Brief Articles

Retro Hydrazino-azapeptoids as Peptidomimetics of Proteasome Inhibitors

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Several groups of proteasome inhibitors are widely used to study the role of the ubiquin proteasome pathway in various cellular processes or as anticancer drugs. Peptidomimetics have been developed to circumvent problems inherent in peptides such as poor bioavailability and protease-mediated degradation, while retaining biological activity. In this study, we introduce new pseudopeptides, the retro hydrazino-azapeptoids, designed as proteasome inhibitor peptidomimetics. Their proteasome inhibitory activity and antiproliferative properties are reported here.

Introduction

The ubiquitin proteasome pathway constitutes the most important system for protein degradation in eukaryotic cells.[1] It prevents the accumulation of nonfunctional, potentially toxic proteins and allows elimination of normal proteins that are no longer required. This pathway depends on the central role of a complex macromolecular structure, the 26S proteasome, which is composed of one 20S and two 19S subunits. The 20S proteasome exhibits multiple proteolytic activities with strict specificities. Three major proteolytic activities can be distinguished: trypsin-like (T-L), chymotrypsin-like (CT-L), and peptidyl-glutamyl peptide hydrolase (PGPH) activities which cleave peptide bonds on the carboxyl side of basic, hydrophobic, and acidic amino acid residues, respectively.[2] These proteolytic activities rely on an unusual mechanism involving the N-terminal threonine residue of particular β-subunits as the catalytic nucleophile.[3] The proton donor/acceptor role in this catalytic process may be performed either by a conserved lysine residue or a water molecule bonded to the α-amino group of the N-terminal threonine.[4]

The exact substrate specificity of the different catalytic sites is still not well understood. Although the distinct activities are generally defined by the amino acid in the P1 position of a synthetic peptide substrate, the specificity determinants go well beyond the P1 position. In fact, the catalytic sites must overlap in specificity to some extent since there are more activities described than there are different catalytic β-subunits.[5] Therefore, characterization of the effects of novel proteasome inhibitors is useful in unraveling the specificity of the different sites. Selective inhibitors of the proteasome are also important tools to study proteasome function in cells. Because proteasome malfunctions are often associated with diseases, proteasome inhibitors constitute a novel class of therapeutic agents. These inhibitors have a potential for treating cancer and neurodegenerative diseases and can also be used to prevent cancer- or AIDS-associated muscle cachexia.[6]

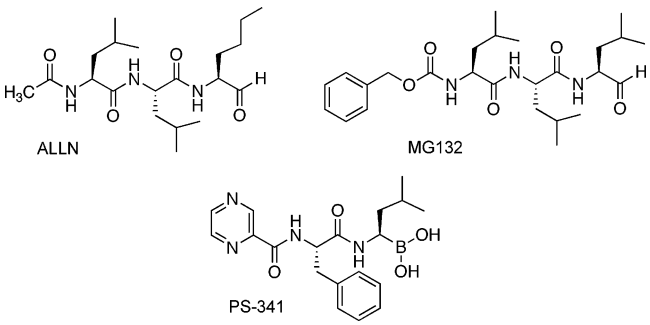

Figure 1. Synthetic peptide aldehyde and boronic acid proteasome inhibitors.

Peptide aldehyde inhibitors (e.g. ALLN and MG132, Figure 1) are still commonly used but they are relatively nonspecific.[7,8] Furthermore, due to the acidity of the α-proton, the substituent adjacent to the aldehyde is not configurationally stable. More recently, peptidyl boronic acids (e.g. PS-341, Figure 1) have been shown to be stable and highly potent inhibitors of the proteasome.[9,10]

Nevertheless, the activity of proteasome peptide-based inhibitors is often hampered by poor bioavailability and protease-mediated degradation. The desire to remedy these disadvantages, while retaining the biological activity of the peptide, has led to the development of peptidomimetics. A particular interesting class of peptidomimetics is formed by peptoids[11−14] and analogues such as azapeptides and azapeptoids,[15−21] urea-peptoids,[22,23] amino-oxypeptoids,[24] β-peptoids,[25] and

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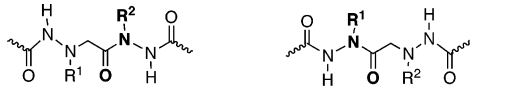
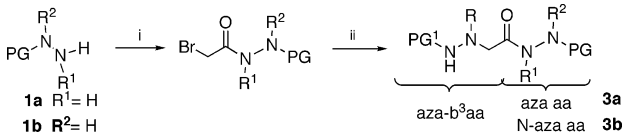


Figure 2. General structure of hydrazino azapeptoids.

Scheme 1[a]



[a] Reagents and conditions: (i) $BrCH_2COBr$, C_5H_5N/CH_2Cl_2, 0 °C; (ii) PG^1NHNHR (**1b**), $CHCl_3$, Δ, 24 h.

hydrazino-azapeptoids,[26] in which the side-chains are linked to nitrogen atoms.

If the carbonyl group contributes to the binding of the target molecule, the peptide bonds and the order of the amino acids have to be reversed ("retro sequence") in a peptoid peptidomimetic to maintain the relative orientation of the carbonyl groups to the R groups. This improved mimicry of the parent peptide may be responsible for the higher biological activity of retropeptoids.[27] The hydrazino-azapeptoids, which are formed from N^{α}-substituted acid hydrazino-acetic monomers or aza-β^3-amino acid units (aza-β^3) and aza-amino acid unit (aza) or N-substituted azaglycine (Naza) monomers are hybrid pseudopeptides with nitrogen-enriched peptidic backbones.[26] As shown in Figure 2, side chains are attached to the nitrogen atom of the analogue monomer instead of the α-carbon atom of the amino acid monomer in peptides. Spectroscopic studies and X-ray crystallography indicated that hydrazino-azapeptoids are capable of adopting eight-membered, hydrogen-bonded turns (N−N turns).[28] In previous work, we have shown that modified C-terminal hydrazino-azapeptoids are potent inhibitors of cell proliferation.[29] As part of an effort to explore new peptoid analogues with potentially useful biological properties, a series of retro hydrazino-azapeptoids, which contain NHNRCO bonds instead of CONRNH bonds, was synthesized and screened for proteasome inhibition and cell cytotoxicity. In short, these materials were designed to mimic the amino acid derived system in Figure 1 and were expected to have similar biological activity.

Results and Discussion

Synthesis. Initially, we examined the submonomer synthesis of hydrazino-azapeptoids backbones of **3** which have been already described (Scheme 1).[29] Finally, we adopted a monomer strategy, which consisted of coupling aza-β^3-amino acids **2** and aza or *N*-aza amino esters **1** (Scheme 2). Aza-β^3-amino acids **2** were prepared as previously described,[30] by nucleophilic substitution of *tert*-butyl or benzyl bromoacetates with N,N′-disubstituted hydrazines (or *N*-aza amino esters) **1b**, or by reductive amination of glyoxylic acid and *N*-aza amino acid **1b.** In addition, dipeptoidic analogues **3** were obtained by a coupling reaction. Tripeptoid analogues (e.g., **7)** and diaza-β^3 peptides (**13b**) were synthesized by the coupling of two readily accessible monomers.

Hybrid pseudopeptoids **3** and **7** and the oligomers **13** were deprotected under standard conditions. Halomethyl ketone was introduced in the free N-terminal position as an electrophilic group to give irreversible interaction with the nucleophilic threonine in position 1 of the proteasome. Analogues **5**, **9,** and **14** were obtained by acylation of the terminal hydrazine of **4** or **8**. Taking account of our previous work,[29] we introduce a bromo acetyl group in N-terminal position to give target inhibitors **5**, **9**, and **14.** Analogues such as **6** were obtained by condensation of substituted hydrazine **4** with 3-bromobenzaldehyde followed by in situ reduction of the hydrazone.

Dipeptidyl boronic acids are new potent proteasome inhibitors.[6,31] Boronic acids act as transition-state analogues for serine proteases because the boron can accept the oxygen lone pair of the active site serine residue. It seems likely that these compounds react similarly with the catalytic N-terminal threonine residue of the proteasome catalytic subunits.[32,33] For these reasons, we also synthesized analogues **10**, **11**, and **12** with a boronic acid in the N-terminal position by condensation of the substituted hydrazine **4** with *o*, *m*, or *p*-formylbenzeneboronic acid.

Biological Evaluation. The synthesized hydrazino-azapeptoids were screened for proteasome inhibition and for cytotoxicity in L1210, B16, and Mel-A cells (Table 1).

Proteasome Inhibition. Among all the hydrazino-azapeptoids studied, only the boronic acid derivatives **10a**, **11a**, **11b**, **11d**, and **11g**, displayed an inhibitory effect on the proteolytic activity of the 20S proteasome. With IC_{50}s ranging from 50 to 500 μM, their ability to inhibit the proteasome CT-L activity remains lower than that of ALLN (IC_{50} = 15 μM), which was used as a control. Like all the other compounds, these boronic acid

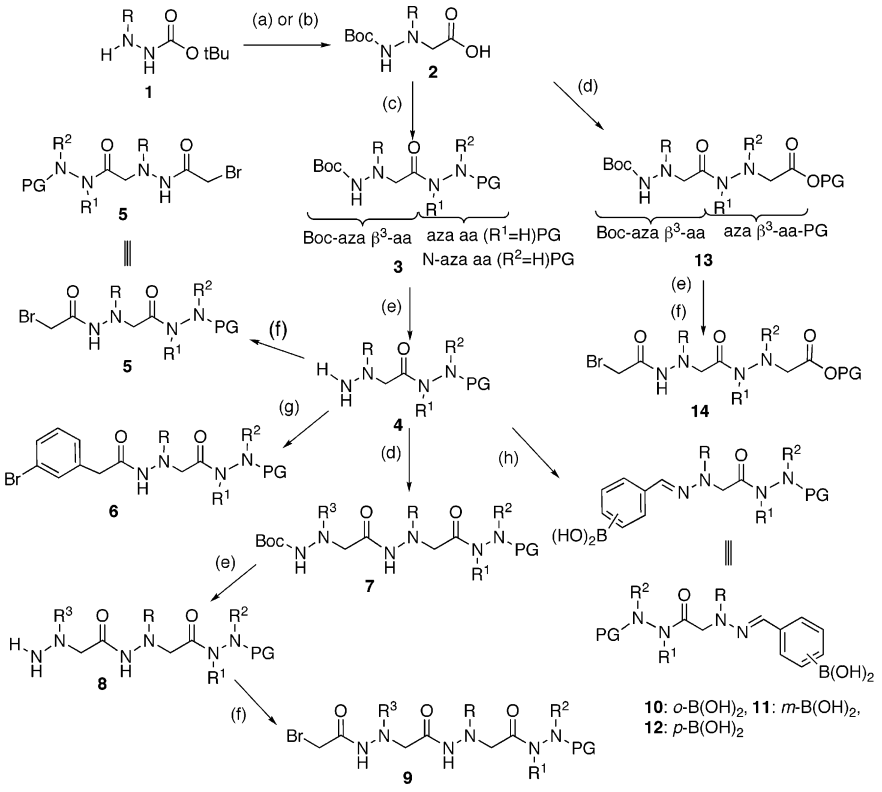
Table 1. Inhibitory Effect on 20S Proteasome CT-L Activity and on Cell Growth

	IC_{50} (μM)			
	inhibition of proteasome	cell growth inhibition		
compd	CT-L activity	L1210	B16	Mel-A
ALLN	15	3.0 ± 0.01	14.5 ± 2.6	ND
PS-341	K_i = 0.62 nM[9,10]			
5a	>1000	6.5 ± 2.2	1.3 ± 0.05	3.3 ± 0.4
5b	>1000	1.3 ± 0.8	1.4 ± 0.1	1.8 ± 0.7
5f	>1000	0.6 ± 0.2	0.7 ± 0.02	1.5 ± 0.2
5h	>1000	24.1 ± 2.9	11.4 ± 5.0	14.8 ± 1.3
6b	>1000	30.0 ± 2.6	>80	15.8 ± 1.9
6h	>1000	45.0 ± 5.5	23.4 ± 0.4	33.3 ± 0.6
9a	>1000	7.6 ± 0.6	2.5 ± 0.1	2.3 ± 1.6
10a	50	16.2 ± 2.3	26.3 ± 0.9	17.3 ± 0.4
10b	>1000	27.9 ± 3.1	65.5 ± 1.2	45.1 ± 1.1
10c	>1000	18.4 ± 1.8	33.5 ± 1.2	25.1 ± 1.9
11a	91	21.2±0.6	53.9 ± 1.6	nd
11b	53	16.2 ± 1.5	20.4 ± 0.8	18.4 ± 0.6
11c	>1000	16.9 ± 0.6	20.5 ± 0.8	19.9 ± 0.8
11d	350	41.6 ± 2.3	21.1 ± 0.9	40.3 ± 1.2
11e	>1000	75.0 ± 7.8	74.9 ± 1.5	60.9 ± 2.8
11g	500	>80	>80	16.3 ± 3.9
11h	>1000	>80	>80	33.7 ± 6.0
12b	>1000	14.7 ± 3.9	32.6 ± 0.6	39.3 ± 2.5
14	>1000	5.0 ± 0.4	2.2 ± 1.1	4.5 ± 0.4

Table 2. Inhibitory Effect of Selected Compounds on the Activity of Calpain I and Cathepsin B

	IC_{50} (μM)	
compd	calpain I	cathepsin B
ALLN	1	12
10a	>1000	>1000
11b	>1000	>1000

Scheme 2. Synthesis of Hydrazino Azapeptoids **11**, **12**, **14**[a]



a: R=R^1=$CH_2CH(CH_3)_2$, R^2= H , PG=Z
b: R= CH_2Ph, R^1=$CH_2CH(CH_3)_2$, R^2= H, PG=Z
c: R=$CH_2CH(CH_3)_2$, R^1= CH_2Ph,,R^2= =H, PG=Z
d: R=$CH_2CH(CH_3)_2$, R^1 =CH_2-(3-C_5H_4N), R^2=H, PG=Z
e: R =CH_2-(3-C_5H_4N), R^1=$CH_2CH(CH_3)_2$, R^2=H, PG=Z
f: R=CH_2Ph, R^1=H, R^2=$CH_2CH(CH_3)_2$, PG=Z
g: R=CH_2Ph, R^1=H, R^2=$CH_2CH(CH_3)_2$,PG=2, 5-Pyrazyle
h: R=$CH_2CH(CH_3)_2$, R^1=H, R^2= CH_2Ph, PG=2, 5-Pyrazyle

[a] Reagents and conditions: (a) $BrCH_2CO_2Bn$, K_2CO_3, toluene, Δ, 24 h; H_2 (1 atm), 5% Pd/C (b) $OHCCO_2H$, methanol, $NaBH_3CN$, HCl (2 N); (c) HNR^{1}NR^{2}PG, EDCI, CH_2Cl_2, rt, 48 h; (d) aza-β^3aa-OPG, EDCI, CH_2Cl_2, rt, 48 h; (e) CF_3CO_2H/CH_2Cl_2; (f) $BrCH_2COBr$, C_5H_5N/CH_2Cl_2, 0 °C; (g) $HCOC_6H_4Br$, methanol, $NaBH_3CN$, HCl (2 N), (h) $HCOC_6H_4B(OH)_2$, Et_2O, rt.

derivatives had no inhibitory effect (up to 1 mM) on either PGPH or T-L activity of the proteasome. Nevertheless, the active boronic acid derivatives were more specific than ALLN, which inhibited the PGPH activity (IC_{50} = 45 μM). As shown in Table 2, the compounds **10a** and **10b** were also more selective than ALLN and did not inhibit cathepsin B and calpain I, two cysteine proteases which were very sensitive to ALLN.

Cell Growth Inhibition/Cytotoxicity. Independent of their ability to inhibit proteasome, the effect of all synthesized compounds on cell growth was tested on murine leukemia L1210, melanoma B16, and normal melanocyte Mel-A cells using the MTT assay which reveals either a cytostatic or a cytotoxic effect of drugs on cultured cells. Most of the compounds showed similar IC_{50}s on the three cell lines. With the exception of **5h**, all bromoacetylated compounds (**5a**, **5b**, **5f**, **9a**, **14**) showed low IC_{50}s (from 0.6 to 7.6 μM) which could be related to the high reactivity of their functional group. In contrast, compounds with bromophenyl or a boronic acid group were less effective with IC_{50}s ranging from 15 to 75 μM. **10a** and **11b**, which are the most potent proteasome inhibitors, had IC_{50}s around 20 μM on the three cell lines while ALLN was more active with IC_{50}s of 3 μM on L1210 and 14.5 μM on B16 cells. None of the compounds displayed any marked selectivity for B16 versus their normal counterpart Mel-A. Surprisingly, the retro analogues **11g** and **11h**, as well as the bromophenyl derivative **6b**, were more active on normal melanocyte than on B16 melanoma cells.

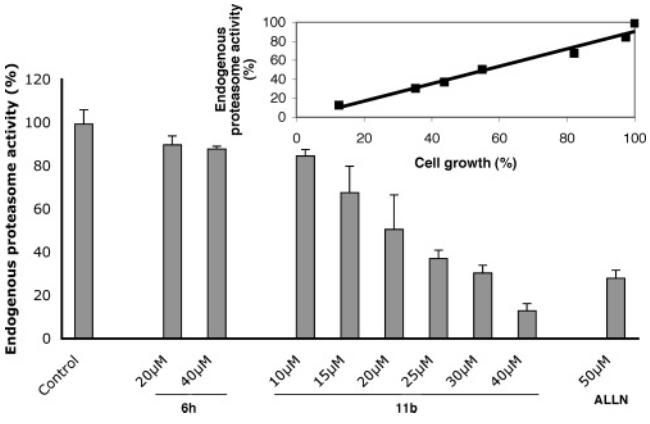


Figure 3. Endogenous proteasome CT-L activity in B16 cells cultured 24 h in the presence of **6h**, **11b**, and ALLN. Insert: Correlation between proteasome and cell growth inhibitions in B16 cells treated with various concentrations of **11b**.

Relation between Cell Growth and Proteasome Inhibition. We wondered if the proteasome is an in vivo target for the hydrazino-azapeptoids with in vitro proteasome inhibitory activity. The activity of endogenous proteasome was measured, using a fluorescent substrate, in lysates of B16 cells cultured in the presence of either **11b** (10 to 40 μM), **6h** (20 and 40 μM), or ALLN (50 μM). As shown in Figure 3, endogenous proteasome

activity was decreased in a dose-dependent manner in B16 cells treated with **11b**, a hydrazino-azapeptoid with in vitro proteasome inhibitory activity. In addition, a linear correlation ($r = 0.975$) was observed between cell growth and endogenous proteasome activity inhibitions (see insert of Figure 3).

As expected, ALLN-treated B16 cells also displayed a reduced proteasome activity. In contrast, **6h**, a compound with an antiproliferative activity similar to **11b** but devoid of any in vitro proteasome inhibitory activity, did not induce any decrease in endogenous proteasome activity even at a dose equivalent to 2 times its IC_{50}. These results suggest that the proteasome inhibitor **11b** may promote cell growth inhibition through inhibition of endogenous proteasome.

Conclusion

The present study is, to our knowledge, the first attempt to synthesize and evaluate the biological activity of peptidomimetics of known proteasome inhibitors. The more active compounds were boronic acid derivatives (e.g. **11b**) that displayed both in vitro and in vivo proteasome inhibitory activity. Despite a high selectivity and specificity, their proteasome inhibitory activity is very low compared to PS-341, the reference boronic acid inhibitor which is active in the nanomolar range. In the present hydrazino-azapeptoid series, the boronic acid is attached to a phenyl group. This structural feature likely causes a steric hindrance which could impair the binding of the compound into the active site and could be responsible for their low affinity for the proteasome. These observations will be taken into consideration in the design of more active peptidomimetics.

Experimental Section

General Procedure for Synthesis of Bromo Analogues 5 and 6. $BrCH_2CO$-aza-β^3-Leu-N-azaLeu-OBz (**5a**, R = $R^1 = CH_2CH(CH_3)_2$, R^2 = H, PG = Z). To a stirred and cool solution (0 °C) of **4a** (1.75 g, 5 mmol) in dichloromethane (10 mL) and pyridine (6 mmol) was added dropwise bromoacetyl bromide (1.2 g, 6 mmol) in 5 mL of dichloromethane. The mixture was stirred and cooled for 5 h. The reaction mixture was washed three times with water (50 mL). The organic layer was dried (Na_2SO_4), filtered, and evaporated in vacuo to give the crude product **5a** as an oil which was purified by chromatography: (1.18 g, 50%); ^{1}H NMR ($CDCl_3$) δ 0.80 (m, 2 × 6H, CH_3), 1.56 (m, 1H, CH), 1.81 (m, 1H, CH), 2.46 (d, $J = 5.5$ Hz, 2H, CH_2), 3.29−3.61 (br, 3 × 2H, CH_2), 5.09 (s, 2H, CH_2), 7.27 (m, 5H, C_6H_5), 8.19 (s br, 1H, NH), 8.42 (s br, 1H, NH); Anal. ($C_{20}H_{31}N_4O_4Br$) C, H, N, Br.

***m*-BrBn-aza-β^3-Phe-N-azaLeu-OBz** (**6b** R = $CH_2C_6H_5$, $R^1 = CH_2CH(CH_3)_2$, R^2 = H). To a stirred solution of **4b** (0.77 g, 2 mmol) in ethanol (5 mL) was added dropwise 3-bromobenzaldehyde (0.38 g, 2.1 mmol) in a solution of ethanol (2 mL). After 1 h of stirring, $NaBH_3CN$ (1.90 g, 30 mmol) was added in small portions at pH 4 (by addition of 2 N HCl), and the reaction mixture was stirred for 1 h. Then, 2 N HCl was added to the solution until pH 1, followed by the addition of $NaHCO_3$ until pH 4. Finally, the solution was extracted twice by CH_2Cl_2 (20 mL). The organic layers were combined and dried with anhydrous Na_2SO_4, filtered, and evaporated under reduced pressure to afford **6b** as an oil which crystallized slowly upon the addition of diethyl ether (4.48 g, 82%): mp 82 °C; ^{1}H NMR ($CDCl_3$) δ 0.94 (d, $J = 6.5$ Hz, 6H, CH_3), 1.90 (m, 1H, CH), 3.40 (d, $J = 6.7$ Hz, 2H, CH_2), 3.84 (s, 2H, CH_2), 4.00 (s, 2H, CH_2), 4.70 (s, 2H, CH_2), 5.13 (s, 2H, CH_2), 7.25−7.45 (m, 15H, C_6H_5 + NH), 7.56 (s, 1H, NH).

General Procedure for Synthesis of Boronic Acid Analogues 10 and 11. *o*-B(OH)$_2$PhCH=-aza-β^3-Leu-N-azaLeu-OBz (**10a**, R = $R^1 = CH_2CH(CH_3)_2$, R^2 = H). To a stirred solution of **4a** (1.8 g, 5 mmol) in diethyl ether (5 mL) was added dropwise 2-formylbenzenzeboronic acid (0.8 g, 5.5 mmol) in a solution of diethyl ether (5 mL). A white precipitate appeared. After stirring for 1 h, the precipitate was filtered and washed with diethyl ether to give **10a** (1.70 g, 82%): mp 142 °C.^{1}H NMR ($CDCl_3$) δ 0.91 (d, $J = 6.0$ Hz, 6H, CH_3), 1.12 (d, $J = 6.2$ Hz, 6H, CH_3), 1.86 (m, 1H, CH), 2.15 (m, 1H, CH), 3.31 (br, 2H, CH_2), 3.81 (br, 2H, CH_2), 4.50 (br, 2H, CH_2), 5.40 (s, 2H, CH_2), 7.01 (t, $J = 7.0$ Hz, 1H, CH), 7.52 (m, 5H, C_6H_5), 8.21 (d, $J = 7.5$ Hz, 1H, CH), 9.30 (s, 1H, NH), 11.26 (s, 2H, OH). Anal. ($C_{25}H_{35}N_4O_5B$) C, H, N.

***m*-B(OH)$_2$PhCH=-aza-β^3-Phe-N-azaLeu-OBz** (**11b** R = $CH_2C_6H_5$, $R^1 = CH_2CH(CH_3)_2$, R^2 = H). To a stirred solution of **4b** (1.92 g, 5 mmol) in diethyl ether (5 mL) was added dropwise 3-formylbenzenzeboronic acid (0.8 g, 5.5 mmol) in a solution of diethyl ether (5 mL). A white precipitate appeared. After stirring for 1 h, the precipitate was filtered and washed with diethyl ether to give **11b** (2.11 g, 82%): mp 157 °C; ^{1}H NMR (DMSO-d_6 δ 0.92 (m, 6H, CH_3), 1.92 (m, 1H, CH), 3.35 (br, 2H, CH_2), 4.23 (br, 2H, CH_2), 4.69 (s, 2H, CH_2), 5.23 (s, 2H, CH_2), 7.23 (s, 1H, CH), 7.40 (m, 12H, C_6H_5), 7.64 (t, $J = 6.0$ Hz, 1H, CH), 7.70 (d, $J = 7.1$ Hz, 1H, CH), 7.85 (s, 1H, CH), 8.13 (s, 2H, NH + OH), 10.1 (s, 1H, OH); Anal. ($C_{28}H_{33}N_4O_5B$) C, H, N, B.

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Supporting Information Available: Synthesis and analytical data for **1**−**14** and biological techniques. This material is available free of charge via the Internet at http://pubs.acs.org.

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