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Synthesis of Conjugates of Muramyl Dipeptide and nor-Muramyl Dipeptide with Retro-Tuftsin (Arg-Pro-Lys-ThrOMe) as Potential Immunostimulants

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The synthesis of retro-tuftsin analogue of sequence Arg-Pro-Lys-ThrOMe (Scheme 1) and it's conjugates containing MDP (muramyl dipeptide) or nor-MDP (nor-muramyl dipeptide) (Scheme 2) are described.

Key words: muramyl dipeptide, MDP, nor-muramyl dipeptide, nor-MDP, retro-tuftsin, tuftsin, synthesis, immunomodulators

Tuftsin, a natural substance of sequence Thr-Lys-Pro-Arg was found in the γ-globulin fraction of the blood of humans and other mammals. It is a constituent of the CH2 domain of the F_c chain of immunoglobulin G (IgG) as a fragment 289–292. Tuftsin was isolated at Tufts University in 1970 by Najjar and Nishioka [1]. It is released from the protein carrier by the action of two specific enzymes: splenic tuftsin endocarboxypeptidase, that splits a Arg^{292}–Glu^{293} peptide bond of leucokinin, and phagocyte membrane enzyme – leucokininase, that clears Lys^{288}–Thr^{289} peptide bond [2,3]. Tuftsin is capable of potentiating granulocyte and macrophage functions such as phagocytosis, motility, somnogenic response, as well as bactericidal and tumoricidal activity [1,4–6]. However, the instability of tuftsin in plasma reduces its efficacy. Therefore, much effort has been undertaken on discover analogues which are more resistant to degradation. Numerous derivatives and analogues of tuftsin have been synthesized and their biological activities were investigated in various laboratories [4–10]. One of tuftsin analogues is Arg-Pro-Lys-Thr with the retro sequence of tuftsin synthesized by Nozaki *et al*. [11]. This tetrapeptide was examined for phagocytosis-stimulating activity [12], however, the obtained data were inconsistent. Yasumura *et al*. [13] reported for it the same activity as tuftsin, whereas other investigators could not observe any activity at all [12].

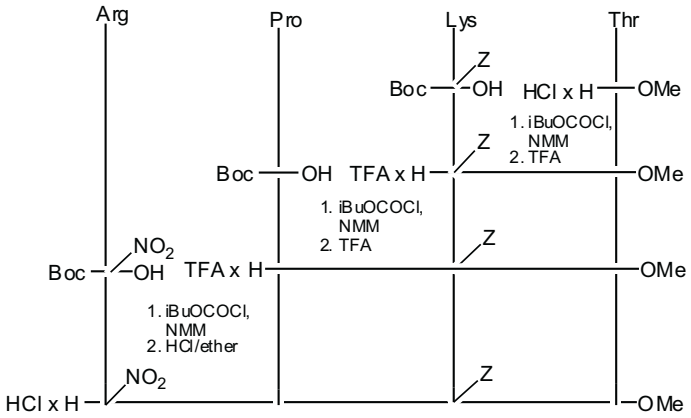
MDP (muramyl dipeptide), a basic unit of the bacterial cell wall peptidoglycan directly derived from the microbial world, is capable of exerting an immunoadjuvant effect on the animal organism. Some muramyl dipeptide derivatives, like murabutide, and lipopeptides, like pimelautide, are under clinical trials [14–17] as immunomodulators and adjuvants. MDP and its analogues have been shown to act synergistically with the several antibiotics, cytostatics and other natural immunomodulators (trehalose dimycolate, cytokines, lipopolysaccharide, Lipid A) [18–20]. In our opinion the strong immunostimulating activity of MDP and its synergistic effects in combination with

some medicines may not only improve their pharmacological properties but also increase the self-defense of the patient organism. It can concern also tuftsin, which is already widely used in immunotherapy protocols aiming at treating patients with immunodeficiencies, cancer and rheumatoid arthritis [6].

In the recent paper [21] we described the synthesis and biological activities of six new conjugates of MDP or nor-MDP with tuftsin. Biological activity of these conjugates was assayed using *in vitro* cultures of human monocytes and lymphocytes. The compounds displayed cytotoxic effects, as was revealed in performed viability tests. It was most probably mediated by induction of the oxidative burst in monocytes and stimulation of redox enzymes in lymphocytes. The conjugates turned out to be also efficient stimulators of TNFα and IL6 secretion by monocytes and lymphocytes. The beneficial properties of the examined compounds (mainly Mur(*N*Ac)-Ala-D-Glu(Thr-Lys-Pro-Arg-OH)-NH_2, Mur(*N*Ac)-Val-D-Glu(Thr-Lys-Pro-Arg-OH)-NH_2 and Thr-Lys-Pro-Arg(NO_2)-OH), which imply their usefulness as potential therapeutic agents, are connected with their quick start of action and more efficient effects comparing to tuftsin. Continuing our studies on syntheses of MDP and nor-MDP conjugates I present the syntheses of MDP and nor-MDP analogues modified at the C-terminus of the muramyl peptide residue with retro-tuftsin (Arg-Pro-Lys-ThrOMe) by the formation of an amide bond between the isoglutamine carboxylic group of MDP and the amine group of the arginine of retro-tuftsin (Arg-Pro-Lys-ThrOMe). In this paper I also describe synthesis of retro-tuftsin analogue of Arg-Pro-Lys-ThrOMe. The results of biological tests of these conjugates and their comparison with muramyl dipeptide and retro-tuftsin will be reported in due course.

RESULTS AND DISCUSSION

The retro-tuftsin analogues (Arg-Pro-Lys-ThrOMe) was synthesized by the conventional chemical procedure using mixed anhydride procedure (Scheme 1). Details of the synthesis are summarized in Table 1. The starting materials of Boc-Lys(Z)-OH, ThrOMe·HCl, Boc-Pro and Boc-Arg(NO_2)-OH were obtained according to standard procedures used in peptide chemistry. For the synthesis of dipeptide Boc-Lys(Z)-ThrOMe **1**, tripeptide Boc-Pro-Lys(Z)-ThrOMe **2** and tetrapeptide Boc-Arg(NO_2)-Pro-Lys(Z)-Thr-OMe **3** the classical mixed anhydride method with isobutyl chloroformate and *N*-methylmorpholine (NMM) in dry DMF was chosen. Reaction was carried out at –15°C for 4 h and then at room temperature for 24 h giving satisfying yields of the respective peptides (Table 1). In each step of the peptide elongation, *t*-butoxycarbonyl amino acids were used as the acylating reagent except in the final stages of the elongation. The *t*-butoxycarbonyl (Boc) group was removed by treatment with trifluoroacetic acid (TFA) prior to the next coupling. The reactions were monitored with TLC (in solvent A, D, E, H or I). After purification of the crude peptide by column chromatography (Silica Gel 60 230–400 mesh ASTM, 0.040–0.063 mm) using chloroform-methanol (4:1), crystalline tetrapeptide **3** was obtained in yield 64%.



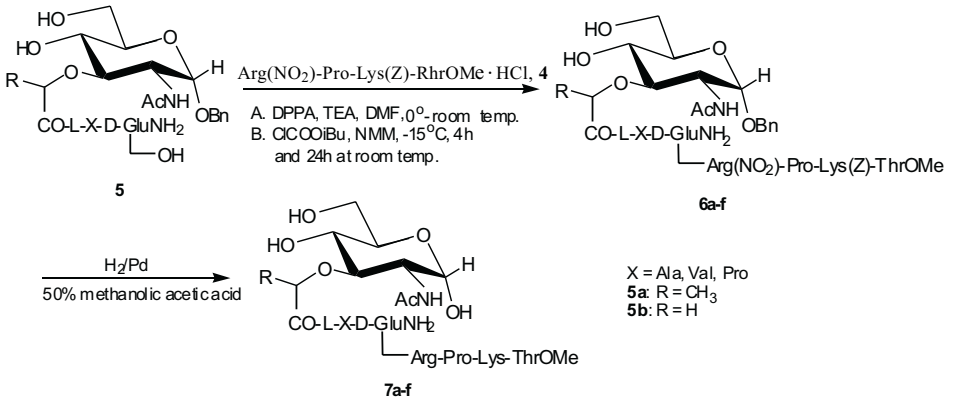
Scheme 1. Preparation of H-Arg(NO_2)-Pro-Lys(Z)-ThrOMe· HCl **4**.

Table 1. Steps of synthesis of tetrapeptide Boc-Arg(NO_2)-Pro-Lys(Z)-Thr-OMe **3** and Arg(NO_2)-Pro-Lys(Z)-ThrOMe·HCl **4**.

Substrates	Products	Yield [%]	M.p. [°C]	$[\alpha]_{546}$ (MeOH)
Boc-Lys(Z)-OH and ThrOMe·HCl	Boc-Lys(Z)-ThrOMe, **1** [26]	76	oil	–14° (*c* 1.0)
TFA·Lys(Z)-ThrOMe and Boc-Pro	Boc-Pro-Lys(Z)-ThrOMe, **2**	73	oil	–48° (*c* 1.0)
TFA·Pro-Lys(Z)-ThrOMe and Boc-Arg(NO_2)OH	Boc-Arg(NO_2)-Pro-Lys(Z)-ThrOMe, **3**	64	86–89	–47.9° (*c* 0.84)
Boc-Arg(NO_2)-Pro-Lys(Z)-ThrOMe, **3**	Arg(NO_2)-Pro-Lys(Z)-ThrOMe·HCl, **4**	93	oil	–

The amino acid composition of the protected tetrapeptide **3** was confirmed by the elemental analysis, analysis of ^{1}H and ^{13}C NMR (500 MHz) spectra and by the TLC qualitative amino acid analysis. The *t*-butoxycarbonyl group was removed with saturated solution of hydrogen chloride in diethyl ether to give tetrapeptide methyl ester **4**. Acylation of Arg amino group of protected retro-tuftsin H-Arg(NO_2)-Pro-Lys(Z)-Thr-OMe· HCl **4** by 1-benzyl-MDP **5a** or 1-benzyl-nor-MDP **5b** was performed according to the following procedures (Scheme 2): **A**. in dry DMF using DPPA as a coupling agent in the presence of TEA or **B**. mixed anhydride method. In the mixed anhydride method crude products were obtained in better yield 48–56% and contained less impurities. The protected conjugates **6a–f** were isolated by radial chromatography on plates covered with Kieselgel 60 PF_{254} and purified with a preparative TLC. The identity of the protected products was confirmed by high resolution ^{1}H NMR (500 MHz) spectroscopy. Identification of the signals corresponding to individual protons of peptide and carbohydrate moieties of the molecules was

Scheme 2



achieved by analysis of COSY, ROESY, NOESY and TOCSY spectra. The final products **7a–f** were hydrogenolysed with H_2/Pd in methanol-acetic acid (1:1), purified with preparative TLC with n-BuOH-AcOH-H_2O (4:2:2) as a solvent and were lyophilized to give hygroscopic solids. The identity of the products were confirmed by high resolution ^{1}H NMR (500 MHz) spectroscopy, TLC qualitative amino acid analysis, and elemental analyses.

EXPERIMENTAL

Melting points (uncorrected) were determined on the Kofler-block apparatus. ^{1}H-NMR spectra were measured in DMSO or $CDCl_3$ solutions with a Varian 500 and 200 NMR spectrometers. Preparative column chromatography and radial chromatography were performed on silica gel (Kieselgel 60, 100–200 mesh) in solvent systems specified in the text. All chemicals and solvents were of reagent grade and were used without further purification. The reactions were monitored by TLC on Merck F_{254} silica gel precoated plates. The following solvent systems (by vol.) were used for TLC, radial and column chromatography development: (A) n-BuOH-pyridine-AcOH-H_2O (60:45:4:30), (B) n-BuOH-pyridine-AcOH-H_2O (15:10:3:12), (C) 2-propanol-H_2O-AcOH (100:50:3), (D) n-BuOH-AcOH-H_2O (4:2:2), (E) n-BuOH-AcOH-H_2O (2:1:1), (F) $CHCl_3$-MeOH-AcOH (90:10:5), (G) $CHCl_3$-MeOH (30:1), (H) $CHCl_3$-MeOH (4:1), (I) $CHCl_3$-MeOH (9:1). All synthesized protected peptides were homogeneous on TLC. Qualitative amino acid analyses of the hydrolyzates of the compounds were accomplished on TLC. Detected by: UV and ninhydrin. The optical rotation values were measured on a Rudolph Research automatic polarimeter Autopol II.

The following abbreviations were applied: A – alanine, Bn – benzyl-, Boc – *tert*-butyloxycarbonyl-, DPPA – diphenyl azidophosphate, G – glucosamine, H – residue of the lactic or acetic acid, K – lysine, Me – methyl-, P – proline, Q – isoglutamine, R – arginine, T – threonine, TEA – triethylamine, TFA – trifluoroacetic acid, Z – benzyloxycarbonyl-, V – valine.

1-*O*-Benzyl-*N*-acetyl-muramyl-L-amino acid-D-isoglutamine **5a** and 1-*O*-benzyl-*N*-acetyl-nor-muramyl-L-amino acid-D-isoglutamine **5b** were prepared in our laboratory [22–25].

Boc-Arg(NO$_2$)-Pro-Lys(Z)-ThrOMe (3). To a stirred solution of Pro-Lys(Z)-ThrOMe trifluoroacetate (0.767 g, 1.267 mmole) in anhydrous DMF (4 ml) cooled to –15°C, NMM (0.14 ml, 1.267 mmole) and isobutyl chloroformate (0.16 ml, 1.267 mmole) were added, followed after 15 min. by an addition of

cool solution of Boc-Arg(NO_2)-OH (0.404 g, 1.267 mmole) in anhydrous DMF (2 ml) and the stirring was continued for 4 h in –15°C and then for 24 h at room temperature. After evaporation of the solvent, the product was dissolved in ethyl acetate and washed with the following cooled liquids: solution of hydrochloric acid (5%), water, solution of potassium hydrogencarbonate (0.5 N), water and saturated sodium chloride solution, and then dried with magnesium sulfate. After evaporation of ethyl acetate in a vacuum rotary evaporator the raw product was purified using column chromatography (Silica Gel 60 230-400 mesh ASTM, 0.040–0.063 mm) in solvent H to obtain compound **3** (Table 1). ^{1}H-NMR (DMSO-d_6) δ: 1.03 (d, J = 6.3 Hz, 3H, T-CH_3), 1.30 (m, 2H, K-γCH_2), 1.39 (m, 2H, K-δCH_2), 1.36 (s, 9H, t-Bu), 1.46 (m, 1H, R-βCH), 1.51 (m, 1H, K-βCH), 1.57 (m, 2H, R-γCH_2), 1.62 (m, 1H, R-βCH), 1.67 (m, 1H, K-βCH), 1.81 (m, 1H, P-βCH), 1.89 (m, 2H, R-γCH_2), 2.02 (m, 1H, P-βCH), 2.97 (q, J = 6.6 Hz, 2H, K-εCH_2), 3.11 (m, 2H, R-δCH_2), 3.53 (m, 1H, P-δCH), 3.60 (m, 1H, P-δCH), 3.61 (s, 3H, $COOCH_3$), 4.11 (ddq, J = 3.2 Hz, J = 5.9 Hz, J = 6.3 Hz, 1H, T-βCH), 4.15 (m, 1H, R-αCH), 4.27 (dd, J = 3.2 Hz, J = 8.4 Hz, 1H, T-αCH), 4.27 (dt, J = 7.9 Hz, J = 6.3 Hz, 1H, K-αCH), 4.37 (dd, J = 3.8 Hz, J = 8.4 Hz, 1H, P-αCH), 4.99 (d, J = 5.9 Hz, 1H, T-OH), 5.0 (s, 2H, Z-CH_2), 7.23 (t, J = 5.7 Hz, 1H, K-εNH), 7.3–7.4 (m, 5H, Ph), 7.80 (d, J = 8.5 Hz, 1H, R-NH), 7.80 (d, J = 8.4 Hz, 1H, T-αNH), 8.01 (d, J = 7.9 Hz, 1H, K-αNH), 8.48 (bs, 1H, R-δNH). ^{13}C NMR (125 MHz, DMSO-d_6) δ: 20.0 (T-CH_3), 28.2 (Boc-CH_3), 40.8 (R-δCH_2), 40.8 (K-εCH_2), 46.7 (P-δCH_2), 51.7 (T-OCH_3), 51.8 (K-αCH), 52.4 (R-αCH), 57.6 (T-αCH), 59.1 (P-αCH), 65.1 (Z-CH_2), 66.3 (T-βCH), 127.8, 128.4, 137.3 (Ph), 156.1–155.4 (Z-C1), 170.5, 171.0, 171.5, 172.2 (four signals of carboxyl group). Anal. Calcd. for $C_{35}H_{55}N_8O_{12}$: C, 53.91; H, 7.11; N, 14.37. Found: C, 54.02; H, 7.22; N, 14.25.

General procedures for the syntheses of conjugates 7a–f. Procedure A. To a stirred solution of MDP **5a** or nor-MDP **5b** derivatives (1 mmol) and Arg(NO_2)-Pro-Lys(Z)-ThrOMe· HCl (1.1 mmol) in anhydrous DMF (4 ml) a solution of DPPA (0.24 ml, 1.1 mmol) was added in anhydrous DMF (1 ml) at 0°C, followed by the addition of TEA (2.2 mmol). The mixture was stirred at 0°C for 3 h and then 48 h at room temperature. After evaporation of the solvent the reaction mixture was purified using radial chromatography and preparative TLC in solvent H or I to obtain compounds **6a–f**.

Procedure B. To a stirred solution of MDP **5a** or nor-MDP **5b** derivatives (0.111 mmol) in anhydrous DMF (1 ml) cooled to –15°C, NMM (0.111 mol) and isobutyl chloroformate (0.1108 mmol) were added, followed after 15 min. by the addition of cooled solution of Arg(NO_2)-Pro-Lys(Z)-ThrOMe hydrochloride (0.129 mmol) and NMM (0.129 mmol) in anhydrous DMF (0.5 ml) and the stirring was continued for 4 h in –15°C and then for 24 h at room temperature. After evaporation of the solvent, the reaction mixture was purified using radial chromatography and preparative TLC in solvent H or I to obtain compounds **6a–f**. Compounds **6a–f** were hydrogenated in 50% methanolic acetic acid containing palladium black for 37 h. The supernatant was decanted and the catalyst washed with water. The colected solutions were combined, filtered and purified using preparative TLC in solvent E.

1-*O*-Bn-Mur(*N*Ac)-Ala-D-Glu(Arg(NO_2)-Pro-Lys(Z)-Thr-OMe)-NH_2 (6a). Yield 47% (procedure A), 50% (procedure B); m.p. 95–99°C. ^{1}H-NMR (DMSO) δ: 1.02 (d, J = 6.4 Hz, 3H, T-CH_3), 1.20 (d, J = 6.8 Hz, 3H, A-CH_3), 1.22 (d, J = 6.8 Hz, 3H, H3-CH_3), 1.29 (m, 2H, K-γCH_2), 1.38 (m, 2H, K-δCH_2), 1.50 (m, 4H, K-βCH, R-βCH, R-γCH_2), 1.64 (m, 3H, R-βCH, K-βCH, Q-βCH), 1.78 (s, 3H, G-CH_3CON), 1.82 (m, 2H, P-γCH_2), 1.90 (m, 2H, P-βCH), 1.92 (m, 1H, Q-βCH), 2.00 (m, 1H, P-βCH), 2.10 (m, 2H, Q-γCH_2), 2.96 (q, J = 6.2 Hz, 2H, K-εCH_2), 3.12 (m, 2H, R-δCH_2), 3.48 (dt, J = 6.2 Hz, J = 9.1 Hz, 1H, G4-CH), 3.52 (m, 2H, G3-CH, G5-CH), 3.53 (m, 1H, G6-CH), 3.55 (m, 1H, P-δCH), 3.62 (m, 1H, P-δCH), 3.60 (s, 3H, T-$COOCH_3$), 3.64 (m, 1H, G6-CH), 3.80 (ddd, J = 3.4 Hz, J = 7.8 Hz, J = 11.2 Hz, 1H, G2-CH), 4.10 (m, 2H, T-βCH, Q-αCH), 4.26 (m, 1H, T-αCH), 4.28 (m, 1H, K-αCH), 4.30 (dq, J = 6.8 Hz, J = 7.1 Hz, 1H, A-αCH), 4.34 (dd, J = 3.9 Hz, J = 8.4 Hz, 1H, P-αCH), 4.42 (d, J = 12.7 Hz, 1H, G-$CH_2\phi$), 4.47 (m, 1H, R-αCH), 4.59 (t, J = 5.9 Hz, 1H, G6-OH), 4.66 (d, J = 12.7 Hz, 1H, G-$CH_2\phi$), 4.73 (d, J = 3.4 Hz, 1H, G1-CH), 4.98 (d, J = 5.4 Hz, 1H, T-OH), 5.0 (s, 2H, K-ZCH_2), 5.29 (d, J = 6.8 Hz, 1H, G4-OH), 6.88 (bs, 2H, R-NH_2), 7.04 and 7.29 (s,s, 1H, 1H, Q-$CONH_2$), 7.20 (t, J = 5.4 Hz, 1H, K-εNH), 7.25–7.40 (m, 5H, ϕ), 7.54 (d, J = 7.3 Hz, 1H, T-NH), 7.78 (d, J = 8.3 Hz, 1H, K-αNH), 7.98 (d, J = 7.8 Hz, 1H, R-αNH), 8.10 (d, J = 8.3 Hz, 1H, G-NH), 8.13 (d, J = 8.3 Hz, 1H, Q-NH), 8.48 (bs, 1H, R-δNH).

1-*O*-Bn-norMur(*N*Ac)-Ala-D-Glu(Arg(NO_2)-Pro-Lys(Z)-Thr-OMe)-NH_2 (6b). Yield 50% (procedure A), 56% (procedure B); m.p. 66–70°C. ^{1}H-NMR (DMSO) δ: 1.03 (d, J = 6.4 Hz, 3H, T-CH_3), 1.24 (d, J = 7.1 Hz, 3H, A-CH_3), 1.29 (m, 2H, K-γCH_2), 1.40 (m, 2H, K-δCH_2), 1.51 (m, 4H, K-βCH, R-βCH, R-γCH_2), 1.66–1.68 (m, 3H, R-βCH, K-βCH, Q-βCH), 1.84 (s, 3H, G-CH_3CON), 1.82 (m, 1H, P-βCH),

1.84 (s, 3H, G-CH_3CON), 1.88 (m, 2H, P-γCH_2), 1.94 (m, 1H, Q-βCH), 2.01 (m, 1H, P-βCH), 2.11 (m, 2H, Q-γCH_2), 2.97 (q, J = 6.6 Hz, 2H, K-εCH_2), 3.12 (m, 2H, R-δCH_2), 3.40 (dt, J = 6.2 Hz, J = 9.1 Hz, 1H, G4-CH), 3.52 (m, 2H, G3-CH, G5-CH), 3.53 (m, 1H, G6-CH), 3.54 (m, 1H, P-δCH), 3.62 (m, 1H, P-δCH), 3.61 (s, 3H, T-$COOCH_3$), 3.66 (m, 1H, G6-CH), 3.85 (ddd, J = 3.8 Hz, J = 8.7 Hz, J = 11.0 Hz, 1H, G2-CH), 4.08 (d, J = 16.2 Hz, 1H, H-CH), 4.11 (m, 2H, T-βCH, Q-αCH), 4.27 (m, 1H, T-αCH), 4.21 (d, J = 16.2 Hz, 1H, H-CH), 4.28 (m, 1H, K-αCH), 4.30 (dq, J = 6.8 Hz, J = 7.1 Hz, 1H, A-αCH), 4.35 (dd, J = 3.9 Hz, J = 8.4 Hz, 1H, P-αCH), 4.44 (d, J = 12.4 Hz, 1H, G-$CH_2\phi$), 4.47 (m, 1H, R-αCH), 4.64 (t, J = 5.8 Hz, 1H, G6-OH), 4.68 (d, J = 12.4 Hz, 1H, G-$CH_2\phi$), 4.71 (d, J = 3.8 Hz, 1H, G1-CH), 5.0 (d, J = 5.6 Hz, 1H, T-OH), 5.0 (s, 2H, K-ZCH_2), 5.79 (d, J = 6.2 Hz, 1H, G4-OH), 6.95 (bs, 2H, R-NH_2), 7.08 and 7.29 (s,s, 1H, 1H, Q-$CONH_2$), 7.21 (t, J = 5.5 Hz, 1H, K-εNH), 7.25–7.40 (m, 5H, ϕ), 7.80 (d, J = 8.4 Hz, 1H, T-NH), 7.99 (d, J = 7.6 Hz, 1H, K-αNH), 8.01 (d, J = 6.8 Hz, 1H, R-αNH), 8.06 (d, J = 6.8 Hz, 1H, A-αNH), 8.15 (d, J = 8.7 Hz, 1H, G-NH), 8.18 (d, J = 8.0 Hz, 1H, Q-NH), 8.49 (bs, 1H, R-δNH).

1-*O*-Bn-Mur(*N*Ac)-Val-D-Glu(Arg(NO_2)-Pro-Lys(Z)-Thr-OMe)-NH_2 (6c). Yield 46% (procedure A), 51% (procedure B); m.p. 75–79°C. ^{1}H-NMR (DMSO) δ: 0.82 and 0.84 (d,d, J = 6.4 Hz, 6H, V-CH_3), 1.03 (d, J = 6.4 Hz, 3H, T-CH_3), 1.23 (d, J = 6.7 Hz, 3H, H3-CH_3), 1.27 (m, 2H, K-γCH_2), 1.38 (m, 2H, K-δCH_2), 1.50 (m, 4H, K-βCH, R-βCH, R-γCH_2), 1.66 (m, 3H, R-βCH, K-βCH, Q-βCH), 1.80 (m, 1H, P-βCH), 1.81 (s, 3H, G-CH_3CON), 1.86 (m, 2H, P-γCH_2), 1.96 (m, 1H, Q-βCH), 2.0 (m, 1H, P-βCH), 2.03 (m, 1H, V-βCH), 2.10 (m, 2H, Q-γCH_2), 2.98 (q, J = 6.3 Hz, 2H, K-εCH_2), 3.12 (m, 2H, R-δCH_2), 3.4 (dt, J = 6.1 Hz, J = 9.2 Hz, 1H, G4-CH), 3.52–3.62 (m, 5H, G3-CH, G5-CH, G6-CH, P-δCH_2), 3.60 (s, 3H, T-$COOCH_3$), 3.63 (m, 1H, G6-CH), 3.82 (ddd, J = 3.1 Hz, J = 8.2 Hz, J = 11.4 Hz, 1H, G2-CH), 4.12 (m, 2H, T-βCH, Q-αCH), 4.18 (t, J = 7.5 Hz, 1H, V-αCH), 4.28 (m, 1H, K-αCH), 4.25 (m, 1H, T-αCH), 4.35 (dd, J = 3.9 Hz, J = 8.4 Hz, 1H, P-αCH), 4.44 (d, J = 12.2 Hz, 1H, G-$CH_2\phi$), 4.46 (m, 1H, R-αCH), 4.64 (t, J = 5.9 Hz, 1H, G6-OH), 4.66 (d, J = 12.2 Hz, 1H, G-$CH_2\phi$), 4.72 (d, J = 3.4 Hz, 1H, G1-CH), 4.98 (s, 2H, K-ZCH_2), 5.0 (d, 1H, T-OH), 5.76 (d, J = 5.4 Hz, 1H, G4-OH), 6.88 (bs, 2H, R-NH_2), 7.04 and 7.29 (s,s, 1H, 1H, Q-$CONH_2$), 7.20 (t, J = 5.4 Hz, 1H, K-εNH), 7.24–7.40 (m, 5H, ϕ), 7.8 (d, J = 8.4 Hz, 1H, T-NH), 8.0 (d, J = 7.8 Hz, 1H, K-αNH), 7.82 (d, J = 8.3 Hz, 1H, V-NH), 8.11 (d, J = 6.9 Hz, 1H, R-αNH), 8.17 (d, J = 8.3 Hz, 1H, G-NH), 8.28 (d, J = 8.3 Hz, 1H, Q-NH), 8.48 (bs, 1H, R-δNH).

1-*O*-Bn-norMur(*N*Ac)-Val-D-Glu(Arg(NO_2)-Pro-Lys(Z)-Thr-OMe)-NH_2 (6d). Yield 50% (procedure A), 54% (procedure B); m.p. 76–80°C. ^{1}H-NMR (DMSO) δ: 0.80 and 0.84 (d,d, J = 6.4 Hz, 6H, V-CH_3), 1.02 (d, J = 6.4 Hz, 3H, T-CH_3), 1.28 (m, 2H, K-γCH_2), 1.38 (m, 2H, K-δCH_2), 1.50 (m, 4H, K-βCH, R-βCH, R-γCH_2), 1.67 (m, 3H, R-βCH, K-βCH, Q-βCH), 1.80 (m, 1H, P-βCH), 1.82 (s, 3H, G-CH_3CON), 1.88 (m, 2H, P-γCH_2), 1.96 (m, 1H, Q-βCH), 2.0 (m, 1H, P-βCH), 2.02 (m, 1H, V-βCH), 2.10 (m, 2H, Q-γCH_2), 2.98 (q, J = 6.4 Hz, 2H, K-εCH_2), 3.12 (m, 2H, R-δCH_2), 3.4 (dt, J = 6.2 Hz, J = 9.0 Hz, 1H, G4-CH), 3.52–3.62 (m, 5H, G3-CH, G5-CH, G6-CH, P-δCH_2), 3.60 (s, 3H, T-$COOCH_3$), 3.64 (m, 1H, G6-CH), 3.82 (ddd, J = 2.9 Hz, J = 8.3 Hz, J = 11.7 Hz, 1H, G2-CH), 4.12 (d, J = 15.6 Hz, 2H, H-CH), 4.11 (m, 2H, T-βCH, Q-αCH), 4.18 (t, J = 7.6 Hz, 1H, V-αCH), 4.26 (m, 1H, T-αCH), 4.28 (m, 1H, K-αCH), 4.35 (dd, J = 3.9 Hz, J = 8.4 Hz, 1H, P-αCH), 4.42 (d, J = 12.2 Hz, 1H, G-$CH_2\phi$), 4.46 (m, 1H, R-αCH), 4.64 (t, J = 5.8 Hz, 1H, G6-OH), 4.66 (d, J = 12.2 Hz, 1H, G-$CH_2\phi$), 4.74 (d, J = 3.4 Hz, 1H, G1-CH), 4.98 (s, 2H, K-ZCH_2), 5.0 (d, 1H, T-OH), 5.76 (d, J = 5.4 Hz, 1H, G4-OH), 6.88 (bs, 2H, R-NH_2), 7.04 and 7.28 (s,s, 1H, 1H, Q-$CONH_2$), 7.20 (t, J = 5.4 Hz, 1H, K-εNH), 7.24–7.40 (m, 5H, ϕ), 7.8 (d, J = 8.4 Hz, 1H, T-NH), 8.0 (d, J = 7.8 Hz, 1H, K-αNH), 7.82 (d, J = 8.3 Hz, 1H, V-NH), 8.1 (d, J = 6.8 Hz, 1H, R-αNH), 8.17 (d, J = 8.3 Hz, 1H, G-NH), 8.28 (d, J = 8.3 Hz, 1H, Q-NH), 8.48 (bs, 1H, R-δNH).

1-*O*-Bn-Mur(*N*Ac)-Pro-D-Glu(Arg(NO_2)-Pro-Lys(Z)-Thr-OMe)-NH_2 (6e). Yield 45% (procedure A), 52% (procedure B); m.p. 71–75°C. ^{1}H-NMR (DMSO) δ: 1.0 (d, J = 6.0 Hz, 3H, T-CH_3), 1.24 (d, J = 6.8 Hz, 3H, H3-CH_3), 1.27 (m, 2H, K-γCH_2), 1.38 (m, 2H, K-δCH_2), 1.50 (m, 4H, K-βCH, R-βCH, R-γCH_2), 1.64–1.67 (m, 3H, R-βCH, K-βCH, Q-βCH), 1.72–2.07 (m, 4H, PP′-βCH, PP′-γCH), 1.80 (m, 2H, PP′-βCH), 1.84 (s, 3H, G-CH_3CON), 1.87 (m, 2H, PP′-γCH), 1.94 (m, 1H, Q-βCH), 2.11 (m, 2H, Q-γCH_2), 2.98 (q, J = 6.2 Hz, 2H, K-εCH_2), 3.12 (m, 2H, R-δCH_2), 3.4 (m, 1H, G4-CH), 3.52 (m, 3H, G3-CH, G5-CH, G6-CH), 3.44–3.64 (m, 4H, PP′-δCH_2), 3.60 (s, 3H, T-$COOCH_3$), 3.66 (m, 1H, G6-CH), 3.78 (ddd, J = 3.3 Hz, J = 7.6 Hz, J = 11.1 Hz, 1H, G2-CH), 4.10 (m, 2H, T-βCH, Q-αCH), 4.27 (m, 1H, T-αCH), 4.28 (m, 1H, K-αCH), 4.34 (m, 2H, PP′-αCH), 4.44 (d, J = 12.2 Hz, 1H, G-$CH_2\phi$), 4.46 (m, 1H, R-αCH), 4.64 (t, J = 5.8 Hz, 1H, G6-OH), 4.65 (d, J = 12.2 Hz, 1H, G-$CH_2\phi$), 4.72 (d, J = 3.4 Hz, 1H, G1-CH), 4.99 (s, 2H, K-ZCH_2), 5.0 (d, 1H, T-OH), 6.00 (d, J = 6.0 Hz, 1H, G4-OH), 6.86 (bs, 2H, R-NH_2), 7.06 and 7.27 (s,s, 1H, 1H, Q-$CONH_2$), 7.20 (t, J = 5.4 Hz, 1H, K-εNH), 7.26–7.39 (m, 5H, ϕ), 7.80 (d, J =

8.3 Hz, 1H, T-NH), 7.99 (d, J = 7.3 Hz, 1H, K-αNH), 8.08 (d, J = 7.4 Hz, 1H, R-αNH), 8.15 (d, J = 8.2 Hz, 1H, G-NH), 8.18 (d, J = 8.3 Hz, 1H, Q-NH), 8.48 (bs, 1H, R-δNH).

1-*O*-Bn-norMur(*N*Ac)-Pro-D-Glu(Arg(NO_2)-Pro-Lys(Z)-Thr-OMe)-NH_2 (6f). Yield 46% (procedure A), 53% (procedure B); m.p. 97–101°C. ^{1}H-NMR (DMSO) δ: 1.01 (d, J = 6.0 Hz, 3H, T-CH_3), 1.28 (m, 2H, K-γCH_2), 1.38 (m, 2H, K-δCH_2), 1.50 (m, 4H, K-βCH, R-βCH, R-γCH_2), 1.66–1.69 (m, 3H, R-βCH, K-βCH, Q-βCH), 1.72–2.07 (m, 4H, PP′-βCH, PP′-γCH), 1.80 (m, 2H, PP′-βCH), 1.84 (s, 3H, G-CH_3CON), 1.88 (m, 2H, PP′-γCH), 1.94 (m, 1H, Q-βCH), 2.11 (m, 2H, Q-γCH_2), 2.98 (q, J = 6.2 Hz, 2H, K-εCH_2), 3.12 (m, 2H, R-δCH_2), 3.4 (m, 1H, G4-CH), 3.52 (m, 3H, G3-CH, G5-CH, G6-CH), 3.44–3.64 (m, 4H, PP′-δCH_2), 3.60 (s, 3H, T-$COOCH_3$), 3.66 (m, 1H, G6-CH), 3.78 (ddd, J = 3.4 Hz, J = 7.8 Hz, J = 11.2 Hz, 1H, G2-CH), 4.08 (d, J = 16.0 Hz, 2H, H-CH), 4.10 (m, 2H, T-βCH, Q-αCH), 4.27 (m, 1H, T-αCH), 4.28 (m, 1H, K-αCH), 4.33 (m, 2H, PP′-αCH), 4.44 (d, J = 12.2 Hz, 1H, G-$CH_2\phi$), 4.46 (m, 1H, R-αCH), 4.64 (t, J = 5.8 Hz, 1H, G6-OH), 4.65 (d, J = 12.2 Hz, 1H, G-$CH_2\phi$), 4.72 (d, J = 3.4 Hz, 1H, G1-CH), 4.99 (s, 2H, K-ZCH_2), 5.0 (d, 1H, T-OH), 6.00 (d, J = 6.0 Hz, 1H, G4-OH), 6.86 (bs, 2H, R-NH_2), 7.05 and 7.28 (s,s, 1H, 1H, Q-$CONH_2$), 7.20 (t, J = 5.4 Hz, 1H, K-εNH), 7.26–7.38 (m, 5H, ϕ), 7.79 (d, J = 8.3 Hz, 1H, T-NH), 7.99 (d, J = 7.3 Hz, 1H, K-αNH), 8.08 (d, J = 7.3 Hz, 1H, R-αNH), 8.16 (d, J = 8.3 Hz, 1H, G-NH), 8.18 (d, J = 8.3 Hz, 1H, Q-NH), 8.48 (bs, 1H, R-δNH).

Mur(*N*Ac)-Ala-D-Glu(Arg-Pro-Lys-Thr-OMe)-NH_2 (7a). Yield 44%; $[\alpha]_{546}^{24}$ = –26° (c 0.5, 5% aqueous acetic acid); amino acid analysis (6 M, 110°C, 20 h): Ala, Arg, Glu, Lys, Pro, Thr. Anal. Calcd. for ($C_{41}H_{72}N_{12}O_{16} \cdot 2CH_3COOH \cdot H_2O$): C, 47.95; H, 7.33; N, 14.91. Found: C, 48.02; H, 7.22; N, 14.86.

nor-Mur(*N*Ac)-Ala-D-Glu(Arg-Pro-Lys-Thr-OMe)-NH_2 (7b). Yield 47%; $[\alpha]_{546}^{24}$ = –12° (c 0.5, 5% aqueous acetic acid); amino acid analysis (6 M, 110°C, 20 h): Ala, Arg, Glu, Lys, Pro, Thr. Anal. Calcd. for ($C_{40}H_{70}N_{12}O_{16} \cdot 2CH_3COOH \cdot H_2O$): C, 47.47; H, 7.24; N, 15.09. Found: C, 47.53; H, 7.18; N, 15.18.

Mur(*N*Ac)-Val-D-Glu(Arg-Pro-Lys-Thr-OMe)-NH_2 (7c). Yield 40%; $[\alpha]_{546}^{24}$ = –18° (c 0.5, 5% aqueous acetic acid); amino acid analysis (6 M, 110°C, 20 h): Ala, Arg, Glu, Lys, Pro, Thr. Anal. Calcd. for ($C_{43}H_{76}N_{12}O_{16} \cdot 2CH_3COOH \cdot H_2O$): C, 48.86; H, 7.50; N, 14.55. Found: C, 48.74; H, 7.41; N, 14.39.

nor-Mur(*N*Ac)-Val-D-Glu(Arg-Pro-Lys-Thr-OMe)-NH_2 (7d). Yield 42%; $[\alpha]_{546}^{24}$ = –22° (c 1.0, 5% aqueous acetic acid); amino acid analysis (6 M, 110°C, 20 h): Ala, Arg, Glu, Lys, Pro, Thr. Anal. Calcd. for ($C_{42}H_{74}N_{12}O_{16} \cdot 2CH_3COOH \cdot H_2O$): C, 48.41; H, 7.42; N, 14.73. Found: C, 48.59; H, 7.37; N, 14.64.

Mur(*N*Ac)-Pro-D-Glu(Arg-Pro-Lys-Thr-OMe)-NH_2 (7e). Yield 38%; $[\alpha]_{546}^{24}$ = –14° (c 1.0, 5% aqueous acetic acid); amino acid analysis (6 M, 110°C, 20 h): Ala, Arg, Glu, Lys, Pro, Thr. Anal. Calcd. for ($C_{43}H_{74}N_{12}O_{16} \cdot 2CH_3COOH \cdot H_2O$): C, 48.95; H, 7.34; N, 14.57. Found: C, 48.86; H, 7.22; N, 14.46.

nor-Mur(*N*Ac)-Pro-D-Glu(Arg-Pro-Lys-Thr-OMe)-NH_2 (7f). Yield 42%; $[\alpha]_{546}^{24}$ = –19.2° (c 0.5, 5% aqueous acetic acid); amino acid analysis (6 M, 110°C, 20 h): Ala, Arg, Glu, Lys, Pro, Thr. Anal. Calcd. for ($C_{42}H_{72}N_{12}O_{16} \cdot 2CH_3COOH \cdot H_2O$): C, 48.49; H, 7.25; N, 14.75. Found: C, 48.35; H, 7.16; N, 14.57.

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