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Large Cyclic Peptides as Cores of Multivalent Ligands: Application to Inhibitors of Receptor Binding by Cholera Toxin

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Received June 17, 2004

Abstract: Large cyclic decapeptides (up to 50-atom ring) were synthesized efficiently on the solid phase with allyl-ester protection of the carboxyl terminus during elongation. Pentavalent ligands, in a "core-linker-finger" modular setup, were assembled by using these cyclic peptide cores to demonstrate large affinity gains for inhibition of surface receptor binding by the cholera toxin B pentamer. The results suggest that the peptide cores retain expanded conformation in solution so that shorter flexible linkers are needed for larger peptide cores to achieve the best inhibitory results.

Multivalent design is an attractive strategy for obtaining high-affinity protein ligands.[1−7] In recent years, significant progress has been made in using the information of the three-dimensional structure of the target protein to guide multivalent ligand design.[8−18] In such structure-based design approaches, it appears that optimal affinity is obtained when the size and geometry of the resulting ligands match to a large extent the binding site distribution on the target protein. Therefore, manipulation of the core of the multivalent ligand can have significant impact on ligand affinity. So far, in most cases of structure-based design of multivalent ligands, relatively small cores are used with long linkers, with the exception of a cyclodextrin-bridged bivalent inhibitor of tryptase[13] and three large peptide cores reported by Ohta et al.[18] Here, we report our investigation on whether large-ring, head-to-tail cyclic peptides can adopt an expanded conformation and be effectively used as the core of multivalent ligands to achieve significant affinity gains.

Head-to-tail cyclic peptides may be excellent cores for the assembly of multivalent ligands, due to the ease of incorporating amino acids with various side chains for attachment of linkers and monovalent ligands. Manipulation of the length and nature of the amino acid sequence of the cyclic peptide provides opportunities to design multivalent ligands that are suitable for different geometric requirements. However, the success of such a cyclic peptide approach will require that large-ring cyclic peptides can retain a predictable, expanded ring conformation in solution to support multivalent ligands. Although cyclic octapeptides (24-atom ring) were found to retain an expanded conformation in the solid state[19] and larger ones (30- to 63-atom ring) were used as scaffolds for multivalent ligands,[18,20] there is no guarantee for larger cyclic peptides that the expanded conformation will be predominant in solution. In fact, Ohta and co-workers found that the α-amino acid sequence of a large-ring cyclic peptide has a marked influence on the peptide conformation in solution.[18] To gain access to large-ring cyclic peptides with a predictable and an expanded conformation, we designed a series of cyclic decapeptides (with 30-, 40-, and 50-atom rings) as the core for pentavalent ligands inhibiting surface receptor binding by cholera toxin (CT). The multivalent inhibitors of this series were compared with inhibitors obtained in our previous work that used the smaller pentacyclen core **1**.[10,21] As shown in Figure 1, three cyclic decapeptides with alternating L-lysine and either glycine (core **2**), γ-aminobutyric acid (Abu) (core **3**), or ϵ-aminohexanoic acid (Ahx) (core **4**) were considered. The key in our design is that we use flexible amino acids without side chains (glycine, Abu, and Ahx) both for achieving the desired ring size variations and for increased likelihood that the peptides might adopt expanded conformations in solution.

Before the synthesis, we performed computer simulations to investigate the conformational freedom of the

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10.1021/jo0489770 CCC: $27.50
Published on Web 09/25/2004

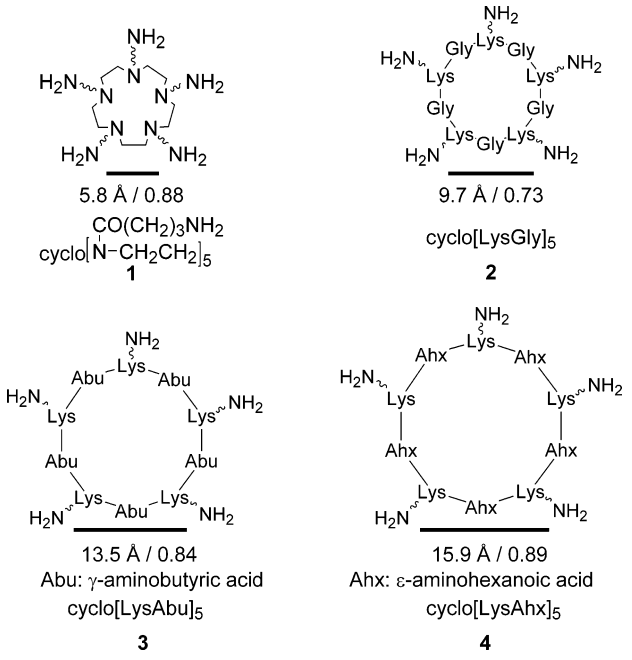

FIGURE 1. Size comparison of the original pentacyclen-based core **1** with cyclic decapeptides cores **2**−**4**. The distances indicated by the black bars are the calculated (see main text), averaged distances between nonadjacent nitrogen atoms of pentacyclen, or in the case of peptides, between the α-carbon atoms of nonadjacent lysines. The unit-less number after the distance is an indicator for the symmetry of each core (see main text), for which a value "1" corresponds to an isotropic ring conformation.

designed peptides. Using the software package QXP,[22] 2000 rounds of torsional Metropolis Monte Carlo at 1000 K were performed for each cyclic peptide core and the pentacyclen core. Then the 300 conformations with the lowest energy for each core were analyzed. In the simulation, the cyclic peptides retained mostly an expanded ring conformation without major twisting or collapsing of the ring. The averaged ring size for each peptide core, defined as the average (Boltzmann-weighted mean) of all pairs of distances between the α-carbon atoms of nonadjacent lysines in the 300 lowest energy conformations, ranged from 10 to 16 Å, as summarized in Figure 1. These sizes are significantly larger than the pentacyclen-based core **1**, where the averaged distance between nonadjacent nitrogen atoms was about 6 Å. To assess how well each core conforms to an expanded ring conformation, we calculated the ratio of the minimum and the maximum distances between the α-carbon atoms of nonadjacent lysines (or between nonadjacent N atoms in the pentacyclen core) in each conformation, and then averaged the ratio with Boltzmann-weighting for all 300 lowest energy conformations for each core. A value of 1 for this ratio indicates that the core is isotropic in the expanded ring conformation. As shown in Figure 1, the peptide-based cores do not deviate very far from an isotropically expanded ring, as the ratio varied from 0.73 (core **2**) to 0.89 (core **4**). The smaller pentacyclen core had a ratio of 0.88. Not surprisingly, core **2**, which incorporates 5 glycines, is the least isotropic on average.

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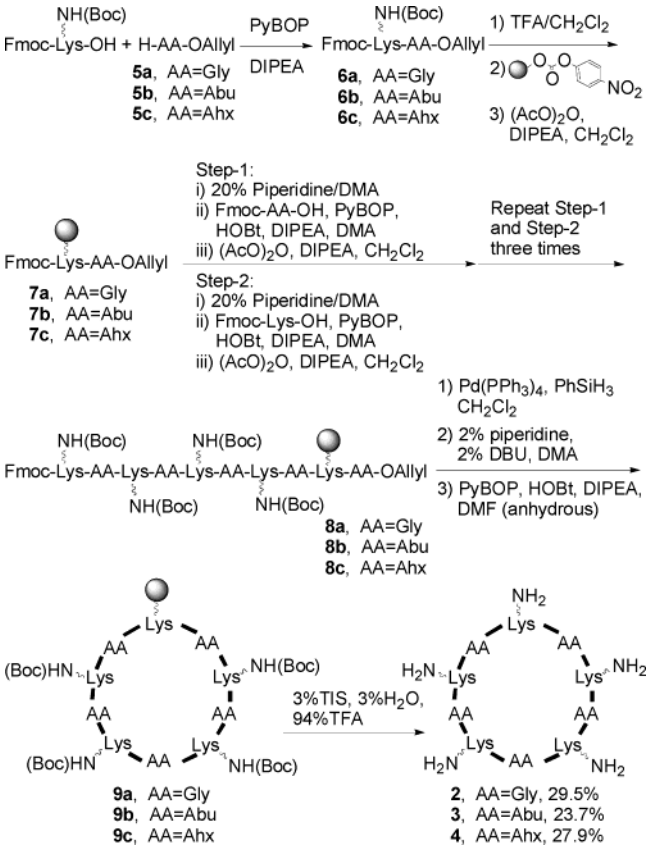
SCHEME 1. Synthesis of Head-to-Tail Cyclic Decapeptides



Computer simulations also revealed that, on average, the lysine side chains on the peptide cores have about an equal chance to be on either side of the peptide ring, and that those side chains mostly point outward. Because we are using long and flexible linkers,[10,15,23] whether the lysine side chains are on the same side of the peptide core or not should not have a major impact on the ligand's affinity. The flexible linkers are expected to easily compensate for this conformational change in the peptide cores.

Synthesis of the cyclic peptide cores is shown in Scheme 1. Following a strategy developed for synthesizing head-to-tail cyclic peptides using Fmoc chemistry,[24] we used the allyl group as the orthogonal protection for the C-terminus. First, allyl-protected amino acids[25] **5** were acylated with Fmoc-Lys to obtain the corresponding dipeptides **6**. After removal of the Boc protection of the lysine side chain, the dipeptides were attached to Wang resin with nitrophenyl carbonate activated resin. The resin-supported dipeptides **7** were then subjected to standard peptide synthesis for linear peptide elongation, using the appropriate N-α-Fmoc protected glycine, Abu, or Ahx, and lysine. Once the resin-supported linear decapeptides **8** were made, the allyl protection was removed by using the condition developed by Teixido et al.,[26] followed by Fmoc removal. On bead cyclization was achieved under standard peptide coupling condition with

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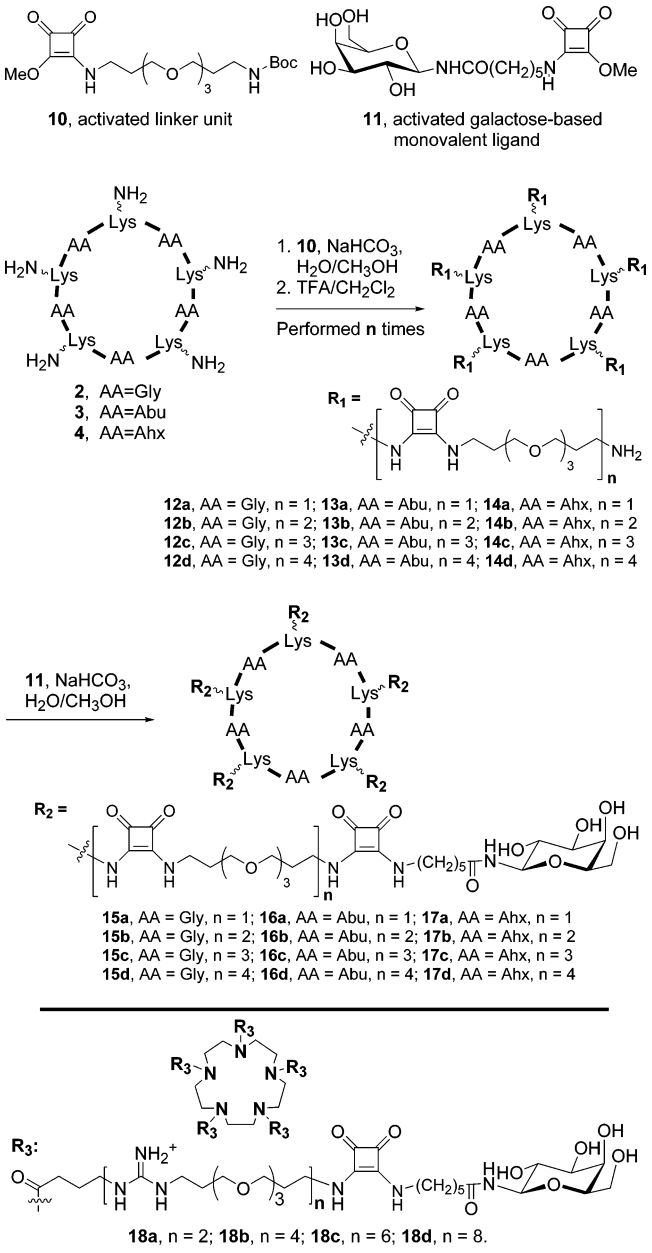
SCHEME 2. Synthesis of Pentavalent Ligands on Cyclic Peptide Cores



PyBop. Final cleavage and HPLC purification gave the desired cyclic decapeptides **2**−**4** in satisfactory yields (24−30%). Although there have been reports of lower yields of formed cyclic peptide for large ring systems with use of the allyl protection strategy,[27,28] this does not appear to be a problem in our hands because the yields of **2**−**4** compared favorably with the yields of cyclic peptides (48- to 87-atom rings) prepared with alternative strategies.[27,28]

As shown in Scheme 2, assembly of the full pentavalent ligands followed our well-developed modular "core-linker-finger" strategy.[10,23] Successive coupling of squarate activated linker unit **10**[10,23] to the peptide cores produced a series of core-linker assemblies **12**−**14** with various linker lengths. Final assembly of the full ligands by coupling of the squarate activated monovalent ligand ("finger") **11**[10,23] gave a set of 12 pentavalent ligands **15**−**17**, with linkers ranging from one to four units of **10**. Excellent isolated yields were obtained after HPLC purification for all intermediates (**12**−**14**) and the final ligands (**15**−**17**), as the yields typically were >70% (see the Experimental Section and the Supporting Information) for each squarate mediated coupling step.

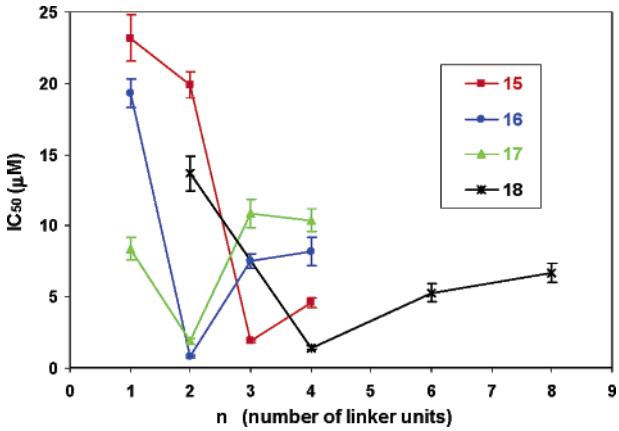


FIGURE 2. Plot of IC_{50} of pentavalent ligands versus the number of linker units in the ligand. The error bars represent the standard deviation of two independent measurements of IC_{50} values, which generally agree within 20%.

These peptide-core-based pentavalent ligands were assayed for their ability to block CT B pentamer binding to ganglioside coated plates.[21,29] The IC_{50} values for ligands **15**−**17** were plotted against the number of linker units in the ligand, as shown in Figure 2. For comparison, IC_{50} data of a series of analogous pentavalent ligands **18**[21] based on a pentacyclen core (Scheme 2) are also included.

It is very exciting to observe that first, compared to pentacyclen-based ligand series **18**, shorter linkers are needed to achieve optimal inhibition in cyclic peptide-based pentavalent ligands. For pentacyclen-supported ligands **18** with a core of about 6 Å, we previously reported that the optimal inhibition is achieved with four linker units.[21] With core **2**-based ligands **15**, for which the core size is about 4 Å larger than the pentacyclen core, only three linker units are needed for optimal inhibition. Another increase in core size by 4 Å, in the case of ligand series **16** compared to **15**, again shows one linker less is needed for optimal inhibition of receptor binding by the CT B pentamer. For the biggest core **4**-based ligands **17** where the core size is 2.5 Å larger than ligand series **16**, it appears that the optimal linkage is between 1 and 2 linker units. Second, in all series of pentavalent compounds, ligands with longer or shorter linkers than optimal all exhibit a loss in inhibitory power. This is consistent with previous observations[8,21] that when a ligand's effective dimension is not matching that of its target, there is a reduction in the ligand's affinity. These experimental data firmly support the results from

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computational simulations that the large head-to-tail cyclic peptides **2**−**4** were able to retain mostly enlarged ring conformations in solution. Again as we have observed before,[10,21] the low to submicromolar IC_{50} values for the best ligands **15c**, **16b**, and **17b** represent a more than 100 000-fold improvement over monovalent galactose, which has an IC_{50} of ∼100 mM in the same surface receptor-binding inhibitory assay.[21,29]

In summary, large head-to-tail cyclic decapeptides (with up to 50-atom ring) were efficiently synthesized on solid support with use of allyl ester for orthogonal protection of the C-terminus. Pentavalent ligands assembled on these cyclic peptide cores were able to exhibit large affinity gains over the monovalent ligand. For effective inhibition, shorter linkers are optimal for larger cyclic peptide-based cores, indicating the nature of an expanded ring conformation of the head-to-tail cyclic peptides. These results validate that large-ring cyclic peptides can be an easily accessible and effective class of backbone support for multivalent ligand design. It is especially significant for blocking cell-surface receptor binding by cholera and related toxins because there is no restriction on the size of the designed multivalent ligands due to the fact that no biological membrane is to be crossed by these ligands.

Experimental Section

Typical Procedure for the Synthesis of Dipeptides 6. Allyl protected amino acids **5** were synthesized according to ref 25 by refluxing allyl alcohol and the related amino acid in the presence of TsOH in benzene with Dean−Stark apparatus. Fmoc-Lys(Boc)-OH (1 equiv), compound **5** (1.3 equiv), PyBOP (1.1 equiv), HOBt (1 equiv), and DIPEA (5 equiv) in anhydrous CH_2Cl_2 were stirred at room temperature overnight. After sequential washing with 5% $NaHCO_3$, water, 10% citric acid, and saturated NaCl and drying over Na_2SO_4, the solvent was removed. The residue was purified via silica gel chromatography with 5% methanol/CH_2Cl_2 to give **6**.

For example, **5a** (3.45 g, 12 mmol) and Fmoc-Lys(Boc)-OH (4.32 g, 9.23 mmol) produced **6a** (4.8 g, 92.0%): ^{1}H NMR ($CDCl_3$) δ 7.75−7.78 (d, J = 7.2 Hz, 2H), 7.58−7.61 (d, J = 7.5 Hz, 2H), 7.38−7.43 (t, J = 7.3 Hz, 2H), 7.29−7.34 (t, J = 7.3 Hz, 2H), 5.83−5.93 (m, 1H), 5.28−5.35 (m, 2H), 4.62−4.67 (m, 4H), 4.45 (b, 1H), 4.19−4.24 (t, J = 6.8 Hz, 1H), 4.06 (s, 2H), 3.66−3.77 (m, 1H), 3.09−3.22 (m, 2H), 1.41−1.50 (m, 15H); ESI-MS m/z 588.4 $(M + Na)^+$, 1153.4 $(2M + Na)^+$.

Solid-Phase Protocol for Head-to-Tail Cyclic Peptide 2. The Boc group of the lysine side chain in compound **6a** (8.2 mmol) was removed in 15 mL of 1:1 TFA:CH_2Cl_2 for 20 min. After the solvent was completely removed in high vacuum, the residue was dissolved in 5 mL of anhydrous CH_2Cl_2, followed by slow addition of DIPEA until the solution became basic. This solution was immediately added into 500 mg of pre-swollen 4-nitrophenyl carbonate Wang resin (loading 0.71 mmol/g) in 5 mL of anhydrous CH_2Cl_2 with 1.3 mL of DIPEA. After the mixture was shaken for 2.5 h, the resin was filtered and washed with CH_2Cl_2 (5 × 5 mL), DMA (5 × 10 mL), and CH_2Cl_2 (5 × 5 mL). The resin was capped with acetic anhydride (0.4 mL, 4.2 mmol) with DIPEA (1.3 mL, 8.43 mmol) in anhydrous CH_2Cl_2 for 40 min to obtain the resin-bound **7a**. Linear peptide elongation was done following standard protocols. Fmoc removal was achieved by treatment with 20% piperidine in DMA (3 × 10 mL) for 10 min each. Coupling was done with 5 equiv of amino acids (Fmoc-Gly-OH or Fmoc-Lys(Boc)-OH), PyBOP (5 equiv), HOBt (5 equiv), and DIPEA (10 equiv) in DMA for 1 h at room temperature. The resin was filtered and washed successively with DMA (5 × 5 mL) and CH_2Cl_2 (5 × 5 mL). The resin was capped with acetic anhydride (0.4 mL, 4.2 mmol) and DIPEA (1.3 mL, 8.43 mmol) in anhydrous CH_2Cl_2 for 40 min after each elongation step. After the resin-supported linear decapeptide **8a** was obtained, the allyl group was removed by $Pd(PPh_3)_4$ (0.1 equiv) and $PhSiH_3$ (10 equiv) in anhydrous CH_2Cl_2 (8 mL) with nitrogen bubbling for 15 min.[26] This treatment was repeated three more times. The last Fmoc group was removed by 2% DBU/2% piperidine/DMA (3 × 10 min). After the resin was washed with DMA (6 × 10 mL), cyclization on the solid phase was performed by shaking the resin-bound **8a**, PyBOP (5 equiv), HOBt (5 equiv), and DIPEA (10 equiv) in anhydrous DMF overnight. This process was repeated once to obtain the resin-bound **9a**. Cleavage of the cyclic decapeptide from the resin was performed with TFA/H_2O/TIS (94:3:3, v/v/v) for 3 h. The filtrate was collected and the solvent was removed. Cold ether was added to precipitate the peptide and the ether was decanted. The solid residue was dissolved in water/methanol mixture and purified by reversed-phase HPLC with 0.1% TFA/water and acetonitrile as solvents. Solutions containing the desired products were combined, and solvents were removed under reduced pressure and lyophilization to obtain cyclic peptide **2** as TFA salt.

Cyclic peptide **2** (0.157 g, 29.5%): ^{1}H NMR (D_2O) δ 4.28−4.35 (t, J = 7.0 Hz, 1 × 5H), 3.92−4.00 (d, 2 × 5H), 2.93−3.01 (t, J = 7.5 Hz, 2 × 5 H), 1.57−1.82 (m, 4 × 5 H), 1.31−1.43 (m, 2 × 5 H); ESI-MS 926.6 $[M + H]^+$, 464.0 $[M + 2H]^{2+}$, 309.6 $[M + 3H]^{3+}$.

Pentavalent Ligand Assembly.[10,23] A cyclic peptide (**2**, **3**, or **4**) (1 equiv) was mixed with *N*-Boc-*N*′-(2-methoxy-3,4-dioxo-1-cyclobuten-1-yl)-4,7,10-trioxa-1,13-tridecane-diamine (**10**)[10,23] (6 equiv for each coupling site) in water/methanol (25/75, v/v) at pH 9 adjusted by $NaHCO_3$ solution. After 2 days of reaction at room temperature, the solution was acidified with dilute aqueous TFA solution and purified by reversed-phase HPLC with 0.1% TFA/water and acetonitrile as solvents (except Boc-protected **12c**, which was purified by size exclusion chromatography (Sephadex LH-20) in DMF, and purity checked by LC-MS to >80% pure). After the solvent was removed, the residue was treated with 10 mL of TFA/CH_2Cl_2 (50/50, v/v) for 40 min to remove five Boc groups. The solvent was removed completely in high vacuum to obtain **12a**, **13a**, or **14a** as the TFA salt. Repeating the procedure produced longer linker attached core-linker assembly.

For example, core **2** (32 mg, 21.4 μmol) gave compound **12a** (50 mg, 78%): ESI-MS m/z 1209.3 $[M + 2H]^{2+}$, 806.8 $[M + 3H]^{3+}$, 605.6 $[M + 4H]^{4+}$, 485.0 $[M + 5H]^{5+}$.

A core-linker assembly (**12**−**14**) was mixed with *N*-(*N*′-(2-methoxy-3,4-dioxo-1-cyclobuten-1-yl)-ϵ-aminocaproyl)-β-D-galactopyranosylamine (**11**)[10] (4 equiv per site) in water/methanol (25/75, v/v) at pH 9 adjusted by $NaHCO_3$ solution. After overnight reaction at room temperature, the product was purified by reversed-phase HPLC, using 0.1%TFA and acetonitrile as solvents. Final ligands **15**−**17** were obtained after lyophilization.

For example, **12a** (20 mg, 6.7 μmol) gave ligand **15a** (26 mg, 91%): ESI-MS m/z 2133.6 $[M + 2H]^{2+}$, 1423.5 $[M + 3H]^{3+}$, 1068.1 $[M + 4H]^{4+}$.

Acknowledgment. This work is supported by the National Institutes of Health (AI44954 and AI34501).

Supporting Information Available: Characterization of new compounds not listed in the main text. This material is available free of charge via the Internet at http://pubs.acs.org.

JO0489770