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SYNTHESIS OF BIOTINYLATED THYMOCARTINS

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SUMMARY

Four biotinylated derivatives of Arg-Lys-Asp-Val immunostimulating tetrapeptide labelled either at the α-amino group of arginyl or the ε-amino moiety of lysyl residue have been synthesized for biological studies using classical methods.

Key words: Arg-Lys-Asp-Val, thymocartin, (+)-biotinyl- and 6-(+)-biotinylaminohexanoyl-labelling

The 49-amino-acid thymopoietins were isolated from bovine thymus [1]. These very similar oligopeptides have an influence on the neuromuscular transmission and a variety of immunostimulating effects, including the induction of early T-cell differentiation, the inhibition of B-cell differentiation and the modulation of mature lymphocytes. Thymopentin [thymopoietin(32-36), all-L Arg-Lys-Asp-Val-Tyr] was reported to be the active center, bearing all the biological properties of the parent hormones [2, 3]. Structure–activity relationships of a number of synthetic – mostly shortened – analogs revealed that even thymotrinan (all-L Arg-Lys-Asp) and thymocartin (al-L Arg-Lys-Asp-Val) possess significant *in vitro* and *in vivo* immunomodulatory effects [4-10]. On the basis of extensive preclinical studies thymocartin was selected for clinical evaluation.

Though thymopoietin oligopeptides have a number of well-defined biological, immunological and pharmacological effects, the exact mechanism of action has not yet been

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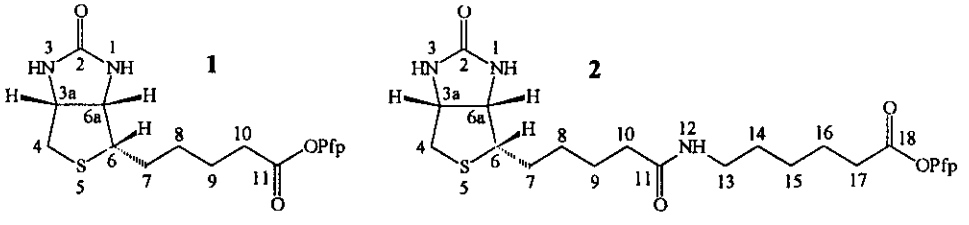
determined. The need to find the biological target of thymocartin prompted us to synthesize labelled derivatives. From among various labelling alternatives we have because of its simplicity selected biotinylation.

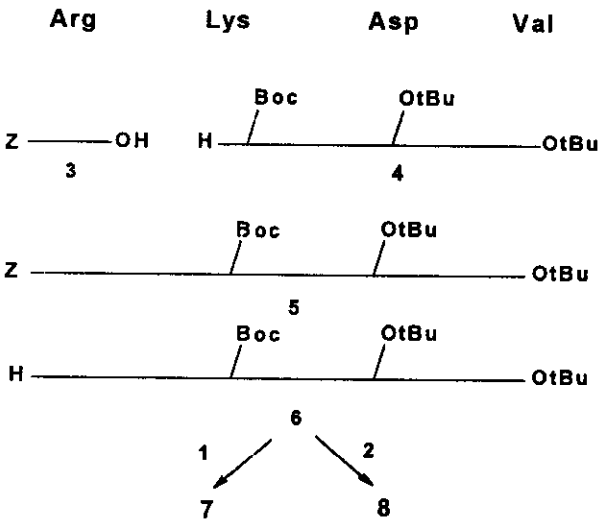
RESULTS and DISCUSSION

Incorporation of an additional moiety into a biologically active substance may lead to an alteration or even disappearance of molecular interactions if interactive functional groups are sterically hindered or blocked by labelling. There are two sites in thymocartin for easy introduction of carboxyl containing compounds, i. e. the α-amino group of Arg or the ε-amino moiety of Lys. We decided to utilize both of them. In addition, to examine the effect of potential steric hindrance at both of the amino groups, we synthesized both the closely connected (+)-biotinylated and the looser hanging 6-(+)-biotinylaminohexanoylated derivatives. Pentafluorophenyl (Pfp) derivatives were used for the introduction of these groups (Figure) [11].

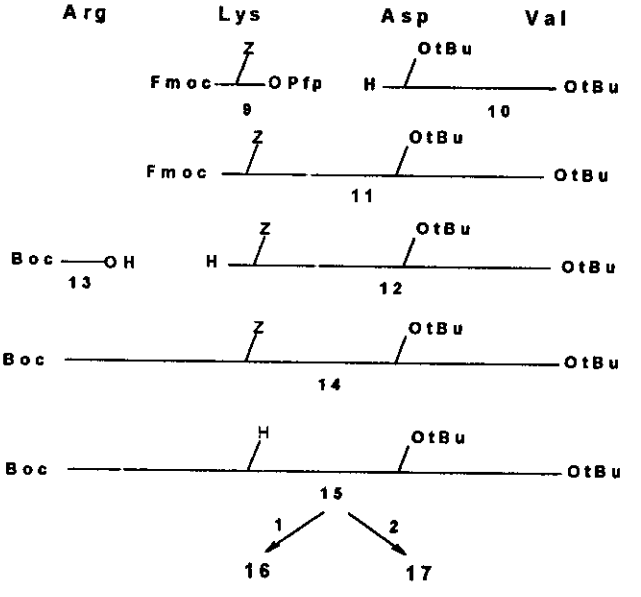


Figure

Protected tetrapeptide derivatives containing a free amino group were designed to utilize intermediates of the large-scale synthesis of thymocartin [12]. Thus, for the synthesis of compounds labelled at the α-amino group of Arg, H-Lys(Boc)-Asp(O^{t}Bu)-Val-O^{t}Bu (**3**) [12] was acylated with Z-Arg-OH (**4**) [13]. Then the protecting group of the resulting tetrapeptide (**5**) was selectively removed by hydrogenolysis, the liberated amino group of the partially protected tetrapeptide (**6**) was acylated with the pentafluorophenyl active esters **1** or **2**, giving protected labelled peptides 7 or **8**, respectively (Scheme 1).



Scheme 1: Synthesis of analogs 7 and 8 labelled at the α-amino group of Arg



Scheme 2: Synthesis of analogs 16 and 17 labelled at the ε-amino group of Lys

For the synthesis of compounds labelled at the ε-amino moiety of lysyl residue, H-Asp(O^{t}Bu)-Val-O^{t}Bu (**9**) [12] was acylated with Fmoc-Lys(Boc)-OPfp (**10**) [14, 15]. Aminolysis of the fully protected tetrapeptide (**11**) gave the partially blocked **12**, which was acylated with Boc-Arg-OH (**13**) [16] to yield **14**. Its debenzyloxycarbonylation by catalytic hydrogenolysis resulted in partially protected **15**, which was acylated either with **1** and **2** to obtain the protected labelled peptides **16** or **17**, respectively (Scheme 2).

18, **19**, **20** and **21** biotinylated thymocartin derivatives were obtained by acidolysis of **7**, **8**, **16** and **17**, respectively. All products were characterized by optical rotations, IR, NMR and MS (see Table 1 and Experimental). Biological activities of **18** to **21** were 20 to 25% of those of the nonbiotinylated thymotrinan and thymocartin in the azathioprine-inhibited E-rosette formation assay. Flow cytometric studies will be published elsewhere.

EXPERIMENTAL

All common L-amino acids were commercial products. Melting points are uncorrected, measured using a Tottoli (Büchi) apparatus. Optical rotations were measured with a Perkin-Elmer 141 digital readout polarimeter, tube length 1 dm. Thin-layer chromatography tests were run on precoated silica gel plates (Merck). The chromatograms were visualized by spraying the plates with ninhydrin, and then with tolidine/KI after chlorination. The progress of the reactions was followed by TLC and all products were tested by TLC. The relative insolubility of the compounds mostly prevented us determining the exact R_f values. However, they gave valuable information on the completion of the reaction and the purity of the intermediates. Solvent systems were made by mixing ethyl acetate and a stock solution of pyridine/acetic acid/water = 20:6:11 in the different portions, so (1) AcOEt/stock=19:1, (2) AcOEt/stock=7:3. Additional solvent systems were made by mixing chloroform and methanol, and chloroform, n-hexane and acetic acid. Column chromatography was performed using silica gel of 0.062-0.20 mm and the appropriate solvent system.

IR spectra were recorded on a Perkin-Elmer 1000 FT-IR spectrophotometer. MS spectra were taken with a Finnigan MAT 95 SQ spectrometer at room temperature using ESI method. All ^{1}H and ^{13}C

NMR spectra were recorded on a Varian Unity-plus 500 spectrometer (500 MHz for ^{1}H and 125 MHz for ^{13}C) with an internal deuterium lock at 30 °C in DMSO-d_6. Chemical shifts are given relative to δ_{TMS}. ^{1}H and ^{13}C assignments were straightforward and made by a concerted use of standard high-field one- and two-dimensional (2D) NMR methods: 2D ^{1}H-^{1}H and ^{1}H-^{13}C shift correlations (DQFCOSY, TOCSY, NOESY, HSQC and HMBC). The obtained scalar and NOE connectivities provided abundant information to ensure unambiguous spectral assignments.

Pentafluorophenyl of (+) biotin (**1**) was prepared as described in Ref. [11], M. p. 182-184 (ethanol), $[\alpha]_D$ +40.2° (c=1, DMF). *MS* m/z: 410 ($M+H^+$). *IR* (KBr, cm^{-1}): 3255, 2942, 2873, 1795, 1709, 1525, 1469, 1291, 1112, 1095, 1006, 987, 894, 742, 605. *^{1}H NMR* (DMSO-d_6) δ: 1.38-1.58 (3H, m, H_2-8, H_2-7x); 1.62-1.76 (3H, m, H_2-7y, H_2-9); 2.59 (1H, d, J=12.4 Hz, H_2-4α); 2.79 (2H, t, J=7.5 Hz, H_2-10); 2.84 (1H dd, J=12.4 Hz and 5.2 Hz, H_2-4β); 3.13 (1H, ddd, J=8.3 Hz, 6.2 Hz and 4.5 Hz, H-6); 4.16 (1H, ddd, J=7.8 Hz, 4.5 Hz and 2 Hz, H-6a); 4.32 (1H, ddt, J=7.8 Hz, 7.5 Hz and 1.2 Hz, H-3a); 6.34 (1H, s, H-3); 6.42 (1H, s, H-1). *^{13}C NMR* (DMSO-d_6) δ: 24.2 (C-9); 27.6 (C-8); 27.9 (C-7); 32.3 (C-10); 39.8 (C-4); 55.2 (C-6); 59.2 (C-3a); 61.0 (C-6a); 124.4 (C-ipso-Pfp)*; 137.4 (C-m-Pfp)*; 138.8 (C-p-Pfp)*; 140.5 (C-o-Pfp)*; 162.6 (C-2); 169.4 (C-11).

* ^{13}C chemical shifts of signals due to the Pfp carbon atoms were measured by using ^{19}F decoupling.

Methyl 6-(+)-biotinylamidohexanoate. Triethylamine (1.4 ml, 10 mmol) was added to a solution of **1** (4.1 g, 10 mmol) and methyl 6-aminohexanoate hydrochloride (1.8 g, 10 mmol, prepared by the usual esterification of 6-aminohexanoic acid in methanol in the presence of thionyl chloride) in DMF. Next day the solvent was removed and a solution of the residue in chloroform was washed with water, thrice with 5% aquaeous $NaHCO_3$, and then with water. The solvent was evaporated after drying and the residue was triturated with diethyl ether to give methyl 6-(+)-biotinylamidohexanoate (2.78 g, 74.8%), m.p. 138-141 °C, $[\alpha]_D$ +49 5° (c=1, EtOH). Anal. calc. for $C_{17}H_{29}N_3O_4S$ (371.5): C 54.96, H 7.87, N 11.31. Found:55.04, H 7.90, N 11.16. *MS* m/z: 372

($M+H^+$). *IR* (KBr, cm^{-1}): 3300, 2929, 1740, 1705, 1642, 1550, 1461, 1265, 1166, 728, 603. *^1H NMR* (DMSO-d_6) δ: 1.20-1.42 (6H, m, H_2-15, H_2-8 H_2-14); 1.42-1.57 (5H, m, H_2-7x, H_2-16, H_2-9); 1.57-1.66 (1H, m, H_2-7y); 2.04 (2H, t, J=7.5 Hz, H_2-10); 2.28 (2H, t, J=7.5 Hz, H_2-17); 2.58 (1H, d, J=12.4 Hz, H_2-4α); 2.82 (1H, dd, J=12.4 Hz and 5.1 Hz, H_2-4β); 3.00 (2H, q, J=6.9 Hz, H_2-13); 3.10 (1H, ddd, J=8.7 Hz, 6.1 Hz and 4.5 Hz, H-6); 3.58 (3H, s, H_3-COO*CH_3*); 4.13 (1H, ddd, J=7.8 Hz, 4.6 Hz and 1.9 Hz, H-6a); 4.31 (1H, ddt, J=7.7 Hz, 5.3 Hz and 1.2 Hz, H-3a); 6.33 (1H, s, H-3); 6.40 (1H, s, H-1); 7.70 (1H, t, J=5.5 Hz, NH-12). *^{13}C NMR* (DMSO-d_6) δ: 24.1 (C-16); 25.3 (C-9); 25.8 (C-15); 28.0 (C-7); 28.1 (C-8); 28.8 (C-14); 33.2 (C-17); 35.2 (C-10); 38.1 (C-13); 39.8 (C-4); 51.1 (C-COO*CH_3*); 55.4 (C-6); 59.2 (C-3a); 61.0 (C-6a); 162.7 (C-2); 171.7 (C-11); 173.2 (C-18).

6-(+)-Biotinylamidohexanoic acid. 1 M aqueous NaOH solution (8.8 ml) was added to a solution of methyl 6-(+)-amidohexanoate (2.1 g, 5.65 mmol) in methanol (50 ml). The reaction mixture was stirred overnight at ambient temperature. The solvent was evaporated and the residue was dissolved in water. Adjustment of the solution pH to 2 resulted in the precipitation of the product. The suspension was filtered to give 6-(+)-biotinylamidohexanoic acid (1.72 g, 85%), m.p. 228-230 °C. *MS* m/z: 358 ($M+H^+$). *IR* (KBr, cm^{-1}): 3378 3292, 2875, 2863, 1711, 1635, 1542, 1265, 1197, 734, 685, 604. *^1H NMR* (DMSO-d_6) δ: 1.20-1.42 (6H, m, H_2-15, H_2-8 H_2-14); 1.42-1.57 (5H, m, H_2-7x, H_2-16, H_2-9); 1.57-1.66 (1H, m, H_2-7y); 2.04 (2H, t, J=7.4 Hz, H_2-10); 2.19 (2H, t, J=7.3 Hz, H_2-17); 2.58 (1H, d, J=12.4 Hz, H_2-4α); 2.82 (1H, dd, J=12.4 Hz and 5.1 Hz, H_2-4β); 3.01 (2H, q, J=6.8 Hz, H_2-13); 3.10 (1H, ddd, J=8.6 Hz, 6.2 Hz and 4.5 Hz, H-6); 4.13 (1H, dd, J=7.7 Hz and 4.6 Hz, H-6a); 4.31 (1H, ddd, J=7.7 Hz, 4.0 Hz and 1.2 Hz, H-3a); 6.20-6.60 (2H, br, H-3, H-1); 7.71 (1H, t, J=5.6 Hz, NH-12). *^{13}C NMR* (DMSO-d_6) δ: 24.2 (C-16); 25.3 (C-9); 25.9 (C-15); 28.0 (C-7); 28.1 (C-8); 28.8 (C-14); 33.6 (C-17); 35.2 (C-10); 38.2 (C-13); 39.8 (C-4); 55.3 (C-6); 59.2 (C-3a); 61.0 (C-6a); 162.6 (C-2); 171.7 (C-11); 174.3 (C-18).

Pentafluorophenyl 6-(+)-biotinylamidohexanoate (**2**) was prepared as described in Ref. [11]. M. p. 154-157 °C (diethyl ether), $[\alpha]_D$ +26.6° (c=1, DMF). *MS* m/z: 524 ($M+H^+$). *IR* (KBr, cm^{-1}): 3315,

2943, 1793, 1699, 1670, 1641, 1528, 1517, 1122, 1103, 1006, 988. *^{1}H NMR* (DMSO-d_6) δ: 1.20-1.40 (4H, m, H_2-8, H_2-15); 1.40-1.57 (5H, m, H_2-7x, H_2-14, H_2-9); 1.57-1.74 (3H, m, H_2-7y, H_2-16); 2.05 (2H, t, J=7.4 Hz, H_2-10); 2.58 (1H, d, J=12.3 Hz, H_2-4α); 2.77 (2H, t, J=7.3 Hz, H_2-17); 2.82 (1H, dd, J=12.4 Hz and 5.1 Hz, H_2-4β); 3.04 (2H, q, J=6.6 Hz, H_2-13); 3.10 (1H, ddd, J=8.5 Hz, 5.9 Hz and 4.5 Hz, H-6); 4.13 (1H, ddd, J=6.4 Hz, 4.6 Hz and 1.6 Hz, H-6a); 4.31 (1H, dd, J=7.4 Hz, and 5.3 Hz, H-3a); 6.33 (1H, s, H-3); 6.39 (1H, s, H-1); 7.73 (1H, t, J=5.4 Hz, NH-12). *^{13}C NMR* (DMSO-d_6) δ: 23.9 (C-16); 25.2 (C-9); 25.5 (C-15); 28.0 (C-7); 28.1 (C-8); 28.6 (C-14); 33.3 (C-17); 35.2 (C-10); 38.0 (C-13); 39.8 (C-4); 55.4 (C-6); 59.2 (C-3a); 61.0 (C-6a); 124.4 (C-ipso-Pfp)*; 137.4 (C-m-Pfp)*; 138.8 (C-p-Pfp)*; 140.5 (C-o-Pfp)*; 162.6 (C-2); 169.4 (C-18); 171.8 (C-11).

* ^{13}C chemical shifts of signals due to the Pfp carbons are measured by using ^{19}F decoupling.

Synthesis of 7 and **8**

Z-Arg(HCl)-Lys(Boc)-Asp(O^{t}Bu)-Val-O^{t}Bu (**5·HCl**). A suspension of H-Lys(Boc)-Asp(^{t}Bu)-Val-O^{t}Bu oxalate in ethyl acetate (160 ml) was treated with 5% aqueous K_2CO_3 solution (80 ml). Evaporation of the solution gave oily **3** which was dissolved in DMF (20 ml).

A solution of Z-Arg(HCl)-OH (**4·HCl**, 10.8 g, 31.2 mmol) in DMF (100 ml) was treated with isobutyl chloroformate (3.8 ml, 28.8 mmol) at -10 °C. Then a solution of N-methylmorpholin (3.2 ml, 28.8 mmol) in DMF (10 ml) was added. The mixed anhydride was stirred for 15 min at -10 °C, then the solution of **3** was added. The reaction mixture was stirred for 20 min at -10 °C, then at ambient temperature. The solvent was removed, and a solution of the residue in water twice extracted with ethyl acetate. The combined organic phase was washed twice with 1 M aqueous HCl solution, then with water and evaporated. Trituration of the oily residue with diethyl ether gave **5·HCl** (17.0 g, 79%), m. p. 130-130 °C.

H-Arg(HCl)-Lys(Boc)-Asp(O^{t}Bu)-Val-O^{t}Bu·HCl (**6·HCl**). **5·HCl** (15.3 g, 17 mmol) in methanol (100 ml) was hydrogenated in the presence of 10% Pd /charcoal (2.5 g) for 4 h. The catalyst was

removed, the filtrate was evaporated, and the residue dissolved in diethyl ether (140 ml). The pH of the solution was adjusted to 4 by addition of 4 M HCl in ethyl acetate. The supernatant was discarded and trituration of the gelatinous residue with n-hexane gave **6·HCl** (12.5 g, 92.0%), m.p. 135 °C (dec.).

[(+)-Biotinyl]-Arg(HCl)-Lys(Boc)-Asp(O^{t}Bu)-Val-O^{t}Bu (**7·HCl**). **1** (0.62 g, 1.5 mmol) and triethylamine (0.28 ml, 2 mmol in two portions) were added to a solution of **6·HCl** (0.80 g, 1.0 mmol) in DMF (10 ml). After 5 h the solvent was removed. Trituration of the residue with ethyl acetate gave the crude product, which was recrystallized twice from ethanol/water to give **7·HCl** (0.47 g, 47%), m.p. 162-167 °C, $[\alpha]_D$ -10.5° (c=1, EtOH).

[6-(+)-Biotinylamidohexanoyl]-Arg(HCl)-Lys(Boc)-Asp(O^{t}Bu)-Val-O^{t}Bu (**8·HCl**). **2** (0.79 g, 1.5 mmol) and triethylamine (0.14 ml, 1 mmol in two portions) were added to a solution of **6·HCl** (0.80 g, 1.0 mmol) in DMF (10 ml). After 5 h the solvent was removed. Precipitation of the residue from ethanol/water gave the crude product (1.32 g) which was washed with hot ethyl acetate to yield **8·HCl** (0.87 g, 78.7%), m.p. 127-129 °C, $[\alpha]_D$ -3.8° (c=1, EtOH).

Synthesis of **16** and **17**

Fmoc-Lys(Z)-Asp(O^{t}Bu)-Val-O^{t}Bu (**11**). Fmoc-Lys(Z)-OPfp (**9**, 2.37 g, 3.55 mmol) and triethylamine (1. ml, 7.1 mmol in two portions) were added to a suspension of **10·HCl** 1.35 g, 3.55 mmol) in chloroform (30 ml). Next day the solvent was removed and a solution of the residue in ethyl acetate was washed with 1 M aqueous HCl, 5% aqueous $NaHCO_3$ solution and water. The organic phase was evaporated, and trituration of the residue with diethyl ether resulted in **11** (2.15 g, 73.1%), m.p. 118-121 °C, R_f (5) 0.70.

Boc-Arg(HCl)-Lys(Z)-Asp(O^{t}Bu)-Val-O^{t}Bu (**14**). 10% dimethylamine in DMF (20 ml) was added to a solution of **11** (2.0 g, 2.4 mmol) in DMF (5 ml). Excess dimethylamine was removed in *vacuo* after 10 min, and the resulting solution of **12** was added dropwise to a mixed anhydride of **13** [prepared from **13·HCl** (1.03 g, 3.12 mmol) in the same way as described previously for the mixed

anhydride of **4**]. The reaction mixture was kept overnight at 4 °C, then diluted with chloroform. The solution was washed in the usual way, then evaporated. Trituration of the residue with hot diisopropyl ether resulted in amorphous **14·HCl** (1.44 g, 66.4%), R_f (30) 0.45.

Boc-Arg(HCl)-Lys[(+)-biotinyl]-Asp(O^{t}Bu)-Val-O^{t}Bu **(16·HCl).** **14·HCl** (2.0 g, 2.22 mmol) in DMF (30 ml) was hydrogenated in the presence of 10% Pd /charcoal (0.4 g) for 4 h. The catalyst was removed, and **1** (1.14 g, 2.77 mmol) was added in three portions to the filtrate. After two days the solvent was removed, a solution of the residue in ethanol was treated with charcoal, then evaporated. The crude product was precipitated from ethanol/water, then chromatographed on a silica gel (50 g) column, using solvent system 2 for elution. Pure fractions were collected and evaporated. Trituration of the residue gave amorphous **14·HCl** (1.0 g, 45%), R_f (30) 0.25.

Boc-Arg(HCl)-Lys[6-(+)-biotinylamidohexanoyl]-Asp(O^{t}Bu)-Val-O^{t}Bu **(17·HCl)** was prepared in the sama way as described for **16·HCl** resulting in amorphous **17·HCl** (0.71 g, 29%), R_f (30) 0.25.

Final removal of the protecting groups

(+)-Biotinyl-Arg-Lys-Asp-Val (18). **7** (0.40 g, 0.40 mmol) was treated with 4 M HCl in acetic acid (5 ml) at ambient temperature for 10 min. The supernatant was discarded and the aqueous solution of the residue was treated with Dowex 2 x 8 resin (acetate cycle). The solvent was evaporated, and trituration of the residue with ethanol gave **18** (Table 1). *MS* m/z: 743 ($M+H^+$). *IR* (KBr, cm^{-1}): 3290, 2960, 2933, 1654, 1547, 1467, 1395, 1264, 652. *^1H NMR* (DMSO-d_6) δ: 0.79, 0.81 (6H, d, J=6.9 Hz, H_3-Val-γ1, H_3-Val-γ2); 1.20-1.80 (16H, m, H_2-7, H_2-8, H_2-9, H_2-Lys-β, H_2-Lys-γ, H_2-Lys-δ, H_2-Arg-β, H_2-Arg-γ); 1.86-1.98 (1H, m, H-Val-β); 2.11 (2H, t, J=7.2 Hz, H_2-10); 2.23 (1H, dd, J=14.9 Hz and 11.1 Hz, H_2-Asp-βx); 2.36 (1H, dd, J=15.2 Hz and 3.2 Hz, H_2-Asp-βy); 2.59 (1H, d, J=12.4 Hz, H_2-4α); 2.66-2.80 (2H, m, H_2-Lys-ε); 2.82 (1H dd, J=12.4 Hz and 5.2 Hz, H_2-4β); 2.88-2.98 (1H, m, H_2-Arg-δx); 3.02-3.14 (2H, m, H_2-Arg-δy, H-6); 3.73 (1H, dd, J=7.9 Hz and 4.9 Hz, H-Val-α); 4.13 (1H, ddd, J=7.7 Hz, 4.4 Hz and 1.7 Hz, H-6a); 4.26 (1H, q, J=7.4 Hz, H-Arg-α); 4.29-4.40 (3H, m, H-3a, H-Lys-α, H-Asp-α); 6.39 (2H, s, H-3, H-1); 6.91 (2H, br s, H_2-Arg-NηH); 7.08 (1H, d, J=7.9 Hz, H-Val-NαH); 7.83 (1H, d, J=7.7 Hz, H-Arg-NαH); 8.03 (1H, d,

J=9.1 Hz, H-Lys-NαH); 8.46 (1H, d, J=8.4 Hz, H-Asp-NαH); 10.6 (1H, t, J=5.0 Hz, H-Arg-NεH). *^{13}C NMR* (DMSO-d_6) δ: 18.3, 19.2 (C-Val-γ1, C-Val-γ2); 22.4 (C-Lys-γ); 24.6 (C-Arg-γ); 25.2 (C-9); 27.2 (C-Lys-δ); 27.9 (C-7); 27.9 (C-8); 30.0 (C-Arg-β); 31.4 (C-Val-β); 32.4 (C-Lys-β); 34.6 (C-10); 38.2 (C-Lys-ε); 39.7 (C-4); 40.5 (C-Asp-β); 40.6 (C-Arg-δ); 52.0 (C-Arg-α); 52.7 (C-Lys-α); 53.1 (C-Asp-α); 55.3 (C-6); 58.8 (C-Val-α), 59.2 (C-3a); 61.0 (C-6a); 157.4 (C-Arg-ζ); 162.7 (C-2); 170.2 (C-Lys-CO); 170.8 (C-Arg-CO); 170.8 (C-Asp-CO); 171.8 (C-11); 173.9 (C-Val-CO); 174.8 (C-Asp-γ).

[6-(+)-Biotinylamidohexanoyl]-Arg-Lys-Asp-Val (**19**) was prepared from **2** and **7** in the same way as described for **18** (Table 1). *MS* m/z: 856 ($M+H^+$). *IR* (KBr, cm^{-1}): 3011, 2931, 2866, 2647, 1650, 1543, 1463, 1394, 1265, 1047, 758, 728, 600. *^{1}H NMR* (DMSO-d_6) δ: 0.82, 0.82 (6H, d, J=6.9 Hz, H_3-Val-γ1, H_3-Val-γ2); 1.18-1.68 (20H, m, H_2-7, H_2-8, H_2-9, H_2-14, H_2-15, H_2-16, H_2-Lys-βx, H_2-Lys-γ, H_2-Lys-δ, H_2-Arg-βx, H_2-Arg-γ); 1.74-1.84 (1H, m, H-Lys-βy); 1.90-2.04 (2H, m, H-Arg-βy, H-Val-β); 2.06 (1H, t, J=7.4 Hz, H_2-10); 2.06-2.20 (2H, m, H_2-17); 2.34 (1H, dd, J=15.9 and 4.6 Hz, H_2-Asp-βx); 2.52 (1H, dd, J=16.1 and 6.1 Hz, H_2-Asp-βy); 2.59 (1H, d, J=12.5 Hz, H_2-4α); 2.75 (2H, t, J=7.4 Hz, H_2-Lys-ε); 2.82 (1H dd. J=12.5 and 5.1 Hz, H_2-4β); 2.92-3.02 (1H, m, H_2-Arg-δx); 3.00 (2H, q, J=5.9 Hz, H_2-13); 3.10 (1H, ddd, J=8.4, 6.0 and 4.4 Hz, H-6); 3.12-3.22 (1H, m, H_2-Arg-δy); 4.01 (1H, dd, J=8.5 and 5.1 Hz, H-Val-α); 4.13 (1H, ddd, J=6.5, 4.3 and 2 Hz, H-6a); 4.20-4.40 (4H, m, H-3a, H-Lys-α, H-Arg-α, H-Asp-α); 6.36 (1H, s, H-3); 6.40 (1H, s, H-1); 7.08 (1H, d, J=8.4 Hz, H-Val-NαH); 7.10 (2H, br s, H_2-Arg-NηH); 7.78 (1H, t, J=5.8 Hz, H-NH12); 7.95 (1H, d, J=7.4 Hz, H-Arg-NαH); 8.36 (1H, d, J=8.3 Hz, H-Lys-NαH); 8.85 (1H, d, J=7.4 Hz, H-Asp-NαH); 10.00 (1H, br, H-Arg-NεH). *^{13}C NMR* (DMSO-d_6) δ: 17.8, 19.0 (C-Val-γ1, C-Val-γ2); 22.2 (C-Lys-γ); 24.8 (C-Arg-γ); 25.0 (C-16); 25.3 (C-9); 26.0 (C-15); 26.5 (C-Lys-δ); 28.0 (C-7); 28.2 (C-8); 28.9 (C-14); 29.6 (C-Arg-β); 30.6 (C-Val-β); 31.0 (C-Lys-β); 34.9 (C-17); 35.2 (C-10); 37.6 (C-Asp-β); 38.3 (C-13); 38.5 (C-Lys-ε); 39.8 (C-4); 40.4 (C-Arg-δ); 50.6 (C-Asp-α); 52.0 (C-Arg-α); 52.5 (C-Lys-α); 55.4 (C-6); 57.5 (C-Val-α); 59.2 (C-3a); 61.0 (C-6a); 157.3

(C-Arg-ζ); 162.7 (C-2); 170.5 (C-Asp-CO); 171.0 (C-Lys-CO); 171.9 (C-11); 172.1 (C-Arg-CO); 172.4 (C-18); 173.0 (C-Val-CO); 174.6 (C-Asp-γ).

Arg-Lys[(+)-biotinyl]-Asp-Val (**20**) was prepared from **1** and **15** in the same way as described for **18** (Table 1). *MS* m/z: 743 ($M+H^+$). *IR* (KBr, cm^{-1}): 3324, 3286, 2933, 1675, 1555, 1397, 1265, 1188, 1048, 685, 605. *^{1}H NMR* (DMSO-d_6) δ: 0.7 (6H, d, J=6.9 Hz, H_3-Val-γ1, H_3-Val-γ2); 1.22-1.84 (15H, m, H_2-7, H_2-8, H_2-9, H_2-Lys-β, H_2-Lys-γ, H_2-Lys-δ, H_2-Arg-βx, H_2-Arg-γ); 1.90-2.02 (2H, m, H_2-Arg-βy, H-Val-β); 2.06 (2H, t, J=7.4 Hz, H_2-10); 2.27 (1H, dd, J=16.0 Hz and 5.1 Hz, H_2-Asp-βx); 2.54 (1H, dd, J=16.0 Hz and 6.0 Hz, H_2-Asp-βy); 2.58 (1H, d, J=12.3 Hz, H_2-4α); 2.83 (1H dd, J=12.5 Hz and 5.2 Hz, H_2-4β); 2.90-3.06 (3H, m, H_2-Arg-δx, H_2-Lys-ε); 3.11 (1H, ddd, J=8.5 Hz, 6.1 Hz and 4.6 Hz, H-6); 3.08-3.20 (1H, br m, H_2-Arg-δy); 3.60 (1H, br, H-Arg-α); 3.91 (1H, dd, J=8.4 Hz and 5.1 Hz, H-Val-α); 4.14 (1H, ddd, J=6.4 Hz, 4.4 Hz and 1.9 Hz, H-6a); 4.22-4.34 (3H, m, H-Lys-α, H-Asp-α, H-3a); 6.34 (1H, s. H-3); 6.43 (1H, s, H-1); 7.05 (1H, d, J=7.9 Hz, H-Val-NαH); 7.15 (2H, br s, H_2-Arg-NηH); 7.86 (1H, t, J=5.8 Hz, H-Lys-NζH); 8.53 (1H, br, H-Lys-NαH); 9.09 (1H, d, J=8.1 Hz, H-Asp-NαH); 10.34 (1H, br, H-Arg-NεH). *^{13}C NMR* (DMSO-d_6) δ: 17.8, 19.0 (C-Val-γ1, C-Val-γ2); 22.8 (C-Lys-γ); 23.8 (C-Arg-γ); 25.3 (C-9); 28.0 (C-7); 28.1 (C-8); 28.8 (C-Lys-δ); 30.6 (C-Val-β); 31.1 (C-Arg-β); 31.2 (C-Lys-β); 35.1 (C-10); 37.7 (C-Asp-β); 38.2 (C-Lys-ε); 39.8 (C-4); 40.6 (C-Arg-δ); 50.7 (C-Asp-α); 52.6 (C-Arg-α); 52.9 (C-Lys-α); 55.3 (C-6); 58.0 (C-Val-α); 59.2 (C-3a); 61.0 (C-6a); 157.4 (C-Arg-ζ); 162.7 (C-2); 170.3 (C-Asp-CO); 170.7 (C-Lys-CO); 171.6 (C-Arg-CO)*; 171.9 (C-11); 173.2 (C-Val-CO); 175.4 (C-Asp-γ).

* Measured at 75 MHz at 30 °C. This signal broadens at 125 MHz to the extent that it escapes detection.

Arg-Lys[6-(+)-biotinylamindohexanoyl]-Asp-Val (**21**) was prepared from **2** and **15** in the same way as described for **18** (Table 1). *MS* m/z: 856 ($M+H^+$). *IR* (KBr, cm^{-1}): 3286, 2932, 1702, 1636, 1551,

1391, 1265, 1152, 728, 685, 596. *^{1}H NMR* (DMSO-d_6) δ: 0.79 (6H, d, J=6.8 Hz, H_3-Val-γ1, H_3-Val-γ2); 1.16-1.42 (10H, m, H_2-8, H_2-14, H_2-15, H_2-Lys-γ, H_2-Lys-δ); 1.42-1.68 (9H, m, H_2-7, H_2-9, H_2-16, H_2-Lys-βx, H_2-Arg-γ); 1.74-1.86 (2H, m, H_2-Arg-βx, H_2-Lys-βy); 1.92-2.04 (2H, m, H_2-Arg-βy, H-Val-β); 2.05 (4H, t, J=7.3 Hz, H_2-10, H_2-17); 2.30 (1H, dd, J=16.4 Hz and 5.0 Hz, H_2-Asp-βx); 2.53 (1H, dd, J=16.3 Hz and 4.7 Hz, H_2-Asp-βy); 2.58 (1H, d, J=12.4 Hz, H_2-4α); 2.83 (1H dd, J=12.4 Hz and 5.1 Hz, H_2-4β); 2.92-3.06 (5H, m, H_2-13, H_2-Arg-δx, H_2-Lys-ε); 3.10 (1H, ddd, J=8.5 Hz, 6.1 Hz and 4.6 Hz, H-6); 3.07-3.20 (1H, br m, H_2-Arg-δy); 3.74 (1H, br t, J=6.0 Hz, H-Arg-α); 3.93 (1H, dd, J=8.3 Hz and 4.7 Hz, H-Val-α); 4.14 (1H, ddd, J=6.7 Hz, 4.6 Hz and 1.7 Hz, H-6a); 4.24-4.34 (3H, m, H-3a, H-Lys-α, H-Asp-α); 6.36 (1H, s, H-3); 6.41 (1H, s, H-1); 7.10 (1H, d, J=7.9 Hz, H-Val-NαH); 7.25 (2H, br s, H_2-Arg-NηH); 7.76 (1H, t, J=5.7 Hz, H-N12H); 7.84 (1H, t, J=5.7 Hz, H-Lys-NζH); 8.68 (1H, d, J=8.2 Hz, H-Lys-NαH); 9.12 (1H, d, J=8.0 Hz, H-Asp-NαH); 10.25 (1H, br, H-Arg-NεH). *^{13}C NMR* (DMSO-d_6) δ: 17.8, 19.0 (C-Val-γ1, C-Val-γ2); 22.7 (C-Lys-γ); 23.5 (C-Arg-γ); 25.0 (C-16); 25.3 (C-9); 26.1 (C-15); 28.0 (C-7); 28.2 (C-8); 28.8 (C-Lys-δ); 28.9 (C-14); 30.2 (C-Arg-β); 30.7 (C-Val-β); 31.3 (C-Lys-β); 35.2 (C-10)*; 35.3 (C-17)*; 37.6 (C-Asp-β); 38.2 (C-Lys-ε); 38.3 (C-13); 39.8 (C-4); 40.6 (C-Arg-δ); 50.8 (C-Asp-α); 52.2 (C-Arg-α); 52.9 (C-Lys-α); 55.4 (C-6); 57.9 (C-Val-α); 59.2 (C-3a); 61.0 (C-6a); 157.5 (C-Arg-ζ); 162.7 (C-2); 170.3 (C-Asp-CO); 170.7 (C-Lys-CO); 170.8 (C-Arg-CO); 171.8 (C-11); 172.0 (C-18); 173.3 (C-Val-CO); 175.3 (C-Asp-γ).

* denotes interchangeable assignments.

Table 1: Biotinylated thymocartin analogs

Product	Yield, %	Melting point, °C	$[\alpha]_D$ (c=1, H_2O)
(+)-Biot-Arg-Lys-Asp-Val, **18**	50.5*	amorphous	-14.0°
[(+)-Biot-Ahe]-Arg-Lys-Asp-Val, **19**	50.0	116-122°	-12.8°
Arg-Lys[(+)-Biot]-Asp-Val, **20**	75.0*	amorphous	+9.2°
Arg-Lys[(+)-Biot-Ahe]-Asp-Val, **21**	54.0	167-171°	+10.4°

*90 to 92% peptide content were thermogravimetrically determined

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