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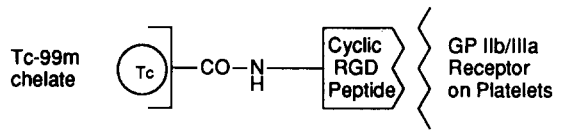
Tc-99m-LABELED FIBRINOGEN RECEPTOR ANTAGONISTS: DESIGN AND SYNTHESIS OF CYCLIC RGD PEPTIDES FOR THE DETECTION OF THROMBI

Thomas D. Harris,* Milind Rajopadhye,* Paul R. Damphousse, Danuta Glowacka, Karmine Yu, Jeffrey P. Bourque, John A. Barrett, David J. Damphousse, Stuart J. Heminway, Joel Lazewatsky, Theresa Mazaika, and Timothy R. Carroll.

Discovery, Radiopharmaceutical Division, The DuPont Merck Pharmaceutical Company, 331 Treble Cove Road, N. Billerica, MA 01862, USA

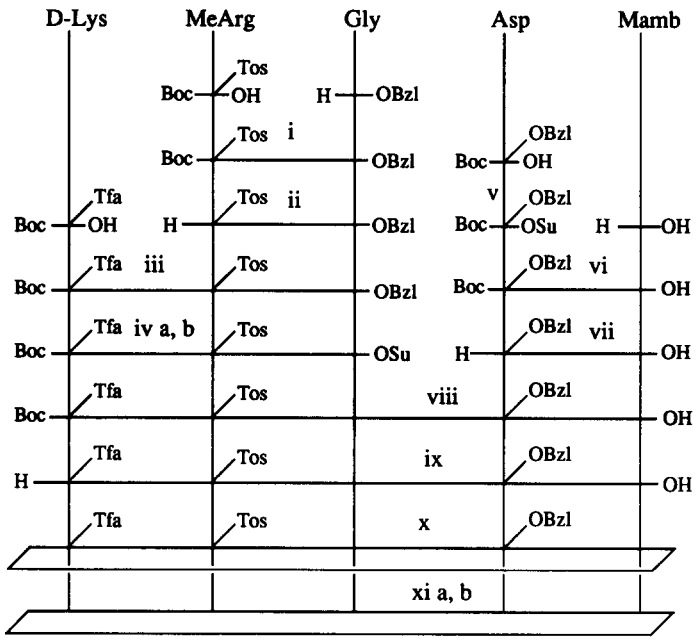
Abstract: Tc-99m labeled derivatives of N-Me-Arg-Gly-Asp containing cyclic peptide GP IIb/IIIa receptor antagonists are potential radiopharmaceuticals for the diagnosis of thrombosis. The design and synthesis of these peptides are described. These compounds are incorporated in rapidly growing thrombi under both arterial and venous conditions in canine models. Thrombi are clearly visible in images acquired at 50 min.

The unequivocal diagnosis,[1] treatment, and prevention of thromboembolic disease has gained considerable attention in the last few years. It has been reported that 33% of the patients in medical intensive care units experience deep vein thrombosis (DVT).[2] A thrombus is an intravascular deposit of fibrin, platelets and red blood cells. The binding site of ligands such as fibrinogen, fibronectin, vitronectin, and von Willebrand's factor to GP IIb/IIIa is the cell adhesive motif Arg-Gly-Asp (RGD). Small molecule antagonists of the GP IIb/IIIa receptor represent a rapidly growing class of potential antithrombotics.[3] Technetium-99m (Tc-99m, 6 h half-life, 140 KeV gamma) is the preferred radiolabel for diagnostic nuclear imaging.[4] Since the platelet GPIIb/IIIa receptor is expressed only on activated platelets intimately involved in thrombus formation, appropriately Tc-99m-labeled RGD-containing molecules that bind with high affinity should provide an approach for the detection and imaging of thrombi.[5] As shown in the drawing below, our approach considered the following aspects: (i) selection of a class of RGD peptides with high binding to GP IIb/IIIa; (ii) identification of a suitable site on the peptide for Tc-99m-labeling; and (iii) selection of a bifunctional chelator for Tc-99m.



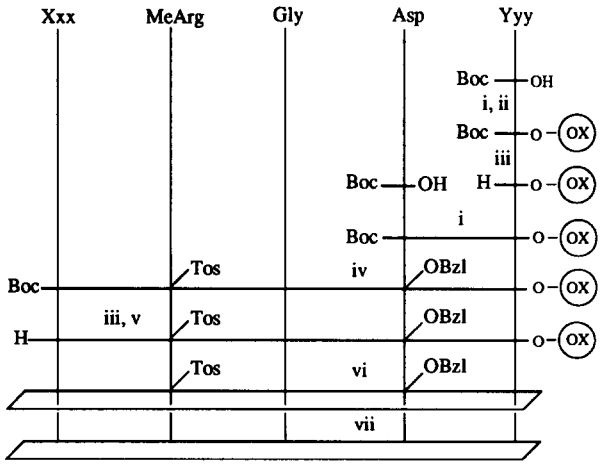
DeGrado and coworkers have described the discovery of a novel class of template constrained fibrinogen receptor antagonist such as DMP757 (*cyclo*(D-Val-NMe-Arg-Gly-Asp-Mamb)).[6] These compounds use a semi-rigid template comprising a D-amino acid and meta-aminomethylbenzoic acid (Mamb) to hold the RGD unit in the preferred conformation for binding. The high receptor affinity of DMP757 (IC_{50} = 6 nM, fibrinogen binding) makes this compound an attractive candidate for modification to design thrombus imaging agents. In this letter we describe the synthesis of "functionalized" cyclic RGD peptides and the evaluation of two sites in DMP757 for the introduction of the Tc-99m radiolabel via a Tc-chelator conjugate. Additionally, since I-125 is the radionuclide utilized in the detection of thrombi using iodinated fibrinogen,[1] we prepared two radioiodinated analogues for comparison with the Tc-99m-labeled compounds.

Scheme 1: Solution-Phase Synthesis of Peptide **2** Using Boc Chemistry



(i) HBTU, DIEA, DCM, 78%;
(ii) TFA, 100%;
(iii) HBTU, DIEA, DCM, 95%;
(iv) a, H_2, Pd/C, MeOH;
b, HOSu, DCC, DMF, 100%;
(v) HOSu, DCC, Dioxane, 73%;
(vi) DIEA, DMF, 92%;
(vii) 4N HCl/ Dioxane, 100%;
(viii) DIEA, DMF, 76%;
(ix) 4N HCl, Dioxane, 98%;
(x) TBTU, DIEA, DMF, 57%;
(xi) a, TFA, TFMSA;
b, 1M piperidine, 45%

Scheme 2: Solid-Phase Synthesis of Peptides **3** and **6** Using Boc Chemistry and Oxime Resin

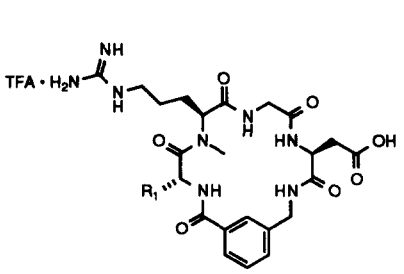


	Xxx	Yyy
3	D-(4-Cbz-NH)-Phe	Mamb
6	D-Val	Mamb(ε–Cbz-Ahx)

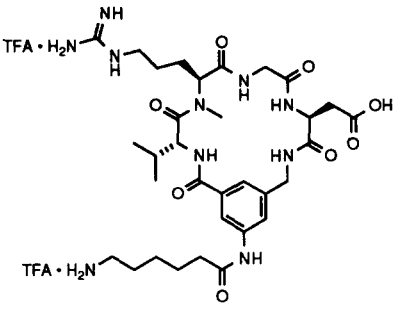
(i) HBTU, DIEA, DMF
(ii) Trimethylacetyl chloride, DIEA, DMF
(iii) 25% TFA/DCM
(iv) Further cycles of deprotection and coupling as in steps iii & i
(v) DIEA, DCM
(vi) HOAc, DMF
(vii) TFA, TFMSA, anisole

Synthesis of the cyclic peptides:[7] The two sites chosen for introduction of the radiolabels were the side chain of the D-amino acid (R_1 below), and the position on the Mamb ring meta to both the carbonyl and aminomethyl groups. In both cases, we elected to use a spacer group terminating in an amine for conjugation to technetium chelators. For example, this was provided by replacement of D-Val in DMP757 with D-Lys (peptide **2**).[8] Peptide **2** was prepared in solution using Boc chemistry, as shown in Scheme 1. Peptide **2** was

conjugated to 6-aminohexanoic acid (Ahx) to test the effect of an additional spacer (peptide **5**) between the peptide and the Tc-99m chelate, and was derivatized with the Bolton-Hunter reagent for radioiodination (peptide **4**). Other analogues synthesized for radioiodination included D-Tyr^8 (peptide **1**), and D-4-NH_2-Phe (peptide **3**), which was also capable of conjugation to technetium chelators. Exploring the second option, we felt that peptide **6**, having the Ahx spacer on the Mamb ring, held the most potential as a thrombus imaging agent because the spacer and Tc-99m complex would be held further from the NMe-Arg-Gly-Asp binding moiety. Peptides **3** and **6** were synthesized by solid phase synthesis on oxime resin[6a] using Boc chemistry, as shown in Scheme 2. Synthesis of the required Mamb unit is shown in Scheme 3.



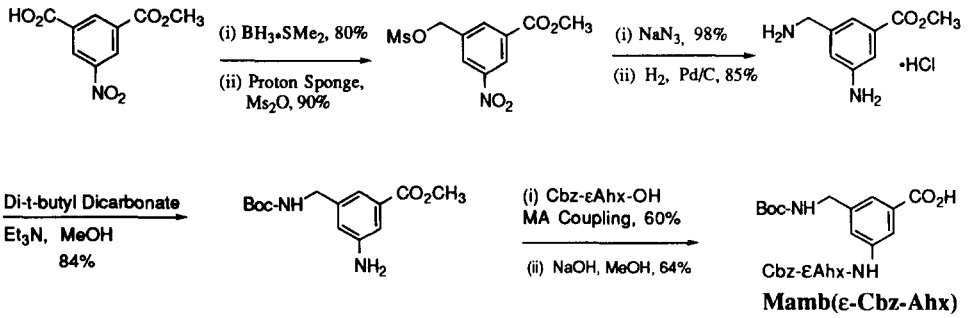
DMP757, **1-5, 7, 8**



6

DMP757 R_1 = iPr; **1** R_1 = -CH_2-(p-OH-C_6H_4); **2** R_1 = -$(CH_2)_4$-NH_2•TFA; **3** R_1 = -CH_2-(p-NH_2-C_6H_4)•TFA; **4** R_1 = -$(CH_2)_4$-NH-CO-$(CH_2)_2$-(p-OH-C_6H_4); **5** R_1 = -$(CH_2)_4$-NH-CO-$(CH_2)_5$-NH_2•TFA; **7** R_1 = -CH_2-(p-OH-C_6H_3-I-125); **8** R_1 = -$(CH_2)_4$-NH-CO-$(CH_2)_2$-(p-OH-C_6H_3-I-125).

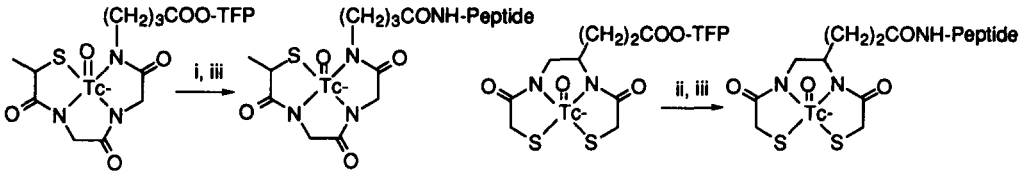
Scheme 3: Synthesis of Linker Mamb(ε-Cbz-Ahx) for Peptide **6**



Mamb(ε-Cbz-Ahx)

Selection of bifunctional chelators: The preformed chelate method of radiolabeling involves the formation of a Tc-99m-chelator complex and the conjugation of this complex to the targeting biological moiety in a separate step at the tracer level. The synthesis and coordination chemistry of the preformed Tc-99m chelates MeMAG$_2$GABA[9a] (**9**[9b]) and MAPT[9a] (**12**[9b-d]) have previously been described in detail. As illustrated below, Tc-99m labeled peptides **10** and **11** were prepared from Tc-99m complex **9** and RGD peptides **2** and **6**, respectively. Similarly, reaction of RGD peptides **2**, **5**, and **6** with Tc-99m complex **12** gave **13**, **14**, and **15**, respectively. All the products were purified by HPLC (>94% pure), the volatiles removed, and diluted to 300 μCi/mL with 0.9% saline for biological evaluation.[9b,10a] Reaction of **12** with peptide **3**

gave a low yield (unreacted **12** present) of the desired product because of the poor nucleophilicity of the aniline-type amine group. Peptides **1** and **4** were iodinated[11] with I-125 to give the iodinated species **7** and **8**, respectively.

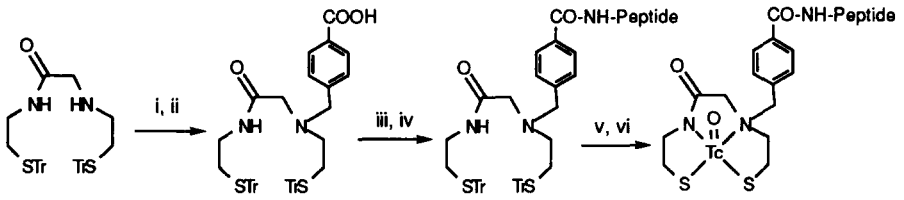


9 **10** (Peptide = **2**) **12** **13** (Peptide = **2**)
11 (Peptide = **6**) **14** (Peptide = **5**)
15 (Peptide = **6**)

(i) Peptides **2** or **6**. (ii) Peptides **2**, **5**, or **6**. (iii) 0.5 M phosphate buffer, pH 9.5-10, 40 °C, 30 min.

The MeMAG2GABA and MAPT complexes described above are anionic complexes of moderate lipophilicity. We have also examined the AADT[9a] ligand, which gives rise to neutral and more lipophilic technetium complexes, to determine what effect the charge and lipophilicity of these peptide conjugates have on their imaging properties. The synthesis of the AADT ligand and conjugation to benzyl mesylates has been described.[12] Using this methodology the functionalized AADT ligand (**16**) in Scheme 4 was prepared and conjugated to peptide **6** to yield **17**, which was radiolabeled[13] with Tc-99m using Tc-99m-glucoheptonate[10a] to give neutral, lipophilic complex **18**. The reverse phase HPLC[10b] retention time of **18** was 20.4 min vs 15.5 min and 16.2 min for complexes **11** and **15**, respectively.

Scheme 4: Synthesis of AADT chelator and conjugation to peptide **6**



AADT **16** **17** **18** (Peptide = **6**)

(i) 4-Carbomethoxybenzyl mesylate, 1,2-dichloroethane, 60%; (ii) 1N NaOH, dioxane, 100%; (iii) HOSu, EDC, 90%; (iv) Peptide **6**, DMF, TEA, 53%; (v) TFA; (vi) Tc-99m-glucoheptonate, 66%

Comparison of Thrombus uptake of Tc-99m and I-125 Labeled Compounds in Canine Thrombus Models:[14] All of the labeled peptides showed good uptake and excellent thrombus/blood ratios under platelet rich arterial conditions. Even under platelet poor venous conditions I-125 labeled peptides **7** and **8**, and Tc-99m labeled peptides **11** and **15** showed moderate to good uptake and good thrombus/blood ratios. The Tc-99m derivatives of peptides **2** and **5** had poor uptake. These data clearly show the superiority of Tc-99m imaging agents in which the technetium chelator is attached to the Mamb ring via the Ahx tether. This is especially evident when comparing the data for compound pairs **10/11**, and **13/15**. Chelators on the

Ahx tether are further removed from the RGD binding region of the peptide than are those on the D-amino acid side chain, and appear to be better tolerated by the receptor. This is presumably due to a combination of steric and electronic effects. These factors are especially evident in the data for peptides derivatized off the side chain of the D-amino acid. Iodinated peptide **8**, having an additional spacer between the peptide and the phenol, shows nearly twice the uptake of peptide **7** under venous conditions, and both of these peptides show much higher uptake than the larger, anionic Tc-99m complexes of peptides **2** and **5**. However, Tc-99m complex **14**, with an additional spacer on lysine, showed very little improvement in venous uptake compared to complex **13**. Replacing the anionic complexes of peptides **11** and **15** with the neutral, more lipophilic AADT complex (as in **18**) led to a reduction in venous uptake.

Table: Comparison of Uptake (% ID/g) of Tc-99m Peptides in the Canine Arteriovenous Shunt Model[10a,15]

		Venous Conditions (V)		Arterial Conditions (A)		
Peptide	Labeled Compound	Uptake	Thrombus/Blood	Uptake	Thrombus/Blood	n
1	**7**	0.25 ± 0.15	19.26 ± 8.96	1.81 ± 0.18	173.40 ± 22.18	5
2	**8**	0.45 ± 0.11	7.82 ± 2.59	2.60 ± 0.01	43.88 ± 4.29	2
2	**10**	0.05 ± 0.02	7.04 ± 4.44	1.28 ± 0.43	158.24 ± 4.69	2
6	**11**	0.21 ± 0.05	6.68 ± 0.39	5.41 ± 0.70	195.20 ± 38.56	A = 4, V = 3
2	**13**	0.04 ± 0.01	12.58 ± 3.27	0.47 ± 0.12	146.67 ± 43.66	3
5	**14**	0.06 ± 0.03	4.00 ± 1.72	1.60 ± 0.12	112.93 ± 1.42	2
6	**15**	0.58 ± 0.22	13.16 ± 4.06	5.75 ± 1.28	141.91 ± 24.43	4
6	**18**	0.16 ± 0.02	7.01 ± 0.58	5.00 ± 0.51	220.87 ± 15.83	2

Tc-99m complex **15** had the highest uptake and was further evaluated in the canine DVT model.[15b] Serial images were acquired using a gamma camera every 5 min for 2 h. The images below demonstrate that Tc-99m labeled conjugate **15** was actively incorporated into the two growing thrombi with images clearly detectable. By 50 min post-injection, thrombus/blood and thrombus/muscle ratios (ROI) were 6-8:1.[14]

In conclusion, the Tc-99m labeled analogues of DMP757 described above, especially those substituted off of the Mamb ring, bind to the GP IIb/IIIa receptor on platelets activated during thrombus formation and are potential radiopharmaceuticals for diagnosing the presence of thrombi by imaging.

Figure 1: Canine Deep Vein Thrombosis Model, Compound **15**

10 min post-injection 50 min post-injection 100 min post-injection

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7. The peptides were purified by reverse phase preparative HPLC and isolated by freeze drying. The analytical HPLC method used a Vydac C_{18} column (4.6 mm x 25 cm) at a flow rate of 1.0 mL/min.; a gradient mobile phase from 98% A (0.1% TFA in water) to 100% B (0.1% TFA in 90% acetonitrile) at 45 min was used. UV detection was set at 220 nm. Purity was greater than 95% (% peak area). HRMS for the peptides was consistent with theoretical calculations.
8. (a) DeGrado, W. F; Mousa, S. A.; Sworin, M.; Barrett, J. A.; Edwards, D. S.; Harris, T. D.; Rajopadhye, M.; Liu, S.; WO 9422494 A1; MARPAT 123:199405; CAPLUS 1995:767392. (b) Peptide **1** was provided by Sharon Jackson and William DeGrado, DuPont Merck.
9. (a) $MeMAG_2GABA$: methyl-mercaptoacetylglycylglycyl-γ-aminobutyric acid; MAPT: bis-mercaptoacetylpentanoate; AADT: amine amide dithiols.[13a] (b) Liu, S.; Edwards, D. S.; Looby, R. J.; Poirier, M. J.; Rajopadhye, M.; Bourque, J. P.; Carroll, T. R. *Bioconj. Chem.* **1996**, *7*, 196. (c) Fritzberg, A. R.; Abrams, P. G.; Beaumier, P. L.; Kasina, S.; Morgan, A. C.; Rao, T. N.; Reno, J. M.; Sanderson, J. A.; Srinivasan, A.; Wilbur, D. S. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 4025. (d) Rao, T. N.; Adhikesavalu, D.; Camerman, A.; Fritzberg, A. R. *J. Am. Chem. Soc.* **1990**, *112*, 5798.
10. (a) Presented at the 1994 Society of Nuclear Medicine meeting. Harris, T. D.; Barrett, J. A.; Bourque, J. P.; Carroll, T. R.; Damphousse, P. R.; Edwards, D. S.; Glowacka, D.; Liu, S.; Looby, R. J.; Poirier, M. J.; Rajopadhye, M.; Yu, K. *J. Nucl. Med.* **1994**, *35*, 245P. (b) 4.6 x 250 mm Vydac C18 column, 1.0 mL/min flow rate, linear gradient from 0% B to 30% B at 15 min to 75% B at 25 min. Solvent A = 10 mM phosphate, pH 6, Solvent B = acetonitrile.
11. Iodination (I-125) of **1** and **4** were performed by DuPont Medical Products, Boston.
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13. For Tc-labeling of AADT systems see also: (a) Mahmood, A.; Wolff, J. A.; Davison, A.; Jones, A. G. in *Technetium and Rhenium in Chemistry and Nuclear Medicine*; Nicolini, M.; Bandoli, G.; Mazzi, U. Eds.; SGE ditoriali: Padova, 1995; pp 211-214. (b) Kung, H. F.; Bradshaw, J. E.; Chumpradit, S.; Zuang, Z. P.; Kung, M. P.; Mu, M.; Frederick, D. *Ibid.*, 1995; pp 293-298.
14. Both femoral arteries and femoral veins of anesthetized adult beagle dogs were cannulated with silicon treated (Sigmacote®), saline filled polyethylene tubing to form extra corporeal arteriovenous shunts (A-V). An occlusive thrombus was formed by the introduction of a thrombogenic surface (4-0 braided silk thread, 5 cm) into one shunt with the other serving as a control. A 1 h shunt period was employed with the test agent administered intravenously as an infusion over 5 min beginning 5 min before insertion of the thrombogenic surface. At the end of the 1 h shunt period the silk was removed, weighed and the uptake (% ID/g) determined via well counting. The thrombus consisted of a platelet rich head on the thrombogenic surface (arterial conditions) and a fibrin rich tail (venous conditions). These were separated and individually counted (see table).
15. (a) Barrett, J.; Edwards, D. S.; Harris, T.; Rajopadhye, M.; Lazewatsky, J.; Liu, S.; Damphousse, D.; Heminway, S.; Mazaika, T.; Thomas, J.; Carroll, T.; Smith, J. *Ibid.* , 1995; pp 275-280. (b) For a detailed description of the thrombus models, see Barrett, J. A.; Damphousse, D. J.; Heminway, S. J.; Liu, S.; Edwards, D. S.; Looby, R. J.; Carroll, T. R. *Bioconj. Chem.* **1996**, *7*, 203.

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