Discovery of a Potent and Novel Motilin Agonist

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A novel series of dihydro- and tetrahydrotriazolopyridazine-1,3-dione-based amino acid derivatives were identified as very potent motilin receptor agonists. Incorporating one additional phenylethyl glycinamide subunit to **1** (EC_{50} = 660 nM) was found to improve in vitro potency approximately 3000-fold, resulting in compound **10** (EC_{50} = 0.22 nM). The more potent enantiomer **11A** has an EC_{50} of 0.047 nM in the motilin receptor functional assay and a K_i of 0.7 nM in the binding assay. In addition, compound **11A** was shown to have a significantly reduced tendency to cause receptor desensitization as compared with the motilin receptor agonist ABT-229.

Introduction

The motilin receptor is a G-protein-coupled receptor (GPCR) expressed on smooth muscle and enteric neurons in the gastrointestinal (GI) tract.[1] The endogenous ligand, motilin, is a 22 amino acid peptide that is secreted by enterochromaffin cells in the small intestine and is involved in the normal regulation of coordinated motility of the GI tract.[2] It has been shown that intravenous infusion of motilin peptide stimulates contractile activity of GI smooth muscle and accelerates gastric emptying.[3−6] Therefore, a motilin agonist could be utilized therapeutically for the treatment of GI hypomotility disorders such as diabetic gastroparesis and constipation type irritable bowel syndrome.[7−8]

Although erythromycin is primarily used as an antibiotic, it can also be used clinically to stimulate GI motility.[9] The prokinetic effects of erythromycin are believed to be mediated by stimulation of the motilin receptor.[10] Long-term use of erythromycin, however, is limited due to its antibiotic activity. Efforts to find erythromycin analogues with functional activity at the motilin receptor and without antibiotic activity have led to the discovery of ABT-229.[11,12] ABT-229, however, failed in several clinical trials due to lack of long-term efficacy.[13,14] Clinical failure might be attributed to tachyphylaxis,[15] i.e., a diminished response upon repeated treatment. Preliminary studies in our laboratory using cellular expression of the cloned human motilin receptor revealed that ABT-229 caused a profound tachyphylaxis after the first treatment, and a long washout period was needed for the receptor to recover. The motilin peptide desensitized the receptor to a much lesser extent, and receptor recovery was faster than that observed for ABT-229. We hypothesize that compounds with a similar agonism profile to that of the natural ligand motilin would be less likely to induce tachyphylaxis and thus offer a more desirable profile as a motilin agonist.

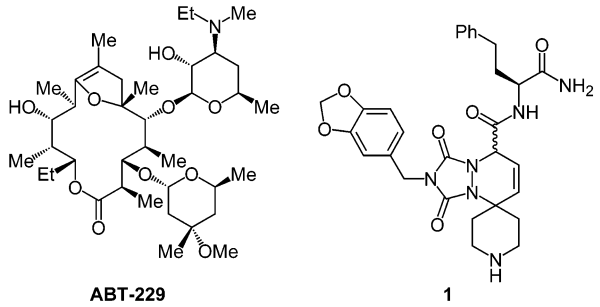

Figure 1. Motilin agonist ABT-229 and HTS lead **1**.

Lead Identification and Chemistry. A number of potent motilin agonists reported in the literature are erythromycin derivatives[16] as exemplified by ABT-229 (EC_{50} = 4.2 nM; K_i = 3.4 nM).[17] Because of the tachyphylaxis displayed by ABT-229 and the potential for other potent erythromycin analogues to share this property, we decided to search for a nonmacrolide lead that might offer a different profile with respect to motilin receptor desensitization. A high throughput screen (HTS) of the BMS compound collection identified a novel (L)-phenylethyl glycinamide derivative **1** (Figure 1) as a modest motilin receptor agonist with an EC_{50} of 660 nM.

The lead compound **1** was originally prepared in a chemical library and was obtained as a mixture of two diastereomers with 90% purity.[18] As part of our HTS validation process, compound **1** was resynthesized as shown in Scheme 1. (L)-Phenylethyl glycinamide **3** was prepared by converting *N*-Boc-(L)-phenylethylglycine **2** to corresponding *N*-Boc-(L)-phenylethylglycine carboxamide, followed by removal of the Boc group with hydrogen chloride in a mixture of dioxane and methanol.

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‖ Lead Discovery.

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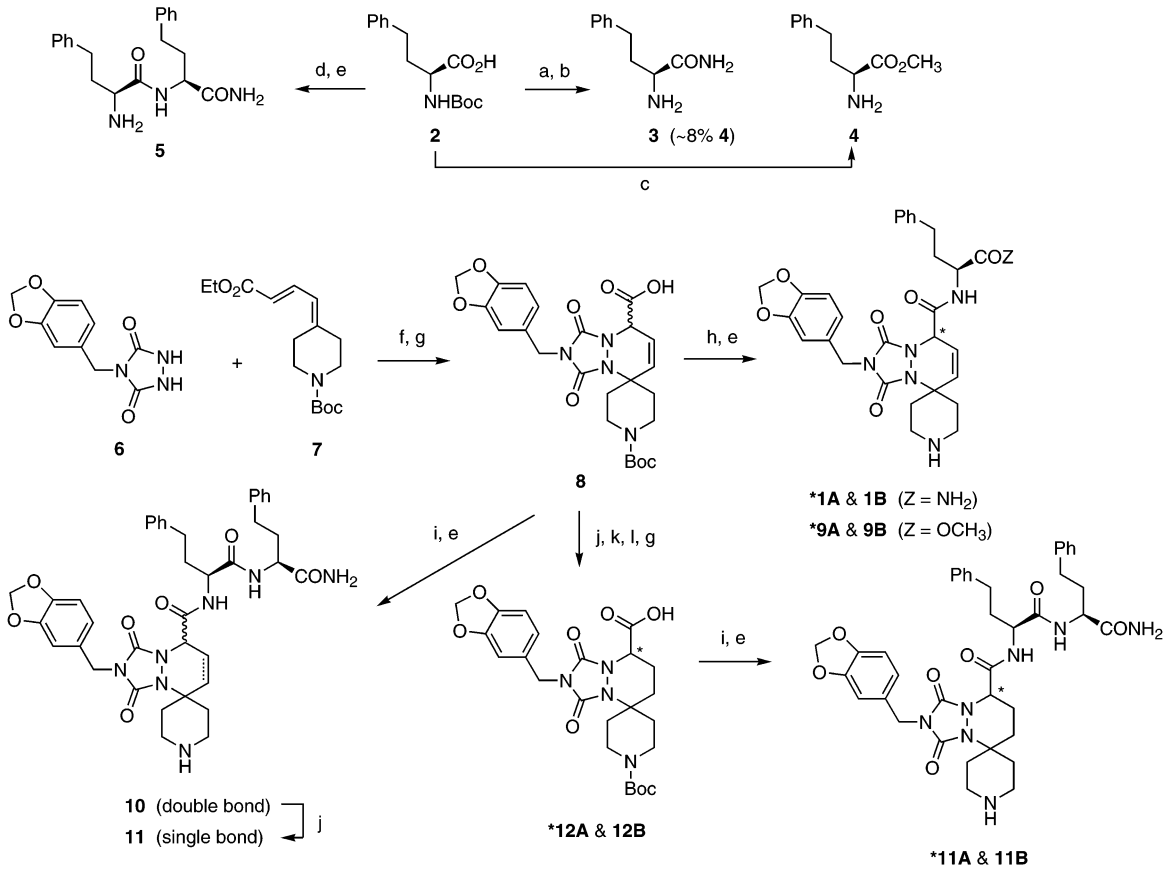
Scheme 1[a]



*isomers were separated.

[a] Reagents and conditions: (a) EDAC, HOAT, NH_4OH; (b) ~ 3 N HCl in dioxane/methanol; (c) saturated HCl in methanol; (d) EDAC, HOAT, **3**; (e) ~15% TFA in CH_2Cl_2; (f) $PhI(CO_2CF_3)_2$; (g) 1 N NaOH; (h) EDAC, HOAT, **3** and/or **4**; (i) EDAC, HOAT, **5**; (j) H_2/Pd−C; (k) $TMSiCHN_2$; (l) chiral separation.

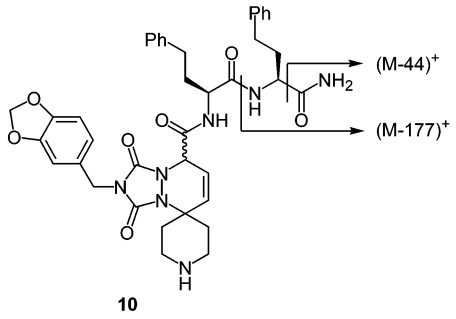
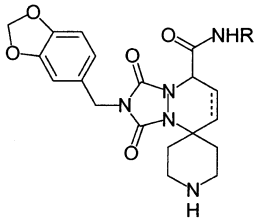


Figure 2. LC/MS/MS fragment pattern for **10**.

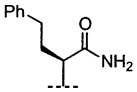
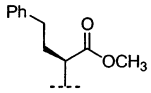
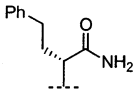
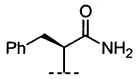
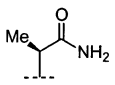
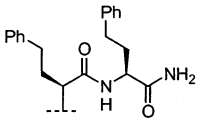
Two key intermediates **6** and **7** were prepared according to a modification of literature procedures.[19,20] The spiro bicyclic dihydrotriazolopyridazine-1,3-dione scaffold was then constructed via a [4 + 2] cycloaddition reaction between **6** and **7** followed by basic hydrolysis of the ester to provide the late-stage intermediate acid **8**.[21,22] Amino acid coupling between acid **8** and amine **3**, followed by Boc deprotection, gave compound **1** as a mixture of two diastereomers. The two isomers were subsequently separated to **1A** and **1B**.[23] Motilin activity resulted primarily from the second eluting isomer **1B** (Table 1).

Because methanol was used as a cosolvent in the Boc removal reaction during the preparation of glycinamide **3** (step b in Scheme 1), a small amount (~8% by HPLC) of phenylethylglycine methyl ester **4** was formed. The mixtures of amide **3** and methyl ester **4** were subsequently used to react with carboxylic acid **8**, resulting in the formation of the corresponding amides **1A** and **1B**, along with methyl esters **9A** and **9B** as side products (~7% yield). Interestingly, the second eluting methyl ester **9B** was found to be a potent, functionally active motilin receptor agonist, with an EC_{50} of 7 nM. Additional amounts of compound **9B** was therefore needed for further evaluation. Pure amino methyl ester **4** was prepared directly from *N*-Boc-phenethylglycine **2** by treatment with a saturated solution of hydrogen chloride in methanol. Subsequent amide formation from amine **4** and acid **8**, followed by Boc deprotection and separation, provided compound **9B**. To our surprise, the second batch of **9B** was inactive in our in vitro motilin assay when tested at concentrations up to 10 μM. A detailed structural analyses between the two batches of **9B** was carried out to determine if there were any differences. Comparison of ^1H, ^{13}C and various 2D NMR spectra (COSY, HMQC, and HMBC) as well as HPLC coinjections confirmed that these two lots had the same major component, compound **9B**, in approximately 95% purity. Chiral HPLC also showed that both batches of **9B** were the same enantiomer. Further HPLC analysis revealed that even though both lots had a similar impurity profile, the first lot of methyl ester **9B** contained a small impurity (0.2% by HPLC) that was not observed in the second inactive lot. Extensive LC/MS/MS analysis indicated that the fragmentation pattern

Table 1. Summary of in Vitro Potency



Compds **1**, **9B, 10**, **13-15** (double bond)
Compds **11** (single bond)

Compd[a]	R	EC_{50} (nM)	K_i (nM)
1A		>10,000	5,800
1B		560	4,100
9B		>10,000	>10,000
13A		>10,000	>10,000
13B		>10,000	>10,000
14A		>10,000	ND
14B		4,200	ND
15A		>10,000	ND
15B		>10,000	ND
10		0.22	2
11		0.35	1
11A		0.047	0.7
11B		7.3	2.7
Motilin		0.15	3.1
ABT-229		4.2	3.4
Erythromycin		400	37,000

[a] Compounds **1**, **10**, and **11** are diastereomeric mixtures; all others are single enantiomers.

for the impurity was consistent with the dipeptide derivative **10**, with a molecular weight of 721 and a loss of 44 mass units for the terminal carboxamide group and 177 for the phenylethylglycinamide moiety (Figure 2). The proposed dipeptide analogue **10** was then synthesized by coupling acid **8** with the dipeptidyl carboxamide **5**, followed by removal of the Boc group (Scheme 1). Compound **10** was subsequently confirmed as a very potent motilin agonist with an EC_{50} of 0.22 nM. It is evident from the result that a small amount of dipeptide **10** present in the first batch of **9B** was responsible for the motilin activity observed.

Reduction of the double bond in **10** provided tetrahydrotriazolopyridazine-1,3-dione analogue **11**. Unfortunately neither **10** nor **11**, both as diastereomeric mixtures, could be separated under a variety of conditions attempted. The problem was circumvented by repeating the last amide formation with enantiomeric pure acid **12A** and **12B**. Reduction of **8** proceeded smoothly. The resultant acid was subsequently converted to methyl ester for ease of prep-scale chiral separation.[24] After both enantiomerically pure methyl esters were obtained,

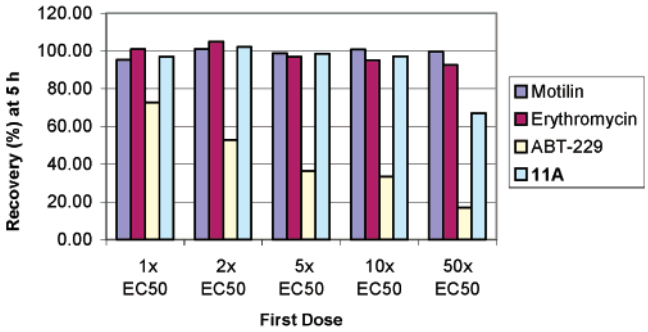


Figure 3. Measurement of receptor desensitization in FLIPR Assay.

they were hydrolyzed to corresponding acids **12A** and **12B** and coupled with carboxamide **5** to yield the dipeptide analogues **11A** and **11B**, both as single isomers (Scheme 1).

Results and Discussion

In Vitro SAR Studies. Initial SAR studies were directed toward simple amino acid analogues of HTS lead **1** (Table 1). To probe the preferred stereochemistry with respect to the amino acid side chain, the corresponding (D)-phenylethyl analogues were prepared and separated as **13A** and **13B**, but both isomers led to a substantial loss of functional activity. Two amino acid derivatives with smaller side chains, (L)-phenylalanine in **14** and (L)-alanine in **15**, were also prepared to probe the optimal size. Similarly this modification resulted in a loss of motilin activity. To our surprise, approximately 3000-fold improvement of in vitro potency (**10** vs **1**) was obtained by adding one additional phenylethyl glycinamide moiety to **1**. The corresponding reduced analogue of dipeptide **10**, tetrahydrotriazolopyridazine-1,3-dione **11**, was also evaluated and showed a comparable in vitro potency (EC_{50} of 0.35 vs 0.22 nM). The more potent isomer **11A** gave an EC_{50} of 0.047 nM in the motilin functional assay and was 3- and 80-fold more potent than natural ligand motilin and macrolide analogue ABT-229 (Table 1). To the best of our knowledge, **11A** is the most potent motilin agonist reported in the literature.

Tachyphylaxis. Tachyphylaxis, or receptor desensitization, is a common phenomenon with G-protein-coupled receptors when treated with an agonist.[25] We suspect the lack of long-term efficacy observed for ABT-229 in humans may be due to motilin receptor desensitization after multiple treatments. Therefore, finding a compound with a lower tendency to cause tachyphylaxis was identified as a key issue. Toward that end, a functional-based fluorescence imaging plate reader (FLIPR) assay was developed to measure the receptor desensitization in a HeLa cell line stably expressing the human motilin receptor. In this assay, the receptor cells were first treated with compounds at a concentration of 1- to 50-fold of their EC_{50}, followed by a washout and recovery period. The maximum functional response for these treated cells was then measured after a challenge using a high dose (i.e., 100-fold EC_{50}) of the testing compound. Treatment with the motilin peptide or erythromycin even at a relatively high concentration (50-fold EC_{50}) showed a complete recovery of receptor to the second stimulation as shown in Figure 3. ABT-

229, on the other hand, gave 73% of recovery when the cells were first treated at a concentration at its EC_{50}. The recovery was reduced to 33−36% when the first dose was at a higher concentration of 5 or 10-fold EC_{50}. It desensitized the receptor further with only 17% recovery after an initial concentration of 50-fold EC_{50}, essentially resulting in a profound down-regulation for the motilin receptor. When **11A** was tested at concentrations of 1- to 10-fold EC_{50}, the receptor showed a complete recovery after 5 h. Desensitization (67% recovery) occurred only at the highest concentration tested (50-fold EC_{50}). These results suggested that ABT-229 would cause a more profound tachyphylaxis whereas compound **11A** will act more like the endogenous ligand motilin, therefore offering a more desirable profile as a motilin agonist.

Conclusion

We have discovered a novel series of dihydro- and tetrahydrotriazolopyridazine-1,3-dione-based amino acid derivatives as potent motilin agonists. Addition of one phenylethyl glycinamide moiety to **1** was found to markedly improve the in vitro potency 3000-fold (i.e., **10** vs **1**). The most potent motilin agonist **11A** has an EC_{50} of 0.047 nM in the functional assay and a K_i of 0.7 nM in the binding assay. In addition, **11A** was shown to have a lower tendency to cause tachyphylaxis of the motilin receptor as compared with ABT-229, thus exhibiting a potentially more desirable profile as a motilin receptor agonist. Motilin agonists with a profile similar to that of **11A** may be able to maintain long-term efficacy for the treatment of GI hypomotility disorders.

Experimental Section

Motilin Receptor Functional Assay (EC_{50}). HeLa-MR9 cell line stably expressing the human motilin receptor was used. The cells were grown in T-150 flask in Dulbecco's modified eagle medium (Gibco, #12320−032) containing 10% fetal bovine serum (Gibco, #16140-071), 1X MEM nonessential amino acids (Gibco, #11140−050), and 3 mg/mL G418 (Gibco, #10131−035). Hela-MR9 cells were plated into a black/clear flat bottom 96-well plate (BD Falcon #353948) at a density of 20 000 per well, and cultured for 20−24 h at 37 °C with 5% CO_2 to make them about 90% to 95% confluent. Compounds were diluted to 5× of final concentration with assay buffer (Hanks balanced salt solution without Ca^{2+} and Mg^{2+}, 10 mM HEPES, pH 7.5, 0.1% BSA), and the final DMSO concentration was kept below 0.5%. When cells were confluent, the medium was aspirated from the cells. To each well was added 100 μL of dye solution made in the assay buffer, consisting of 5 μg/mL fluo-3 (Molecular Probes, #F-1242), 2.5 mM probenecid (Sigma, #P-8761), and 0.5% pluronic acid (Molecular Probe #P-3000) and incubated at 37 °C. After incubation for 60 min, the dye solution was aspirated, and 120 μL of assay buffer was added to each well. The cell and compound plates were loaded in a fluorescence imaging plate reader (FLIPR), and 30 μL of compound was added to the cell plate. The intracellular Ca^{2+} signal was measured at a 1 s interval for first 90 s and 3 s interval for the next 3 min in the FLIPR.

Measurement of Tachyphylaxis for Motilin Receptor. Effect of compounds on HeLa-MR9 cell FLIPR functional response was used to measure the receptor desensitization (tachyphylaxis). The HeLa-MR9 cells were first treated with compounds at a concentration of 1- to 50-fold of their EC_{50} followed by a 5-h washout and recovery period. The cells were stimulated with second addition of the compound at maximum effective dose (100× EC_{50}) and Ca^{2+} signal was then measured in FLIPR.

Motilin Binding Assay (K_i). A homogeneous scintillation proximity assay (SPA) in HeLa-MR9 cell line was used to measure the motilin receptor binding constant. To a 96-well OptiPlate (Packard) were added 40 μL of 2X SPA binding buffer (1X: 50 mM Tris-HCl, 5 mM $MgCl_2$, 50 mM NaCl, 5 mM KCl, 1 mM PMSF, 2 mM CHAPS, and 0.1% BSA, pH 7.6), 10 μL of 1 mg/mL cell membranes (10 μg), 15 μL of 2 nM [^{125}I]-motilin (NEN NEX 378, 2200 Ci/mmol; or Amersham Pharmacia IM337, 2000 Ci/mmol), 15 μL of 10X compound or unlabeled 2 μM motilin, and 0.5 mg of PVT-WGA SPA beads in 70 μL of 1X SPA binding buffer. Contents in 150 μL of total volume were incubated at room temperature for 2 h. The plate was counted in a TopCount (Packard) for 1 min/well to determine radioactive ligand bound to the receptor.

General Chemistry Methods. Proton NMR spectra were recorded on a JEOL 400 or 500 MHz instrument. Analytical HPLC was performed on a Shimadzu instrument using YMC C18 5 micron 4.6 × 50 mm column with a 4-min gradient of 0−100% solvent A (90%MeOH/10%H_2O/0.2% H_3PO_4) and 100−0% solvent B (10%MeOH/90%H_2O/0.2% H_3PO_4) with a 1-min hold. LC-MS spectra were obtained on a Shimadzu HPLC and Micromass Platform using electrospray ionization. HRMS were obtained on a Micromass LCT in lockspray with electrospray ionization. The preparative HPLC was performed on an automated Shimadzu system using the YMC ODS C18 5 μm preparative columns with mixtures of solvent C (10% MeOH/90%H_2O/0.2% TFA) and solvent D (90% MeOH/10% H_2O/ 0.2% TFA) or mixtures of solvent E (10% CH_3CN/ 90%H_2O/0.2% TFA) and solvent F (90% CH_3CN/10% H_2O/ 0.2% TFA). Preparation of intermediate compounds **3**−**8** and **12A** are described in the Supporting Information. All other reagents and solvent were obtained from commercial sources and were used without further purification.

Compounds 1A, 1B, 9A, and 9B. To a stirred solution of compound **8** (140 mg, 0.28 mmol) in 2 mL of dichloromethane were added EDAC (107 mg, 0.56 mmol), HOAT (76 mg, 0.56 mmol), Et_3N (78 μL, 0.56 mmol), and mixtures of **3** and **4** (120 mg), and the reaction was stirred at RT overnight. The mixtures were diluted with EtOAc, washed with water (2×), 1 N NaOH (2×), and brine, dried over Na_2SO_4, and concentrated. The Boc-intermediate was deprotected with 3 mL of 15% TFA/CH_2Cl_2 solution at RT for 3 h. The final compounds were separated on an automated Shimadzu HPLC system to give compounds **1A**, **1B**, **9A**, and **9B**, all as TFA salts. The first eluting isomer was assigned as **A**, and the second eluting isomer was assigned as **B**.

Compound **1A** (38 mg), HPLC: 98% purity; LCMS *m*/*z* 561 (M + H); HRMS calcd for (M + H) 561.2462, found 561.2466; ^{1}H NMR (MeOH-d_4) δ 1.87−2.04 (m, 3H), 2.09−2.19 (m, 1H), 2.49 (dt, J = 5, 14 Hz, 1H), 2.55−2.71 (m, 2H), 3.21 (dt, J = 3, 14 Hz, 1H), 3.29−3.52 (m, 4H), 4.26−4.33 (m, 1H), 4.61 (dd, J = 15, 39 Hz, 2H), 5.12 (dd, J = 2, 5 Hz, 1H), 5.90 (s, 2H), 6.24 (dd, J = 5, 11 Hz, 1H), 6.61 (dd, J = 2, 10 Hz, 1H), 6.73 (d, J = 8 Hz, 1H), 6.84 (dd, J = 2, 8 Hz, 1H), 6.88 (d, J = 1 Hz, 1H), 7.12−7.27 (m, 5H), 8.58 (d, J = 8 Hz, 1H).

Compound **1B** (34 mg), HPLC: 98% purity; LCMS *m*/*z* 561 (M + H); HRMS calcd for (M + H) 561.2462, found 561.2465; ^{1}H NMR (MeOH-d_4) δ 1.90−2.12 (m, 4H), 2.47 (dt, J = 5, 14 Hz, 1H), 2.58−2.77 (m, 2H), 3.16−3.52 (m, 5H), 4.32−4.39 (m, 1H), 4.63 (dd, J = 15, 39 Hz, 2H), 5.13 (dd, J = 2, 5 Hz, 1H), 5.90 (s, 2H), 6.25 (dd, J = 5, 11 Hz, 1H), 6.57 (dd, J = 2, 10 Hz, 1H), 6.71 (d, J = 8 Hz, 1H), 6.85 (dd, J = 2, 8 Hz, 1H), 6.89 (d, J = 1 Hz, 1H), 7.12−7.26 (m, 5H), 8.56 (d, J = 8 Hz, 1H).

Compound **9A** (15 mg), HPLC: 95% purity; LCMS *m*/*z* 576 (M + H); HRMS calcd for (M + H) 576.2458, found 576.2460; ^{1}H NMR (MeOH-d_4) δ 1.90−2. 05 (m, 3H), 2.06−2.18 (m, 1H), 2.42 (dt, J = 5, 14 Hz, 1H), 2.54−2.72 (m, 2H), 3.20 (dt, J = 3, 14 Hz, 1H), 3.33−3.52 (m, 4H), 3.68 (s, 3H), 4.28−4.36 (m, 1H), 4.61 (dd, J = 15, 42 Hz, 2H), 5.12 (dd, J = 2, 5 Hz, 1H), 5.90 (s, 2H), 6.25 (dd, J = 5, 11 Hz, 1H), 6.60 (dd, J = 2, 11 Hz, 1H), 6.73 (d, J = 8 Hz, 1H), 6.85 (dd, J = 1, 8 Hz, 1H), 6.88 (d, J = 1 Hz, 1H), 7.13−7.27 (m, 6H), 8.66 (d, J = 8 Hz, 1H).

Compound **9B** (15 mg), HPLC: 95% purity; LCMS *m*/*z* 576 (M + H); HRMS calcd for (M + H) 576.2458, found 576.2482; ^{1}H NMR (MeOH-d_4) δ 1.90−2.20 (m, 4H), 2.44 (dt, J = 5, 14

Hz, 1H), 2.58−2.76 (m, 2H), 3.21 (dt, J = 3, 14 Hz, 1H), 3.34−3.52 (m, 4H), 3.67 (s, 3H), 4.33−4.40 (m, 1H), 4.63 (dd, J = 15, 42 Hz, 2H), 5.13 (dd, J = 2, 5 Hz, 1H), 5.90 (s, 2H), 6.25 (dd, J = 5, 11 Hz, 1H), 6.60 (dd, J = 2, 11 Hz, 1H), 6.72 (d, J = 8 Hz, 1H), 6.85 (dd, J = 1, 8 Hz, 1H), 6.88 (d, J = 1 Hz, 1H), 7.12−7.28 (m, 6H), 8.80 (d, J = 8 Hz, 1H).

Compound 10. To a stirred solution of compound **8** (50 mg, 0.1 mmol) in 0.8 mL of DMF were added EDAC (19.2 mg, 0.1 mmol), HOAT (14 mg, 0.1 mmol), DIEA (18 μL, 0.1 mmol), and **5** (34 mg, 0.09 mmol), and the mixtures were stirred at RT overnight. The reaction was diluted with EtOAc, washed with water (2×), 1 N NaOH (2×), and brine, dried over Na_2SO_4 and concentrated. The intermediate was purified on HPLC, and then the Boc group was deprotected with 1 mL of 50% TFA/CH_2Cl_2 solution at RT for 30 min. It was concentrated to give 31 mg (40%) of compound **10** as foam solid. HPLC: 97% purity; LCMS m/z 722 (M + H); HRMS calcd for (M + H) 722.3302, found 722.3328; ^{1}H NMR (MeOH-d_4) δ 1.85−2.20 (m, 6H), 2.37−2.80 (m, 5H), 3.15−3.50 (m, 5H), 4.25−4.70 (m, 4H), 5.10−5.16 (m, 1H), 5.83 and 5.85 (s, total 2H), 6.20−6.28 (m, 1H), 6.48−6.90 (m, 4H), 7.10−7.26 (m, 10H), 8.12 and 8.23 (d, J = 7 Hz, total 1H), 8.67 and 8.81 (d, J = 7 Hz, total 1H).

Compound 11A. To a stirred solution of **12A** (80 mg, 0.2 mmol) in 2 mL of DMF were added PyAOP (115 mg, 0.22 mmol), **5** (97 mg, 0.22 mmol), and DIEA (80 μL, 0.44 mmol) at RT, and the reaction was stirred at RT overnight. The reaction was diluted with EtOAc, washed with water (2×), dried over Na_2SO_4, and concentrated. The Boc intermediate was deprotected with 1 mL of 30% TFA in dichloromethane (v/v) at RT for 2 h. It was purified on HPLC to give a TFA salt which was washed with $NaHCO_3$ solution to give 87 mg (60%) of compound **11A** as a white powder. HPLC: 95% purity; LCMS m/z 724 (M + H); HRMS calcd for (M + H) 724.3459, found 724.3466; ^{1}H NMR (MeOH-d_4) δ 1.75−2.33 (m, 12H), 2.55−2.80 (m, 4H), 3.00−3.24 (m, 3H), 3.26−3.30 (m, 1H), 4.31−4.40 (m, 2H), 4.57 (dd, J = 15, 49 Hz, 2H), 4.75 (t, J = 6 Hz, 1H), 5.85 and 5.86 (s, total 2H), 6.70 (d, J = 8 Hz, 1H), 6.84 (d, J = 8 Hz, 1H), 6.88 (s, 1H), 7.10−7.30 (m, 10H), 8.27 (d, J = 7 Hz, 1H), 8.43 (d, J = 7 Hz, 1H). Anal. ($C_{39}H_{45}N_7O_7 \cdot 0.39H_2O$) C, H, N.

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Supporting Information Available: Preparation of intermediate compounds **3**−**8** and **12A** as well as detailed biological assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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