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Synthesis of Fragments of the Peptide Component of Pseudobactin

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Abstract: Pseudobactin is a structurally complex and physiologically important siderophore (microbial iron chelator) from *Pseudomonas putida-fluorescens*. Various fragments of the unusual peptide component of pseudobactin listed below were prepared by solution-phase peptide synthesis.

L-Lys·D-*threo*-β-OH Asp·L-Ala·D-*allo*-Thr·L-Ala
L-Lys·D-*threo*-β OH Asp·L-Ala·D-*allo*-Thr
D-*threo*-β-OH Asp·L-Ala·D-*allo*-Thr·L-Ala·D-*N*-OH-cycloOrn
D-*threo*-β-OH-Asp·L-Ala·D-*allo*-Thr·L-Ala
L-Ala·D-*allo*-Thr·L-Ala·D-*N*-OH-cycloOrn

A class of related peptides named pseudomycins have shown promising antifungal activity. To examine if these peptide fragments above would elicit similar activity, the fragments were tested and found to have no antifungal activity in limited bioassays.

Keywords: sideropore; pseudobactin; pseudomycin; solution-phase peptide synthesis; unusual amino acids

Siderophores are low molecular weight iron chelators synthesized and utilized by most microorganisms to overcome iron deprivation in their natural environments. These compounds, which most often contain hydroxamates and/or catecholates, bind ferric ion and facilitate its transport into cells through specific iron transport channels. Most representatives of the genus *Pseudomonas* synthesize and utilize fluorescent yellow-green pigments called pyoverdines or pseudobactins, the simplest of which is pseudobactin (**1**, Figure 1), as their siderophores [1–3]. Pseudobactin (**1**) is thought to play an important role in the beneficial plant growth-promoting ability of *Pseudomonas putida* and *Pseudomonas fluorescens*. These fluorescent pseudomonads are considered to exert their beneficial effects in part by the production of pseudobactin and its release into surrounding soil where it strongly complexes ferric ion, thus depriving deleterious microorganisms of this vital element [4,5].

In the course of our synthetic studies towards pseudobactin (**1**) our attention was drawn to the potential applications of pseudomonads as agricultural [4–8] and therapeutic agents [9,10]. Recently, an enzyme-linked immunosorbent assay (ELISA) using monoclonal antibodies to ferric pseudobactin has been reported to be sensitive and specific for

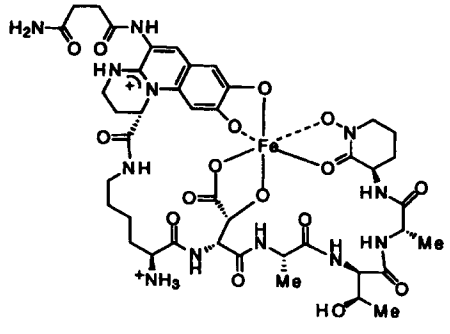

Figure 1 Pseudobactin (1), a siderophore from *Pseudomonas putida-fluorescens*.

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pseudobactin detection [11,12]. We anticipated that fragments of the peptide component of pseudobactin may be useful as probes for the epitope fragment of antibodies to pseudobactin, or as antigens to raise antibodies that bind pseudobactin (private communication and collaboration with Dr J. S. Buyer of the United States Department of Agriculture, Maryland) [13]. Interestingly too, a class of related peptides conjugated with fatty acids, named pseudomycins, from *Pseudomonas syringae* have demonstrated broad-spectrum antifungal activity *in vivo* [14,15]. In view of these interesting reports, and as part of our research program on the synthesis and biological study of pseudobactin [16–18], we decided to synthesize different fragments of the peptide component of pseudobactin to evaluate their biological utility.

The synthesis of different fragments of the unusual peptide component of pseudobactin presented numerous challenges. Three of the constituent amino acids, D-*threo*-β-hydroxyaspartic acid, D-*allo*-threonine and δ-*N*-hydroxy-D-cycloornithine, are unusual, are unavailable or are very expensive commercially. The β-hydroxy amino acids required careful handling to avoid competing acylation and elimination reactions during formation of peptide bonds, and protection and deprotection protocols. Our synthetic approach to the peptide fragments of pseudobactin and the selective protection of the β-carboxylate of D-*threo*-β-hydroxyaspartic acid also had to take into consideration observations that β-hydroxyaspartates may be prone to lactamization [19]. By following the synthetic methodology we reported in an earlier publication, and using some of the peptide intermediates prepared earlier [18], we were able to overcome these problems and synthesize several fragments of the peptide component of pseudobactin.

A convenient approach to most of the fragments of the peptide component of pseudobactin started with a common tripeptide template, *N*-Cbz-L-Ala-D-*allo*-Thr-L-Ala-O*t*-Bu (**2**), which was then elaborated by coupling to appropriate residues (Figure 2). The synthesis of protected tripeptide **2**, β-benzyl D-*threo*-β-hydroxyaspartate (**3**), and δ-*N*-benzyloxy-D-cycloornithine·HBr (**4**) was reported earlier [18]. As shown in Figure 2, hydrogenolysis of the Cbz

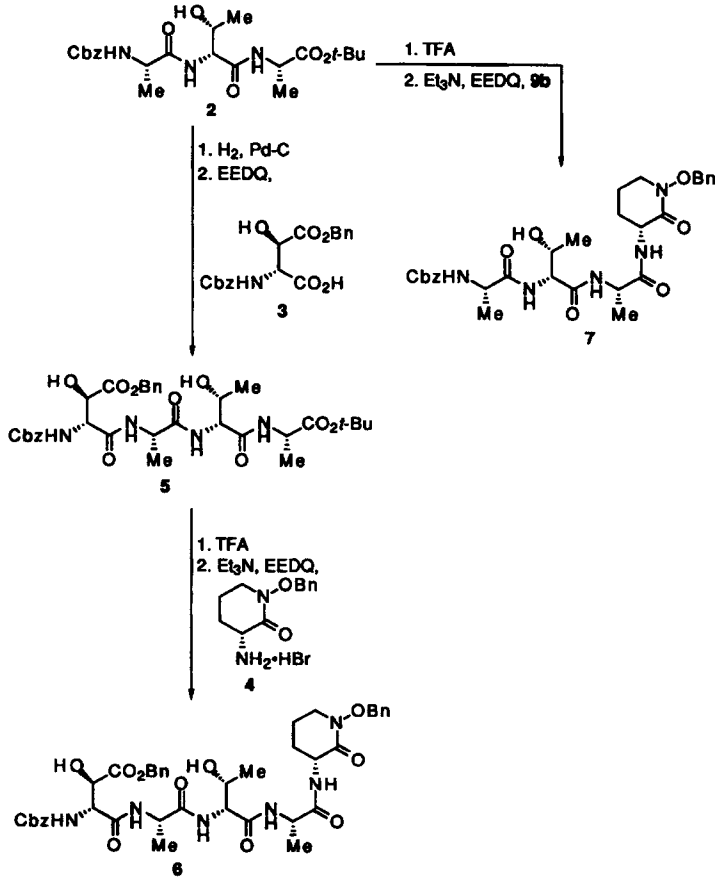

Figure 2 Synthesis of the protected peptide fragments of pseudobactin.

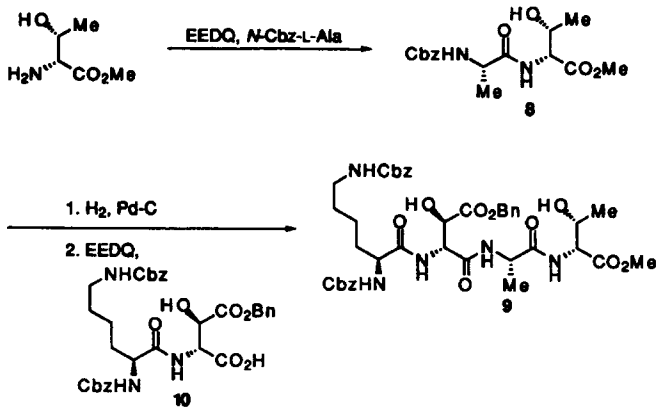

Figure 3 Synthesis of protected N-terminal tetrapeptide fragment of pseudobactin.

protective group in protected tripeptide **2** followed by EEDQ-mediated coupling to β-benzyl *N*-Cbz-D-*threo*-β-hydroxyaspartate (**3**) provided protected tetrapeptide **5** in 74% yield. Treatment of **5** with TFA in CH_2Cl_2 (1:1) to deprotect the *t*-Bu ester and subsequent EEDQ coupling to δ-*N*-benzyloxy-D-cycloornithine·HBr (**4**) provided protected C-terminal pentapeptide fragment **6**. Similarly, protected tripeptide **2** was treated with TFA in CH_2Cl_2, and the resulting acid coupled to cycloornithine derivative **4** to afford protected C-terminal tetrapeptide fragment **7**. Aqueous acid and base work-up during the synthesis of fragments **6** and **7** led to opening up of the δ-*N*-benzyloxy-D-cycloornithine residue and low yields of desired products. The products were therefore purified by omitting the aqueous work-up and charging the crude products directly on to a silica gel column. Fragments **6** and **7** were obtained in 65% and 81% yields, respectively.

Protected *N*-terminal tetrapeptide fragment **9** was synthesized as shown in Figure 3. D-*allo*-Thr·OMe [20] was coupled to *N*-Cbz-L-Ala to form dipeptide **8** in 61% yield. Deprotection of **8** by hydrogenolysis followed by EEDQ-mediated coupling to protected dipeptide **10** provided **9** in 37% yield. Protection of the Thr carboxylate was required since access to the N-terminal tetrapeptide **9** by initial activation of *bis*-*N,N'*-Cbz-L-lysyl-β-benzyl D-*threo*-β-hydroxyaspartate (**10**) with *N*-hydroxysuccinimide then attaching L-Ala-D-*allo*-Thr was unsuccessful.

Appropriate deprotections of the protected peptides synthesized provided the fragments of the peptide component of pseudobactin listed in Figure 4. The *t*-butyl ester **5** and N-terminal pentapeptide, *bis*-*N,N'*-Cbz-L-Lysyl-D-*threo*-β-hydroxyaspartyl, β-benzyl ester-L-alanyl-D-*allo*-threonyl-L-alanine, *t*-butyl ester [18], were treated with TFA in CH_2Cl_2 (1:1) to cleave *t*-butyl ester, then hydrogenolysed to remove benzyl and Cbz protective groups to obtain fragments **15** and **12**, respectively. Both benzyl and Cbz protective groups in peptides **6** and **7** were hydrogenolysed to give fragments, **14** and **16**, respectively. Saponification of the Me ester of **9** using aqueous LiOH, followed by catalytic hydrogenolysis provided fragment **13**.

L-Lys•D-*threo*-β-OH Asp•L-Ala•D-*allo*-Thr•L-Ala•D-*N*-OH-cycloOrn (**11**)
L-Lys•D-*threo*-β-OH Asp•L-Ala•D-*allo*-Thr•L-Ala (**12**)
L-Lys•D-*threo*-β-OH Asp•L-Ala•D-*allo*-Thr (**13**)
D-*threo*-β-OH Asp•L-Ala•D-*allo*-Thr•L-Ala•D-*N*-OH-cycloOrn (**14**)
D-*threo*-β-OH Asp•L-Ala•D-*allo*-Thr•L-Ala (**15**)
L-Ala•D-*allo*-Thr•L-Ala•D-*N*-OH-cycloOrn (**16**)

Figure 4 Pseudobactin peptide fragments synthesized.

A class of related peptides called pseudomycin have shown promising antifungal activity [14]. To examine if the peptide fragments **11** [18], **12** and **14**–**16** (Figure 4) would elicit similar activity, the fragments were screened for antifungal activity, but they were found to be inactive. Application of the fragments of the peptide component of pseudobactin as probes for the epitope fragment of antibodies to pseudobactin, or as antigens to raise antibodies that bind pseudobactin is still under consideration.

EXPERIMENTAL PART

Most instruments and general methods used have been described previously [21]. ^1H and ^{13}C-NMR spectra were obtained on a General Electric GN-300

spectrometer. 1,4-Dioxane was used as reference in ^{13}C-NMR spectra recorded in D_2O. Solvents used were dried and purified by standard methods [22]. The term 'dried' refers to the drying of an organic layer over anhydrous $MgSO_4$.

The stereogenic integrity of the protected peptides was determined using a Beckman HPLC system consisting of a model 110 A pump, a model 420 controller, a model 332 injector, and an Alltech Econosil column (25 cm × 4.6 mm, 5 μm silica) run at a flow rate of 1.5 ml/min. The diastereomeric purity of fragments **12** and **14** was also confirmed by analysis of their *o*-phthalaldehyde (OPA) derivatives using reversed-phase (C-18) HPLC with fluorescence detection [23].

N-Cbz-D-*threo*-β-Hydroxyaspartyl, β-benzyl ester-L-alanyl-D-*allo*-threonyl-L-alanine, *t*-butyl ester (5)

Protected tripeptide **2** [18] (0.86 g, 1.9 mmol) in 40 ml of MeOH was hydrogenolysed over 10% Pd–C (0.17 g) at atmospheric pressure for 3 h. The catalyst was removed by filtration and the filtrate was concentrated. The residue was dissolved in 30 ml of CH_3CN and added to a solution of β-benzyl *N*-Cbz-D-*threo*-β-hydroxyaspartate (**3**, 0.70 g, 1.9 mmol) in 30 ml of CH_3CN. EEDQ (0.52 g, 2.1 mmol) was then added to the solution, and the resulting mixture was stirred at room temperature (r.t.) for 20 h. The solvent was evaporated under reduced pressure and the residue dissolved in EtOAc. The EtOAc solution was washed with 10% citric acid, 10% $NaHCO_3$, saturated NaCl, and then dried, filtered and concentrated. The crude product was purified by column chromatography on silica gel with EtOAc as eluent and recrystallized from EtOAc to afford 0.91 g (74%) of tetrapeptide **5** as a white solid: m.p. 151–154°C; HPLC R_t 11 min (10% 2-propanol in hexanes); ^{1}H-NMR ($CDCl_3$) δ1.15 (d, J=6.3 Hz, 3H, Thr CH_3), 1.30 (d, J=7.2 Hz, 3H, Ala CH_3), 1.32 (d, J=7.0 Hz, 3H, Ala CH_3), 1.44 (s, 9H, C(CH_3)$_3$), 3.88 (m, 1H, Thr β-H), 4.40 (overlapping m, 2H, Thr α-H and Ala α-H), 4.56 (m, J=7.2 Hz, 1H, Ala α-H), 4.86 (s, 1H, β-OH-Asp α-H), 4.88 (s, 1H, β-OH-Asp β-H), 4.92–5.15 (m, 4H, benzylic H), 6.62 (d, J=8.9 Hz, 1H, NH), 7.30 (m, 10H, aromatic H), 7.50 (overlapping m, 3H, NH); ^{13}C-NMR ($CDCl_3$) δ 17.1, 17.9, 19.4, 27.9, 48.7, 49.2, 57.3, 58.2, 67.2, 67.6, 68.4, 71.1, 82.5, 127.9, 128.1, 128.5, 128.5, 134.9, 136.1, 156.0, 169.8, 170.0, 171.6, 172.5, 172.8; IR (KBr) 3700–3140 (br), 1745 (br), 1710–1600 (br) cm^{-1}; HRMS (FAB) calcd for $C_{33}H_{45}N_4O_{11}$ (MH^+) 673.3085, found 673.3086.

N-Cbz-D-*threo*-β-Hydroxyaspartyl, β-benzyl ester-L-alanyl-D-*allo*-threonyl-L-alanyl-δ-*N*-benzyloxy-D-cycloornithine (6)

N-Cbz-D-*threo*-β-OH-Asp·β-OBn-L-Ala-D-*allo*-Thr-L-Ala·O*t*-Bu (**5**, 0.34 g, 0.50 mmol) was suspended in 5 ml of anhydrous CH_2Cl_2 under N_2. The suspension was cooled to 0°C and 5 ml of TFA was added. The resulting solution was stirred at r.t. for 3 h. The solvent was evaporated under reduced pressure. Toluene (5 ml) was added to the residue and evaporated under reduced pressure to completely remove TFA. The residue was dissolved in 10 ml of $CHCl_3$. EEDQ (0.19 g, 0.75 mmol), Et_3N (0.05 g, 0.07 ml, 0.5 mmol) and δ-*N*-benzyloxy-D-cycloornithine·HBr [16,18] (**4**, 0.15 g, 0.50 mmol) were added and the solution was stirred at r.t. for 48 h. The solvent was evaporated under reduced pressure and the residue was chromatographed on silica gel eluting with $CHCl_3$/EtOH (9:1) to provide **6** as a white solid (0.33 g, 79%) after triturating with $CHCl_3$/hexanes. The product was purified further by preparative TLC in $CHCl_3$/EtOH (20:1) to provide a sample of **6** for microanalysis as a white solid (0.27 g, 65%): m.p. 123–125°C; HPLC R_t 11 min (10% 2-propanol in CH_2Cl_2); ^{1}H-NMR ($CDCl_3$) δ 1.15 (d, J=5.8 Hz, 3H, Thr CH_3), 1.27 (d, J=8.3 Hz, 3H, Ala CH_3), 1.32 (d, J=7.1 Hz, 3H, Ala CH_3), 1.40–2.30 (m, 4H, *c*Orn δ-CH_2), 3.40 (m, 2H, *c*Orn δ-H), 3.80 (m, 1H, Thr β-H), 4.35 (m, 1H, Thr α-H), 4.55 (quintet, J=7.1 Hz, 1H, *c*Orn α-H), 4.70 (overlapping m, 2H, Ala α-H), 5.00 (overlapping m, 8H, β-OH-Asp α and β-H and benzylic H), 5.25 (br s, 1H, NH), 7.10 (d, J=9.4 Hz, 1H, NH), 7.30 (m, 15H, aromatic H), 7.95 (d, J=8.5 Hz, 1H, NH), 8.20 (d, J=7.0 Hz, 1H, NH), 8.30 (m, 1H, NH); ^{13}C-NMR ($CDCl_3$) δ 17.1, 18.6, 19.8, 20.7, 27.7, 48.8, 49.2, 50.6, 50.7, 57.2, 58.4, 67.2, 67.6, 69.2, 71.1, 75.8, 128.06, 128.12, 128.38, 128.44, 128.5, 128.9, 129.5, 134.7, 134.9, 136.1, 156.1, 168.2, 169.5, 170.3, 171.6, 171.7, 172.7; IR (KBr) 3600–3250 (br), 1720, 1680–1620 (br) cm^{-1}; HRMS (FAB) calculated for $C_{41}H_{51}N_6O_{12}$ MH^+) 819.3565, found 819.3565; Anal. Calcd for $C_{41}H_{50}N_6O_{12}$: C, 60.14; H, 6.15; N, 10.26. Found: C, 59.84; H, 6.19; N, 10.04.

N-Cbz-L-Alanyl-D-*allo*-threonyl-L-alanyl-δ-*N*-benzyloxy-D-cycloornithine (7)

N-Cbz-L-Ala-D-*allo*-Thr-L-Ala·O*t*-Bu [18] (**2**, 0.45 g, 1.0 mmol) was suspended in 6 ml of anhydrous CH_2Cl_2 under N_2. The suspension was cooled to 0°C and 6 ml of TFA was added. The resulting solution was stirred at r.t. for 3 h. The solvent was

evaporated under reduced pressure. Toluene (6 ml) was added to the residue and evaporated under reduced pressure to completely remove TFA. The residue was dissolved in 20 ml of $CHCl_3$. EEDQ (0.27 g, 1.1 mmol), Et_3N (0.10 g, 0.14 ml, 1.0 mmol) and *δ-N*-benzyloxy-D-cycloornithine·HBr [16,18] (**4**, 0.30 mg. 1.0 mmol) were added and the solution was stirred at r.t. for 48 h. The solvent was evaporated under reduced pressure and the residue chromatographed on silica gel eluting with $CHCl_3$/EtOH (19:1) to provide **7** as a white solid (0.48 g, 81%) after recrystallization from MeOH/EtOAc: m.p. 223–225°C; HPLC R_t 22 min (10% 2-propanol in CH_2Cl_2); ^{1}H-NMR (CD_3OD) δ 1.22 (d, J=6.0 Hz, 3H, Thr CH_3), 1.33 (d, J=7.1 Hz, 3H, Ala CH_3), 1.41 (d, J=7.1 Hz, 3H, Ala, CH_3), 1.70–2.00 (m, 4H, *c*Orn β- and γ-CH_2), 3.40 (m, 1H, *c*Orn δ-CH_2), 3.50 (m, 1H, *c*Orn δ-CH_2), 4.01 (m, 1H, Thr β-H), 4.16 (overlapping m, 2H, Thr α-H and *c*Orn α-H), 4.40 (overlapping m, 2H, Ala α-H), 4.90 (m, 2H, benzylic H), 5.07 (s, 2H, benzylic H), 7.35 (m, 10H, aromatic H); ^{13}C-NMR (CD_3OD) δ 17.6, 18.1, 22.0, 28.6, 50.7, 51.7, 52.0, 52.1, 52.2, 60.8, 67.7, 68.4, 76.7, 128.9, 129.1, 129.5, 129.8, 130.7, 130.7, 136.6, 168.6, 172.5, 174.8, 176.0; IR (KBr) 3300 (br), 1685, 1660 (br), 1650 cm^{-1}; HRMS (FAB) calculated for $C_{30}H_{40}N_5O_8$ (MH^+) 598.2877, found 598.2877; Anal. Calcd for $C_{30}H_{39}N_5O_8$: C, 60.29; H, 6.58; N, 11.72; Found: C, 60.46; H, 6.38; N, 11.79.

N-Cbz-L-Alanyl-D-*allo*-threonine, methyl ester (8)

D-*allo*-Thr·OMe [19] (0.50 g, 3.8 mmol) and *N*-Cbz-L-Ala (Aldrich, 0.22 g, 3.8 mmol) were dissolved in 20 ml of CH_3CN. EEDQ (1.16 g, 4.52 mmol) was added and the solution stirred at r.t. for 18 h. The solvent was evaporated under reduced pressure and the residue dissolved in EtOAc. The EtOAc solution was washed with 10% citric acid, 10% $NaHCO_3$, saturated NaCl, and then dried, filtered and concentrated. The crude product was purified by column chromatography on silica gel with EtOAc/hexanes (4:1) as eluent and recrystallized from EtOAc/hexanes to afford 0.77 g (61%) of dipeptide **8** as white needle-shaped crystals: m.p. 119–120°C; HPLC R_t 17 min (10% 2-propanol in hexanes); ^{1}H-NMR ($CDCl_3$) δ 1.17 (d, J=6.4 Hz, 3H, Thr CH_3), 1.42 (d, J=7.1 Hz, 3H, Ala CH_3), 3.25 (d, J=6.4 Hz, 1H, OH), 3.75 (s, 3H, OCH_3), 4.15 (m, 1H, Thr β-H), 4.34 (quintet, J=6.0 Hz, 1H, Thr α-H), 4.64 (dd, J=7.1 and 3.1 Hz, 1H, Ala α-H), 5.15 (s, 2H, $ArCH_2$), 5.50 (br s, 1H, NH), 7.10 (br s, 1H, NH), 7.40 (m, 5H, ArH); ^{13}C-NMR ($CDCl_3$) δ 18.5, 18.8, 50.7, 52.6, 58.1, 67.2, 68.7, 128.1, 128.2, 128.5, 136.0, 156.1, 170.5, 173.0; IR (KBr) 3520, 3350, 3300, 1740, 1680, 1645 cm^{-1}; HRMS (FAB) calculated for $C_{16}H_{23}N_2O_{16}$ (MH^+) 339.1556, found 339.1540.

bis-*N,N'*-Cbz-L-Lysyl-D-*threo*-β-hydroxyaspartyl, β-benzyl ester-L-alanyl-D-*allo*-threonine, methyl ester (9)

N-Cbz-L-Ala-D-*allo*-Thr·OMe (**8**) (0.34 g, 1.0 mmol) in 10 ml of MeOH was hydrogenolysed over 10% Pd–C (0.08 g) at atmospheric pressure for 3 h. The catalyst was removed by filtration and the filtrate was concentrated. The residue was dissolved in 10 ml of CH_3CN and added to a solution of *bis-N,N'*-Cbz-L-Lysyl-D-*threo*-β-hydroxyaspartyl, β-benzyl ester [18] (**10**, 0.64 g, 1.0 mmol) in 10 ml of CH_3CN. EEDQ (0.25 g, 1.0 mmol) was then added to the solution, and the resulting mixture was stirred at r.t. for 20 h. The solvent was evaporated under reduced pressure and the residue dissolved in EtOAc. The EtOAc solution was washed with 10% citric acid, 10% $NaHCO_3$, saturated NaCl, and then dried, filtered and concentrated. The crude product was purified by column chromatography on silica gel with EtOAc/hexanes (9:1) as eluent to afford 0.31 g (37%) of tetrapeptide **9** as a clear syrup that solidified on standing for several weeks (attempts to recrystallize product were unsuccessful): HPLC R_t 12 min (10% 2-propanol in CH_2Cl_2); ^{1}H-NMR ($CDCl_3$) δ 1.20 (d, J=6.5 Hz, 3H, Thr CH_3), 1.25 (m, 2H, Lys CH_2), 1.40 (d, J=7.2 Hz, 3H, Ala CH_3), 1.42–1.55 (m, 4H, Lys CH_2), 2.00 (overlapping br s, 2H, OH), 3.07 (q, J=6.2 Hz, 2H, Lys ε-CH_2), 3.68 (s, 3H, OCH_3), 4.00 (m, 1H, Lys α-H), 4.20 (m, 1H, Thr β-H), 4.40 (m, J=6.9 Hz, 1H, Thr α-H), 4.60 (m, 1H, Ala α-H), 4.70 (br, 1H, β-OH-Asp α-H), 4.90–5.40 (overlapping m, 7H, β-OH-Asp β-H and $ArCH_2$), 5.55 (d, J=5.2 Hz, 1H, NH), 7.30 (m, 15H, ArH), 7.65 (d, J=8.7 Hz, 1H, NH), 7.73 (d, J=7.6 Hz, 1H, NH); ^{13}C-NMR ($CDCl_3$) δ 16.7, 19.1, 22.4, 29.5, 30.7, 40.3, 50.3, 52.4, 55.7, 55.9, 57.9, 66.9, 67.3, 67.9, 68.7, 70.6, 127.8–128.8 (multiple peaks), 134.8, 135.8, 136.5, 156.68, 156.71, 169.8, 170.8, 171.8, 172.5, 172.6; IR (KBr) 3300 (br), 1730–1610 (br) cm^{-1}; HRMS (FAB) calcd for $C_{41}H_{52}N_5O_{13}$ (MH^+) 822.3562, found 822.3575.

L-Lysyl-D-*threo*-β-hydroxyaspartyl-L-alanyl-D-*allo*-threonyl-L-alanine (12)

bis-N,N'-Cbz-L-Lys-D-*threo*-β-OH-Asp·β-OBn-L-Ala-D-*allo*-Thr-L-Ala·O*t*-Bu [18] (0.22 g, 0.23 mmol) was

suspended in 7 ml of anhydrous CH_2Cl_2 under N_2. The suspension was cooled to 0°C, and 7 ml of TFA was added. The resulting solution was stirred at r.t. for 3 h. The solvent was evaporated under reduced pressure. Toluene (7 ml) was added to the residue and evaporated under reduced pressure to completely remove TFA. Recrystallization from EtOAc/ether provided a white solid (0.17 g, 82%), which was dissolved in 8 ml of MeOH and hydrogenolysed over 10% Pd–C (0.03 g) at atmospheric pressure for 3 h. The catalyst was removed by filtration and washed with H_2O. The filtrate was concentrated and lyophilized to give pentapeptide **12** as a white fluffy solid (0.10 g, 82%): m.p. melts slowly with decomposition above 195°C; reversed-phase HPLC [24] R_t 24 min; ^{1}H-NMR (D_2O) δ 1.14–1.44 (three d, J=6.2, 7.1 and 7.1 Hz, 11H, three CH_3 and Lys CH_2), 1.68 (br s, 2H, Lys CH_2), 1.85 (br s, 2H, Lys CH_2), 3.00 (m, 2H, Lys CH_2), 4.00–4.20 (m, 3H, Thr β-H, Lys α-H, Ala α-H), 4.35 (d, J=6.3 Hz, 1H, Thr α-H), 4.43 (q, J=7.0 Hz, 1H, Ala α-H), 4.52 (br s, 1H, β-OH Asp α-H), 4.92 (br s, 1H, β-OH Asp β-H); ^{13}C-NMR (D_2O) δ 17.2, 18.2, 19.1, 21.8, 27.05, 31.1, 39.9, 50.8, 53.9, 57.3, 59.6, 67.4, 68.1, 170.9, 171.2, 172.1, 175.6; IR (KBr) 3700–2300 (br), 1800–1480 (br) cm^{-1}; HRMS calcd for $C_{20}H_{37}N_6O_{10}$ (MH^+) 521.2571, found 521.2572.

L-Lysyl-D-*threo*-β-hydroxyaspartyl-L-alanyl-D-*allo*-threonine (13)

bis-*N*,*N*′-Cbz-L-Lys-D-*threo*-β-OH-Asp·β-OBn-L-Ala-D-*allo*-Thr·OMe (**9**, 0.05 g, 0.06 mmol) was dissolved in 2 ml of THF and treated with 2 ml of 1 N aq. LiOH at 0°C. The mixture was stirred at 0°C for 2 h. THF was removed by evaporation under reduced pressure and the residue acidified to pH 3 with 10% citric acid. The resulting precipitate was extracted with EtOAc, washed with saturated NaCl solution, dried and concentrated. The residue was dissolved in 10 ml of MeOH and hydrogenolysed over 10% Pd–C (0.01 g) at atmospheric pressure for 4 h. The catalyst was removed by filtration and washed with H_2O. The filtrate was concentrated to afford tetrapeptide **13** as a white solid (0.02 g, 93%): ^{1}H-NMR (D_2O) δ 1.14 (d, J=6.0 Hz, 3H, Thr CH_3), 1.36 (m, 2H, Lys CH_2), 1.40 (d, J=7.0 Hz, 3H, Ala CH_3), 1.64 (m, 2H, Lys CH_2, 1.84 (m, 2H, Lys CH_2), 3.00 (m, 2H, Lys ε-CH_2), 4.20 (two overlapping m, 2H, Lys α-H and Thr β-H), 4.33 (d, J=4.9 Hz, 1H, Thr α-H), 4.42 (m, J=7.2 Hz 1H, Ala α-H), 4.69 (d, J=2.1 Hz 1H, β-OH-Asp α-H), 4.80 (1 H obscured by H_2O peak, β-OH-Asp β-H); ^{13}C-NMR (D_2O) δ 17.6, 18.3, 21.8, 27.0, 31.0, 39.9, 50.8, 53.9, 57.4, 60.7, 68.8, 72.7, 170.9, 172.0, 173.7, 174.6, 175.1; HRMS (FAB) calculated for $C_{17}H_{32}N_5O_9$ (MH^+) 450.2200, found 450.2213.

D-*threo*-β-Hydroxyaspartyl-L-alanyl-D-*allo*-threonyl-L--δ-*N*-hydroxy-D-cycloornithine (14)

N-Cbz-D-*threo*-β-hydroxyaspartyl, β-benzyl ester-L-alanyl-D-*allo*-threonyl-L-alanyl-δ-*N*-benzyloxy-D-cycloornithine (**6**, 0.11 g, 0.13 mmol) in 20 ml of MeOH was hydrogenolysed over 10% Pd–C (0.02 g) at atmospheric pressure for 3 h. The catalyst was removed by filtration and washed with H_2O. The filtrate was concentrated and lyophilized to give **14** as a white fluffy solid (0.07 g, 100%): m.p. melts slowly with decomposition above 195°C, ^{1}H-NMR (D_2O) δ 1.21 (d, J=6.4 Hz, 3H, Thr α-H), 1.41 (d, J=7.2 Hz, 6H, Ala CH_3), 1.80 (m, 1H, *c*Orn γ-H), 2.00 (m, 3H, *c*Orn CH_2), 3.62 (m, 2H, *c*Orn δ-CH_2), 4.14 (quintet, J=6.3 Hz, 1 H, Thr β-H), 4.29–4.47 (overlapping m, 4H, α-H's), 4.49 (d, J=4.2 Hz, 1H, β-OH Asp α-H), 4.80 (1H obscured by H_2O, β-OH Asp β-H); ^{13}C-NMR (D_2O) δ 17.4, 17.5, 19.3, 20.8, 27.5, 50.7, 50.9, 51.1, 52.5, 56.0, 59.9, 67.8, 71.0, 167.5, 168.2, 172.1, 175.3, 175.7; HRMS (FAB) calculated for $C_{19}H_{33}N_6O_{10}$ (MH^+) 505.2258, found 505.2254.

D-*threo*-β-Hydroxyaspartyl-L-alanyl-D-*allo*-threonyl-L--alanine (15)

N-Cbz-D-*threo*-β-OH-Asp·β-OBn-L-Ala-D-*allo*-Thr-L-Ala·O*t*-Bu (**5**, 0.18 g, 0.26 mmol) was suspended in 7 ml of anhydrous CH_2Cl_2 under N_2. The suspension was cooled to 0°C and 7 ml of TFA was added. The resulting solution was stirred at r.t for 3 h. The solvent was evaporated under reduced pressure. Toluene (7 ml) was added to the residue and evaporated under reduced pressure to completely remove TFA. Recrystallization from EtOAc/ether provided a white solid (0.13 g, 74%), which was dissolved in 8 ml of MeOH and hydrogenolysed over 10% Pd–C (0.03 g) at atmospheric pressure for 3 h. The catalyst was removed by filtration and washed with H_2O. The filtrate was concentrated and lyophilized to give tetrapeptide **15** as a white fluffy solid (0.08 g, 74% from **5**): m.p. melts slowly with decomposition above 175°C; reversed-phase HPLC [24] R_t 10 min; ^{1}H-NMR (D_2O) δ 1.17 (d, J=6.3 Hz, 3H, Thr CH_3), 1.37 (d, J=6.3 Hz, 6H, Ala CH_3), 4.12 (m, 1H, Thr β-H), 4.25–4.50 (overlapping m, 5H, α-H's and β-H); ^{13}C-NMR (D_2O) δ 17.3, 17.5, 18.9, 50.9, 59.6,

67.8, 171.8, 175.7; IR (KBr) 3700–2300 (br), 1780–1600 (br) cm^{-1}; HRMS calculated for $C_{14}H_{25}N_4O_9$ (MH^+) 393.1622, found 393.1613.

L-Alanyl-D-*allo*-threonyl-L-alanyl-δ-*N*-hydroxy-D-cycloornithine (16)

N-Cbz-L-Ala-D-*allo*-Thr-L-Ala-δ-*N*-BnO-D-cycloOrn (**7**, 0.10 g, 0.090 mmol) in 20 ml of MeOH was hydrogenolysed over 10% Pd–C (0.02 g) at atmospheric pressure for 3 h. The catalyst was removed by filtration and washed with H_2O. The filtrate was concentrated and lyophilized to give **16** as a white fluffy solid (0.06 g, 100%); m.p. melts slowly with decomposition above 194°C; ^{1}H-NMR (D_2O) δ 1.22 (d, J=6.4 Hz, 3H, Thr α-H), 1.37 (d, J=7.0 Hz, 3H, Ala CH_3), 1.39 (d, J=7.2 Hz, 3H, Ala CH_3), 1.75 (m, 1H, cOrn γ-CH_2), 1.95 (m, 3H, cOrn CH_2), 3.60 (m, 2H, cOrn δ-CH_2), 3.82 (q, J=7.0 Hz, 1H, cOrn α-H), 4.10 (quintet, J=6.4 Hz, 1 H, Thr β-H), 4.30 (d, J=6.4 Hz, 1H, Thr αH), 4.32–4.45 (overlapping m, 2H, Ala α-H); ^{13}C-NMR (D_2) δ 17.4, 19.2, 19.5, 20.7, 27.6, 50.3, 50.9, 51.1, 52.8, 59.9, 67.8, 165.5, 172.3, 175.1, 175.9; HRMS (EI) calculated for $C_{15}H_{27}N_5O_6$ 373.1961, found 373.1949.

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