Brief Articles

Dihydropyrrolopyrazole Transforming Growth Factor-β Type I Receptor Kinase Domain Inhibitors: A Novel Benzimidazole Series with Selectivity versus Transforming Growth Factor-β Type II Receptor Kinase and Mixed Lineage Kinase-7

Hong-yu Li,*,† Yan Wang,† Charles R. Heap,‡ Chi-Hsin R. King,‡ Sreenivasa R. Mundla,§ Matthew Voss,§ David K. Clawson,† Lei Yan,# Robert M. Campbell,|| Bryan D. Anderson,|| Jill R. Wagner,|| Karen Britt,|| Ku X. Lu,# William T. McMillen,† and Jonathan M. Yingling#

Discovery Chemistry Research and Technology, Process Chemistry Research, Cancer Research, and Lead Optimization Biology, Lilly Research Laboratory, A Division of Eli Lilly and Company, Lilly Corporate Center, Indianapolis, Indiana 46285, and Medicinal Chemistry Department, Albany Molecular Research, Inc., P.O. Box 15098, Albany, New York 12212

Received March 15, 2005

Novel dihydropyrrolopyrazole-substituted benzimidazoles were synthesized and evaluated in vitro as inhibitors of transforming growth factor-β type I receptor (TGF-β RI), TGF-β RII, and mixed lineage kinase-7 (MLK-7). These compounds were found to be potent TGF-β RI inhibitors and selective versus TGF-β RII and MLK-7 kinases. Benzimidazole derivative **8b** was active in an in vivo target (TGF-β RI) inhibition assay.

Introduction

The transforming growth factor-β (TGF-β) type I receptor is a member of a large family of growth factors involved in the regulation of a diverse array of biological processes including cell growth and differentiation, matrix modulation, and embryonic development.[1] Extensive research has indicated that inhibition of the TGF-β type I receptor may treat a number of diseases involving the TGF-β signaling pathway, including fibrosis and cancer.[2]

For identification of the TGF-β inhibitors, high-throughput screening (HTS) has been an effective and validated approach.[3d] The HTS identified leads and through SAR determined that the minimum requirements for tight binding at the active site included the presence of a 2-pyridyl group and an adjacent aryl or heteroaryl substituent featuring a hydrogen bond acceptor from the central ring.[3] This SAR conclusion was supported by the X-ray crystal structures of TGF-β RI inhibitors bound to the ATP site.[3f] During our continuing efforts to examine other heterocyclic pharmacophores, we have discovered that incorporation of a benzimidazole ring system provides a series of novel and potent TGF-β RI inhibitors. In light of previously published SAR studies of TGF-β RI inhibitors, the p38 MAP kinase selectivity was no longer a major focus of our current TGF-β effort. However, internal kinase panel profiles of known TGF-β RI inhibitors revealed that mixed lineage kinase-7 (MLK-7), a kinase in the MAP kinase signal pathway and closely related to the TGF-β RII, which potentially interrupts the TGF-β RI pathway, was potently inhibited. This paper will focus on the selectivity of our inhibitors against MLK-7 and TGF-β RII. The p38 MAP kinase activity was checked for new imidazole analogues **8a** and **11a** (20−50 × selectivity vs p38 MAP kinase).

Chemistry

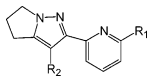
The compounds appearing in Table 1 were prepared according to Scheme 1. The starting materials 6-methylpyridinyl DHP (dihydropyrrolopyrazole)-bromide **2a**, pyridinyl DHP bromide **2b**, 6-methyl pyridinyl DHP boronic acid **7a**, and pyridinyl DHP boronic acid **7b** were synthesized by following known procedures.[3e] Three different Suzuki reaction conditions were used for small-scale screening. The best yielding conditions as judged by LC−MS analysis were scaled up to a 60−250 mg reaction: (1) refluxing **1** and **2b** with 10% $Pd_2(dba)_2$, 10% Pd(DIPHOS), and K_2CO_3 in THF overnight gave the desired isolated product in 5% yield; (2) microwave-assisted heating of **4** with **2a** and **2b** with 3% Pd_2Cl_2 (ddpf), 6% (*o*-biphenyl)P(*t*-Bu)$_2$, and KOH in dioxane produced the desired products **5a** and **5b** in 68% and 10% yields, respectively; (3) microwave-assisted heating of compounds **6** with **7a** and **7b** catalyzed by $Ph(PP_3)_4$ and $NaHCO_3$ in DMSO/H_2O afforded **8a** and **8b** in 56% and 32% yields, respectively.[4]

The synthesis of N-substituted benzimidazole compounds is summarized in Scheme 2. The alkylation of 5-iodobenzimidazole with base NaH in DMF went smoothly and yielded a 1/1 mixture of two regioisomers in good yields (96% for **9a**/**9b** and 83% for **10a**/**10b**). In both cases the two regioisomers were very difficult to separate in a practical manner. As a result, the regioisomer mixtures were used for a small-scale Suzuki reaction screening, and the conditions of 5% $Pd(PPh_3)_4$ and $NaHCO_3$ in DMSO/H_2O assisted with microwave-heating were identified as best. Subsequently, the regioisomer mixtures **11a**/**11b**, **12a**/**12b**, **13a**/**13b**, and **14a**/**14b** were synthesized. At this stage, the separation of the two regioisomers was better on TLC screening than their precursors but still challenging. To improve the efficiency of the separation, we explored MPLC conditions and found that by eluting with 20% THF/80% CH_2Cl_2, the regioisomer mixture of **11a**/**11b** was perfectly separated. It is noted

* To whom correspondence should be addressed. Phone: 317-433-3349. Fax: 317-276-7600. E-mail: li_hong-yu@lilly.com.
† Discovery Chemistry Research and Technology, Lilly Research Laboratory.
‡ Albany Molecular Research, Inc.
§ Process Chemistry Research, Lilly Research Laboratory.
# Cancer Research, Lilly Research Laboratory.
|| Lead Optimization Biology, Lilly Research Laboratory.

10.1021/jm058209g CCC: $33.50
Published on Web 02/21/2006

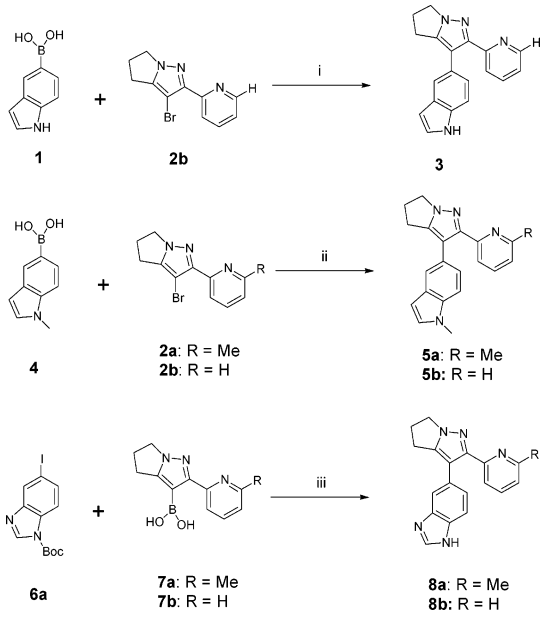
Table 1. Kinase and Cellular Activity of Indole and Benzimidazole[a]



compd	R1	R2	TGF-β RIK, IC_{50}, μM	TGF-β RIIK IC_{50}, μM	MLK-7K IC_{50}, μM	p3TP-Lux IC_{50}, μM	NIH 3T3 IC_{50}, μM
LY364947	H	See ref. 3e	0.059 ± 0.023	0.4	1.4	0.04 ± 0.022	0.081 ± 0.067
3	H		>20	>20	>20	NT	NT
5a	Me		4.10	>20	>20	NT	NT
5b	H		12.23 ± 7.59	>20	16.61	NT	NT
8a	Me		0.047	1.30	15.94	0.015	0.042
8b	H		0.068 ± 0.043	0.409	9.45	0.057 ± 0.050	0.118 ± 0.090

[a] All IC_{50} values were from 10 point determinations. Mean values ± SEM for a minimum of two determinations.

Scheme 1[a]

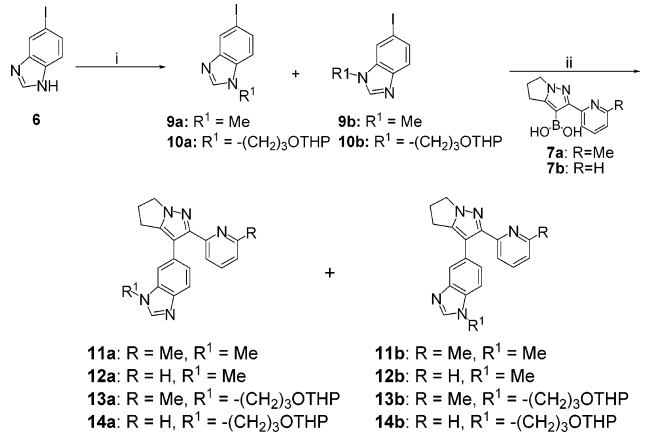


[a] Conditions: (i) 10% $Pd_2(dba)_3$, 10% Pd(DIPHOS), K_2CO_3, THF, 4%; (ii) 3% Pd_2Cl_2(ddpf), 6% (*o*-diphenyl)P(*t*-Bu)$_2$, KOH, dioxane/EtOH (1:1), microwave (at 120 °C, 25 min), 68% (for **5a**), 10% (for **5b**); (iii) 5% $Pd(PPh_3)_4$, $NaHCO_3$, DMSO/H_2O, 56% (for **8a**), 32% (for **8b**).

that the N-1-substituted benzimidazole eluted faster than N3-substituted benzimidazole. Under solvent conditions of CH_2Cl_2 to 20% THF/80% CH_2Cl_2, the regioisomer mixture was separated to give the faster eluting **11a** (42%) followed by **11b** (36%). The structures of the regioisomers **11a** and **11b** were assigned from NOE experiments. Irradiation of the methyl group at δ 3.73 for **11a** gave an enhancement of the singlet proton H4 at δ 7.31, while irradiation of the methyl group for **11b** at δ 3.81 gave an enhancement of the doublet proton H7 at δ 7.26. Similarly, the regioisomer mixtures **12a**/**12b**, **13a**/**13b**, and **14a**/**14b** were separated and structurally determined by the NOE experiments.

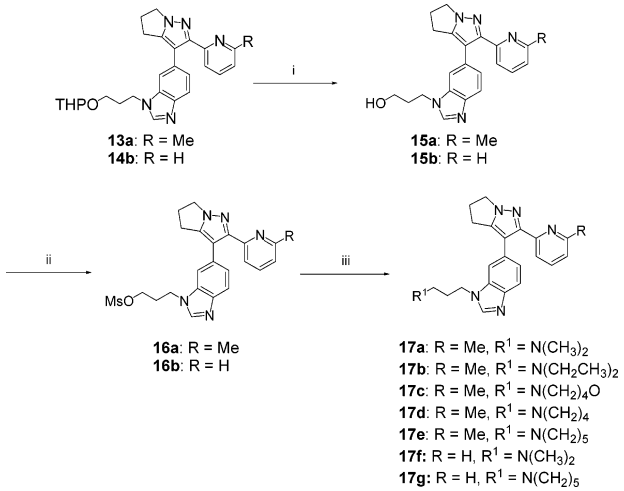
Transformation of the THP-protected key intermediates (**13a** and **14b**) are described in Scheme 3. Deprotection with the standard conditions of AcOH, THF, and H_2O followed by mesylate formation with MsCl in CH_2Cl_2 catalyzed by pyridine

Scheme 2[a]



[a] Conditions: (i) MeI, NaH, DMF, 95% (for **9a**/**9b**); BrPrOTHP, NaH, DMF, 83% (for **10a**/**10b**); (ii) 5% $Pd(PPh_3)_4$, $NaHCO_3$, DMSO/H_2O (1:1), microwave (at 100 °C, 10 min), separation, 42% (for **11a**), 36% (for **11b**), 37% (for **12a**), 41% (for **12b**), 36% (for **13a**), 35% (for **13b**), 32% (for **14a**), 34% (for **14b**).

Scheme 3[a]



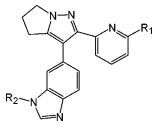
[a] Conditions: (i) AcOH, THF, H_2O, refluxed, 77% (for **15a**), 75% (for **15b**); (ii) MsCl, pyridine, CH_2Cl_2, 85% (for **16a**), 55% (for **16b**); (iii) RNH/or RNH_2, THF, 95% (for **17a**), 96% (for **17b**), 89% (for **17c**), 90% (for **17e**), 100% (for **17f**), 100% (for **17g**).

gave **16a** and **16b** in 85% and 53% yields, respectively. Heating the mesylate with various amines neatly produced the final compounds (Scheme 3).

Selective Inhibition of TGF-β RI by Benzimidazole Compounds

4-[3-(Pyridin-2-yl)-1*H*-pyrazol-4-yl]-quinoline (LY364947)[3e] was used as a standard compound for the assays. Table 1 summarizes the TGF-β RI inhibitory activity of unsubstituted benzimidazoles and indoles (**3**, **5a**/**5b**, and **8a**/**8b**). In view of the key interaction as a hydrogen-bond acceptor of the quinoline-4-yl nitrogen in LY364947 with the active site His283 residue,[3e] the N-1 of the benzimidazole group potentially acts through the same pharmacophore. To support this hypothesis, we have investigated the SAR of the indole ring system in which neither the unsubstituted or substituted N-1 can serve as a hydrogen-bond acceptor. As expected, indoles showed reduced potency in inhibiting the TGF-β RI kinase domain. In view of the dramatically different potency observed between indole **3** (IC_{50} > 20 000 nM) and imidazole **8a** (IC_{50} = 47 nM), the binding conformation would likely be 1,2-conjugated, with N-1 as a

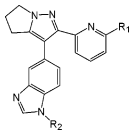
Table 2. Kinase and Cellular Activity of N-1-Substituted Benzimidazole[a]



compd	R_1	R_2	IC_{50}, μM TGF-β RI	TGF-β RII	MLK-7	p3TP-Lux	NIH 3T3
LY364947	H	see ref 3e	0.059 ± 0.023	0.4	1.4	0.04 ± 0.022	0.081 ± 0.067
11a	−Me	−Me	0.022	0.668	2.34	0.029	0.096
12a	−H	−Me	0.075	0.395	1.31	0.008	0.032
15a	−Me	$-(CH_2)_3OH$	0.041	0.690	2.81	0.078	0.371
15b	−H	$-(CH_2)_3OH$	0.079	0.352	0.708	0.079 ± 0.007	0.371 ± 0.182
17a	−Me	$-(CH_2)_3N(CH_3)_2$	0.085	>20	>20	0.053 ± 0.033	0.139
17b	−Me	$-(CH_2)_3N(CH_2CH_3)_2$	0.122	>20	>20	0.1 ± 0.005	0.274 ± 0.113
17c	−Me	$-(CH_2)_3N(CH_2)_4O$	0.069	19.75 ± 0.3	8.48	0.186 ± 0.062	0.277 ± 0.001
17d	−Me	$-(CH_2)_3N(CH_2)_4$	0.074	>20	>20	0.117 ± 0.041	0.249 ± 0.133
17e	−Me	$-(CH_2)_3N(CH_2)_5$	0.155 ± 0.110	>20	>20	0.051 ± 0.027	0.150 ± 0.054
17f	−H	$-(CH_2)_3N(CH_3)_2$	0.398	>20	15.88	0.104	1.001
17g	−H	$-(CH_2)_3N(CH_2)_4$	0.246	10.84 ± 4.59	4.88	0.226 ± 0.112	0.854 ± 0.224

[a] All IC_{50} values were from 10 point determinations. Mean values ± SEM for a minimum of two determinations.

Table 3. Kinase and Cellular Activity of N-3-Substituted Benzimidazole[a]



compd	R_1	R_2	IC_{50}, μM TGF-β RI	TGF-β RII	MLK-7	p3TP-Lux	NIH 3T3
LY364947	H	See ref 3e	0.059 ± 0.023	0.4	1.4	0.04 ± 0.022	0.081 ± 0.067
11b	−Me	−Me	1.70	8.73	>20	0.422	0.872
12b	−H	−Me	4.74	19.63	>20	1.373	1.622
13b	−Me	$-(CH_2)_3OTHP$[b]	1.83	14.54	>20	2.43 ± 0.264	NT[c]
14b	−H	$-(CH_2)_3OTHP$[b]	6.58	>20	>20	5.89 ± 1.28	4.70 ± 2.30

[a] All IC_{50} values were from 10 point determinations. Mean values ± SEM for a minimum of two determinations. [b] THP: tetrahydropyran. [c] NT: not tested.

hydrogen-bond acceptor, rather than the 2,3-conjugated system with NH-1 as a hydrogen-bond donor. This conclusion was consistent with the X-ray cocrystal structure analysis of **8b** in the active site: the N-1 of imidazole acts as a hydrogen bond acceptor from the backbone N−H of residue His238 (Supporting Information).

To identify a better compound for in vivo evaluation, our medicinal chemistry effort was focused on evaluating the effect of substitutions at N-1 and N-3 of benzimidazole on TGF-β RI enzyme activity, inhibition of TGF-β-dependent luciderase activation (p3TP-Lux),[5] inhibition of TGF-β-stimulated proliferation in NIH3T3 cells,[6] selectivity vs TGF-β RII and MLK-7,[5] and inhibition of cytochrome P-450 2D6. As expected, N-1-substituted benzimidazoles (**11b**, **12b**, **13b**, **14b**) (Table 3) are much less active as inhibitors of TGF-β RI. In contrast, the N-3-substituted benzimidazoles (Table 2) with N-1 as a hydrogen-bond acceptor are potent inhibitors of TGF-β RI with IC_{50} of 22−398 nM. Importantly, the compounds with large substitutions at N-3 of the benzimidazole such as **17c**−**d** showed excellent TGF-β RI inhibitions, indicating that the N-3 substituent may orientate away from the binding pocket toward solvent. In general, the N-3 position of benzimidazole tolerates a large array of substitutions (Table 2).

The cellular inhibitory activity (NIH3T3 and p3TP-Lux) of these benzimidazole compounds is shown in Tables 1−3. Compounds **11a**, **12a**, **15a**, **15b**, and **17a**−**g** displayed IC_{50} values between 8 and 226 nM in mink lung cells (p3TP-Lux) and between 32 and 854 nM in NIH3T3 cells. There is a strong correlation ($R = 0.73$) between TGF-β RI activity and the p3TP-Lux reporter and NIH3T3 proliferation cell-based assays. Therefore, these compounds inhibit TGF-β signaling in two different cell lines and their activity correlates with TGF-β RI enzyme inhibition.

Besides the potent activity of the benzimidazoles against TGF-β RI in enzymatic and cell-based assays, they were also very selective versus MLK-7 kinase (>50-fold) and moderately selective versus TGF-β RII kinase (>10-fold) (Tables 1−3). The selectivity for inhibiting TGF-β RI versus TGF-β RII was improved to >50-fold with amine substitutions (**17a**−**g**). Because of the relatively weaker inhibition of **8a** (IC_{50} = 27 μM) in comparison with the 5−10 μM activity of N-3 substituted compounds in the cytochrome P-450 2D6 enzyme assay, **8b** was selected for in vivo study.

In Vivo Target Inhibition of TGF-β RI in the Tumor Xenograft Model

The TGF-β in vivo target inhibition study was designed using Calu6 human anaplastic carcinoma lung cells. Calu6 cells were implanted in the Charles River nude mouse at 10^6 cells subcutaneously. On day 11, **8b** was administrated by oral gavage. Tumors were harvested 2 h postdose (50 mg/kg) and processed by Western blot for phospho-Smad2 level. Briefly, the tumor was pulverized in liquid nitrogen and lysed with 600 μL of lysis buffer containing 50 mM Tris-HCl at 7.5, 500 mM NaCl, 1% NP-40, 0.25% Na-dexydolate, 20 mM NaF, protease inhibitor (Roach), and phosphatase inhibitor cocktails I and II (Sigma). Then 80 μg of protein was loaded onto 10% SDS Tris-glycine gel. Western blot was performed with a proprietary

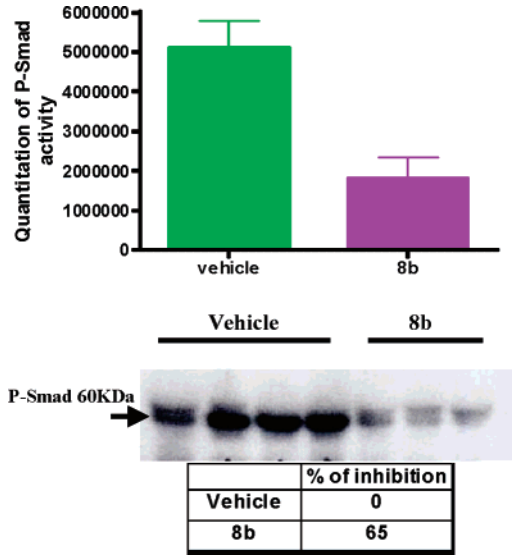


Figure 1. In vivo target inhibition Western Blot data: (vehicle) dosing with cmc/SLS/PVP/AF; (**8b**) oral dosing at 50 mg/kg. Data are the mean and standard errors (65% ± 13%) determined from three treated animals; $p = 0.012$.

phospho-Smad2 antibody. Compound **8b** showed significant TGF-β RI target inhibition (65% compared to a vehicle) with a p value of 0.012 (Figure 1).

In summary, we have disclosed a series of benzimidazole-substituted dihydropyrrolopyrazoles as a new class of TGF-β RI inhibitors. These compounds are selective versus TGF-β RII and MLK-7 kinases and capable of inhibiting TGF-β signaling in cells. Benzimidazole derivative **8b** was active in in vivo target (TGF-β RI) inhibition assays and may represent a new treatment for TGF-β-mediated diseases.

Experimental Section

Flash column chromatography was carried out using prepacked silica gel columns from Biotage or Isco. Ion exchange chromatography was carried out using SCX BondElut columns from Varian. ^{1}H NMR spectra were collected on Varian 400 MHz or Bruker 300 MHz instruments. The synthesis of key final compounds **8b**, **11a**, and **11b** are indicated below. For more information, see Supporting Information.

6-(2-Pyridin-2-yl-5,6-dihydro-4*H*-pyrrolo[1,2-*b*]pyrazol-3-yl)-1*H*-benzoimidazole (8b). A round-bottom flask was charged with **6b** (1.04 g, 2.83 mmol) in MeCN/H_2O (10:1) (12 mL), and tetrakis-(triphenylphosphine)palladium (165 mg, 0.14 mmol) and sodium bicarbonate (249 mg, 2.97 mmol) were subsequently added. Then **7b** (809 mg, 3.53 mmol) was added in three equal portions (270 mg each), and the mixture was heated at 50 °C for 1 h and at 65 °C for 4 h and then refluxed for 5 h. The precipitate was formed and isolated by filtration. The crude mixture contained about the same amounts of product and impurities and was chromatographed on a silica gel column, eluting with EtOAc, MeOH/EtOAc, and 2% NH_4OH to yield **8b**. Further purification of this compound was conducted on an alumina column, eluting with 2.5% MeOH/97.5% $CHCl_3$ to give pure **8b** (139.0 mg, 16%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.55 (d, $J = 4.8$ Hz, 1H), 7.99 (s, 1H), 7.77 (t, $J = 7.6$ Hz, 1H), 7.66 (d, $J = 8.0$ Hz, 1H), 7.39 (d, $J = 8.5$ Hz, 1H), 7.35−7.29 (m, 2H), 7.27 (m, 1H), 4.28 (t, $J = 7.3$ Hz, 2H), 3.01 (t, $J = 7.3$ Hz, 2H), 2.69 (m, 2H). TOF MS ES$^+$ (m/z) 302.3 $(M + 1)^+$. HPLC method A: >99%. HPLC method B: >99%.

1-Methyl-6-[2-(6-methylpyridin-2-yl)-5,6-dihydro-4*H*-pyrrolo-[1,2-*b*]pyrazol-3-yl]-1*H*-benzoimidazole (11a) and 1-Methyl-5-[2-(6-methylpyridin-2-yl)-5,6-dihydro-4*H*-pyrrolo[1,2-*b*]pyrazol-3-yl]-1*H*-benzoimidazole (11b). To a solution of 6-bromoimidazo-[1,2-*a*]pyridine (0.060 g, 0.44 mmol), 2-pyridin-2-yl-5,6-dihydro-4*H*-pyrrolo[1,2-*b*]pyrazole-3-boronic acid (0.1 g, 0.44 mmol), and sodium bicarbonate (0.037 g, 0.44 mmol) in dimethyl sulfoxide/water (1.0:1.0 mL/mL) in a microwave tube (10 mL) was added tetrakis(triphenylphosphine)palladium(0) (0.025 g, 0.022 mmol). After the mixture was stirred for 1 min, the tube was capped. The reaction was monitored by LC−MS after irradiation of the mixture in a microwave reactor set at 110 °C and 50 W for 10 min. Upon the completion of the reaction, methanol/water (1:1, 6 mL) was added. The resulting solution was passed over an SCX column (prewashed with methanol) and further washed with more methanol/water. The desired compounds eluted with the 2 M NH_3-in-methanol fraction. The two regioisomers were separated by ISCO and eluted gradually from CH_2Cl_2 to 20% THF/80% CH_2Cl_2 to give **11a** (23.6 mg, 42%) and **11b** (20.3 mg, 36%). For **11a**: TOF MS ES$^+$ (m/z) 330.2 $(M + 1)^+$. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.81 (s, 1H), 7.67 (d, $J = 8.4$ Hz, 1H), 7.36 (t, $J = 7.6$ Hz, 1H), 7.32 (bs, 1H), 7.17 (dd, $J = 8.4$, 1.6 Hz, 1H), 7.07 (d, $J = 7.6$ Hz, 1H), 6.99 (d, $J = 7.6$ Hz, 1H), 4.26 (t, $J = 7.2$ Hz, 2H), 3.73 (s, 3H), 3.01 (t, $J = 7.2$ Hz, 2H), 2.65 (m, 2H), 2.60 (s, 3H). ^{1}H NOE ($CDCl_3$, 400 MHz): δ 3.73 (*Me*-1) → 7.81 (*H*-2 on benzimidazole), 7.32 (*H*-4 on benzimidazole). HPLC method A: >99%. HPLC method B: >99%. For **11b**: TOF MS ES$^+$ (m/z) 330.2 $(M + 1)^+$. ^{1}H NMR ($CDCl_3$) δ 7.82 (s, 1H), 7.71 (s, 1H), 7.30 (t, $J = 8.0$ Hz, 1H), 7.26 (d, $J = 8.4$ Hz, 1H), 7.18 (dd, $J = 8.4$, 1.6 Hz, 1H), 7.03 (d, $J = 7.6$ Hz, 1H), 6.99 (d, $J = 7.6$ Hz, 1H), 4.26 (t, $J = 7.2$ Hz, 2H), 3.81 (s, 3H), 3.00 (t, $J = 7.2$ Hz, 2H), 2.64 (m, 2H), 2.57 (s, 3H). HPLC method A: >99%. HPLC method B: >99%. ^{1}H NOE ($CDCl_3$, 400 MHz): δ 3.73 (*Me*-1) → 7.82 (*H*-2 on benzimidazole), 7.26 (*H*-7 on benzimidazole).

Acknowledgment. Thanks are given to Dr. J. Scott Sawyer for critically reading the manuscript and to Dr. Philip W. Iversen for statistical analysis.

Supporting Information Available: Experimental procedures and characterization data for compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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JM058209G