. Compounds 10a–d were prepared by first deprotecting

Figure 1. Substituted indole and indolizine sPLA2 inhibitors.

Abbreviations: hGIIA, human group IIA secreted phospholipase A2; mGIIA, mouse group IIA secreted phospholipase A2; sPLA2, secreted phospholipase A2.
the 4-methoxy substituent on \(9a\) and \(9b\) using \(\text{BBr}_3\) followed by addition of the appropriate alkyl bromoacetate or 2-bromo-N-(arylsulfonyl)acetamide with sodium hydride as the base.

Addition of the oxalamide group to the indole was carried out by treating \(10a\)–\(d\) with oxalyl chloride followed by addition of ammonia gas to give compounds \(11a\)–\(d\). Deprotection of the indole esters \(11a\) and \(11b\) was carried out with \(\text{NaOH}\) to give \(12a\) or with trifluoroacetic acid to yield \(12b\).

Preparation of the 6,7-benzoindole inhibitors \(11g\), \(11h\), \(12e\), and \(12f\) was done using identical routes described for the substituted indole inhibitors (Scheme 2). Compounds \(14a\) and \(14b\), N-methyl amides \(15a\) and \(15b\), and all \(11d\) derivatives were prepared using analogous steps to those outlined in Scheme 2. All Indoxam derivatives (\(15c\) and \(16a–c\)) were prepared using similar techniques to those outlined in Scheme 2.

Results and Discussion

Molecular Modeling. We recently reported that compound 1 was 30 times more potent than the 2-methyl indole against hGX.\(^7\) We explored this gain in selectivity by docking indole compounds with larger 2-alkyl groups into the hGIIA and hGX sPLA2 active sites of existing X-ray crystal structures\(^13,16\) using the FLO/QXP docking program.\(^20\) An overlay of the hGIIA and hGX enzyme structures (rms \(C_R\) = 0.98 Å) revealed a region of extra space in the hGX active site not present in hGIIA. This difference in hydrophobic space results mostly from a change in one amino acid residue. hGIIA has an isoleucine whereas hGX has a valine in the active site region which is contacted by the 2-position substituent on the indole ring (Figure 2). Larger 2-alkyl substituents would clash with this portion of the hGIIA active site but not in the case of hGX. Our designs were supported by data from workers at Shionogi showing that 2-isobutyl indole and indole-like inhibitors selectively inhibited the hGX enzyme.\(^21\) However, this report only included IC\(_{50}\) values for these compounds against hGIB, hGIIA, hGV, and hGX. As a group X specific inhibitor would be extremely useful, we wanted to test 2-isobutyl indole derivatives against all human and mouse sPLA2 enzymes.

In Vitro Inhibition. Using a fluorometric sPLA2 assay,\(^16\) the substituted indoles, 6,7-benzoindoles, and indolizines were tested against the full panel of human and mouse sPLA2 enzymes, with the exception of mGIIIC (because humans contain a group IIC pseudogene) and mGXIIA, which has 94% sequence identity to hGXIIA.\(^15\) All reported compounds in this study except \(13a–i\), \(14b\), and \(15a–c\) were tested against hGII and hGXIIA sPLA2 enzymes, and gave <50% inhibition for both enzymes at 1.6 \(\mu\)M concentrations. The active sites of GIII and GXIIA sPLA2 are predicted to be significantly different than those of the other mammalian sPLA2s, and this probably explains why the indole/indolizine set of inhibitors lack potency on GIII and GXIIA enzymes. IC\(_{50}\) values generated against hGIID were

\[\text{IC}_{50}\]
obtained using the \[^{3}H\]oleic acid-labeled \textit{E. coli} membrane assay, which was preferred for this enzyme because of the higher sensitivity achieved over the fluorometric assay. Data in Table 1 show that 11d and 12b are highly selective for hGX over all other human and mouse sPLA2s. Thus, the large isobutyl group is well tolerated only by hGX, which is consistent with modeling studies. Interestingly, these compounds lack potency against mGX despite the fact that hGX and mGX share 72% sequence identity. Structural alignment reveals that mGX does not contain a valine in the active site region that contacts the indole 2-position like hGX, but rather a leucine. This extra hydrophobic bulk sterically excludes the 2-isobutyl indoles from the mGX active site in similar fashion as with GIIA. Other sPLA2s such as GIB, GIIE, and GV also have an isoleucine in this region like the GIIA enzyme. However, GIID and GIIF have a valine in this region like human GX, which supports the fact that the 2-isobutyl compounds 11h and 12f display somewhat increased potency against GIID and GIIF enzymes.

A small subset of 11d derivatives were synthesized and tested against hGX sPLA2 (Table 2). As initial docking studies predicted that the phenylsulfonamide group would extend out of the active site, it was surprising to see a 38-fold difference in inhibition for compounds 13b–d when the phenyl ring was substituted with a chlorine at the para-, meta-, and ortho-positions (Table 2). Compounds 13d, and 13f, with substitutions at the ortho-position with a chloro- or methyl- group, resulted in higher inhibition potency over 11d (Table 2). It is possible that the extra methyl or chlorine groups pack into a small pocket of the active site, which would increase the binding affinity. However, replacing the phenylsulfonamide on 11d with a methylsulfonamide (13h) also increases potency against hGX (Table 2). Without a crystal structure, it is difficult to conclude how this phenylsulfonamide is contacting the enzyme active site.

The 6,7-benzoindole inhibitors display general potency against all tested human and mouse sPLA2 enzymes (Table 3). Because the extra hydrophobic bulk is predicted not to make direct contact with the enzyme, the increased potency is likely due to increased partitioning of the inhibitor into the phospho-

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**Table 1. IC\(_{50}\) Values of Substituted Indole Inhibitors against Human and Mouse sPLA2s**

<table>
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<tr>
<th>Comp</th>
<th>R1</th>
<th>R2</th>
<th>hGIB</th>
<th>mGIB</th>
<th>hGIIA</th>
<th>mGIIA</th>
<th>hGIID</th>
<th>mGIID</th>
<th>hGIIE</th>
<th>mGIIE</th>
<th>hGIIF</th>
<th>mGIIF</th>
<th>hGV</th>
<th>mGV</th>
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\(\text{IC}_{50}\) values are reported as the mean of duplicate or triplicate analysis with standard deviations. Each compound was screened at 1660 nM and reported as >1600 nM if the inhibition was <50%. \(\text{IC}_{50}\) values for GIB, GIIA, GIIE, GV, and GX from ref 17. Retest of this compound against hGV and mGV gave 110±30 and 160±20 nM, respectively. \(\text{IC}_{50}\) values obtained using \textit{E. coli} membrane assay. Each compound was screened at 1330 nM and reported as >1300 nM if the inhibition was <50%.

**Table 2. IC\(_{50}\) Values of 11d Derivatives against hGX sPLA2s**

<table>
<thead>
<tr>
<th>Comp</th>
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<td>13c</td>
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<td>7±2</td>
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<tr>
<td>13i</td>
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<td>30±10</td>
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</table>

\(\text{IC}_{50}\) values are reported as the mean of duplicate or triplicate analysis with standard deviations.
lipid substrate vesicles, which increases the ratio of $X_I/K_I^*$ ($X_I$ is the mole fraction of inhibitor in the interface and $K_I^*$ is the interfacial dissociation constant).22,23 Of particular note is compound 12e that inhibited human and mouse groups IB, IIA, IID, IIE, IIF, V, and X sPLA2s with an IC$_{50}$ of less than 350 nM (Table 3). We also sought structurally similar compounds that would be devoid of sPLA2 binding activity because such compounds are useful as controls in cellular studies. The X-ray structure of an Indoxam analogue bound to hGIIA and Me-Indoxam bound to hGX show that the carboxyl group of the substituent at the 4-position of the indole directly coordinates to the active site Ca$^{2+}$.16,24 We thus synthesized 14a and 14b with only a methoxy group at the 4-position to remove the interaction made between the inhibitor and Ca$^{2+}$. Surprisingly, while 14b (Figure 3) gave an IC$_{50}$ of 1000 nM against hGX (data not included in table), 14a had an IC$_{50}$ of 14 and 34 nM against human and mouse GIIA, respectively (Table 3).

Table 3. IC$_{50}$ Values of Substituted Benzo-Fused Indole Inhibitors against Human and Mouse sPLA2s$^a$

<table>
<thead>
<tr>
<th>Comp</th>
<th>R1</th>
<th>R2</th>
<th>hGIIA</th>
<th>mGIIA</th>
<th>hGIIA</th>
<th>mGIIA</th>
<th>hGIIA</th>
<th>mGIIA</th>
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<td></td>
<td></td>
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$^a$ IC$_{50}$ values are reported as the mean of duplicate or triplicate analysis with standard deviations. Each compound was screened at 1660 nM and reported as >1600 nM if the inhibition was <50%. $^b$ IC$_{50}$ values obtained using E. coli membrane assay. Each compound was screened at 1330 nM and reported as >1300 nM if the inhibition was <50%.

Compound 14a was also potent against hGIIE and mGIIE, consistent with trends observed for other potent group IIA indole-based inhibitors. Poor inhibiting control compounds were successfully designed by introduction of an N-methyl group on the oxalamide of the indole scaffold to give compounds 15a–15c (Figure 3). Analysis of the co-crystal structure containing Me-Indoxam in the hGX active site reveals that the introduced N-methyl group disrupts a key hydrogen bond with either a histidine or aspartate residue, while also introducing extra hydrophobic bulk into the active site.16 All N-methyl oxalamide control compounds had IC$_{50}$ values that were >30-fold higher than their parent compound (Figure 3).

The 2-isobutyl Indoxam derivative 16a was synthesized and found to poorly inhibit sPLA2 enzymatic activity (Table 4). Since Indoxam does not inhibit hGX in the low nanomolar range (Table 4), it is not surprising that 16a fails to inhibit hGX. This result suggests that poor inhibition of hGX activity by Indoxam or its derivatives has more to do with the indolizine heterocycle and not the substituents present on the ring. Interestingly, the 8-oxopropanone derivative 16b and the 8-methoxy derivative 16c were selectively potent against hGIIE and hGIIE which was similar to the gain in selectivity displayed by 14a. We also prepared 15c (Figure 3), which did not significantly inhibit hGIIE at concentrations below 1600 nM.

Conclusion

A series of indole- and indolizine-based compounds were synthesized and tested against the full set of human and mouse sPLA2 enzymes. Compound 11d was found to be selectively potent against hGX over all other human and mouse sPLA2 enzymes. Derivatives of 11d, such as 13h, were also found to bind with higher affinity to the hGX enzyme active site and may help in further studies of hGX sPLA2 function. An inhibitor selective for mouse and human GIIE and GIIE sPLA2 (14a) as well as selective human GIIE and GIIE inhibitors (16b and 16c) were also identified from this group of compounds. Compound 12e is potent against human and mouse groups IB, IIA, IID, IIE, IIF, V, and X and is the most generally potent sPLA2 inhibitor reported to date. It is also the first reported potent...
inhibitor of groups IID and IIF sPLA2s. The inhibitors we describe may be useful in probing the roles of sPLA2s in inflammatory diseases such as asthma and arthritis.

Experimental Section

Enzyme Inhibition Assays. For compounds with IC50 < 1600 nM in the fluorometric assay or < 1300 nM in the E. coli membrane assay, inhibitor concentrations were varied with five different concentrations used to determine IC50 values. All IC50 values were obtained by nonlinear regression curve-fitting of percent inhibition versus log [inhibitor] using the Kaleidograph software.

Fluorometric Assay. Microtiter plate assay of sPLA2s using pyrene-labeled phosphatidylglycerol as the substrate was performed as described previously with the exception that seven wells were used per assay instead of eight.

E. coli Membrane Assay. IC50 values calculated for hGIID were done using a modified procedure from that reported previously. See Supporting Information for details.

Synthesis. All reagents were purchased from Sigma-Aldrich and used directly unless otherwise stated. Reactions were performed under an atmosphere of dry nitrogen in oven-dried glassware. Reactions were monitored for completeness by thin layer chromatography (TLC) using Merck 60F254 silica plates, and column chromatography was done with 60 Å silica gel purchased from E. Merck. Preparative reverse phase HPLC was performed on an automated Varian Prep Star system using a YMC S5 ODS column (20 × 100 mm, Waters Inc.).

Representative Procedure for Synthesis of 6,7-Benzindole Inhibitors (Compound 12e): Preparation of 1-Benzyl-2-carbamoyl-6,7-benzoindole (6b). Compound 5b (485 mg, 1.41 mmol) was suspended in 15 mL of 30% KOH/MeOH/THF (2:1:1) and refluxed for 2.0 h (all the solid dissolved during reflux). After refluxing, the reaction was cooled on ice and the pH was made acidic using 2 N HCl, causing the product to precipitate. The white solid was collected by vacuum filtration and washed with 10 mL of cold water and 2 × 10 mL of cold hexanes to give a white solid (400 mg, 86% yield). 1H NMR (300 MHz, CDCl3) δ 3.85 (s, 3H), 6.34 (bs, 2H), 6.77 (s, 1H), 7.09 (d, J = 7.2 Hz, 2H), 7.16–7.31 (m, 4H), 7.37 (t, J = 7.2 Hz, 1H), 7.68 (s, 1H), 7.78 (d, J = 8.1 Hz, 1H), 8.06 (d, J = 8.4 Hz, 1H).

Preparation of 1-Benzyl-2-carboxylic acid-4-methoxy-6,7-benzoindole (6b). Compound 5b (485 mg, 1.41 mmol) was dissolved in 10 mL dry DMF and stirred at 0 °C and sodium hydride (140 mg, 5.5 mmol) was added. After stirring for five minutes at 0 °C, benzylbromide (820 μL, 6.90 mmol) was added and the reaction was stirred for 30 min at room temperature. The reaction mixture was poured onto 20 mL of H2O and 20 mL of EtOAc in a separatory funnel. The layers were separated and the organic layer was washed with 3 × 10 mL H2O and the combined aqueous layer was back-extracted with 1 × 20 mL EtOAc. The combined organic layer was dried over MgSO4 and filtered, and the solvent was removed by rotary evaporation. The crude white solid was purified by column chromatography on silica gel (20% EtOAc/80% hexanes) to give a white solid (820 mg, 75% yield).

Table 4. IC50 Values of Substituted Indolizine Inhibitors against Human and Mouse sPLA2s

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1 IC50 values are reported as the mean of duplicate or triplicate analysis with standard deviations. Each compound was screened at 1660 nM and reported as > 1600 nM if the inhibition was < 50%. IC50 values obtained using E. coli membrane assay. Each compound was screened at 1330 nM and reported as > 1300 nM if the inhibition was < 50%.

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Preparation of 1-(1-Benzyl-4-methoxy-1H-6,7-benzoindol-2-yl)-ethanol (8c). Compound 7c (366 mg, 1.11 mmol) was dissolved in 30 mL of 75% EtOH/25% THF, and NaBH₄ (100 mg, 3.33 mmol) was added to the mixture and stirred at room temperature for 16 h. The reaction mixture was then poured onto 30 mL of EtOAc and 30 mL of H₂O in a separatory funnel. The layers were separated and the water phase was washed with 2 × 20 mL of EtOAc. The organic layers were combined and washed with 2 × 20 mL of H₂O and 1 × 20 mL of NaCl. The organics were dried over MgSO₄ and filtered, and the solvent was removed by rotary evaporation to give 8c as a white solid that was used without further purification (355 mg, 96% yield).

3 H NMR (300 MHz, CDCl₃) δ 1.72 (d, J = 6.3 Hz, 3H), 4.08 (s, 3H), 4.99 (m, 1H), 5.96 (d, J = 20.7 Hz, 1H), 6.09 (d, J = 20.7 Hz, 1H), 6.81 (s, 1H), 6.85 (s, 1H), 7.05 (d, J = 6.9 Hz, 2H), 7.15 (t, J = 6.9 Hz, 1H), 7.20–7.31 (m, 4H), 7.79 (d, J = 8.1 Hz, 1H), 7.93 (d, J = 8.7 Hz, 1H).

Preparation of 1-Benzyl-2-ethyl-4-methoxy-1H,6,7-benzoindole (9c). Compound 8c (420 mg, 1.26 mmol) was dissolved in 20 mL of dry CH₂Cl₂ and added dropwise to a mixture of 14 mL of 99% trifluoroacetic acid (TFA) and NaBH₄ (243 mg, 6.3 mmol) at 20 °C. Ammonia gas was then bubbled into the reaction mixture for five minutes. The reaction mixture then was poured into a separatory funnel containing 20 mL of 2 N HCl. The layers were separated and the aqueous layer was extracted with 2 × 10 mL of CH₂Cl₂. The organic layers were combined, dried over MgSO₄ and filtered, and the solvent was removed by rotary evaporation. The crude material was purified by column chromatography over silica gel (70% EtOAc/30% hexanes) to give a white yellow solid (10.9 mg, 41% yield).

3 H NMR (300 MHz, CDCl₃) δ 1.23 (t, J = 7.5 Hz 3H), 2.94 (q, J = 7.5 Hz 2H), 3.81 (s, 3H), 4.88 (s, 2H), 5.42 (bs, 1H), 5.81 (s, 2H), 6.72 (bs, 1H), 6.81 (s, 1H), 7.10 (d, J = 6.9 Hz, 2H), 7.17 (t, J = 6.9 Hz, 1H), 7.20–7.31 (m, 4H), 7.74 (d, J = 7.8 Hz, 1H), 7.92 (d, J = 8.4 Hz, 1H). MS (ESI, pos. ion) m/z: 467 (M + Na⁺).

Preparation of 2-(3-(2-Amino-2-oxoacetyl)-1-benzyl-2-ethyl-1H,6,7-benzoindol-4-yl)acetic acid (12e). Compound 11e (10.9 mg, 0.024 mmol) was dissolved in 5 mL of MeOH/THF (5:1) with 0.5 mL of 1.5 M NaOH added to the reaction mixture and stirred for 2.5 h at room temperature. The reaction mixture was then poured onto 20 mL of 2 N HCl and 20 mL of CH₂Cl₂ in a separatory funnel. The layers were separated and the aqueous layer was extracted with 2 × 10 mL of CH₂Cl₂. The combined organic layer was dried over MgSO₄ and filtered, and the solvent was removed by rotary evaporation to yield 12e quantitatively. A portion of 12e was purified by HPLC using the following program (eluting solvents each contained 0.08% TFA): 0–5 min 30% MeOH/70% H₂O, 5–30 min 30% MeOH/70% H₂O–70% MeOH/30% H₂O, 30–32 min 70% MeOH/30% H₂O–100% MeOH, 32–35 min 100% MeOH. The product eluted at 24.5 min and the solvent was removed by Speed-Vac to afford a white/yellow solid (4.9 mg). 3 H NMR (300 MHz, MeOD) δ 1.26 (t, J = 7.2 Hz 3H), 3.04 (q, J = 7.5 Hz 2H), 4.93 (s, 2H), 5.99 (s, 2H), 6.96 (s, 1H), 7.15–7.23 (m, 3H), 7.27–7.39 (m, 4H), 7.83 (d, J = 6.9 Hz, 1H), 8.07 (d, J = 8.4 Hz, 1H). MS (ESI, pos. ion) m/z: 431 (M+).

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Supporting Information Available: Details of synthetic methods, including NMR and MS data, for all other described compounds, HPLC traces showing purity of key target compounds, molecular modeling details, and E. coli membrane enzyme assay procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

References
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