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SYNTHESIS, EVALUATION AND Tc-99m COMPLEXATION OF A HYDRAZINONICOTINYL CONJUGATE OF A GP IIb/IIIa ANTAGONIST CYCLIC PEPTIDE FOR THE DETECTION OF DEEP VEIN THROMBOSIS

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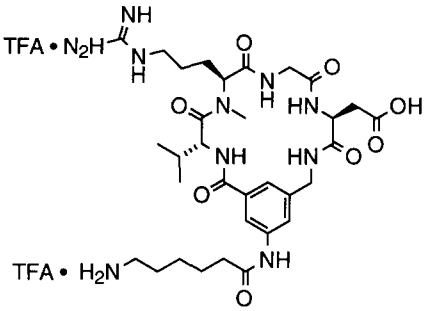
Abstract: A cyclic peptide GP IIb/IIIa receptor antagonist containing the N-Me-Arg-Gly-Asp motif has been derivatized with the technetium chelating hydrazinonicotinyl group (Hynic). The Hynic derivative, and the Tc-99 diazenido complex, retain the high receptor affinity of the parent peptide. The Tc-99m complex shows high thrombus uptake, and rapid clearance of background, producing excellent images in under 1 h.

The incidence of deep vein thrombosis (DVT) in medical intensive care unit patients is reported to be as high as 33 of 100 patients.[1] Current methods of detecting DVT in the clinic include venography, fibrinogen scanning, impedence plethysmography, duplex ultrasonography, and Doppler blood flow studies. But these have inadequacies, and unequivocal diagnosis[2] of DVT is still a difficult task.[3] Mechanism based incorporation of a radiopharmaceutical in a growing thrombus is perhaps the most logical approach for their detection. For example, in iodine-125 labeled fibrinogen scanning, the fibrinogen binds, via an Arg-Gly-Asp (RGD) motif, to the GP IIb/IIIa receptor on platelets during thrombus formation. However, this procedure requires about 24 h for detection, and I-125 is not a suitable radionuclide for diagnostic imaging. Appropriately technetium-99m-labeled (Tc-99m, 6 h half-life, 140 KeV gamma)[4] RGD-containing small molecules that bind with high affinity to activated platelet GP IIb/IIIa intimately involved in thrombus formation, have provided an approach for the detection and imaging of thrombi.[5,6]

We have previously communicated the synthesis and thrombus uptake of several Tc-99m labeled cyclic peptides, such as **1** and **2**, based on DuPont Merck's **DMP757** (*cyclo*(D-Val-N-Me-Arg-Gly-Asp-Mamb)),[7] and prepared using N_2S_2 or N_3S chelators such as MAPT, $MeMAG_2GABA$, and AADT.[5,8] This earlier work evaluated two different sites for attachment of the technetium chelator: (i) the 6-aminohexanamide (6-Ahx) tether on the Mamb ring of peptide **1** (Mamb = meta-aminomethylbenzoic acid); (ii) the lysine side chain of peptide **2**. The data clearly demonstrated the superior imaging characteristics of labelled peptides having the technetium chelator attached to the 6-Ahx tethered analogue of **DMP757** (e.g., peptide **1**). Uptake at the growing thrombi was good even under the platelet poor venous conditions of the canine DVT model, and venous thrombi were clearly visible using a gamma camera in under 1 h.

In this Letter we wish to report the synthesis of 6-Ahx tethered GP IIb/IIIa receptor antagonist **1** conjugated to a hydrazinonicotinoyl[9a] (Hynic) group, which serves as a chelator for Tc-99m. The binding affinity of the conjugated peptides to the GP IIb/IIIa receptor is reported herein, along with the biodistribution and evaluation of thrombus uptake of the Tc-99m labeled complex of the Hynic-peptide.



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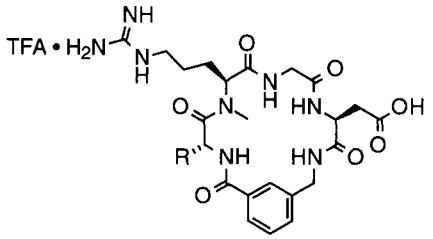
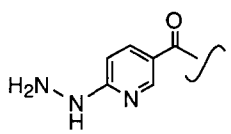
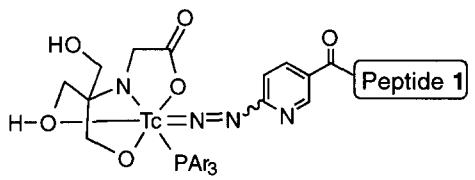


DMP757, R = *i*-Pr
2, R = -$(CH_2)_4$-NH_2 • TFA

High binding fibrinogen receptor antagonists inhibit clot formation, and if present in sufficient amount could result in pharmacological side effects undesirable in a diagnostic agent. Therefore it is essential that the minimum amount of the peptide-chelator conjugate be used in the preparation of the radiopharmaceutical kit. This entails the use of a chelator which can be complexed with Tc-99m in high specific activity. It is known that chelators such as MAPT, $MeMAG_2GABA$, and AADT require use of about 0.15 μmol of peptide-chelator conjugate for labeling with 25 mCi of Tc-99m to >90% radiochemical purity (RCP).[8] Administration of this amount of a high affinity fibrinogen receptor antagonist is likely to produce a pharmacological response. The Hynic group, on the other hand, can be rapidly labeled with 25 mCi of Tc-99m to >90% RCP using 0.02 μmol of the peptide-Hynic conjugate.[9b] The resulting technetium-diazenido complexes have been utilized in the Tc-99m labeling of polyclonal IgG,[9c,d] derivatives of the natural chemotactic peptide fMLF,[9e] tumor specific monoclonal antibody fragments,[9f] and fragment E_1 for imaging thrombi.[9g] Another attractive feature of the Hynic chelator is the ability to modify the pharmacokinetics of the complexes by changing the ancillary ligands.[9b] The Hynic group therefore appeared to us to be an ideal technetium chelator for the development of a thrombus imaging agent, and we herein report the extension of our earlier work to the synthesis and evaluation of Hynic-conjugated cyclic RGD peptide **5**, which, as Tc-99m ternary ligand complex **RP444**, shown below, is the biologically active piece in a thrombus imaging agent currently in early clinical trials.

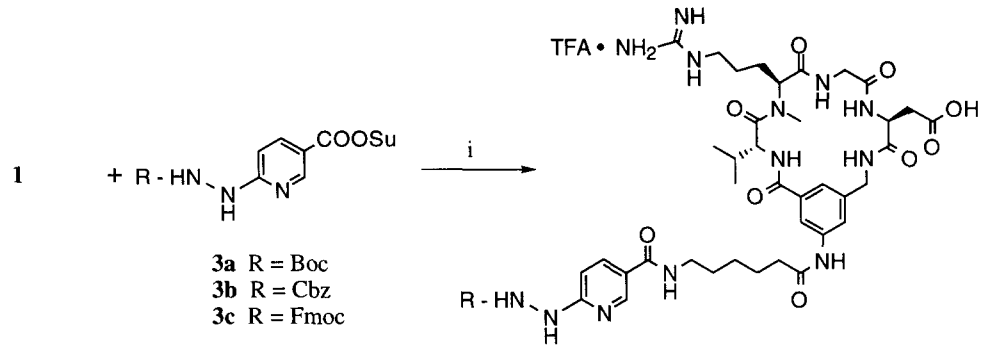


Hynic group



RP444, Ar = 3-sulfonatophenyl

Preparation of Peptide-Hynic Conjugates: Reaction of peptide **1** with Boc-Hynic-OSu[9a] (**3a**) in DMF in the presence of triethylamine at room temperature gave Boc-Hynic-peptide conjugate **4a** in 80% yield after work up.[10] Deprotection of **4a** was accomplished using either TFA-anisole 95:5, or HBr/acetic acid.[9a,f] Preparative HPLC gave purified Hynic conjugate **5** in low yield.[10] The conditions of deprotection must be carefully controlled to obtain a clean product, and we therefore examined alternative protection for Hynic which can be removed under non-acidic conditions. Cbz-Hynic-OSu (**3b**) was prepared in two steps from Hynic by



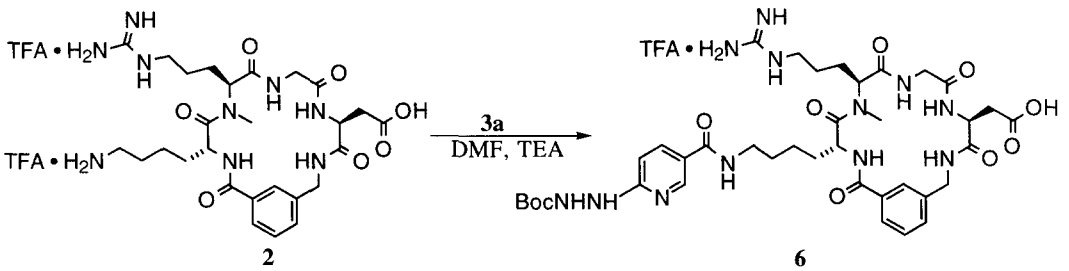
(i) DMF, TEA, stir rt; prep. HPLC
(ii) 95% TFA, 5% Anisole, stir rt; prep. HPLC or HBr/HOAc, stir rt, prep. HPLC
(iii) H_2, $PdCl_2$, MeOH, 1 Atm; prep HPLC
(iv) Piperidine; prep. HPLC

4a R = Boc $\xrightarrow{ii}$ **5** R = H • TFA

4b R = Cbz $\xrightarrow{iii}$ **5** R = H • TFA

4c R = Fmoc $\xrightarrow{iv}$ **5** R = H • TFA

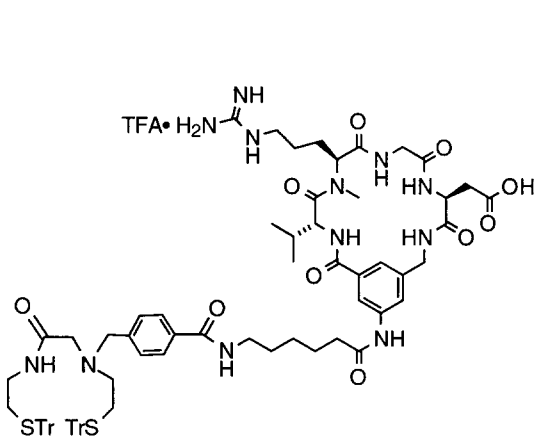
treatment with *N*-(benzyloxycarbonyloxy)succinimide in aqueous bicarbonate pH 8, followed by treatment with water soluble carbodiimide and *N*-hydroxysuccinimide in DMF. Overall yield of **3b** was 59% after purification by flash chromatography. Conjugation with peptide **1** and HPLC purification[10] gave a 67% yield of **4b**. Catalytic hydrogenation of **4b** gave Hynic conjugate **5** in near quantitative yield, but the crude purity of 85% (by HPLC) was equivalent to that obtained by starting with Boc protected conjugate **4a**. We also briefly examined the use of Fmoc-Hynic-OSu (**3c**), but dropped this approach due to low yields in the formation of conjugate **4c**, and in the deprotection step. Thus, despite its limitations, **3a** remains the preferred reagent of the three investigated for preparing Hynic-labeled peptides. Hynic conjugate **6**, derived from lysine peptide **2**, was prepared in order to allow a comparison of the two derivatization sites of **DMP757** in the Hynic conjugate series. Preparative HPLC gave purified **6** in 64% yield.[10]



2 **6**

IC_{50} Data of Peptide-Chelator Conjugates: The utility of these RGD peptide-Hynic conjugates as thrombus imaging agents is determined in part by their affinity for the GP IIb/IIIa receptor. In our design of Hynic-peptide conjugate **5** we have been successful in retaining the GP IIb/IIIa binding activity of the non-functionalized cyclic RGD peptide **DMP757**, as shown by the IC_{50} data (determined using activated canine platelets) in Table 1. It is readily apparent that **DMP757** is very tolerant of substituents on the Mamb ring. The IC_{50} values change very little with the addition of a 6-Ahx tether, the protected, or unprotected form of Hynic.

The excellent receptor affinity of Hynic conjugate **6** and MAPT conjugate **7**[8b] demonstrate that substitution at the lysine side chain of peptide **2** is also well tolerated by the receptor. Our earlier finding that the Tc-99m complexes of MAPT conjugates **8** and **7** differ markedly in thrombus uptake,[5a] suggests that the lower thrombus uptake of the conjugates of lysine peptide **2** can be attributed to the pharmacokinetics of these complexes. Taken together these data confirm our earlier conclusion that the optimum site for derivatization of **DMP757** is on the Mamb ring. The IC_{50} value that will ultimately determine the binding to activated platelets is that of the technetium complex. Peptide **5** can be coordinated to Tc-99 using tricine and tris(3-sulfonatophenyl)phosphine (TPPTS) as ancillary ligands to give Tc-99-**RP444**, the ternary ligand complex [Tc-99(**5**)(tricine)(TPPTS)].[11] Tc-99-**RP444**, which has nearly twice the MW of Hynic conjugate **5**, also binds to the GP IIb/IIIa receptor with nearly the same affinity as the parent peptide. The poor binding of $AADT(Tr)_2$ conjugate **9** is presumably the result of the extreme lipophilicity of the two trityl protecting groups, since the Tc-99m complex of the detritylated chelator conjugate has moderate thrombus uptake as reported earlier by us.[5a]



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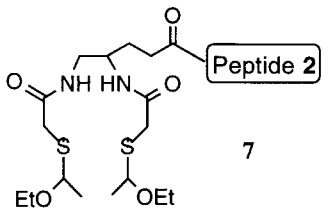

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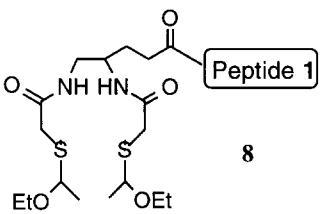

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Table 1: IC_{50} (nM) of Cyclic Peptides in GP IIb/IIIa Binding Assay[12]

Peptide	Type	IC_{50}(IIb/IIIa)	n
DMP757	Untethered Parent Peptide	6 ± 2	3
1	Peptide **1**	5 ± 1	3
10	Peptide **1**-Boc-Hynic	9	1
5	Peptide **1**-Hynic	8 ± 1	3
RP444	Peptide **1**-Hynic-Tc	13	3
8	Peptide **1**-MAPT	13	1
9	Peptide **1**-$AADT(Tr)_2$	>1000	1
7	Peptide **2**-MAPT	11	1
6	Peptide **2**-Boc-Hynic	16/11	1
RGDS	Standard	9600 ± 700	3

Biodistribution and Thrombus Imaging of Tc-99m Complex RP444 in the Canine Deep Vein Thrombosis (DVT) Model: The biodistribution of **RP444** (the Tc-99m ternary complex of **5**) in the canine DVT model[5b,11b] is shown in Table 2, and contrasted with the biodistribution of **RP419**, the Tc-99m complex

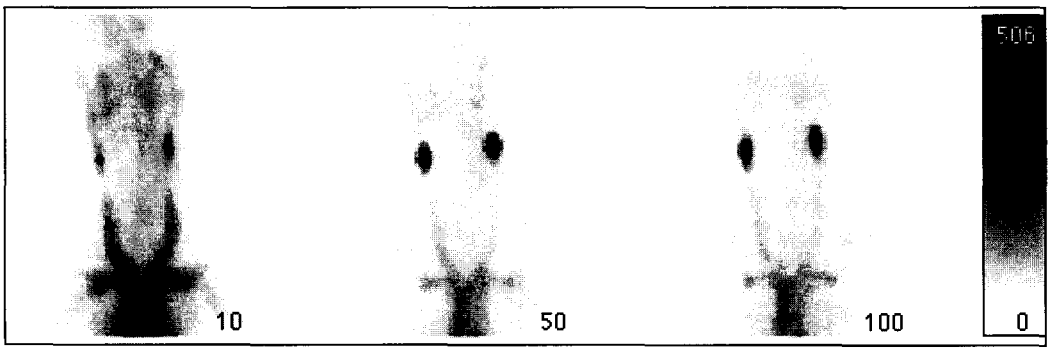
of **8**. These two complexes follow very different excretion pathways, with **RP444** being excreted primarily via the kidneys, and **RP419** being excreted primarily via the hepatobiliary pathway. Renal clearance is most desired in a thrombus imaging agent, as the reduced activity in the abdominal cavity increases the ability to detect pulmonary embolism. Both complexes show low uptake in the heart, general musculature, and lungs. **RP444** shows venous uptake of 0.54% ID/g in the canine AV shunt model,[5b,11b] which compares favorably with **RP419** (0.58% ID/g).[5]

Table 2: Biodistribution of Tc-99m Complexes **RP419** and **RP444** (Kit), %ID/g, 2 h Post-Injection

Complex	Liver	Kidney	Bile	Spleen	Lung	Heart
RP419 (n = 4)	0.038 ± 0.004	0.007 ± 0.001	2.498 ± 0.649	0.101 ± 0.010	0.021 ± 0.005	0.004 ± 0.001
RP444 (n = 4)	0.026 ± 0.001	0.088 ± 0.059	0.022 ± 0.009	0.191 ± 0.034	0.028 ± 0.004	0.007 ± 0.002

RP444 was evaluated in the canine DVT model, with serial images being acquired using a gamma camera every 5 min for 2 h. The images below demonstrate that **RP444** was actively incorporated into the two growing thrombi with thrombi first visible 10 min post-injection. By 50 min post-injection, thrombus/blood and thrombus/muscle ratios (ROI) were 9.20:1 and 12.77:1, respectively.

Figure 1: Canine Deep Vein Thrombosis Model, RP444



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

In conclusion, this work, and previous papers in the series, have demonstrated the ability of Tc-99m-labeled GP IIb/IIIa antagonists based on Mamb-functionalized cyclic peptide **DMP757** to rapidly detect growing thrombi under both mixed arterial and venous conditions. The present work has highlighted the specific advantages of Hynic-peptide conjugate **5**. These advantages include: (i) high affinity for the GP IIb/IIIa receptor; (ii) efficient coordination with Tc-99m at concentrations that do not affect hemodynamics, hematology, the coagulation cascade, and platelet function; (iii) rapid renal clearance from the blood; (iv) high thrombus uptake; and (v) excellent images as early as 50 min postinjection. Ternary ligand complex [Tc-99m(**5**)(tricine)(TPPTS)] (**RP-444**) is a developmental candidate currently in early clinical trials for the rapid diagnosis of thromboembolic events occuring under both arterial and venous conditions.

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10. The peptides were purified by reverse phase HPLC on a Vydac C_{18} column (21.2 x 250 mm) at a flow rate of 15 mL/min; linear mobile phase gradients consisting of 0.1% TFA in water (A) and 0.1% TFA in 90% acetonitrile, HPLC grade (B); UV detection was set at 220 nm. Products were isolated from eluant by freeze drying. The analytical HPLC method used a Vydac C_{18} column (4.6 x 250 mm) at a flow rate of 1.0 mL/min; a gradient mobile phase from 98% A to 100% B at 45 min was used. UV detection was set at 220 nm. Compound **3b**; post HPLC purity: 95%; HRMS-FAB: for $C_{18}H_{17}N_4O_6$ + H, *m/z* calc. 385.1148, found 385.1141. Compound **4a**; post HPLC purity: 100%; HRMS-FAB: for $C_{43}H_{63}N_{13}O_{11}$ + H, *m/z* calc. 938.4848, found 938.4849. Compound **4b**; post HPLC purity: 100%; HRMS-FAB: for $C_{45}H_{60}N_{13}O_{11}$ + H, *m/z* calc. 958.4535, found 958.4542. Compound **4c**; post HPLC purity: 100%; HRMS-FAB: for $C_{53}H_{65}N_{13}O_{11}$ + H, *m/z* calc. 1060.5005, found 1060.5001. Compound **5**; purity: 100%; HRMS-FAB: for $C_{38}H_{55}N_{13}O_9$ + H, *m/z* calc. 838.4324, found 838.4324. Compound **6**; post HPLC purity: 100%; HRMS-FAB: for $C_{38}H_{54}N_{12}O_{10}$ + H, *m/z* calc. 839.4164, found 839.4153.
11. (a) For the coordination chemistry of this reaction and confirmation of structure, see Edwards, D. S.; Liu, S.; Looby, R. J.; Harris, A. R. *J. Nucl. Med.* **1996**, *37*, 29P. (b) Barrett, J. A.; Damphousse, D.; Crocker, A.; Mazaika, T.; Bresnick, M.; Kagan, M.; Lazewatsky, J.; Edwards, D. S.; Liu, S.; Harris, T.; Rajopadhye, M.; Carroll, T. *J. Nucl. Med.* **1996**, *37*, 129P.
12. Binding of I-125-fibrinogen to platelets was performed as described by Plow, E. F.; Marguerie, G.; Ginsberg, M. *Biochem. Pharmacol.* **1987**, *36*, 4035. All concentrations of test article in each assay were run in triplicate. Percent inhibition of I-125-fibrinogen binding to activated platelets was calculated by dividing the specific binding (total binding - nonspecific binding) obtained in the presence of inhibitors by that obtained in the absence of the inhibitors. IC_{50} values were calculated by fitting the percent inhibition values to a regression line.

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