Brief Articles

Dicarboxylic Acid *bis*(L-Prolyl-pyrrolidine) Amides as Prolyl Oligopeptidase Inhibitors

Erik A. A. Wallén,*,[†] Johannes A. M. Christiaans,[†,‡] Markus M. Forsberg,[§] Jarkko I. Venäläinen,[§]
Pekka T. Männistö,[§,||] and Jukka Gynther[†,||]

*Department of Pharmaceutical Chemistry and Department of Pharmacology and Toxicology, University of Kuopio,
P.O. Box 1627, FIN-70211 Kuopio, Finland, and Finncovery, Ltd., Kuopio, Finland*

New dicarboxylic acid *bis*(L-prolyl-pyrrolidine) amides were synthesized, and their inhibitory activity against prolyl oligopeptidase from pig brain was tested in vitro. As compared with earlier described prolyl oligopeptidase inhibitors, these new compounds have in common an L-prolyl-pyrrolidine moiety, but the typical lipophilic acyl end group is replaced by another L-prolyl-pyrrolidine moiety connected symmetrically with a short dicarboxylic acid linker. These compounds are a new type of peptidomimetic prolyl oligopeptidase inhibitor.

Introduction

The serine protease prolyl oligopeptidase (POP, previously called prolyl endopeptidase or postproline cleaving enzyme, EC 3.4.21.26) is a large intracellular enzyme (80 kDa) that preferentially hydrolyses proline-containing oligopeptides at the carboxyl side of a prolyl residue. It is presumably involved in the maturation and degradation of peptide hormones and neuropeptides.[1]

A low level of substance P, which is a substrate of POP, is characteristic in the brains of Alzheimer's patients. Supportingly, administration of β-amyloids decreases substance P levels in the brain. Vice versa, administration of substance P is able to alleviate amyloid peptide-induced toxicity.[2]

There is no firm evidence of increased POP activity in Alzheimer's patients. In contrast, rather low enzyme activities are correlated with the severity of Alzheimer's disease, which is thought to reflect the degree of neuronal damage.[3] However, in aged rats, POP gene levels are manyfold increased in several brain areas.[4] Correspondingly, in mice subjected to an enriched environment, the POP gene levels are decreased.[5] As a whole, it seems that enhanced levels of neuroactive peptides such as substance P, angiotensins, vasopressin, oxytocin, and thyrotropine-releasing hormone, which are all substrates of POP, may be beneficial in old age and in patients with cognitive disturbances. Indeed, POP inhibitors have been able to reverse scopolamine-induced amnesia in rats.[6–8] Centrally acting POP inhibitors that increase neuropeptide concentrations[9] may therefore be worthy of testing in Alzheimer's disease.

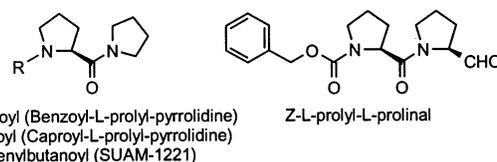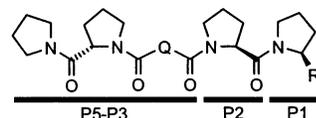* To whom correspondence should be addressed. E-mail: wallen@
uku.fi.
  † Department of Pharmaceutical Chemistry, University of Kuopio.
  ‡ Present address: Altana Pharma, P.O. Box 61, NL-1160 AB
Zwanenburg, The Netherlands.
  § Department of Pharmacology and Toxicology, University of Kuopio.
  || Finncovery, Ltd.

Chart 1

R = benzoyl (Benzoyl-L-prolyl-pyrrolidine)
R = caproyl (Caproyl-L-prolyl-pyrrolidine)
R = 4-phenylbutanoyl (SUAM-1221)

Z-L-prolyl-L-prolinal

Chart 2

1a Q = -(CH₂)₂-, R = H	**1f** Q = *m*-phenylene, R = H
1b Q = -(CH₂)₃-, R = H	**1g** Q = *o*-phenylene, R = H
1c Q = -(CH₂)₄-, R = H	**2a** Q = *m*-phenylene, R = CHO
1d Q = -CH₂C(CH₃)₂CH₂-, R = H	**2b** Q = *m*-phenylene, R = CN
1e Q = *p*-phenylene, R = H	**2c** Q = *m*-phenylene, R = COCH₂OH

Most described low molecular weight POP inhibitors have an acyl-L-prolyl-pyrrolidine backbone, wherein a lipophilic acyl end group has been reported to be important for high inhibitory activity.[10] Four reference compounds of this type with different lipophilic acyl end groups are benzoyl-L-prolyl-pyrrolidine,[11] caproyl-L-prolyl-pyrrolidine,[12] SUAM-1221,[11–13] and Z-L-prolyl-L-prolinal.[14] SUAM-1221 and Z-L-prolyl-prolinal are the two most potent ones, and the latter has the pyrrolidine group replaced by an L-prolinal group, which increases the activity further. The lipophilic acyl end group is the P3 site, since it binds to the S3 binding site of the active site of the enzyme (the P2 site is the L-prolyl residue in the center of the molecule, and the P1 site is the pyrrolidine and the L-prolinal end groups, respectively) (Chart 1).

In a new series of POP inhibitors **1a**−**g**, two L-prolyl-pyrrolidine groups were connected with a short dicarboxylic acid linker. These compounds do not have the typical lipophilic acyl end group found in the most
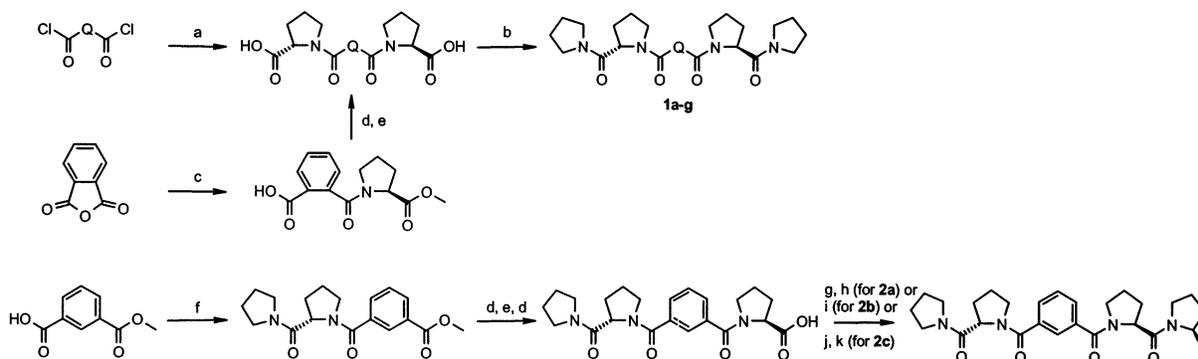
Scheme 1[a]

[Scheme: reaction sequence showing synthesis of compounds 1a–g and 2a–c]

[a] Reagents: (a) L-Proline, NaOH/H$_2$O, diethyl ether. (b) (1) Pivaloyl chloride, Et$_3$N/DCM; (2) pyrrolidine, Et$_3$N/DCM. (c) L-Proline methyl ester HCl salt, Et$_3$N/DCM. (d) (1) Pivaloyl chloride, Et$_3$N/DCM; (2) L-proline methyl ester HCl salt, Et$_3$N/DCM. (e) LiOH/MeOH, H$_2$O. (f) (1) Pivaloyl chloride, Et$_3$N/DCM; (2) L-Prolyl-pyrrolidine TFA salt, Et$_3$N/DCM. (g) (1) Pivaloyl chloride, Et$_3$N/DCM; (2) L-Prolinol, Et$_3$N/DCM. (h) SO$_3$ pyridine complex, Et$_3$N, DMSO. (i) (1) Pivaloyl chloride, Et$_3$N/DCM; (2) 2(*S*)-Cyanopyrrolidine TFA salt, Et$_3$N/DCM. (j) (1) Pivaloyl chloride, Et$_3$N/DCM; (2) 2(*S*)-(acetoxyacetyl)pyrrolidine TFA salt, Et$_3$N/DCM. (k) K$_2$CO$_3$/MeOH, H$_2$O.

potent POP inhibitors. The linking dicarboxylic acid in the center of the molecule might act as the lipophilic P3 moiety, although the lipophilic chain is not very long. The second L-prolyl-pyrrolidine group will reach the S4 and most probably also the S5 binding sites of the enzyme. As compared with an oligopeptide substrate, which is also bound to the same active site, the amide bond direction in the new compounds is reversed in the center of the molecule. The new compounds are thereby a new type of peptidomimetic POP inhibitors (Chart 2).

A series of substituted derivatives of compound **1f** were also synthesized, where the P1 pyrrolidine group was replaced by either L-prolinal, 2(*S*)-cyanopyrrolidine, or 2(*S*)-(hydroxyacetyl)pyrrolidine, resulting in compounds **2a**, **2b**, and **2c**, respectively. These P1 groups have earlier been reported to increase the inhibitory activity.[14−18]

Synthetic Chemistry

The used synthesis routes are outlined in Scheme 1. The synthesis of **1a** was started from succinyl dichloride, which was reacted with 2 equiv of L-proline. The resulting compound was activated with 2 equiv of pivaloyl chloride and then reacted with 2 equiv of pyrrolidine. The compounds **1b−f** were synthesized using the same synthesis route, starting from glutaryl dichloride, adipoyl dichloride, 3,3-dimethyl glutaryl dichloride, terephthaloyl dichloride, and isophthaloyl dichloride, respectively.

Compound **1g** was synthesized starting from phthalic anhydride, which was reacted with L-proline methyl ester. The resulting compound was activated with pivaloyl chloride and then reacted with L-proline methyl ester. The methyl ester groups were hydrolyzed with LiOH in water−methanol. The resulting compound was activated with 2 equiv of pivaloyl chloride and then reacted with 2 equiv of pyrrolidine.

The synthesis of compounds **2a−c** was started from isophthalic acid monomethyl ester, which was activated with pivaloyl chloride and then reacted with L-prolyl-pyrrolidine. The methyl ester group was hydrolyzed with LiOH in water−methanol. The resulting compound was activated with pivaloyl chloride and then reacted with L-proline methyl ester. The methyl ester group was hydrolyzed with LiOH in water−methanol. The result-

Table 1. Inhibitory Activity (IC$_{50}$) of the Compounds **1** (R = H) against POP from Pig Brain (95% Confidence Intervals Given in Parentheses)

compd	Q	IC$_{50}$ (nM)
1a	-(CH$_2$)$_2$-	77 (59−100)
1b	-(CH$_2$)$_3$-	48 (38−62)
1c	-(CH$_2$)$_4$-	68 (44−110)
1d	-CH$_2$C(CH$_3$)$_2$CH$_2$-	13 (12−14)
1e	*para*-phenylene	81 (72−92)
1f	*meta*-phenylene	26 (21−32)
1g	*ortho*-phenylene	21 000 (7800−54 000)

ing compound was activated with pivaloyl chloride and then reacted with L-prolinol, 2(*S*)-cyanopyrrolidine, or 2(*S*)-(acetoxyacetyl)pyrrolidine, respectively. Compound **2a** was obtained after the product from the reaction with L-prolinol was oxidized with SO$_3$ pyridine complex in dimethyl sulfoxide. Compound **2b** was obtained directly from the reaction with 2(*S*)-cyanopyrrolidine. Compound **2c** was obtained after the product from the reaction with 2(*S*)-(acetoxyacetyl)pyrrolidine was hydrolyzed with K$_2$CO$_3$ in water−methanol.

In Vitro Assay of POP Activity

The inhibitory activity of the compounds against POP from pig brain was determined by a method based on that of Toide et al.[19] Suc-Gly-Pro-7-amido-4-methylcoumarin was used as substrate, and the formation of 7-amido-4-methylcoumarin was determined fluorometrically with a microplate fluorescence reader.

Results and Discussion

In the series of compounds **1,** the linking group Q was varied (Table 1). A flexible alkylene linker gives very potent compounds **1a−c**, the glutaric acid derivative **1b** being the most active one. The optimum chain length seems to be three carbon atoms. A branched alkylene with the optimum chain length results in an even more potent compound, as is indicated by the 3,3-dimethyl glutaric acid derivative **1d**. Compounds with rigid *para*-, *meta*-, and *ortho*-phenylene linkers have a wider range of inhibitory activities. The terephthalic acid derivative **1e** and the isophthalic acid derivative **1f** are very potent compounds, the latter being the more active one. On the other hand, the phthalic acid derivative **1g** is almost inactive. These rigid compounds seem to require a

Table 2. Inhibitory Activity (IC_{50}) of the Compounds **2** (Q = *m*-Phenylene) against POP from Pig Brain (95% Confidence Intervals Given in Parentheses)

compd	R	IC_{50} (nM)
2a	CHO	1.3 (1.1−1.6)
2b	CN	1.5 (1.1−2.2)
2c	$COCH_2OH$	0.39 (1.1−2.2)

Table 3. Inhibitory Activity (IC_{50}) of Reference Compounds against POP from Pig Brain (95% Confidence Intervals Given in Parentheses)

ref compd	IC_{50} (nM)
benzoyl-L-prolyl-pyrrolidine	66 (38−110)
SUAM-1221	2.2 (1.9−2.5)
Z-L-prolyl-L-prolinal	0.33 (0.24−0.47)

correct geometry for high activity. In the series of compounds **1**, the 3,3-dimethylglutaric acid derivative **1d** and the isophthalic acid derivative **1f** are the most potent inhibitors, with IC_{50} values of 13 and 26 nM, respectively.

The second series of compounds **2** are derivatives of compound **1f**, wherein one of the pyrrolidine ends is replaced by either L-prolinal, 2(*S*)-cyanopyrrolidine, or 2(*S*)-(hydroxyacetyl)pyrrolidine, resulting in compounds **2a**, **2b**, and **2c**, respectively (Table 2). All three compounds **2a**−**c** are very potent inhibitors with IC_{50} values ranging from 0.39 to 1.5 nM.

The increased potency of the compounds of series **2** results from the interaction of the formyl, cyano, and hydroxyacetyl groups, respectively, with the active serine residue (Ser554) of the POP enzyme. The formyl group is known to form a hemiacetal adduct with the active serine residue.[20] The hemiacetal adduct mimics the tetrahedral transition state of the enzyme-catalyzed reaction. Therefore, it can be classified as a transition state analogue inhibitor. It is very likely that the cyano and hydroxyacetyl groups interact via a similar mechanism with the active serine residue, forming an imino ether and a hemiketal adduct, respectively. The compounds of series **1** mimic the substrate, and they do not form a covalent adduct with the enzyme, which makes these compounds substrate analogue inhibitors.

The impact of the second L-prolyl-pyrrolidine moiety is best seen by comparing the compounds **1e**−**g** (Table 1) with benzoyl-L-prolyl-pyrrolidine, which has an IC_{50} value of 66 nM in our in vitro assay (Table 3). The only difference between the new compounds and this reference compound is the second L-prolyl-pyrrolidine moiety connected from a second carboxylic acid group. The meta-substituted compound **1f** has an IC_{50} value of 26 nM, and it is thereby more active than the reference compound. On the other hand, the para-substituted compound **1e** is slightly less active and the ortho-substituted compound **1g** is almost inactive as compared to the reference compound. The two very potent reference compounds SUAM-1221 and Z-L-prolyl-L-prolinal have also been tested in our in vitro assay, and their IC_{50} values are 2.2 and 0.33 nM, respectively (Table 3). These reference compounds and our best new compounds are equipotent.

Conclusions

New potent POP inhibitors with a symmetrical peptidomimetic structure were synthesized. These com-

pounds are structurally made of two L-prolyl-pyrrolidine moieties connected symmetrically with a short dicarboxylic acid linker. The dicarboxylic acid linker in the center of the molecule has a strong effect on the inhibitory activity. The two best dicarboxylic acids are 3,3-dimethylglutamic acid and isophthalic acid. Replacing one of the pyrrolidine ends of the symmetrical molecule with L-prolinal, 2(*S*)-cyanopyrrolidine, or 2(*S*)-(hydroxyacetyl)pyrrolidine increases the inhibitory activity strongly. The most potent new inhibitor has an IC_{50} value of 0.39 nM.

Experimental Section

Coupling a Dicarboxylic Acid Dichloride with L-Proline. A solution of 1.0 mmol dicarboxylic acid dichloride in a suitable organic solvent was added to a solution of 2.0−2.2 mmol L-proline and 4.0−6.0 mmol NaOH in water at 0 °C. The reaction was stirred vigorously for several hours at room temperature. The organic phase was separated, and the aqueous phase was made acidic. The aqueous phase was evaporated, and the residue was dissolved in dichloromethane or chloroform, or alternatively, the product was extracted from the aqueous phase with ethanol−chloroform. The organic phase was dried and evaporated.

Amide Formation Using Pivaloyl Chloride Activation. A 1.0 mmol amount of pivaloyl chloride was added to a solution of 1.0 mmol of carboxylic acid (or 0.5 mmol of dicarboxylic acid) and 1.1 mmol of triethylamine in dichloromethane at 0 °C. The reaction was stirred at 0 °C for 1 h. A solution of 1.1 mmol of triethylamine and 1.0−1.1 mmol of amine in dichloromethane was added slowly at 0 °C, or if the amine was in the form of a trifluoroacetic acid salt or HCl salt, then 2.2−3.3 mmol of triethylamine was used and the triethylamine was added separately before the addition of the amine. The reaction mixture was stirred for several hours at room temperature. The dichloromethane solution was washed with 30% citric acid, saturated NaCl, and saturated $NaHCO_3$. The dichloromethane phase was dried and evaporated.

Coupling of Phthalic Anhydride with L-Proline Methyl Ester. A 1.5 g (10 mmol) amount of phthalic anhydride was added to a solution of 1.5 g (10 mmol) of L-proline methyl ester HCl salt and 3.1 mL (22 mmol) of triethylamine in dichloromethane at 0 °C. The reaction was stirred for 2 h at room temperature. The organic phase was extracted with saturated $NaHCO_3$. The aqueous phase was made acidic, and the aqueous phase was extracted with chloroform. The chloroform phase was dried and evaporated.

Hydrolysis of a Methyl Ester. A 1.1−1.5 mmol amount of LiOH was added to a solution of 1.0 mmol of the methyl ester (or 3.0 mmol of LiOH in the case of a dicarboxylic acid dimethyl ester) in water−methanol. The reaction was stirred overnight or longer at room temperature. The methanol was evaporated, and the residue was dissolved in water. The aqueous phase was washed with dichloromethane. The aqueous phase was made acidic, and the product was extracted with dichloromethane. The dichloromethane phase was dried and evaporated.

Oxidation with SO_3 Pyridine Complex in Dimethyl Sulfoxide. A 370 mg (0.75 mmol) amount of isophthalic acid L-prolyl-pyrrolidine L-prolyl-L-prolinol amide and 0.31 mL (2.3 mmol) of triethylamine were dissolved in 5 mL of anhydrous dimethyl sulfoxide. A solution of 360 mg (2.3 mmol) of SO_3 pyridine complex dissolved in 5 mL of anhydrous dimethyl sulfoxide was added at room temperature, and the reaction was stirred for 2 h. The reaction mixture was poured in 50 mL of ice−water. The product was extracted with chloroform. The chloroform phase was washed with 30% citric acid, saturated NaCl, and saturated $NaHCO_3$. The chloroform phase was dried and evaporated.

Hydrolysis of an Acetyl Ester. A 270 mg (1.94 mmol) amount of K_2CO_3 was added to a solution of 1.0 g (1.76 mmol) of isophthalic acid L-prolyl-2(*S*)-(acetoxyacetyl)pyrrolidine L-

prolyl-pyrrolidine amide in 10 mL of 50% methanol in water at 0 °C. The reaction was stirred for 1 h at room temperature. The methanol was evaporated, and the residue was dissolved in dichloromethane and saturated NaCl. The phases were separated, and the dichloromethane phase was washed with saturated NaCl. The dichloromethane phase was dried and evaporated.

Succinic Acid *bis*(L-Prolyl-pyrrolidine) Amide (1a). MS (ESI) $m/z = 419$ (MH$^+$). ^{13}C NMR (CDCl$_3$): δ 24.11, 24.67, 26.21, 28.80, 28.87, 45.90, 46.22, 47.15, 57.83, 170.69 (same shift for both carbonyls). Anal. ($C_{22}H_{34}N_4O_4 \cdot 1.4H_2O$) C, H, N.

Glutaric Acid *bis*(L-Prolyl-pyrrolidine) Amide (1b). MS (ESI) $m/z = 433$ (MH$^+$). ^{13}C NMR (CDCl$_3$): δ 19.80, 24.08, 24.81, 26.16, 28.86, 33.28, 45.86, 46.24, 47.32, 57.69, 170.65, 171.27. Anal. ($C_{23}H_{36}N_4O_4 \cdot 0.7H_2O$) C, H, N.

Adipic Acid *bis*(L-Prolyl-pyrrolidine) Amide (1c). MS (ESI) $m/z = 447$ (MH$^+$). ^{13}C NMR (CDCl$_3$): δ 24.14, 24.29, 24.84, 26.21, 28.87, 34.26, 45.87, 46.28, 47.28, 57.63, 170.75, 171.38. Anal. ($C_{24}H_{38}N_4O_4 \cdot 1.0H_2O$) C, H, N.

3,3-Dimethyl Glutaric Acid *bis*(L-Prolyl-pyrrolidine) Amide (1d). MS (ESI) $m/z = 461$ (MH$^+$). ^{13}C NMR (CDCl$_3$): δ 24.13, 24.94, 26.22, 28.39, 28.86, 34.30, 44.32, 45.91, 46.20, 48.12, 57.72, 170.74, 170.78. Anal. ($C_{25}H_{40}N_4O_4 \cdot 1.0H_2O$) C, H, N.

Terephthalic Acid *bis*(L-Prolyl-pyrrolidine) Amide (1e). MS (ESI) $m/z = 467$ (MH$^+$). ^{13}C NMR (CDCl$_3$): δ 24.19, 25.57, 26.24, 28.98, 46.03, 46.41, 50.24, 58.15, 127.29, 138.00, 168.71, 170.35. Anal. ($C_{26}H_{34}N_4O_4 \cdot 0.4H_2O$) C, H, N.

Isophthalic Acid *bis*(L-Prolyl-pyrrolidine) Amide (1f). MS (ESI) $m/z = 467$ (MH$^+$). ^{13}C NMR (CDCl$_3$): δ 24.18, 25.57, 26.25, 28.99, 46.04, 46.42, 50.30, 58.23, 126.06, 128.24, 129.12, 136.60, 168.61, 170.34. Anal. ($C_{26}H_{34}N_4O_4 \cdot 0.2H_2O$) C, H, N.

Phthalic Acid *bis*(L-Prolyl-pyrrolidine) Amide (1g). MS (ESI) $m/z = 467$ (MH$^+$). ^{13}C NMR (CDCl$_3$): δ 23.30, 23.97, 24.13, 25.19, 25.89, 26.23, 29.09, 30.54, 45.64, 45.79, 45.99, 46.21, 46.86, 49.93, 57.51, 59.19, 126.89, 127.37, 128.26, 128.87, 134.78, 135.60, 168.75, 168.78, 170.45, 170.80. Anal. ($C_{26}H_{34}N_4O_4 \cdot 0.7H_2O$) C, H, N.

Isophthalic Acid L-Prolyl-pyrrolidine L-Prolyl-L-prolinal Amide (2a). MS (ESI) $m/z = 495$ (MH)$^+$. ^{13}C NMR (CDCl$_3$) δ 24.19, 25.04, 25.52, 25.55, 25.55, 26.24, 28.98, 29.26, 46.04, 46.40, 47.08, 50.27, 50.29, 58.05, 58.24, 64.89, 126.04, 128.30, 128.99, 129.19, 136.34, 136.69, 168.53, 168.73, 170.29, 171.38, 198.92. Anal. ($C_{27}H_{34}N_4O_5 \cdot 2.2H_2O$) C, H, N.

Isophthalic Acid L-Prolyl-2(S)-cyanopyrrolidine L-Prolyl-pyrrolidine Amide (2b). MS (ESI) $m/z = 492$ (MH)$^+$. ^{13}C NMR (CDCl$_3$) δ 23.01, 24.20, 25.43, 25.56, 26.25, 28.98, 29.80, 30.84, 46.05, 46.42, 46.50, 46.63, 50.27, 50.30, 58.03, 58.25, 118.63, 126.09, 128.36, 128.93, 129.29, 136.12, 136.77, 168.50, 168.83, 170.31, 171.13. Anal. ($C_{27}H_{33}N_5O_4 \cdot 1.0H_2O$) C, H, N.

Isophthalic Acid L-Prolyl-2(S)-(hydroxyacetyl)pyrrolidine L-Prolyl-pyrrolidine Amide (2c). MS (ESI) $m/z = 525$ (MH)$^+$. ^{13}C NMR (CDCl$_3$) δ 24.20, 25.39, 25.51, 25.55, 26.25, 28.25, 28.77, 28.99, 46.04, 46.41, 47.14, 47.20, 50.28, 58.04, 58.21, 61.16, 67.15, 126.08, 128.29, 128.96, 129.18, 136.32, 136.73, 168.52, 168.73, 170.30, 170.91, 209.03. Anal. ($C_{28}H_{36}N_4O_6 \cdot 0.4H_2O$) C, H, N.

The yields for all synthesis routes mentioned above varied between 7 and 55%.

Supporting Information Available: Complete experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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