Thrombus Imaging Using Technetium-99m-Labeled High-Potency GPIIb/IIIa Receptor Antagonists. Chemistry and Initial Biological Studies

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Platelet-specific compounds which are radiolabeled with γ-emitting radionuclides may be particularly useful for the noninvasive *in vivo* detection of thrombi. The synthesis of peptides which are potent inhibitors of platelet aggregation and which contain a chelator for the radionuclide technetium-99m are described. The target compounds were designed such that stable, oxotechnetium(V) species could be prepared where the site of metal coordination was well defined. A strategy was employed where the pharmacophore -Arg-Gly-Asp-(RGD), or RGD mimetic, was constrained in a ring which was formed by the S-alkylation of a cysteine residue with an N-terminal chloroacetyl group. Binding affinities were enhanced by the replacement of arginine with the arginine mimetics *S*-(3-aminopropyl)cysteine and 4-amidinophenylalanine. Further enhancements could be obtained by the synthesis of oligomers which contained two or more rings containing receptor binding regions. The increase in binding affinity seen was more than that expected from a simple stoichiometric increase of pharmacophore. The most potent compounds described had IC_{50}s of approximately 0.03 μM for the inhibition of human platelet aggregation. Two of the more potent peptides (P280 and P748) were labeled with technetium-99m and assessed in a canine thrombosis model. The ^{99m}Tc complexes of the peptides prepared in this work hold promise as thrombus imaging agents due to their high receptor binding affinity, ease of preparation, and expected rapid pharmacokinetics.

Introduction

Deep vein thrombosis (DVT) and pulmonary embolism (PE) are thromboembolic disorders which constitute a major health risk. In the United States, approximately 100 000 deaths per year result from the estimated 5 million patients who experience one or more episodes of DVT and the 500 000 estimated cases of PE.[1] It is generally agreed that untreated PE is associated with high mortality (18−35%) which can be reduced substantially (to 2−8%) with prompt treatment.[2] But, prompt treatment requires prompt diagnosis. Unfortunately, the current state-of-the-art medicine does not provide rapid, noninvasive, cost-effective, definitive diagnosis of all cases of PE. Pulmonary angiography is the most sensitive and specific method for the diagnosis of PE.[2a] However, it is a technically demanding procedure which, despite relatively low morbidity and mortality (1.96 and 0.2%[3]), is often avoided. The most common diagnostic procedure for PE is the V/Q scan, which is a combination of a scintigraphic lung perfusion scan and a scintigraphic lung ventilation scan in which a mismatch in image defects is considered to be diagnostic of PE.[4] A recent study[5] showed that V/Q scans had high sensitivity but low specificity, and an editorial which followed this report stated that "One of the most difficult diagnoses to make in medicine today is that of pulmonary embolic disease".[6]

It has been estimated that approximately 70% of cases of PE arise from DVT of the lower extremities.[7] Therefore, the accurate detection of DVT is important both to allow prompt treatment to prevent formation of an embolus and as an adjunct to the diagnosis of PE. For the diagnosis of DVT of the lower extremities above the knee, Duplex ultrasonography is considered highly accurate[8] and has become widely used over impedance plethysmography.[9] For diagnosis of DVT below the knee, Duplex US is also used. However, approximately 40% of studies are technically inadequate, requiring either a confirming contrast venogram or a repeat study after 2−3 days.[5] Although the contrast venogram is considered the most accurate method for detecting DVT, this uncomfortable procedure has significant associated complications and is decreasingly used.

Anticoagulant therapy is available for the treatment of thromboembolic disorders, but poses additional risks such as hemorrhaging and thrombocytopenia. It has been estimated that heparin is the most common cause of drug-related death in reasonably healthy patients[10] and that heparin-associated morbidity and mortality is quite high (30 and 2%, respectively[11]). Therefore, it is undesirable to unnecessarily anticoagulate.

Two major conclusions can be drawn from this review of the literature: (i) that a rapid, noninvasive, cost-effective, and accurate method for the diagnosis of PE is highly desirable and (ii) that a rapid, noninvasive, cost-effective, and accurate method for the diagnosis of DVT in any part of the lower extremities is also highly desirable, both to indicate treatment and to aid in the diagnosis of PE.

Radionuclide imaging offers considerable hope for a successful diagnostic agent which would address all of the above criteria. ^{99m}Tc is the radionuclide of choice due to the fact that it has a convenient 6 h physical half-life, is relatively inexpensive, and is available 24 h a day as a solution of [^{99m}Tc]pertechnetate from an in-house ^{99}Mo/^{99m}Tc generator. In addition, ^{99m}Tc yields greater photon flux per unit of radiation dose delivered

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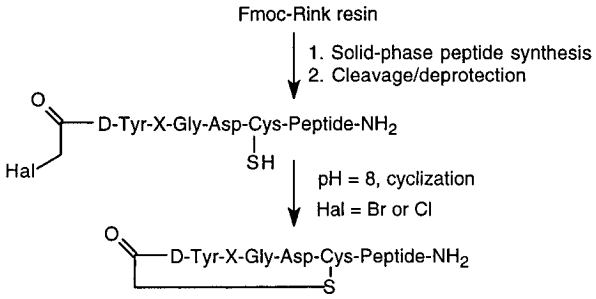
to the patient than other radionuclides used clinically. Early work in thrombus imaging focused on the radiolabeling of fibrinogen, fibrin, or platelets.[12] However, the success of these approaches was thwarted by the slow blood clearance of the large macromolecules or platelets, resulting in poor target to background ratios over the course of several hours after injection. Frequently, useful images could not be obtained until 24 h post injection. Monoclonal antibodies have been raised against fibrin and the Fab′ fragments labeled with ^{99m}Tc.[13] Recently, encouraging results have been obtained from a monoclonal antibody reactive with the DD domain of cross-linked fibrin.[14] However, even Fab′ fragments have been shown to leave the blood pool too slowly to provide a reliably rapid diagnosis.[15] In addition, it is apparent that the development of radiopharmaceuticals from monoclonal antibodies faces a number of technical and regulatory barriers which make it unlikely that this approach will easily lead to a commercial product.

An alternate approach which has been investigated recently involves the radiolabeling of smaller bioactive peptides. Since the discovery of the platelet glycoprotein IIb/IIIa complex (GPIIb/IIIa) as the final common pathway for all agonists in platelet adhesion,[16] there has been much effort toward the development of small molecule GPIIb/IIIa antagonists for the treatment of thromboembolic disorders. Most of the structure−activity work has revolved around the arginine−glycine−aspartic acid (RGD) sequence, or mimetics of this tripeptide sequence.[17] New approaches aimed at identifying a commercially viable radiotracer to image both PE and DVT have also involved radiolabeling of peptides containing the RGD sequence. Promising results were seen when bitistatin, an RGD-containing 9000 Da polypeptide derived from a snake venom, was labeled with the γ-emitting radionuclide ^{123}I and tested in a dog model of PE/DVT.[18] However, although the IC_{50} of bitistatin for inhibition of dog platelet aggregation is 0.028 μM, the IC_{50} for inhibition of human platelet aggregation is 0.24 μM,[19] indicating 10 times less potency in binding to human platelets. Furthermore, the biological origin of this compound, and the fact that it contains 14 pairs of cysteine residues and so may be difficult to label with ^{99m}Tc without loss of receptor affinity, combine to make it unclear whether this approach will lead to a commercial product. Also, some smaller peptides (17−32 residues) have been synthesized which contained one or more RGD sequences in addition to containing a chelator for ^{99m}Tc.[20] While blood clearance of the ^{99m}Tc-labeled peptides was rapid, with excretion through the kidneys, thrombus imaging was poor. We believe this may have been a consequence of their low affinity for the GPIIb/IIIa receptor ($IC_{50} \geq$ 10 μM for the inhibition of human platelet aggregation).

We have directed our research to the preparation of high-potency GPIIb/IIIa receptor-binding peptides labeled with ^{99m}Tc for evaluation as thrombus-imaging agents. In addition to its role in thrombus formation, this receptor is an attractive target for imaging blood clots due to the fact that its behavior on the activated platelets found in thrombi is different than that on quiescent platelets circulating in the blood stream.[21] This allows for the design of peptides in which their affinity for this receptor on activated human platelets is high enough that sufficient thrombus uptake for imaging purposes could be achieved, potentially without interference from the bloodstream background. We also designed these peptides to incorporate chelators for ^{99m}Tc at discrete locations such that the coordination of this radionuclide would not interfere with receptor binding. For this purpose, we have developed peptide sequences which are effective technetium chelators.[22] The details of the synthesis of these GPIIb/IIIa receptor-binding peptides which contain sites for the chelation of ^{99m}Tc and their activity *in vitro* and *in vivo* are described herein.

Scheme 1. Preparation of GPIIb/IIIa-Binding Analogs Containing ^{99m}Tc Chelator



1 X = Arg, Peptide = Gly-Gly-Gly-Cys_{Acm}-Gly-Cys_{Acm}-NH_2
2 X = Arg, Peptide = Gly-Gly-Cys_{Acm}-Gly-Cys_{Acm}-NH_2
3 X = Apc, Peptide = Gly-Gly-Gly-Cys_{Acm}-Gly-Cys_{Acm}-NH_2
4 X = Apc, Peptide = Lys-Gly-Cys_{Acm}-Gly-Cys_{Acm}-NH_2
5 X = Apc, Peptide = Apc-Gly-Asp-Phe-Lys-Cys_{Acm}-Gly-Cys_{Acm}-NH_2
6 X = Apc, Peptide = Lys-Cys-Gly-NH_2
7 X = Amp, Peptide = Lys-Gly-Cys-Gly-NH_2
8 X = Apc, Peptide = $(Gly)_2$Lys-$(Gly)_2$Lys-(ε-Lys)-Gly-Cys-Gly-NH_2
9 X = Apc, Peptide = Lys-$(Gly-Gly)_2$Lys-(ε-Lys)-Gly-Cys-(β-Ala)-NH_2

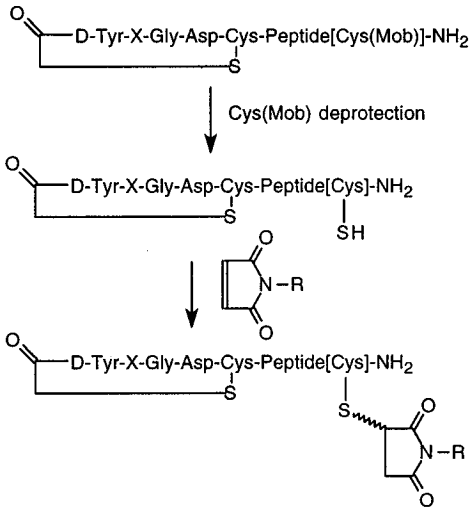
Apc = S-(3-aminopropyl)cysteine

Amp = p-amidinophenylalanine

Chemistry

All compounds were prepared by the routes outlined in Schemes 1−3. For peptide synthesis, standard solid-phase methodology[23] was used in which the *N*-α-amino groups were Fmoc-protected and the side chains (except cysteines not involved in cyclization) were protected with TFA-labile groups. It should be noted that in several cases a branched lysine approach was taken in which both the α- and ϵ-amines were Fmoc-protected. Synchronous removal of the Fmoc groups followed by continuation of the synthesis resulted in identical sequences attached to lysine at both the α- and ϵ-amines. At the end of the synthesis, the N-terminal D-tyrosine was capped with either a bromo- or chloroacetyl moiety. After cleavage and deprotection, the portion of the peptide containing the XGD sequence was cyclized by subjecting the peptide to basic conditions, thereby affecting the intramolecular S-alkylation between the free cysteine and the haloacetyl.[24] Cysteines not involved in this cyclization were S-protected with either the acetamidomethyl (Acm) group or the *p*-methoxybenzyl (Mob) group. Acm protection was used on those cysteines which could later comprise part of the ^{99m}Tc-chelator donor set in Tc-labeling experiments. In those cases where a cysteine was to be used to further

Scheme 2. Preparation of Multimers via S-Alkylation of Maleimides



10 X = Apc, Peptide = Gly-Gly-Cys_{Acm}-Gly-Cys_{Acm}-Gly-Gly-[Cys]-NH_2, R = $-(CH_2)_2O$ (**P280**)

11 X = Apc, Peptide = Gly-Gly-Cys_{Acm}-Gly-Cys_{Acm}-Gly-Gly-[Cys]-NH_2, R = $-CH_2CH_2)_3N$

12 X = Apc, Peptide = Lys-Gly-Cys_{Acm}-Gly-Cys_{Acm}-Gly-Gly-[Cys]-NH_2, R = $-CH_2)_2O$

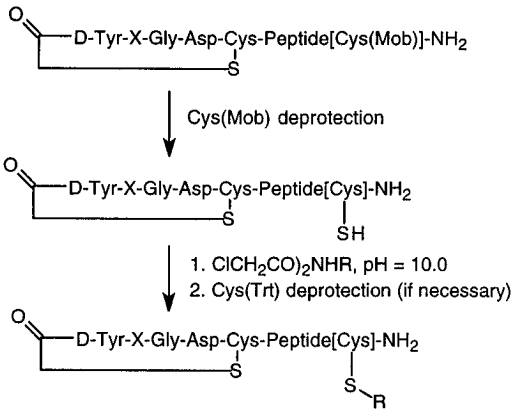
13 X = Apc, Peptide = Gly-Gly-Cys_{Acm}-Gly-Cys_{Acm}-Gly-Gly-[Cys]-NH_2, R = $-CH_2CH_2CH_2)_2$

Apc = S-(3-aminopropyl)cysteine

elaborate the peptide via an S-alkylation, the 4-methoxybenzyl (Mob) protecting group was used. In this orthogonal protection scheme, the peptide was first cyclized as described above, followed by removal of the Mob group with boron trifluoride. The resulting free cysteine was reacted with either maleimide-containing compounds (Scheme 2) or compounds containing chloroacetyl moieties (Scheme 3). In those cases where the chloroacetyl groups resided at the end of a peptide sequence (precursors to compounds **15** and **17**−**20**), the same solid-phase protocol described above was used to prepare these intermediates. In addition to the chloroacetyl groups, these peptides contained an *S*-trityl-protected cysteine which remained protected through the subsequent S-alkylation reaction. Removal of the trityl group with TFA at the end of the synthesis provided the final products. In addition to X = arginine in the XGD sequence, we also used the arginine mimetics *S*-(3-aminopropyl)cysteine (Apc) and *p*-amidinophenylalanine (Amp) at the X position. The syntheses of these intermediates, suitably protected for peptide synthesis, are shown in Schemes 4 and 5.

Oxorhenium(+5) complexes were prepared by reacting peptides **15** and **20** with $Bu_4NReOBr_4$[25] in DMF. The resulting compounds were purified by reversed-phase chromatography, characterized by electrospray mass spectroscopy, and found to be different from the uncomplexed peptides by coinjection on analytical HPLC. The technetium-99m complexes of compounds **10** (P280) and **20** (P748) were prepared by reacting these peptides with ^{99m}Tc-labeled glucoheptonate in a buffered solution. The radiochemical purity of these complexes was measured by analytical HPLC.

Scheme 3. Preparation of Multimers via S-Alkylation of Haloacetyls



14 X = Apc, Peptide = Gly-Gly-Cys_{Acm}-Gly-Cys_{Acm}-Gly-Gly-[Cys]-NH_2, R = $-CH_2CONHCH_2CH_2OCH_2)_2$

15 X = Apc, Peptide = Gly-Gly-Cys_{Acm}-Gly-Cys_{Acm}-Gly-Gly-[Cys]-NH_2, R = $-CH_2CO)_2$Lys-(ε-Lys)-Gly-Cys-NH_2

16 X = Apc, Peptide = Gly-Gly-Cys_{Acm}-Gly-Cys_{Acm}-Gly-Gly-[Cys]-NH_2, R = $-CH_2CONHCH_2CH_2)_3N$

17 X = Apc, Peptide = Gly-Gly-[Cys]-Gly-NH_2, R = $-CH_2CO)_2$Lys-(ε-Lys)-Gly-Cys-NH_2

18 X = Apc, Peptide = Gly-Gly-Cys_{Acm}-Gly-Cys_{Acm}-Gly-Gly-[Cys]-NH_2, R = $-CH_2CO)_2$Lys)$_2$Lys-(ε-Lys)-Gly-Cys-NH_2

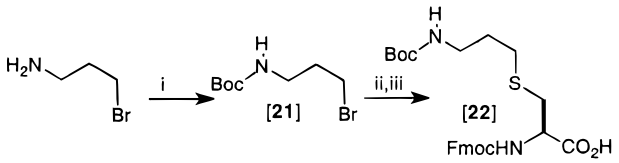
19 X = Amp, Peptide = Gly-Gly-Cys_{Acm}-Gly-Cys_{Acm}-Gly-Gly-[Cys]-NH_2, R = $-CH_2CO)_2$Lys-(ε-Lys)-Gly-Cys-NH_2

20 X = Amp, Peptide = Lys-Gly-[Cys]-Gly-NH_2, R = $-CH_2CO)_2$Lys-(ε-Lys)-Gly-Cys-NH_2 (**P748**)

Apc = S-(3-aminopropyl)cysteine

Amp = p-amidinophenylalanine

Scheme 4.[a] Preparation of Fmoc-Apc(Boc)-OH



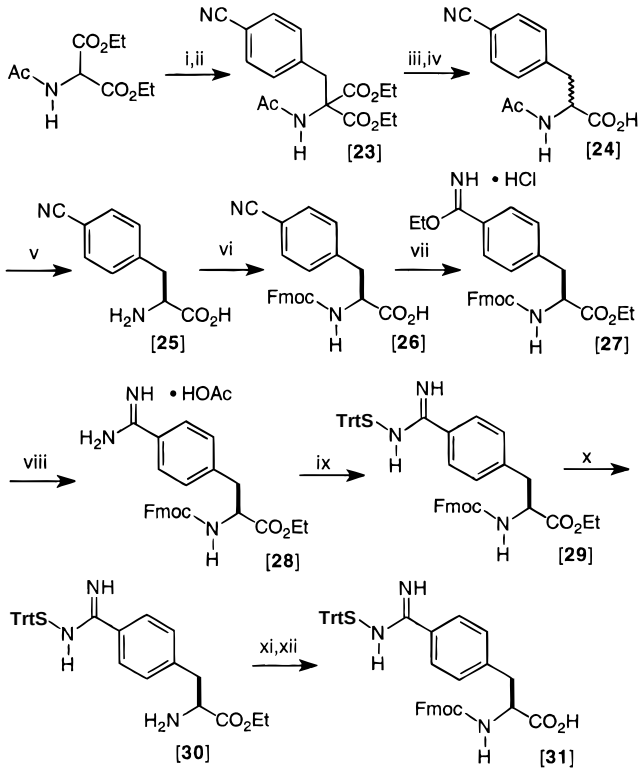
[a] (i) Boc_2O, DIEA, CH_3CN; (ii) L-cysteine, NaOMe, MeOH; (iii) FmocOSu, dioxane/water, pH = 10.

Biology

The inhibition of ADP-induced platelet aggregation by compounds **1**−**20** was measured in human platelet-rich citrated plasma (PRP) using 10−20 μM ADP. The progress and the extent of the platelet aggregation reaction was monitored by measuring transparency of the platelet suspension in an aggregometer. The results are summarized in Table 1.

The ^{99m}Tc complexes of compounds **10** and **20**, along with ^{99m}Tc-labeled glucoheptonate as a negative control, were assessed in a canine thrombosis model. The radiotracers were administered to anesthetized dogs which contained an induced thrombus in a femoral vein, and the animals were sacrificed after 4 h. The thrombus-containing vessels were carefully dissected out, weighed, and, along with known fractions of the injected doses, counted in a γ well counter. A tissue sample of a similar

Scheme 5.[a] Preparation of Fmoc-Amp(STrt)-OH



[a] (i) EtONa, EtOH; (ii) 4-cyanobenzyl bromide; (iii) NaOH, EtOH; (iv) HCl, Δ; (v) porcine kidney acylase; (vi) FmocOSu, dioxane/aqueous Na_2CO_3; (vii) HCl, EtOH; (viii) NH_3, NH_4OAc, EtOH; (ix) TrtSCl, DIEA, DMF; (x) 50% Et_2NH/DMF; (xi) 1 M LiOH(aq)/MeOH; (xii) FmocOSu, dioxane/aqueous Na_2CO_3.

Table 1. IC_{50}'s of Compounds **1**−**20** in Platelet Aggregation Experiments

compd	IC_{50}[a] (μM)	compd	IC_{50}[a] (μM)
1	1.4	**12**	0.04
2	1.3	**13**	0.14
3	0.3	**14**	0.07
4	0.16	**15**	0.11
5	0.17	**15**·ReO	0.09
6	0.14	**16**	0.03
7	0.08	**17**	0.11
8	0.11	**18**	0.03
9	0.08	**19**	0.05
10 (P280)	0.09	**20** (P748)	0.03
11	0.04	**20**·ReO	0.04

section of vein of the contralateral (control) legs and samples of thigh muscle were also dissected out and counted. Percent injected dose (%ID)/g in the thrombus, muscle, and blood obtained just prior to euthanasia were determined and thrombus-to-blood and thrombus-to-muscle ratios were calculated from the %ID/g values. Tissue data are presented in Table 2.

Results and Discussion

Our goal was to synthesize peptides containing a technetium chelator which would also have a high binding affinity for the GPIIb/IIIa receptor. An additional consideration was the fact that the target

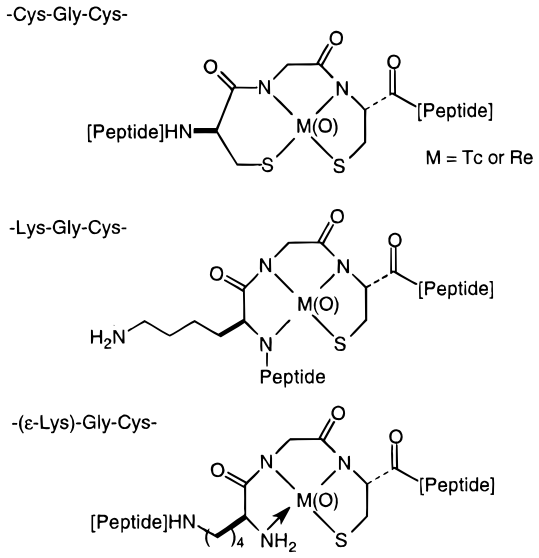


Figure 1. Peptidyl chelating systems.

compounds could not be reductively labile due to the reducing conditions necessary to convert Tc(VII) to Tc(V) in the labeling process. Cyclic RGD-containing peptides have already been described in the literature in which the ring is formed via a thioether linkage.[26] Recognizing that this approach to constraining the pharmacophore in a nonreducible cyclic structure fit our needs, we synthesized novel analogs modified to incorporate peptide sequences to serve as technetium chelators. The use of the synthetic strategy described above insured that the chelator could be incorporated at known positions during the synthesis. Several different chelating systems were utilized. These chelators included (see Figure 1) bisamide bisthiols, such as are provided by the novel chelating sequence -Cys-Gly-Cys- (e.g. compound **1**), and triamide thiols, such as are provided by the chelating sequence -Lys-Gly-Cys- (e.g. compound **7**). Bisamide bisthiols and triamide thiols have been shown to form anionic oxotechnetium complexes.[27] A novel diamide−amine−thiol chelator system of the type -(ϵ-Lys)-Gly-Cys- (e.g. compound **8**) was also prepared. In this system it is postulated that the α-amine of lysine becomes part of the coordination complex while the ϵ-amine is used to form an amide bond to the next amino acid in the sequence. This system is similar to the triamide thiols above in that the metal chelator contains three nitrogens and one sulfur. However, unlike the triamide thiols, one of the nitrogens is a less readily ionizable primary amine. We expect metal coordination by this amine to be through its lone pair electrons, thereby forming neutral oxotechnetium(+5) complexes.

The binding affinities of these compounds to the GPIIb/IIIa receptor were measured as a function of their ability to inhibit platelet aggregation. Although an indirect measure of receptor binding, this *in vitro* assay employs activated platelets and we postulated it to be a reasonable measure of the ability or our peptides to

Table 2. 4 h Femoral Vein Data in the Canine Thrombosis Model

compound	%ID/g thrombus	%ID/g blood	thrombus/blood	thrombus/muscle
[^{99m}Tc]glucoheptonate	0.0026 ± 0.0002	0.0015 ± 0.0007	2.2 ± 0.8	4.3 ±2.4
[^{99m}Tc]-**10** (P280)	0.0059 ± 0.0025	0.0012 ± 0.0003	4.4 ± 0.7	11 ± 7
[^{99m}Tc]-**20** (P748)	0.017 ± 0.0086	0.0025 ± 0.0006	7.2 ± 4.1	41 ± 24

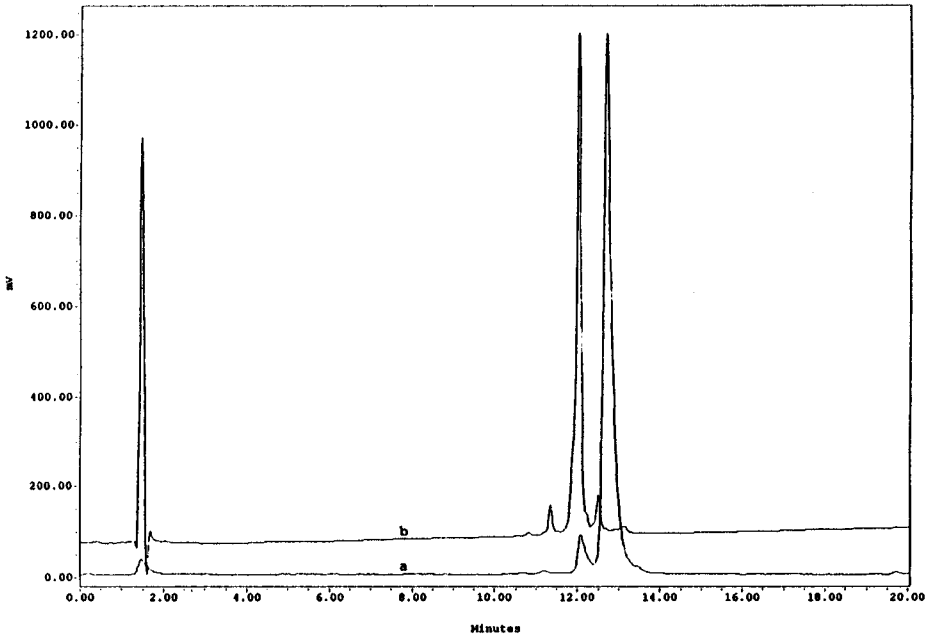


Figure 2. Labeling of P748 with ^{99m}Tc. The bottom chromatogram (a) is after 15 min incubation with [^{99m}Tc]glucoheptonate. The top chromatogram (b) is the oxorhenium complex of P748.

localize at regions of active thrombus formation. This assay is also reliable for very potent GPIIb/IIIa antagonists whereas it has been reported[26] that measurement of the binding of RGD-containing peptides to the isolated GPIIb/IIIa receptor in an ELISA assay shows some discrepancies in the range in which we were most interested ($IC_{50} < 0.5\ \mu M$ in platelet aggregation experiments). As seen from the results (Table 1), receptor binding affinity was not compromised by the incorporation of technetium-binding peptide sequences. To further validate our approach, metal complexes of compounds **15** and **20** were prepared. Because only radioactive nuclides of technetium exist, rhenium, which is very similar to technetium in its (+5) oxo coordination chemistry,[28] was used as a surrogate for the corresponding technetium complex in the *in vitro* assay. As seen in Table 1, the oxorhenium complexes retained the receptor binding affinity of the parent peptide.

In order to produce higher binding antagonists, we used *S*-(3-aminopropyl)cysteine (Apc) in place of arginine in the pharmacophore. This substitution gave a 5-fold increase (compound **3** vs compound **1**) in binding affinity so this substitution was retained in the majority of the compounds which we prepared. We also prepared dimers, trimers, and tetramers of the XGD-containing pharmacophore in an effort to boost receptor binding by increasing the local concentration of this tripeptide sequence. Indeed, we found that peptides containing dimeric Apc-Gly-Asp's had more than the expected 2-fold increase in activity in platelet aggregation experiments (e.g. compound **4** vs compound **12**). This was also shown to be the case by others in a recent study on a different family of RGD-containing molecules.[29] We also found that the substitution of the *p*-amidinophenylalanine (Amp) for arginine[30] in the RGD sequence increased the binding affinity by another factor of 2 relative to compounds containing Apc (e.g. compound **19** vs compound **15**).

Compound **10** (P280) was labeled with ^{99m}Tc within 15 min at 100 °C by ligand exchange using [^{99m}Tc]-glucoheptonate. The resulting ^{99m}Tc complex was a single species with a radiochemical purity of >90% by reversed-phase HPLC analysis. Compound **20** (P748) was labeled using the same ligand exchange technology but at room temperature. An example of an early HPLC trace of [^{99m}Tc]P748 is shown in Figure 2. An HPLC comparison of the oxorhenium complex of compound P748 (lower trace) showed it to elute close to the ^{99m}Tc-labeled species.

In a canine thrombosis model, the ^{99m}Tc complex of P280 showed thrombus-to-blood and thrombus-to-muscle ratios of 4.4 and 11 (see Table 2). In comparison, [^{99m}Tc]glucoheptonate, studied as a negative control, gave much lower uptake and lower thrombus-to-blood and thrombus-to-muscle ratios (2.2 and 4.3, respectively). The more potent GPIIb/IIIa antagonist, P748, when administered as the ^{99m}Tc complex, showed much higher uptake and higher thrombus-to-blood and thrombus-to-muscle ratios (7.2 and 41, respectively) compared to P280. The ratios seen above for both P280 and P748 are well within the range of that expected for a successful imaging agent.

Conclusions

Low molecular weight peptides were prepared which possessed both a high affinity for the GPIIb/IIIa receptor and a chelator for the radioisotope ^{99m}Tc. In experiments inhibiting the aggregation of human platelets, our best compounds had an IC_{50} of 0.03 μM, which is comparable to the most potent fibrinogen antagonists prepared to date. Furthermore, the technetium-99m complexes of P280 and P748 studied above were found to have good thrombus uptake in canine *in vivo* experiments. The ^{99m}Tc complexes of the peptides prepared in this work offer promise as useful thrombus imaging agents due to their high receptor binding affinity, ease in preparation, and expected rapid pharmacokinetics.

Experimental Section

Symbols and abbreviations generally follow the IUPAC−IUB recommendations (*Int. J. Pept. Protein Res.* **1984**, *24*, 9−37). Amino acids used were of the L-configuration unless

stated otherwise. All protected amino acids used (except *N*-Fmoc-*S*-tritylhomocysteine and *N*-α-Boc-*N*-β-Fmoc-diaminopropionic acid) were purchased from either Midwest Bio-Tech, Bachem, CA, Novabiochem, or Advanced ChemTech and used as is. Bis-maleimidomethyl ether was purchased from Cal Biochem. Bis(1,6-maleimido)hexane was purchased from Pierce. Other chemicals and solvents were purchased from either J. T. Baker, Advanced ChemTech, Fluka, or Aldrich and used as is. Aldrich SureSeal solvents were used when anhydrous conditions were necessary. When indicated, an automated Applied Biosystems 431A peptide synthesizer was used. For manual peptide synthesis, the reaction vessel used was purchased from Safe-Lab, Santee, CA. Analytical HPLCs were performed on a Waters system using a Delta-Pak C18 column (300 Å, 5 μm, 3.9 × 150 mm) at a flow rate of 1.2 mL/min or on a Waters system using a radial compression Nova-Pak C18 column (4 μm, 8 × 100 cm) at a flow rate of 3.0 mL/min. Preparative HPLCs were performed on a Waters LC 4000 system using a 4 × 32.5 cm C18 Delta-Pak preparative HPLC column. For normal-phase chromatographic separations Merck grade 9385 silica gel was used with a mesh of 230−400. R_f values were determined with silica gel TLC plates (Kieselgel 60 F_{254}, 0.25 mm layer thickness, Merck). ^{1}H-NMR spectra were obtained on a Varian Gemini 200 spectrometer at 200 MHz using TMS as an internal standard. ^{13}C-NMR spectra were obtained on a Varian Gemini 200 spectrometer at 100 MHz using TMS as an internal standard. Fast atom bombardment mass spectra (FABMS) were obtained by M-Scan using a VG Analytical ZAB 2-SE or by Scripps Research Institute using a VG ZAB VSE. Electrospray mass spectra (ESMS) were obtained at Scripps Research Institute using an API III Pe Sciex triple−quadrupole mass spectrometer.

Platelet Aggregation Assay. A 27 mL sample of blood was drawn into a 30 mL syringe, containing 3 mL of 3.8% sodium citrate, from the antecubital vein of a donor using a 19 G butterfly. The blood was mixed gently in the syringe and then placed in a 50 mL polypropylene centrifuge tube and spun at 800 rpm (ca. 100*g*) for 10 min in an IEC Centra-8 centrifuge. The platelet rich plasma (PRP) was drawn off using a pipette, the residual blood was recentrifuged at 3800 rpm (ca. 200*g*), and the supernatant platelet poor plasma (PPP) was drawn off and placed in a 50 mL centrifuge tube. PRP was stored at room temperature during the course of the assay.

Ex vivo platelet aggregation was determined by recording the increase in light transmission through a stirred suspension of PRP maintained at 37 °C. Peptide solution (or control) (15−40 μL) was added to 450 μL of PRP, followed by the addition of 25 μL of 0.2 mM ADP (in phosphate buffered saline) to give a final solution volume of approximately 0.5 mL which was 10 μM in ADP. Platelet aggregation was recorded for 3 min. Percent inhibition was calculated as follows: 100 × [maximum percent aggregation (control) − observed percent aggregation (inhibitor)] divided by [maximum percent aggregation (control)].

Radiolabeling of P280 and P748 with Technetium-99m. Peptide (0.1 mg) was dissolved in 0.1 mL of 0.9% saline. [^{99m}Tc]Glucoheptonate was prepared by reconstituting a Glucoscan vial (E. I. Dupont de Nemours, Inc; this kit contains stannous chloride as a reducing agent and glucoheptonate as a transfer ligand) with 1.0 mL of [^{99m}Tc]sodium pertechnetate (from a ^{99}Mo/^{99m}Tc generator) containing up to 200 mCi. The vial was allowed to stand at room temperature for 15 min. A 25 μL aliquot of [^{99m}Tc]gluceptate from this vial was then added to the peptide solution above and the reaction allowed to proceed at room temperature for 15−30 min (for compound **20**) or at 100 °C for 15 min (for compound **10**). After filtration through a 0.2 μm filter, the purity of the ^{99m}Tc-labeled peptide was assessed by analytical reversed-phase HPLC (10−40% B/A over 20 min using a Waters Delta-Pak C18 column, 5 μm, 39 × 150 mm; A = 0.1% TFA in water, B = 0.1% TFA in 90% acetonitrile/water). Radioactive components were detected using an in-line radiometric detector linked to an integrating recorder. [^{99m}Tc]Gluceptate and [^{99m}Tc]sodium pertechnetate elute between 1 and 4 min under these conditions, whereas the ^{99m}Tc-labeled peptides eluted later (retention time = 11.65 min) as one species for compound **10**. HPLC analysis of the ^{99m}Tc complex of compound **20** showed a peak which eluted at 12.7 min. This compares with a retention time of 12.0 min for the ReO complex of **20**.

Canine Thrombosis Study. The model used was that reported by Knight.[31] Mongrel dogs (25−57 lb, fasted overnight) were sedated (ketamine and acepromazine, im) and then anesthetized with sodium pentobarbital iv. In each animal, an 18 gauge angiocath was inserted into the distal half of the right femoral vein and an 8 mm Dacron-entwined stainless steel embolisation coil (Cook Co., Bloomington, IN) was placed in the femoral vein at approximately the mid-femur. The catheter was removed, the wound was sutured, and the placement of the coil was documented by X-ray. The animals were allowed to recover overnight. On the day following coil placement, each animal was re-anesthetized, iv saline drips were placed in each foreleg, and a urinary bladder catheter was inserted to collect urine. ^{99m}Tc-labeled **10** (0.2−0.4 mg at 5−10 mCi, 6 dogs) or ^{99m}Tc-labeled **20** (0.05−0.4 mg at 10−40 mCi, 7 dogs) was injected as a bolus into a foreleg iv line and flushed in with saline. As a negative control, two dogs were studied with [^{99m}Tc]glucoheptonate (8 mCi). After 4 h each animal was deeply anesthetized with pentobarbital. Two blood samples were collected by cardiac puncture using a heparinized syringe followed by a euthanising dose of saturated potassium chloride solution administered by bolus iv injection. The femoral vein containing the thrombus, a similar section of vein of the contralateral (control) leg, and samples of thigh muscle were carefully dissected out. The thrombus, coil, and coil fibers were dissected free of the vessel. The thrombus, saline-washed vessel samples, coil, and coil Dacron fibers were separated, weighed, and, along with known fractions of the injected doses, counted in a γ well counter in the ^{99m}Tc channel. Fresh thrombus weight, percent injected dose (%ID)/g in the thrombus and blood obtained just prior to euthanasia, %ID in the coil, excised blood vessels, muscle, and blood were determined. Thrombus-to-blood and thrombus-to-muscle ratios were calculated from %ID/g values.

cyclo-[CH_2CO-D-Tyr-Apc-Gly-Asp-Cys(S-)]-Lys-Gly-Cys-(Acm)-Gly-Cys(Acm)-NH_2 (4). Fmoc-protected Rink resin (0.25 mmol) was placed in the reaction vessel of an automated peptide synthesizer. The standard FastMoc protocol[32] was used, and appropriately protected amino acids were added sequentially until the desired sequence had been synthesized. Briefly, the N-terminal Fmoc was removed with 20% piperidine/DMF, and the resin or resin-supported peptide was washed with NMP. Coupling was affected in NMP by reacting 4 equiv (1 mmol) of amino acid derivative with 4 equiv (1 mmol) of 1:1 HBTU/HOBt solution (0.45M in DMF) and 8 equiv (2 mmol) of DIEA. After adding the activated amino acid to the reaction vessel, the resin was agitated for an appropriate amount of time (usually 40 min). The resin was then washed with additional NMP and any unreacted amine capped with excess acetic anhydride. The order of addition of protected amino acids for the synthesis of **4** was Fmoc-Cys-(Acm)-OH, Fmoc-Gly-OH, Fmoc-Cys(Acm)-OH, Fmoc-Gly-OH, Fmoc-Lys(Boc)-OH, Fmoc-Cys(Trt)-OH, Fmoc-Asp(tBu)-OH, Fmoc-Gly-OH, Fmoc-Apc(Boc)-OH (**22**), and Fmoc-D-Tyr(tBu)-OH. After the last amino acid in the sequence had been added, the N-terminal Fmoc protecting group was once again removed as indicated above and the resulting free amine reacted with excess chloroacetic anhydride, thus placing a chloroacetyl moiety at the N-terminus. After additional washes with NMP and then methylene chloride, the resin was transferred to a round bottom flask and 10 mL of trifluoroacetic acid/water/triethylsilane (92.5:5:2.5) was added. The deprotection/cleavage mixture was stirred at room temperature for 1.5 h and filtered through a sintered glass funnel. The resin was washed with TFA (2 mL) which was added to the filtrate. Cold ethyl ether (200 mL) was added to precipitate out the chloroacetyl intermediate. The solid was filtered and washed with ethyl ether to yield 123 mg of crude peptide. This peptide was taken up in 1.2 L of argon-degassed water, and 1 M ammonium hydroxide was added until a pH of 8.0 was achieved. The reaction mixture was stirred for 20 h and concentrated *in vacuo* to a volume of about 100 mL. The solution was loaded

onto a preparative reversed-phase HPLC column at a flow rate of 20 mL/min. The peptide was eluted as follows at 75 mL/min (solvent A = 0.1% TFA in water, solvent B = 0.1% TFA in 90% acetonitrile water): 0−5 min, 0% B/A; 5−10 min, 0−20% B/A; 10−30 min, 20−50% B/A; 30−34 min, 50% B/A. Fractions were collected while monitoring at 230 nm and analyzed by analytical HPLC (Delta-Pak, 0−100% B/A over 20 min, **4** had a retention time of 9.28 min). Fractions containing pure product were combined, and the acetonitrile was removed *in vacuo* on the rotovap. Lyophilization yielded **4** (100 mg) as the trifluoroacetate salt. FABMS indicated a molecular ion peak (MH^+) at 1247 which corresponds to the molecular formula of $C_{48}H_{75}N_{15}O_{16}S_4$ with a mass of 1246. Compounds **1**−**3** and **5**−**9** were produced by the same protocol outlined above and were confirmed by FAB mass spectral analysis unless otherwise noted: compound **1** ($C_{46}H_{69}N_{17}O_{17}S_3$) expected 1229, found 1229 ($M + H^+$); compound **2** ($C_{44}H_{66}N_{16}O_{16}S_3$) expected 1172, found 1171 ($M + H^+$); compound **3** ($C_{46}H_{69}N_{15}O_{17}S_4$) expected 1233, found 1232 ($M + H^+$); compound **5** ($C_{67}H_{101}N_{19}O_{21}S_5$) expected 1670, found 1669 ($M + H^+$); compound **6** ($C_{37}H_{57}N_{11}O_{12}S_3$) expected 945, found 945 ($M + H^+$); compound **7** ($C_{43}H_{58}N_{13}O_{13}S_2$) expected 1030, found 1030 ($M + H^+$); compound **8** ($C_{147}H_{216}N_{42}O_{49}S_9$) ESMS, expected 3644.1, found 3643.5 (M^+); compound **9** ($C_{92}H_{144}N_{28}O_{29}S_5$) expected 2268, found 2267 ($M + H^+$).

cyclo-[CH_2CO-D-Tyr-Apc-Gly-Asp-Cys(S-)]-Gly-Gly-Cys-(Acm)-Gly-Cys(Acm)-Gly-Gly-Cys-NH_2. The title peptide was synthesized on a 0.25 mmol scale using the same solid-phase peptide synthesis protocol as that used above in the preparation of compound **4**. The order of addition of protected amino acids was Fmoc-Cys(Mob)-OH, Fmoc-Gly-OH, Fmoc-Gly-OH, Fmoc-Cys(Acm)-OH, Fmoc-Gly-OH, Fmoc-Cys(Acm)-OH, Fmoc-Gly-OH, Fmoc-Gly-OH, Fmoc-Cys(Trt)-OH, Fmoc-Asp(tBu)-OH, Fmoc-Gly-OH, Fmoc-Apc(Boc)-OH (**24**), and Fmoc-D-Tyr(tBu)-OH. After the last amino acid in the sequence had been added, the N-terminal Fmoc protecting group was once again removed as indicated above and the resulting free amine reacted with excess chloroacetic anhydride, thus placing a chloroacetyl moiety at the N-terminus. After additional washes with NMP and then methylene chloride, the resin was transferred to a round bottom flask and 10 mL of trifluoroacetic acid/water/triethylsilane (92.5:5:2.5) was added. The deprotection/cleavage mixture was stirred at room temperature for 1.5 h and filtered through a sintered glass funnel. The resin was washed with TFA (2 mL) which was added to the filtrate. Cold ethyl ether (200 mL) was added to precipitate out the chloroacetyl intermediate. The solid was filtered and washed with ethyl ether to produce 190 mg of crude peptide. Then 100 mg of this peptide was taken up in 1 L of argon-degassed water, and 1 M ammonium hydroxide was added until a pH of 8.0 was achieved. The reaction mixture was stirred for 20 h followed by concentrating it *in vacuo* to a volume of about 200 mL. Lyophilization yielded 100 mg of crude Mob-protected material. This material was treated with 10 mL of TFA/*m*-cresol/BF_3·OEt_2 (8:1:1) for 1 h to remove the Mob protecting group. Cold ethyl ether (200 mL) was added to precipitate the product, and the solid was filtered, washed with ether, and dissolved in acetonitrile/water (1:1). The solution was loaded onto a preparative reversed-phase HPLC column at a flow rate of 20 mL/min. The peptide was eluted as follows at 75 mL/min (solvent A = 0.1% TFA in water, solvent B = 0.1% TFA in 90% acetonitrile water): 0−5 min, 0% B/A; 5−25 min, 10−40% B/A; 25−35 min, 50% B/A. Fractions were collected while monitoring at 230 nm and analyzed by analytical HPLC (Delta-Pak, 10−40% B/A over 20 min, the product had a retention time of 8.83 min). Fractions containing pure product were combined, and the acetonitrile was removed on the rotovap. Lyophilization yielded 41.5 mg of product. FABMS indicated a molecular ion peak at 1392 which corresponds to the molecular formula of $C_{51}H_{77}N_{17}O_{19}S_5$ with a monoisotopic mass of 1391.4.

(cyclo-[CH_2CO-D-Tyr-Apc-Gly-Asp-Cys(S-)]-Gly-Gly-Cys(Acm)-Gly-Cys(Acm)-Gly-Gly-Cys[S-Maleimido-CH_2-]-NH_2)$_2$O (10) [P280]. A 126 mg (0.09 mmol) portion of the cyclo-[CH_2CO-D-Tyr-Apc-Gly-Asp-Cys(S-)]-Gly-Gly-Cys(Acm)-Gly-Cys(Acm)-Gly-Gly-Cys-NH_2 produced above was dissolved in 5 mL of acetonitrile and 7 mL of water. Then, 38 mL of 50 mM sodium phosphate buffer (pH = 7.0) containing 0.5 mM EDTA was added and the solution degassed with argon. Bismaleimidomethyl ether (11.8 mg, 0.045 mmol) in 150 μL of DMF was added, and the reaction mixture was stirred at room temperature under an atmosphere of argon. When HPLC analysis indicated that the reaction was complete (retention time shift from 8.83 min to 12.09 min in 10−40% B/A, 20 min) the reaction mixture was loaded onto a preparative reversed-phase HPLC column at a flow rate of 20 mL/min. The peptide was eluted as follows at 75 mL/min (solvent A = 0.1% TFA in water, solvent B = 0.1% TFA in 90% acetonitrile water): 0−5 min, 0% B/A; 5−25 min, 10−40% B/A; 25−35 min, 50% B/A. Fractions were collected while monitoring at 230 nm and analyzed by analytical HPLC (Delta-Pak, 10−40% B/A over 20 min, the product had a retention time of 12.09 min). Fractions containing pure product were combined, and the acetonitrile was removed on the rotovap. Lyophilization yielded 169 mg (62% yield) of product. ESMS indicated a molecular ion peak at 3021 which corresponds to the molecular formula of $C_{112}H_{162}N_{36}O_{43}S_{10}$ with an average mass of 3021.3.

Compounds **11**−**13** were produced by the same protocol outlined above by reacting the appropriate free cysteine-containing peptides (see Scheme 2) with the following maleimide electrophiles. For compound **11** tris(2-maleimidoethyl)-amine (preparation below) was used. For compound **12** bismaleimidomethyl ether was used. For compound **13** bis-(1,6-maleimido)hexane was used. Final products were confirmed by FAB mass spectral analysis: compound **11** ($C_{171}H_{249}N_{55}O_{63}S_{15}$) expected 4461, found 4586 ($M + Na^+$); compound **12** ($C_{120}H_{180}N_{38}O_{43}S_{10}$) expected 3162, found 3163 ($M + H^+$); compound **13** ($C_{116}H_{170}N_{36}O_{42}S_{10}$) expected 3060, found 3062 ($M + H^+$).

(cyclo-[CH_2CO-D-Tyr-Apc-Gly-Asp-Cys(S-)]-Gly-Gly-Cys(Acm)-Gly-Cys(Acm)-Gly-Gly-Cys-[S-CH_2CO-]-NH_2)$_2$-Lys-(ϵ-Lys)-Gly-Cys-NH_2 (15). ($ClCH_2CO)_2$Lys-(ϵ-Lys)-Gly-Cys(Trt)-NH_2 was synthesized on a 0.25 mmol scale by the same solid-phase peptide synthesis protocol used above in the synthesis of **4**. The order of addition for the synthesis of this intermediate was Fmoc-Cys(Trt)-OH, Fmoc-Gly-OH, *N*-α-Boc-*N*-ϵ-Fmoc-Lys-OH, and *N*-α-Fmoc-*N*-ϵ-Fmoc-Lys-OH. After the last amino acid in the sequence had been added, the N-terminal Fmoc protecting groups were removed as indicated above and the resulting free amines reacted with excess chloroacetic anhydride, thus placing a chloroacetyl moiety at the α- and ϵ-amines of the N-terminal lysine. The resin-supported peptide was treated with 10 mL of TFA/water (95:5) for 1 h. The resin was filtered and washed with 5 mL of $CHCl_3$. The filtrates were combined, and the volatiles were removed *in vacuo* on the rotovap. The crude product was taken up in 20 mL of $CHCl_3$, and this was also removed *in vacuo*. This chloroform treatment was repeated several times until the color of the crude peptide in solution was no longer red. This color change indicates removal of trace amounts of TFA and concomitant reattachment of the trityl protecting group to the cysteine sulfhydryl. This material was used directly in the next reaction. Therefore 13.9 mg (16.7 μmol) of this intermediate and 47 mg (33.5 μmol) of cyclo-[CH_2CO-D-Tyr-Apc-Gly-Asp-Cys(S-)]-Gly-Gly-Cys(Acm)-Gly-Cys(Acm)-Gly-Gly-Cys-NH_2 were dissolved in 1 mL of acetonitrile and 3 mL of 0.1 M Na_2CO_3 buffer (pH = 10.0) containing 0.5 mM EDTA. The reaction mixture was stirred for 4 h under an atmosphere of argon followed by removal of the volatiles on the rotovap. The crude product was treated with 10 mL of TFA/water/triethylsilane (10:0.5:0.2) for 1.5 h. Ethyl ether (200 mL) was added to precipitate the product which was filtered and washed with ethyl ether. Crude product was then dissolved in 5 mL of acetonitrile/water (1:1), and the resulting solution was diluted with 10 mL of 0.1% TFA/water. The solution was loaded onto a preparative reversed-phase HPLC column at a flow rate of 20 mL/min. The peptide was eluted as follows at 75 mL/min (solvent A = 0.1% TFA in water, solvent B = 0.1% TFA in 90% acetonitrile water): 0−5 min, 0% B/A; 5−25 min, 10−30% B/A; 25−35 min, 50% B/A. Fractions were collected while monitoring at 220 nm and

analyzed by analytical HPLC (Delta-Pak, 10−40% B/A over 20 min, the product had a retention time of 11.41 min). Fractions containing pure product were combined, and the acetonitrile was removed on the rotovap. Lyophilization yielded 32 mg of product. ESMS indicated a molecular ion peak at 3298 which corresponds to the molecular formula of $C_{123}H_{187}N_{41}O_{44}S_{11}$ with an average mass of 3296.7.

Compounds **14** and **16**−**20** were produced by the same protocol outlined above by reacting the appropriate free cysteine-containing peptides (see Scheme 2) with the following bischloroacetyl electrophiles. For compound **14** 2,2′-(ethylenedioxy)bis(chloroacetamidoethane) [$(ClCH_2CONHCH_2CH_2OCH_2)_2$] was used. For compound **16** tris(2-chloroacetamidoethyl)amine [$(ClCH_2CONHCH_2CH_2)_3N$] was used. For compounds **17**−**20** $(ClCH_2CO)_2$Lys-(ϵ-Lys)-Gly-Cys(Trt)-NH_2 was used. Final products were confirmed by electrospray mass spectral analysis: compound **14** ($C_{112}H_{170}N_{36}O_{42}S_{10}$) expected 3013, found 3013 (M^+); compound **16** ($C_{165}H_{249}N_{55}O_{60}S_{15}$) expected 4444, found 4443 (M^+); compound **17** ($C_{91}H_{137}N_{29}O_{32}S_7$) expected 2374, found 2373 (M^+); compound **18** ($C_{243}H_{370}N_{80}O_{87}S_{21}$) expected 6477, found 6478 (M^+); compound **19** ($C_{131}H_{187}N_{43}O_{44}S_9$) expected 3357, found 3357 (M^+); compound **20** ($C_{107}H_{151}N_{33}O_{32}S_5$) expected 2572, found 2573 (M^+).

(cyclo-[CH_2CO-D-Tyr-Apc-Gly-Asp-Cys(S-)]-Gly-Gly-Cys(Acm)-Gly-Cys(Acm)-Gly-Gly-Cys[S-CH_2CO-]-NH_2)$_2$Lys-(ϵ-Lys)-Gly-Cys-NH_2·Oxorhenium Complex (15·ReO). Compound **15** (20 mg, 6 μmol) and 5.6 mg (7.2 μmol) of $Bu_4NReOBr_4$ were taken up in 1 mL of DMF, and the reaction mixture was stirred at room temperature under argon for 16 h. The solution was diluted with 1 mL of water and loaded directly onto a preparative reversed-phase HPLC column at a flow rate of 2 mL/min. The peptide was eluted as follows at 75 mL/min (solvent A = 0.1% TFA in water, solvent B = 0.1% TFA in 90% acetonitrile water): 0−5 min, 0% B/A; 5−25 min, 10−30% B/A; 25−35 min, 50% B/A. Fractions were collected while monitoring at 220 nm and analyzed by analytical HPLC (Delta-Pak, 10−40% B/A over 20 min, the product had a retention time of 11.20 min). Fractions containing pure product were combined, and the acetonitrile was removed on the rotovap. Lyophilization yielded 9.0 mg of product. ESMS indicated a molecular ion peak at 1166.9 [$(M + 3H^+)^{3+}$, deconvoluting to 3497.7] which corresponds to a molecular formula of $C_{123}H_{184}N_{41}O_{45}S_{11}Re$ with an average mass of 3495.9. The oxorhenium complex of compound **20** was produced by the same protocol outlined above. ESMS analysis of this compound indicated a molecular ion peak (M) of 2772 which corresponds to a molecular formula of $C_{107}H_{150}N_{33}O_{33}S_5Re_1$with an average mass of 2773.1. By analytical HPLC (Delta-Pak, 10−40% B/A over 20 min) the oxorhenium complex of **20** had a retention time of 11.65 min as compared to the free peptide which had a retention time of 10.97 min.

***N*-Boc-3-amino-1-bromopropane (21).** 3-Aminopropyl bromide hydrobromide (50.0 g, 0.228 mol) was weighed out in a 500 mL round bottom flask, and the solid was dissolved in 200 mL of anhydrous acetonitrile. The solution was cooled to 0 °C with an ice bath, and 39.7 mL (0.228 mol) of diisopropylethylamine was added with stirring. This was followed by the addition of 49.7 g (0.228 mol) of di-*tert*-butyl dicarbonate. The ice bath was removed, and the reaction mixture was stirred for 3 h. At this time TLC (10% EtOAc/hexane) indicated that the reaction was complete. The volatiles were removed *in vacuo* on the rotovap to produce an oil, and 500 mL of diethyl ether was added. The salts were filtered off and washed with an additional 250 mL of ether. The combined organics were washed with 1 M NaH_2PO_4 (250 mL), 5% $NaHCO_3$ (250 mL), and saturated NaCl (250 mL). The organics were dried over $MgSO_4$ and filtered and the volatiles removed *in vacuo* on the rotovap to yield an oil. Hexanes were added to the crude product, and the solution was left standing at −20 °C for 20 h. The resulting solid was filtered and washed with hexanes. Trace solvents were removed under high vacuum to yield 41.55 g (77% yield) of product: ^{1}H NMR ($CDCl_3$) δ 1.45 (9H, s), 2.06 (2H, quint, J = 7 Hz), 3.28 (2H, quart, J = 7 Hz), 3.45 (2H, t, J = 7 Hz), 4.73 (1H, br s).

***N*-α-Fmoc-*S*-(*N*-Boc-3-aminopropyl)-L-cysteine (22).** A 500 mL round bottom flask was charged with 19.97 g (0.165 mol) of cysteine and 100 mL of anhydrous methanol. The atmosphere of the reaction was flushed with argon followed by the addition of 65 mL (0.35 mol) of a 5.4 M solution of sodium methoxide in methanol. After stirring for 10 min, 39.27 g (0.165 mol) of **21** was added. The reaction mixture was stirred under an atmosphere of argon for 5 h. TLC analysis (AcOH/MeOH/CH_2Cl_2, 5:20:75) indicated that the reaction was complete. $NaHCO_3$ (1.9 g) was added followed by the dropwise addition of acetic acid until a pH of 11 was achieved. Then 55.6 g (0.165 mol) of FmocOSu was dissolved in 200 mL of anhydrous THF, and this solution was added to the reaction mixture. Stirring was continued under argon for an additional 16 h. The volatiles were then removed *in vacuo*, and the resulting slurry was taken up in 500 mL of ethyl acetate. The organics were washed with 1 M H_3PO_4 (250 mL), water (250 mL), and saturated NaCl (250 mL). After drying over $MgSO_4$, the organics were filtered and concentrated *in vacuo* on the rotovap to yield an oil. The product was crystallized from EtOAc/hexanes to yield 63.9 g (77% yield) of pure product with a melting point of 116 −18 °C: ^{1}H NMR (CD_3OD) δ 1.40 (9H, s), 1.70 (2H, quint, J = 7 Hz), 2.56 (2H, t, J = 7 Hz), 2.7−3.2 (4H, m), 4.15−4.4 (4H, m), 7.2−7.8 (9H, m); ^{13}C NMR (CD_3OD) δ 29.1, 30.8, 31.1, 35.6, 40.7, 48.7, 56.4, 68.4, 80.3, 121.1, 126.6, 128.5, 129.1, 142.8, 145.5, 158.7, 176.3; FABMS ($C_{26}H_{32}N_2O_6S$) expected 501.6, found 501.6 ($M + H^+$); $[\alpha]^{22}_D = -26.3 \pm 0.7$ (c = 1.0, DMF).

***N*-Acetyl-α-carboethoxy-4-cyano-D,L-phenylalanine, Ethyl Ester (23).** A 5-L 3-neck round bottom flask was equipped with a mechanical stirrer and a reflux condenser. The apparatus was flame-dried under a stream of argon. After cooling, the flask was charged with 1400 mL of absolute ethanol. Sodium metal (46.0 g 2.00 mol) was weighed out in hexane and cut up into cubic portions. The sodium was then added to the ethanol portionwise over 1 h under a blanket of argon. After all of the sodium was added, the solution was stirred until sodium ethoxide formation was complete. The reflux condenser was removed and the flask fitted with a thermometer. The ethoxide solution was then cooled to 0 °C (ice bath), and diethyl acetamidomalonate (434 g, 2.00 mol) was added portionwise under an atmosphere of argon, keeping the temperature between 0 and 5 °C. After all the malonate was added, the reaction mixture was stirred an additional 45 min. The resulting white slurry was kept at 0−5 °C, and α-bromo-*p*-toluidine (392 g, 2.00 mol) was added. The ice bath was removed, and a heating mantle was fitted on. The reaction mixture was brought to a gentle reflux and held there for 2.5 h. At this time the progress of the reaction was checked by TLC (50% EtOAc/hexane), and it was found to be complete. The reaction mixture was cooled, and 1.4 L of water was added. Stirring was momentarily discontinued and the solid broken up with a large spatula. Stirring was initiated again and continued until a mostly fine slurry resulted (about 1 h). The solid was filtered, and the any chunks that were remaining in the reaction flask were removed and crushed with a mortar and pestle. The resulting slurry was suspended in water and filtered with the rest of the crude product. This crude product was then washed with water (2 × 400 mL) and left standing overnight in the filter funnel to dry. The crude product was divided approximately in half and each half dissolved in about 1300 mL of hot ethanol. After slow cooling overnight the needles were filtered, and any residual solvent was removed *in vacuo* at 50 °C. The product (605.7 g, 91% yield) was produced as white needles with a melting point of 170−72 °C: ^{1}H NMR ($CDCl_3$) δ 1.30 (6H, t, J = 7 Hz), 2.04 (3H, s), 3.72 (2H, s), 4.28 (4H, q, J = 7 Hz), 6.54 (1H, s), 7.12 (2H, d, J = 8 Hz), 7.56 (2H, d, J = 8 Hz); ^{13}C NMR ($CDCl_3$) δ 13.9, 22.9, 37.9, 62.9, 66.9, 111.4, 118.5, 130.6, 132.0, 141.1, 167.1, 169.2.

***N*-Acetyl-4-cyano-D,L-phenylalanine (24).** Sodium hydroxide (146 g, 3.64 mole) was taken up in 950 mL of absolute ethanol in a 5-L three-neck round bottom flask equipped with a mechanical stirrer. The mixture was stirred until all of the hydroxide went into solution, followed by the addition of 605.4 g (1.82 mol) of **23** as a solid. The reaction mixture was then refluxed for 18 h with stirring. After this time, stirring was discontinued and the reaction mixture was cooled in an ice

bath. A 400 mL sample of 6 M HCl was added slowly while the precipitate was broken up with a large spatula. After all of the HCl was added (pH = 3) the precipitate was sufficiently broken up to continue mechanical stirring again. Concentrated HCl was added in 25 mL portions until a pH of 2 was reached (four portions). The reaction mixture was heated and 800 mL of ethanol was distilled off. The solution was left to cool overnight, seed crystals were added in the morning, and the solution was cooled in an ice bath. After crystallization was well underway the ice bath was removed and the solution was allowed to stand at room temperature for 3 h. The resulting white solid was filtered off and washed with 1 L of water. Drying *in vacuo* at 50 °C yielded 546.4 g of a white solid. A second crop of 50.0 g was collected from the filtrate. The two crops were combined and divided into approximately two equal portions. Each was dissolved in about 1200 mL of hot ethyl acetate with stirring. Hot hexane was added (about 200 mL) and the solution set aside to cool. A first crop of 170.5 g of white needles were collected by filtration (mp 174−76 °C). After concentration of the filtrate and cooling, a second crop was taken of 31.6 g (mp 173−76 °C) for a total of 202.1 g of product (48% yield): ^{1}H NMR (acetone-d_5) δ 1.91 (3H, s), 3.11 (1H, q, J = 15, 9 Hz), 3.32 (1H, q, J = 15, 5.5 Hz), 4.80 (1H, m), 7.42 (1H, d, J = 8 Hz), 7.51 (2H, d, J = 8 Hz), 7.73 (2H, d, J = 8 Hz); ^{13}C NMR (acetone-d_5) δ 22.3, 38.0, 53.5, 111.1, 119.1, 131.0, 132.6, 144.1, 170.0, 172.3.

4-Cyano-L-phenylalanine (25). In a 5-L three-neck flask equipped with a mechanical stirrer was suspended 196.6 g (0.846 mol) of **24** in 2 L of distilled water at 40 °C(water bath). As the solution was being deoxygenated by sparging with argon gas the pH was adjusted with 1 M LiOH to a pH of 7.2−7.5. As the hydroxide was added, the suspension gradually went into solution. Next, 500 mg of porcine kidney acylase was added and the reaction mixture was stirred for 16 h. After this time the reaction was monitored by HPLC (Delta-Pak column, 0−100% B/A over 20 min, 100% B 20−25 min; solvent A = 0.1% TFA in water, solvent B = 0.1% TFA in 90% acetonitrile/10% water) and found to be 50% complete (the retention time of starting material was 5.55 min, and the retention time of product was 4.64 min). The pH was adjusted from 6.5 to 7.3 with 1 M LiOH, and another 200 mg of enzyme was added. After another 24 h the progress was again checked by HPLC. Since it appeared as if the progress had slowed down considerably, 714 mg of cobolt chloride hexahydrate was added to make the reaction mixture 1 mM in Co^{2+}. The pH was adjusted to 7.5, and another 300 mg of enzyme was added. The reaction was allowed to proceed as before for an additional 16 h. After this time HPLC analysis indicated that the ratio of H-L-Phe(CN)OH to Ac-Phe(CN)OH was 46:54. The pH was adjusted to 5.0 with concentrated HCl, and the reaction mixture was heated to 60 °C. About 6 g of activated carbon was added, and the reaction mixture was stirred at 60 °C for 10 min. It was then filtered through a pad of Celite while still warm, and the pH of the filtrate was adjusted to 1.5 with concentrated HCl. The aqueous solution was split into two 1800 mL portions, and each was extracted with EtOAc (6 × 300 mL). The aqueous fractions (HPLC analysis indicated that there was still 2.6% *N*-acetyl compound) were then recombined and applied to a column of Dowex 50 × 8 (1 kg of Dowex). The column was washed with water until pH = 4 was reached, and then the desired amino acid was eluted with 1 M aqueous ammonia. Fractions containing product were concentrated *in vacuo* to yield the desired product as a white solid (56.9 g, 70.7% yield based on theoretical amount of *N*-Ac-L-isomer in racemic mixture) with a melting point of 242 °C (sublimed): ^{1}H NMR (D_2O, dioxane ref = δ 3.63) δ 3.08 (1H, q, J = 15, 9 Hz), 3.21 (1H, q, J = 15, 5.5 Hz), 3.89 (1H, m), 7.33 (2H, d, J = 8 Hz), 7.65 (2H, d, J = 8 Hz); ^{13}C NMR (D_2O, dioxane ref = δ 67.8) δ 37.7, 56.8, 111.4, 120.9, 131.4, 134.2, 142.6, 174.4.

***N*-Fmoc-4-cyano-L-phenylalanine (26).** Sodium carbonate (27.9 g, 0.263 mol) was dissolved in 260 mL of water, and **25** (25.0 g, 0.131 mol) was added with stirring. After all solids went into solution, the reaction mixture was cooled with an ice bath, and FmocOSu (42.1 g, 0.125 mol) in 520 mL of anhydrous dioxane was added dropwise over 2 h. After addition was complete the ice bath was removed and the reaction mixture was allowed to come to room temperature over 1.5 h. TLC analysis (BuOH/HOAc/water, 4:1:1) indicated that the reaction was complete so the reaction mixture was transferred to a 4 L Ehrlenmeyer equipped with mechanical stirring and cooled once again in an ice bath. About 1 L of ethyl acetate was added, and 1 M HCl was added until the pH of the aqueous layer was 1.5. The ethyl acetate separated off in a separatory funnel, and the aqueous layer was washed with 600 mL of additional EtOAc. The combined organics were washed with water (500 mL) and saturated NaCl (500 mL). After drying over sodium sulfate, the organics were decanted and the volatiles removed *in vacuo* on the rotovap to yield a clear oil. This was taken up in about 300 mL of ethyl acetate and heated to reflux with stirring. Hot hexane (about 300 mL) was added until the solution was cloudy. Cooling yielded the product as white crystals (49.15 g, 89% yield, mp 191−92 °C): ^{1}H NMR (acetone-d_6) δ 3.18 (1H, q, J = 17, 9.5 Hz), 3.40 (1H, q, J = 17, 4.5 Hz), 4.28 (3H, m), 4.60 (1H, m), 6.84 (1H, d, J = 8.5 Hz), 7.3−7.9 (12H, m); ^{13}C NMR (acetone-d_6) δ 37.8, 47.6, 55.3, 66.8, 111.0, 119.1, 120.5, 125.7, 127.6, 128.2, 131.0, 132.6, 141.8, 144.1, 144.6, 156.5, 172.5.

***N*-α-Fmoc-4-(ethoxyimino)-L-phenylalanine, Ethyl Ester (27). 26** (49.11 g, 0.119 mol) was suspended in 1420 mL of anhydrous ethanol in a 5-L three-necked flask equipped with a mechanical stirrer and a reflux condenser. Anhydrous HCl gas was bubbled in (absorption of HCl caused the reaction mixture to reflux) until the solution was saturated. Reflux was maintained for 6 h with a heating mantle followed by slow cooling overnight. Two liters of absolute ether were added, and the reaction mixture was left standing for 0.5 h. Filtration yielded the product as a white solid (34.8 g) after drying under high vacuum. The filtrate was concentrated *in vacuo* to a volume of about 500 mL and diluted with 1200 mL of ethyl ether. A second crop was obtained which was dried under high vacuum to yield an additional 22.5 g of product for a total of 57.3 g (92% yield): ^{1}H NMR (DMSO-d_6) δ 1.15 (3H, t, J = 7 Hz), 1.45 (3H, t, J = 7 Hz), 3.12 (1H, q, J = 17, 9.5 Hz), 3.20 (1H, q, J = 17, 4.5 Hz), 4.0−4.4 (6H, m), 4.61 (2H, t, J = 7 Hz), 7.25−8.10 (13H, m); ^{13}C NMR (DMSO-d_6) δ 13.8, 18.6, 36.2, 46.5, 55.2, 60.5, 65.6, 69.3, 119.9, 125.0, 126.9, 127.3, 127.5, 128.8, 132.5, 140.6, 140.7, 143.6, 155.7, 167.6, 171.5.

***N*-α-Fmoc-4-amidino-L-phenylalanine, Ethyl Ester (28). 27** (55.9 g, 0.107 mol) and 150 g of ammonium acetate were suspended in 1.5 L of absolute ethanol in a three-necked flask equipped with a mechanical stirrer. To this stirred suspension was added 40 mL of a saturated ethanolic ammonia solution (the pH was checked and found to be 8). The reaction mixture was brought to reflux and held there for 30 min, at which time the reaction was judged complete by TLC analysis (5% MeOH/CH_2Cl_2). The reaction mixture was cooled in a water bath to room temperature, and 2 L of water was added. After stirring for 10 min, the resulting suspension was filtered and the white solid which was collected was washed with 700 mL of water followed by 1 L of ether. Residual solvent was removed under high vacuum to yield 31.97 g of product as a very slightly yellow solid. A second crop (8.75 g) was collected from the aqueous filtrate above for a total of 40.7 g of product (74% yield) as the acetate salt: ^{1}H NMR (DMSO-d_6) δ 1.13 (3H, t, J = 7 Hz), 1.78 (HOAc, 3H, s), 2.8−3.2 (2H, m), 4.0−4.4 (6H, m), 7.25−8.0 (13H, m), 9.15 (3H, br s); ^{13}C NMR (DMSO-d_6) δ 13.9, 23.0 (HOAc), 36.1, 36.9, 55.3, 60.0, 60.6, 109.4, 119.8, 121.2, 127.2, 128.7, 129.6, 137.3, 139.3, 142.5, 143.9, 159.6, 165.7, 172.6, 174.2 (HOAc).

***N*-α-Fmoc-4-[(N_{am}-tritylsulfenyl)amidino]-L-phenylalanine, Ethyl Ester (29). 28** (14.64 g, 28.3 mmol) was suspended in 150 mL of anhydrous DMF, and tritylsulfenyl chloride (11.43 g, 36.8 mmol) was added as a solid with stirring. Most of the suspension went into solution after the addition of the sulfenyl chloride. The reaction mixture was stirred at room temperature under argon, and diisopropylethylamine (14.7 mL, 84.6 mmol) was added dropwise via syringe. The reaction mixture was stirred at room temperature for 1 h. The volatiles were removed *in vacuo* on the rotovap, and the crude product was taken up in 400 mL of ethyl acetate. The organics were washed briefly with 1 M $KHSO_4$ (100 mL), water (100 mL), saturated $NaHCO_3$ (100

mL), and saturated NaCl (50 mL). The organics were quickly passed through sodium sulfate and concentrated *in vacuo* to yield a yellow foam (23.19 g). This was taken up in a minimum amount of methylene chloride and applied to a silica gel column which had been equilibrated with 20% EtOAc/hexane, with the top 1 cm equilibrated with methylene chloride. The column was eluted with 20−30% ethyl acetate/hexane. Fractions containing product were pooled and the volatiles removed *in vacuo*. The resulting yellow oil was taken up in chloroform, and this was likewise removed *in vacuo* to rid the product of any trace amounts of ethyl acetate. Product was produced as a yellow foam (17.90 g, 86% yield): ^{1}H NMR ($CDCl_3$) δ 1.25 (3H, t, J = 7 Hz), 3.10 (2H, m), 4.1−4.7 (6H, m), 4.93 (2H, br s), 5.28 (1H, d, J = 8 Hz), 6.9 −7.8 (27H, m); ^{13}C NMR ($CDCl_3$) δ 14.1, 38.1, 47.3, 54.7, 61.6, 66.9, 70.1, 120.0, 125.0, 126.1, 126.8, 127.1, 127.6, 127.7, 129.7, 130.2, 134.2, 137.5, 141.4, 143.8, 144.8, 152.3, 155.4, 171.2.

4-[(N_{am}-Tritylsulfenyl)amidino]-L-phenylalanine, Ethyl Ester (30). 29 (17.90 g, 24.5 mmol) was dissolved in 200 mL of anhydrous DMF, and 40 mL of diethylamine was added. After 45 min the solvents were removed on the rotovap. The crude yellow oil remaining was triturated with hexanes (3 × 200 mL). A solid formed after the second trituration. After the third trituration, the solid was pumped on under high vacuum to remove any residual hexane yielding 10.64 g (85% yield) of product: ^{1}H NMR ($CDCl_3$) δ 1.25 (3H, t, J = 7 Hz), 1.51 (2H, br s), 2.83 (1H, q, J = 16, 8 Hz), 3.17 (1H, q, J = 16, 5.5 Hz), 3.68 (1H, q, J = 8, 5.5 Hz) 4.27 (2H, t, J = 7 Hz), 4.92 (2H, br s), 7.05−7.45 (19H, m); ^{13}C NMR ($CDCl_3$) δ 14.2, 40.9, 55.8, 61.0, 70.1, 126.1, 126.8, 127.7, 129.2, 130.2, 134.1, 139.2, 144.9, 152.4, 174.9.

***N*-α-Fmoc-4-[(N_{am}-tritylsulfenyl)amidino]-L-phenylalanine (31). 30** (10.13 g, 19.9 mmol) was taken up in 200 mL of dioxane, and this was added dropwise over 90 min to a solution of 120 mL of 0.5 M LiOH(aq) and 120 mL of dioxane. The reaction mixture was stirred for 30 min after addition was complete. Sodium bicarbonate (10.0 g, 120 mmol) was added, and the pH was checked and found to be 10.5. FmocOSu (6.70 g, 19.9 mmol) was added portionwise as a solid while the reaction mixture was being stirred. The pH was checked after addition was complete and found to be 8.5. The reaction mixture was stirred an additional 30 min, 140 mL of 1 M HCl was added, and the pH was checked and found to be 7. The solvents were removed *in vacuo*, and the crude product was partitioned between 400 mL of 20% 2-propanol/chloroform and 100 mL of 0.5 M $KHSO_4$. The organics were washed with water (2 × 200 mL), brine (100 mL) and dried briefly over $MgSO_4$. Filtration and concentration *in vacuo* yielded a yellow foam (15.99 g) which was taken up in 50 mL of chloroform and added dropwise to 600 mL of ether with stirring. The dropping funnel used was washed with an additional 10 mL of chloroform. The solid formed on addition was filtered and washed with 200 mL of ether, and residual solvent was removed under high vacuum to yield the product (12.41 g, 89% yield) as a white solid: ^{1}H NMR (1:1 $CD_3OD/CDCl_3$) δ 3.02 (1H, q, J = 16, 8 Hz), 3.22 (1H, q, J = 16, 5.5 Hz), 3.68 (1H, q, J = 8, 5.5 Hz) 4.05−4.50 (4H, m), 7.1−7.8 (27H, m); ^{13}C NMR (1:1 $CD_3OD/CDCl_3$) δ 37.4, 46.6, 54.9, 66.2, 74.2, 119.3, 124.4, 125.5, 126.5, 127.2, 127.4, 127.9, 129.0, 129.6, 140.8, 141.0, 143.2, 143.3, 144.3, 155.9, 169.1, 172.9.

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