Bromination and Subsequent Catalytic Tritiation of Thienylalanine and 4-Methyltyrosine Residues in the Bradykinin Analog RMP-7

*Alan Akiyama, Gary F. Musso, Janet L. Smart, Christopher Lang and Julie Ann Straub**

Alkermes, Inc., 64 Sidney Street, Cambridge, MA 02139, U.S.A.

SUMMARY

A two step strategy was devised for the synthesis of ^{3}H-RMP-7 for use in pharmacokinetic studies. First, RMP-7 was brominated predominantly on the thiophene ring of thienylalanine using Br_2 in AcOH. Then, reductive tritiation of the brominated RMP-7 using $^{3}H_2$ and Pd/C yielded ^{3}H-RMP-7 (specific activity 1 Ci/mmol; 96% radiochemical purity). An HPLC based assay using on-line radioactivity detection was developed for *in vivo* pharmacokinetic studies of ^{3}H-RMP-7. Rapid clearance from the blood and metabolism of RMP-7 to *des*-Arg^{1}-RMP-7 was observed *in vivo* in rats.

Key words: RMP-7, thienylalanine, tritiation, metabolism

INTRODUCTION

RMP-7 is currently being evaluated as a therapeutic for use in facilitating entry of drugs into the brain and brain tumors for the treatment of a number of CNS diseases (1). RMP-7, a nonapeptide analog of bradykinin, was modified to increase selectivity for the bradykinin B_2 receptor and to decrease the likelihood of enzymatic degradation (2-3). Bradykinin is metabolized in the lungs by angiotensin converting enzyme, and in plasma by carboxypeptidases (4). The structure of RMP-7 (H-Arg-Pro-Hyp-Gly-Thi-4-MeTyrΨ(CH_2NH)Arg-OH) contains a reduced dipeptide isostere, 4-MeTyrΨ(CH_2NH)Arg for increased stability to those enzymes (5). As part of the pre-clinical development effort for RMP-7, we studied the *in vivo* metabolism and biodistribution of RMP-7. Radiolabelled RMP-7 and an assay capable of distinguishing RMP-7 from its metabolites were required for these studies.

Literature reports indicated that the aromatic ring components of thienylalanine and 4-methyltyrosine could be brominated using Br_2 (6-7), and that the bromininated amino acids could be catalytically hydrogenated to restore the hydrogen to these amino acids (8-9). This paper describes the application of this synthetic strategy to prepare ^{3}H-RMP-7, labelled primarily on thienylalanine, and development of an HPLC assay to study the *in vivo* metabolism of ^{3}H-RMP-7 in rats.

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RESULTS AND DISCUSSION

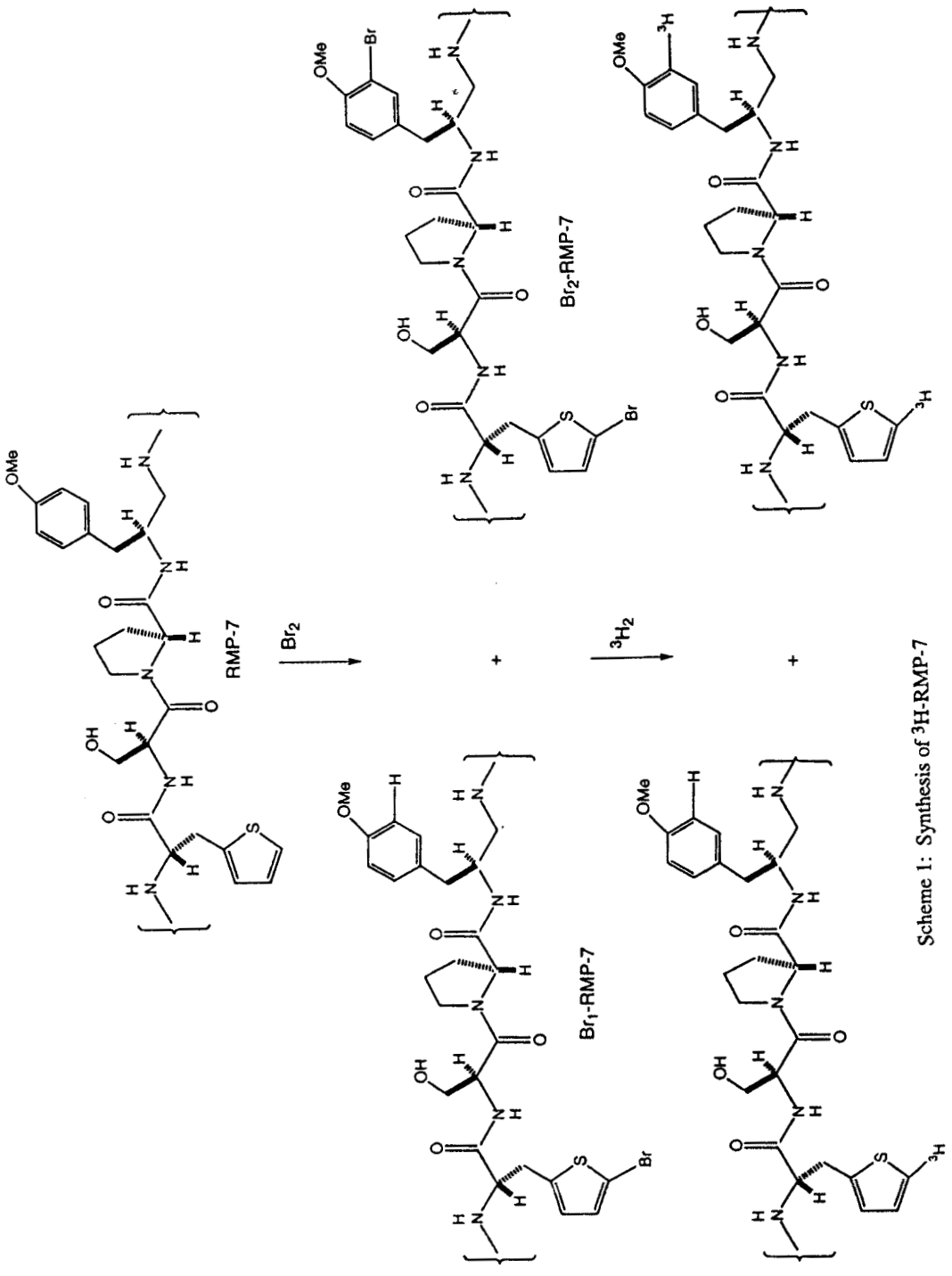
RMP-7 contains two aromatic amino acid side chains (thiophene and a methoxyphenyl) that could be utilized for radiolabelling by halogenation, followed by reductive tritiation. Preliminary studies of this chemistry were performed using the amino acids thienylalanine (contains a thiophene ring) and 4-methyltyrosine (contains a methoxyphenyl ring) as models. Initially, bromination conditions were developed using a 1:1 mixture of D,L-thienylalanine and L-4-methyl-tyrosine with varying quantities of Br_2. The extent of the reaction was monitored by ^1H NMR spectroscopy. Bromination proceeded rapidly on the thienylalanine to give 5-bromo-thienylalanine. Bromination occurred more slowly on the methyl-tyrosine to yield 3-bromo-4-methyl-tyrosine. In the reverse direction, hydrogenation of the brominated compounds proceeded to completion.

The synthetic route to ^3H-RMP-7 is shown in Scheme 1. Two types of bromination conditions were developed for RMP-7. One method used one equivalent of Br_2, and resulted in a mixture of mono-bromo-Thi-RMP-7 (Br_1-RMP-7; 78%) and RMP-7 (22%) as determined by HPLC analysis (Method #1 retention times: 19.8 min (RMP-7); 23.1 min (Br_1-RMP-7); 25.7 min (Br_2-RMP-7)). As the presence of non-brominated RMP-7 (^1H-RMP-7) would only slightly reduce specific activity of ^3H-RMP-7, no attempt to separate the RMP-7 from Br_1-RMP-7 was made. A second method (Scheme 1) used 10 equivalents of Br_2, and yielded a mixture (Br_m-RMP-7) consisting of bromo-Thi-bromo-Me-Tyr-RMP-7 (Br_2-RMP-7; 37%) and mono-bromo-Thi-RMP-7 (Br_1-RMP-7; 63%) as determined by HPLC analysis (Method # 2 retention times: 14.4 min (RMP-7); 17.7 (Br_1-RMP-7)). Attempts at increasing the level of bromine incorporation onto the 4-methyltyrosine were unsuccessful due to decomposition of the peptide upon extended exposure to the bromination conditions. ^1H NMR analysis confirmed that the bromination occurred on RMP-7 on the 5-position of the thiophene ring of Thi and on one of the meta positions of the phenyl ring of Me-Tyr. Hydrogenation (H_2 with Pd/C) of Br_m-RMP-7 to ^1H-RMP-7 was demonstrated, indicating that reductive tritiation was likely to be successful.

Reductive tritiation of Br_m-RMP-7 was performed by Amersham Corporation (Arlington Heights, IL) on 13 mg of brominated RMP-7, which resulted in 22 mCi of crude radioactivity. The crude ^3H-RMP-7 was first purified by C_{18} Sep-Pak®, and then by HPLC using a system capable of separating Br_1-RMP-7 and Br_2-RMP-7 from RMP-7 itself.

The final solution of ^3H-RMP-7 was found to have a radioactive concentration of 0.11 μCi/μL (1.3 mCi total). The UV absorbance at 273 nm of the same solution was 0.150, which corresponded to a concentration of 0.115 mg RMP-7/mL (1.38 mg, 11% yield based on starting quantity of Br_m-RMP-7), or 1.05×10^{-4} mmol/mL. The specific activity of the tritiated sample was therefore 1.0 Ci/mmol. The specific activity of ^3H-RMP-7 was lower than anticipated with, on average, only one in thirty molecules labelled with tritium. The reasons for such low specific activity are unclear, although it can be speculated that the water present as solvent in the reaction exchanged ^1H for ^3H on the catalyst. The yield based on Br_m-RMP-7 was low as a result of losses that occurred during HPLC purification associated with emphasis on radiochemical purity rather than total yield.

HPLC analysis of the tritiated RMP-7 using an HPLC system capable of resolving RMP-7 from brominated derivatives (Figure 1) showed a single retained UV adsorbing component with a retention time equivalent to that of ^1H-RMP-7. The radiochemical purity of the material based on radioactivity associated with RMP-7 HPLC peak was 96%. Thin layer chromatography of the tritiated



Scheme 1: Synthesis of ^{3}H-RMP-7

RMP-7, visualized both with fluorescamine and by auto-radiography also revealed that the majority of the radioactivity in the ^{3}H-RMP-7 preparation co-eluted with ^{1}H-RMP-7.

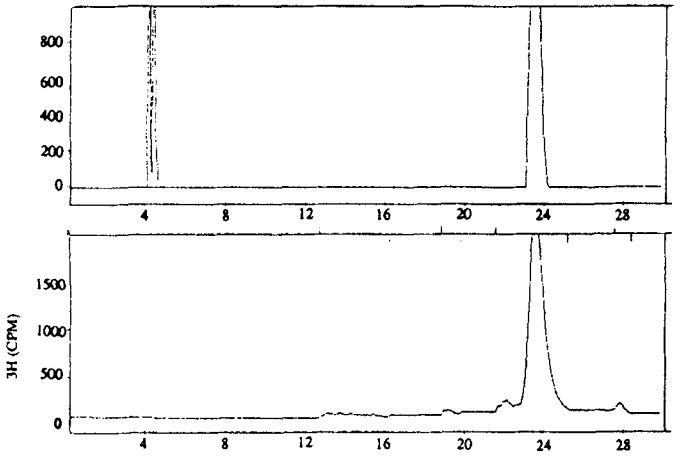


Figure 1: HPLC profile of a co-mix of ^{3}H-RMP-7 and ^{1}H-RMP-7 (HPLC Method #5). The upper chromatogram shows 210 nm detection. The lower chromatogram shows ^{3}H cpm detection.

Pronase digestion of ^{3}H-RMP-7 followed by HPLC analysis showed the expected HPLC pattern based on UV detection (HPLC Method #4 ret. time = 9 min. for the thienylalanine, 26 min. for the C-terminal tetrapeptide which contains the 4-methyltyrosine) (3). The radioactivity associated with these two peaks was determined (91% associated with thienylalanine, 9% associated with Me-Tyr). The difference in the location of the bromine in the starting material and the location of the tritium in the product could be due to incomplete reduction of the bromine on the 4-methyltyrosine in Br_m-RMP-7. Any remaining material that was mono-brominated on the Me-Tyr portion of the molecule would have been removed in the purification.

Based on the analytical data, the ^{3}H-RMP-7 produced from the multi-brominated RMP-7 yielded material suitable for the *in vivo* studies. *In vitro* metabolism studies indicated the potential production of *des*-Arg^{1}-RMP-7 and the C-terminal tetrapeptide (Ser-Pro-4-MeTyrΨ(CH_2NH)Arg) as metabolites (5). As ^{3}H-RMP-7 was labelled on the thienylalanine portion of the molecule, any *des*-Arg^{1}-RMP-7 generated from ^{3}H-RMP-7 would also be radioactive. Therefore, HPLC Method #5 was developed to separate ^{3}H-RMP-7 from ^{3}H-*des*-Arg-RMP-7. In addition, a method for stopping the *in vitro* metabolism of RMP-7 by blood was developed that allowed for storage of pharmacokinetic samples prior to analysis (data not shown). Typical chromatograms that were generated in a pharmacokinetic study in rats are seen in Figure 2. *Des*-Arg^{1}-RMP-7 was found to be generated *in vivo* in rats (Table 1). RMP-7 was cleared from blood rapidly ($t_{1/2}$ < 1 min.).

RMP-7 was designed to be protected from carboxypeptidases and angiotensin converting enzyme. *In vitro* assays showed that RMP-7 was significantly less susceptible to angiotensin converting enzyme than bradykinin (5). However, the structural modifications incorporated into RMP-7 did not protect RMP-7 from enzymes that cleave the amino-terminus of the molecule *in vivo*. The study presented here also indicated that distribution as well as metabolism was important.

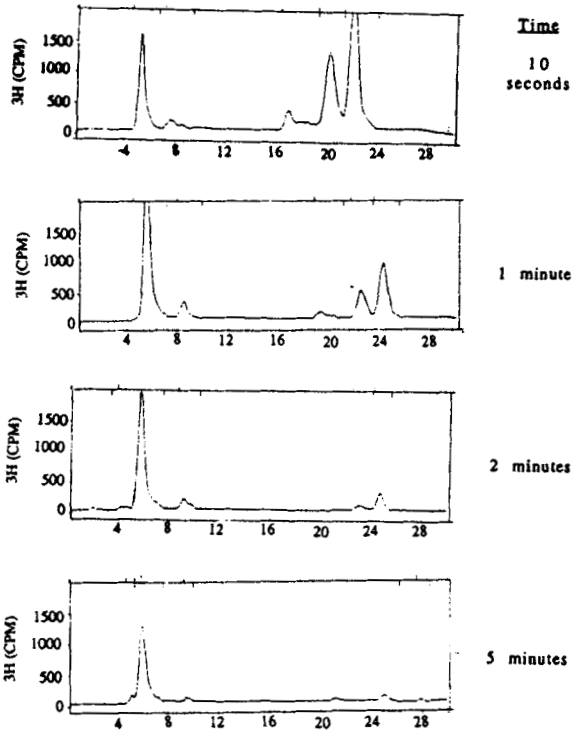


Figure 2: HPLC study of *in vivo* rat metabolism (HPLC Method #5). The x-axes are time (min), and the y axes are radioactivity (in cpm).

Table 1: ^{3}H-RMP-7 and ^{3}H-*des*-Arg$_1$-RMP-7 as a Percentage of the Radioactivity Found in Blood

Time After Injection (min)	Percent RMP-7	Percent *des*-Arg^1-RMP-7	N
0.17	31.2 ± 5.5	38.1 ± 2.8	3
1.00	15.9 ± 5.0	23.5 ± 4.5	4
2.00	8.0 ± 1.8	15.6 ± 4.0	4
5.00	3.8 ± 3.9	8.6 ± 6.7	3

EXPERIMENTAL

Materials

RMP-7 triacetate salt was prepared for Alkermes, Inc. by Peninsula Laboratories, Inc. (Belmont, CA). Bromine and palladium on carbon were purchased from Aldrich Chemical Co. (Milwaukee, WI). 4-Methyl-tyrosine and thienylalanine were purchased from Sigma Chemical Co. (St. Louis, MO) and Bachem California (Torrance, CA), respectively. Pronase from *Streptomyces griseus* was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). EN^{3}Hance®

was from NEN Research Products (Boston, MA). Hydrofluor from National Diagnostics (Manville, NJ) was used for scintillation counting. Palladium on carbon (10% palladium content) and tritium gas were used by Amersham Laboratories (Arlington Heights, IL) in the catalytic halogen displacement reaction. C_{18} Sep-Pak® cartridges were from Waters Associates (Millford, MA). TLC plates (silica gel 60-F254, pre-coated, 5 x 10 cm) were purchased from EM Science (Cherry Hill, NJ).

General Equipment

Scintillation counting was performed on a Beckman LS5000TA scintillation counter. Nuclear magnetic resonance spectra were acquired with either a Bruker AM300 (Harvard University, Cambridge, MA), a Bruker AM400 (Harvard University, Cambridge, MA), or a Bruker AM600 (National Center for NMR Applications, Colorado State University, Fort Collins, CO). Elemental analysis was performed by Oneida Research Services (Whitesboro, NY). Amino acid analysis was performed by Analytical Biotechnology Services (Boston, MA).

HPLC Assay Methods

HPLC Method #1: Hewlett-Packard 1090M system; a Vydac C_{18} column (4.6 mm x 250 mm); Eluant A (0.1% TFA in water); Eluant B (0.065% TFA in acetonitrile); gradient: 10% B to 40% B over 30 minutes; 1 mL/min flow rate; 210 nm detection.

HPLC Method # 2: Beckman HPLC system; Waters μBondapak® 10 C_{18} column (3.9mm x 300mm); Eluant A (0.1% TFA in water); Eluant B (0.065% TFA in acetonitrile); gradient: 10% B to 40% B over 30 minutes; 1.2 mL/min flow rate; 210 nm detection.

HPLC Method # 3: Beckman HPLC system; Phenomenex Bondclone® C18 column (3.9 mm x 300 mm) with a Phenomenex Bondclone® 10 C18 guard column (3.9 x 30 mm); Eluant A (0.1% TFA in water); Eluant B (acetonitrile); gradient: 15% B to 30% B over 40 minutes; 1 mL/min flow rate; 210 nm detection.

HPLC Method # 4: Beckman HPLC system; Phenomenex Bondclone® C18 column (3.9 mm x 300 mm) with a Phenomenex Bondclone® 10 C18 guard column (3.9 x 30 mm); Eluant A (0.1M sodium perchlorate/0.1% phosphoric acid (85%), in water, pH 2.50); Eluant B (acetonitrile); gradient of 0% B to 40% B over 40 minutes; 1 mL/min flow rate; 210 nm detection.

HPLC Method # 5: Beckman HPLC system with three 110B pumps (two for the elution gradient, one for the scintillant, which was Beckman Ready Flow III flowing through the radioactivity detector at a rate of 3 mL/min) and a 171 radioactivity detector; Phenomenex Bondclone® C18 column (3.9 mm x 300 mm) with a Phenomenex Bondclone® 10 C18 guard column (3.9 x 30 mm); Eluant A (0.1% TFA in water); Eluant B (0.1% TFA in water-acetonitrile (60-40)); gradient of 35% B to 70% B over 30 minutes; 1 mL/min flow rate; 210 nm was monitored along with ^3H cpm.

Synthesis of 5-Br-Thienylalanine and 3-Br-4-Me-Tyrosine

Bromine (128 μL) was added to glacial acetic acid (4.87 mL). Thienylalanine (21.4 mg, 0.125 mmol) and 4-methyl-tyrosine (24.4 mg, 0.125 mmol) were dissolved in 5 mL glacial acetic acid, and distributed into 5 equal (1 mL; 0.025 mmols of each amino acid) portions in 10 mL amber vials. The bromine/HOAc solution was added to each of the five vials in increasing amounts (50 μL, 100 μL, 150 μL, 200 μL and 250 μL; 0.025 mmol, 0.05 mmol, 0.075 mmol, 0.10 mmol and 0.125 mmol, respectively). The samples were kept at room temperature overnight; then transferred to 5 mL round

bottom flasks and the solvent removed *in vacuo*. The solid residues were redissolved in deuterium oxide for NMR analysis. The solutions were then frozen and lyophilized for use in hydrogenation studies.

Br-Thienylalanine: ^{1}H NMR (D_2O) δ 6.92 (d, 1H, C4 on thiophene ring), 6.70 (d, 1H, C3 on thiophene ring), 3.94 (dd, 1H, C_α), 3.34 (m, 2H, C_β); TLC (silica, n-BuOH : AcOH : H_2O 12 : 3 : 5) R_f = 0.53.

3-Br, 4-Me-Tyrosine: ^{1}H NMR (D_2O) δ 7.40 (s, 1H, C_{ortho} on phenyl ring, α to Br), 7.12 (d, 1H, C_{ortho} on phenyl ring, γ to Br), 6.95 (d, 2H, C_{meta} on phenyl ring), 3.95 (dd, 1H, C_α), 3.77 (s, 3H, CH_3O), 3.03 (ddd, 2H, C_β); TLC (silica, n-BuOH : AcOH : H_2O 12 : 3 : 5) R_f = 0.53.

Hydrogenation of 5-Br-Thienylalanine and 3-Br-4-Me-Tyrosine

A 1:1 mixture of bromo-thienylalanine and bromo-methyltyrosine was dissolved in water (2 mL) at a concentration of 0.5 mg/mL (2.7 μmol of bromo-thienylalanine, 2.7 μmol of bromo-methyltyrosine). Palladium on carbon (5% palladium content, 0.8 mg; 0.38 μmol of Pd) was added. The vessel was stirred under an atmosphere of hydrogen (1 atm) for 1 hour. The reaction mixture was filtered through Celite and the filtrate centrifuged at 16,000 x g for 5 minutes. The supernatant was lyophilized, dissolved in deuterium oxide and analyzed by NMR (only signals from thienylalanine and methyltyrosine were observed).

Thienylalanine: ^{1}H NMR (D_2O) δ 7.25 (d, 1H, C5 on thiophene ring), 6.88 (m, 2H, C3 + C4 on thiophene ring), 3.94 (dd, 1H, C_α), 3.34 (m, 2H, C_β); TLC (silica, n-BuOH : AcOH : H_2O 12 : 3 : 5) R_f = 0.43.

Me-tyrosine: ^{1}H NMR (D_2O) δ 7.12 (d, 2H, C_{ortho} on phenyl ring), 6.85 (d, 2H, C_{meta} on phenyl ring), 3.95 (dd, 1H, C_α), 3.70 (s, 3H, CH_3O), 3.03 (ddd, 2H, C_β); TLC (silica, n-BuOH : AcOH : H_2O 12 : 3 : 5) R_f = 0.43.

Mono-brominated RMP-7

Bromine (64 μL; 1.24 mmol) was added to glacial acetic acid (2.436 mL) resulting in a solution of Br_2 at 0.5 M. RMP-7 (42 mg peptide, 38.5 μmol) was dissolved in glacial acetic acid (6 mL), and the bromine/acetic acid solution (75.4 μL; 37.7 μmol) was added. The reaction mixture was stirred at room temperature for 30 minutes. The solvent was removed *in vacuo*. The solid residue was dissolved in 5 mL water (15 μL removed for HPLC analysis by HPLC Method #1) and lyophilized, yielding 50.4 mg of a white solid. HPLC analysis indicated that the material was 78% mono-brominated RMP-7 (Br_1-RMP-7) and 22% non-brominated RMP-7. Based on nitrogen content (by elemental analysis) and the HPLC-derived value for the ratio of Br_1-RMP-7 and RMP-7, the percent peptide (anhydrous free base, excludes weight of bound acetate and water) was determined to be 77.3% (39.0 mg peptide; 30.4 mg Br-RMP-7 and 8.6 mg RMP-7). FAB-MS showed two molecular ions: 1177 (major, Br_1-RMP-7) and 1097 (minor, RMP-7). Amino acid analysis yielded the following: 1 x Arg, 2 x Pro, 1 x Hyp, 1 x Gly, 0.46 x Thi, 1 x Ser. The method used for amino acid analysis could not detect Br-Thi and H-4-Me-TyrΨ(CH_2NH)-ArgOH. The following are the NMR assignments for resonances observed in the aromatic region of Br_1-RMP-7: ^{1}H NMR (D_2O) δ 7.05 (d, 2H, C_{ortho} on methyltyrosine ring), 6.85 (d, 1H, C4 on thiophene ring), 6.80 (d, 2H, C_{meta} on methyltyrosine ring), and 6.55 (d, 1H, C3 on thiophene ring).

Multi-brominated RMP-7

Bromine (128 μL) was added to 4.87 mL of glacial acetic acid in an amber vial, resulting in a 0.5 M solution of Br_2. RMP-7 (8.3 mg peptide, 7.6 μmol) was dissolved in 2 mL glacial acetic acid, the bromine/acetic acid solution (156 μL, 78 μmol) was added, and the reaction mixture was allowed to stand at room temperature for 45 minutes. A 25 μL aliquot was analyzed by HPLC (Method #2). The remainder of the solution was transferred to a 5 mL round bottom flask and the solvent removed *in vacuo*. A second batch of RMP-7 (4 mg peptide) was brominated using the same protocol, and the product combined with the material from the bromination of 8.3 mg of RMP-7. The combined material was dissolved in 5 mL of water and lyophilized yielding 13 mg of an off-white powder. HPLC analysis indicated that the material was 37% mono-brominated RMP-7 (Br_1-RMP-7) and 63% di-brominated RMP-7 (Br_2-RMP-7). No attempt to separate the Br_1-RMP-7 from Br_2-RMP-7 was made.

NMR data for aromatic region of Br_m-RMP-7 had resonances for both Br_1-RMP-7 and Br_2-RMP-7 . Bromo-thienylalanine resonances (aromatic region): ^1H NMR (D_2O) δ 6.82 (d, C4 on thiophene ring), 6.55 (d, C3 on thiophene ring). Bromo-methyltyrosine resonances (aromatic region): ^1H NMR (D_2O) δ 7.33 (s, C_{ortho} on phenyl ring, α to Br), 7.10 (d, C_{ortho} on phenyl ring, γ to Br), 6.93 (d, C_{meta} on phenyl ring). Methyltyrosine (aromatic region): ^1H NMR (D_2O) ∂ 7.05 (d, C_{ortho} on phenyl ring), 6.82 (d, C_{meta} on phenyl ring).

Hydrogenation of Multi-brominated-RMP-7

Br_m-RMP-7 (2.4 mg; 2.2 μmol) was dissolved in 2 mL of water. Palladium on carbon (3.8 mg of 5% Pd/C; 1.8 μmol Pd) was added. The reaction mixture was treated with H_2 (1 atm) for 2 h. The catalyst was removed by centrifugation and the supernatant analyzed by HPLC (Method #1). As the reaction was not complete, a new aliquot of 3.8 mg of 5% Pd/C (1.8 μmol Pd) was added. The resulting mixture was hydrogenated for an additional 2.5 h. The Pd/C was removed by centrifugation, and the supernatant analyzed by HPLC (Method #2), which indicated a complete reaction.

Synthesis of 3H-RMP-7

The multi-brominated RMP-7 was tritiated by catalytic halogen displacement at Amersham Corporation (Arlington Heights, IL). Br_1-RMP-7 (13 mg) was dissolved in water and placed in a 4 mL glass vessel. The catalyst of 10% Pd/C (10 mg) was added to the solution and the vessel sealed. The vessel was flushed with tritium gas, then kept under an atmosphere of tritium gas (1 atm, 10 Ci) for 4 hours. The catalyst and any remaining labile tritium gas were removed and the reaction solution then lyophilized to dryness several times. The solid residue was redissolved (2 x 11 mCi samples, each in 1 mL of 2% ethanol in water) and sent to Alkermes, Inc.

Purification of 3H-RMP-7

The crude ^3H-RMP-7 was initially purified by C_{18} Sep-Pak®. Each 11 mCi sample was purified separately. The sample was applied to a pre-conditioned C_{18} Sep-Pak® and eluted using a series of step gradients, collecting the flow-through from each eluant as a fraction. The eluants consisted of the following mixtures of 0.1% aqueous TFA with acetonitrile (Acn) : 15 mL of TFA (fraction 1), 15 mL of 95:5 TFA-Acn (fraction 2), 15 mL 90:10 TFA-Acn (fraction 3), 15 mL of 89:11 TFA-Acn (fraction 4), 15 mL of 88:12 TFA-Acn (fraction 5), 15 mL of 88:12 TFA-Acn (fraction 6),

15 mL of 87:13 TFA-Acn (fraction 7), and 10 mL of Acn (fraction 8). The fractions of ^{3}H-RMP-7 from the Sep-Pak® runs were analyzed by TLC (n-butanol:pyridine:acetic acid:water, 5:5:1:4, v/v). The TLC plates were spotted with 1 µL of sample, eluted, allowed to dry (3 h), sprayed with EN^{3}HANCE®, allowed to dry again. X-ray film was exposed to the plates for 24 hours (-80°C), and then developed. Based on the TLC results, fractions 3 - 5 were used for further purifications. Each of these fractions was lyophilized and redissolved in 1 mL of ethanol:water (2:98, v/v) for further processing.

The material was then purified by HPLC (Method #3) in 11 separate batch purifications (0.25 to 0.6 mL of sample solution per batch). Fractions were collected over 1 minute intervals, and analyzed by liquid scintillation counting. The bulk of the radioactivity was associated with a single HPLC peak with a retention time equivalent to the retention time for RMP-7, and those fractions collected from the earliest eluting portion of this peak were combined, lyophilized to dryness and redissolved in water:ethanol (1:1, v/v, 1 mL per batch purification sample). The isolates from all of the batch purifications were then combined to yield a total of 1.31 mCi in 12 mL of water-ethanol.

Characterization of 3H-RMP-7

Purified ^{3}H-RMP-7 was characterized with respect to its specific activity (based on dpm/mL and UV absorbance/mL at 273 nm), HPLC chromatographic properties (Method #5), and by TLC (n-butanol:pyridine: acetic acid:water, 5:5:1:4, v/v, fluorescamine visualization and autoradiography). For autoradiography of the TLC plate, the plate was lightly sprayed with EN^{3}HANCE and exposed to X-ray film for 24 hours at -80°C.

Pronase digestion to assess label position

A solution of pronase was prepared by dissolving pronase solid (7 mg) in buffer (1400 µL; 0.2M Tris, 5 mM calcium chloride, pH 7.5). Pronase solution (400 µL), and ^{3}H-RMP-7 (11.5 µg, 100 µL) were mixed, and incubated at 37°C overnight. An aliquot (50 µL) was analyzed by HPLC (Method #4). Fractions were collected at 1 minute intervals and aliquots were counted to determine the radioactivity associated with thienylalanine and the C-terminal tetrapeptide (contains the 4-methyltyrosine portion of the molecule).

Metabolism of 3H-RMP-7 in Rats

An aliquot of the purified ^{3}H-RMP-7 containing 2.4 x 10^{7} dpm was dried in a Speed-Vac, and then reconstituted with 1 mL sterile saline. Unlabelled RMP-7 (101 µL of 2 mg/mL in saline) and additional saline (699 µL) were added to the solution of ^{3}H-RMP-7. The total concentration of RMP-7 (^{3}H-labelled and unlabelled) was 0.12 mg/mL, at 1.3 x 10^{7} dpm/mL. Young adult female Sprague-Dawley rats (125-175 g, from Harlan Sprague Dawley, Inc. (Indianapolis, IN)) were used in the study. The dosing solution containing ^{3}H-RMP-7 was administered intravenously to the animals via a lateral tail vein. The RMP-7 dose was 0.4 mg/kg and the dose volume was 3.2 mL/kg. Animals were anesthetized with chloral hydrate before administration of RMP-7. Following dosing with RMP-7, blood was collected at the following times: 10 seconds and 1, 2, and 5 minutes after injection. One mL of heparinized whole blood was collected from each rat and added immediately to 1.5 mL of ethanol to quench the metabolic activity of the blood. This mixture was centrifuged at 10,000 x g for

10 minutes, after which the supernatant was transferred to another container and 30 µL of 1 N HCl was added to adjust the pH of the solution to 3.5. The sample was then frozen at -80°C until assayed by HPLC Method #5.

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