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Chemistry of α-hydroxymethylserine: problems and solutions[‡]

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Abstract: Further improvements related to the synthesis of peptides containing HmS are presented. Efficient synthetic protocols have been developed to synthesize "difficult" sequences containing a *C*-terminal HmS residue, MeA–HmS or consecutive HmS. Preparative methods for orthogonal *N*- and/or *C*-protected HmS(Ipr) derivatives are described. Their compatibility with standard solution or solid-phase peptide chemistry protocols allows synthetic flexibility toward HmS-containing peptides. In the synthesis of the sterically hindered dipeptides with the *C*-terminal HmS(Ipr) residue, HATU proves the highest efficiency, as compared with the fluoride and PyBroP/DMAP coupling methods. The HATU method also outperforms the fluoride activation in the solid-phase assembly of HmS homosequence. Specific protocols are described to overcome an undesired cyclization to diketopiperazines that occurs during the removal of Fmoc from dipeptides with the *C*-terminal HmS(Ipr) or HmS residues, thus precluding their $C \rightarrow N$ elongation. The successful protocols involve: (i) the $2+1$ condensation using mixed anhydride activation yielding the desired product with the highest optical integrity or (ii) use of the 2-chlorotrityl resin as a solid support sterically suppressing the undesired cleavage due to diketopiperazine formation. The latter approach allows the mild conditions of peptide cleavage from solid support, preserving the isopropylidene protection and minimizing the undesired $N \rightarrow O$-acyl migration that was observed under prolonged acid treatment used for cleaving the HmS peptide from the Wang resin. Copyright © 2008 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: α-hydroxymethylserine; *O*,*O*-isopropylidene protective group; diketopiperazine formation; $N \rightarrow O$-acyl shift; HATU

INTRODUCTION

The $C^{\alpha,\alpha}$-disubstituted glycines play an important role as building blocks for the design and synthesis of peptides with predetermined three-dimensional structure and modified biological activity [1]. As a member of this family, HmS remains neglected by peptide chemists, despite its potential to generate specific constraints on the conformational freedom of a peptide chain. Another point relevant to the potential usefulness of this nonproteinogenic residue in biochemical applications is that this amino acid could furnish peptides resistant to enzymatic attack and with an enhanced solubility in water. In addition, HmS was found to be a much more efficient chelating ligand for transition metals than serine. Potentiometric and spectroscopic studies have shown that HmS exerts a significant electronic effect on neighboring residues. The theoretical calculations supported the evaluated deprotonation microconstants indicating that in the HmS–HmS–His tripeptide, the *N*-terminal amine is more acidic than the His- imidazole. The hydrogen bond formation between the *N*-terminal amino group and imidazole nitrogen stabilizes the cyclic conformation of the metal-free peptide. A tripeptide amide HmS–HmS–His–NH_2 was found to be the strongest peptidic Cu(II) chelator known to date, due to the steric shielding of the chelate plane as well as the aforementioned electronic effects [2–7].

Only few examples of the incorporation of HmS into a molecule of biological relevance have been reported so far. In the total synthesis of the heptapeptide antibiotic antrimycin D_v accomplished by Schmidt and Riedl [8] and independently by Nakamura *et al.* [9], HmS was incorporated as the *N*-terminal residue. Cappi *et al.* used that amino acid to mimic the D-galactose residue in the synthesis of sialyl Lewis X glycopeptide mimetics [10]. More recently, it has been shown that incorporation of α-hydroxymethylserine residue in substrate specificity P1 position of trypsin inhibitor SFTI-1 from sunflower seeds produced analogs with a retained trypsin inhibitory activity [11].

Sterically hindered $C^{\alpha,\alpha}$-disubstituted glycines, particularly when contiguous in sequence, have proven a synthetic challenge for incorporation into a peptide chain due to their poor coupling efficiency. In the case of HmS, this is further complicated by the presence of the side-chain hydroxyl groups. Our laboratory has

Abbreviations: HmS, α-hydroxymethylserine; IBCF, isobutyl chloroformate; Ipr, *O*,*O*-isopropylidene; MeA, α-methylalanine; PG, protective group. HATU, *N*-[(dimethylamino)-1H-1,2,3-triazolo-[4,5-b]pyridin-1-yl-methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide; PyBrop, bromo-tris-pyrrolidinophosphonium hexafluorophosphate

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undertaken a systematic investigation of the routes to peptides containing a single or consecutive HmS residues with the aim to optimize solution [12–14] and solid-phase synthesis [15]. We concluded that the *O*,*O*-protection of the HmS residue in the form of an Ipr derivative (see Figure 1, PG = H, R = OH) is of key importance for the efficient synthesis of peptides containing HmS [13,14]. Because both side-chains are tied up in a 1,3-dioxane ring, severe steric hindrance typical for the acyclic $C^{\alpha,\alpha}$-dialkyl glycines should be reduced, and as a consequence, the protection and coupling reactions proceed more satisfactorily than with the *O*,*O*-unprotected HmS. The HmS(Ipr) (5-amino-2,2-dimethyl-1,3-dioxane-5-carboxylic acid) represents the first example of a heterocyclic $C^{\alpha,\alpha}$-disubstituted glycine and may be interesting, by itself, as a peptide building block. Here, we report further examples of our synthetic methodology. In particular, methods to overcome encountered side reactions will be discussed.

MATERIALS AND METHODS

Melting points were determined on a Gallenkamp capillary melting-point apparatus and are uncorrected. Optical rotations were measured on a Horiba polarimeter in a 1 dm cell (1 ml) at 589 nm (Na D line) at room temperature. TLC was carried out on the 250 mm silica gel GF precoated uniplates (Analtech) with the following solvent systems: **I** $CHCl_3$/MeOH/AcOH (80 : 20 : 3); **II** $CHCl_3$/MeOH/AcOH (20 : 1 : 1); **III** $CHCl_3$/MeOH (1 : 1); **IV** $CHCl_3$/MeOH (10 : 1); **V** AcOEt/hexane (1 : 1); **VI** AcOEt/heptane (1 : 1); **VII** AcOEt/heptane (1 : 2); **VIII** AcOEt/heptane (2 : 3). The UV light, chlorine followed by starch/KI spray, or spraying with ninhydrin was used for visualization. HPLC was performed on a thermoseparation instrument using the C_{18} Vydac (0.46 cm × 25 cm) column thermostated at 36 °C, at a flow rate 1 ml/min, and UV detection at 220 nm. The following solvent system (with gradient change over 25 min) was used: **A** 0.05% TFA in water; **B** 0.038% TFA in acetonitrile/water (90 : 10). The NMR spectra were obtained on a 250 MHz instrument (Bruker Avance DPX) with TMS as an internal standard (unless otherwise noted). FAB mass spectra were recorded on an APO Electron (Ukraine) Model MI 1201E mass spectrometer equipped with a FAB ion source. For the flash chromatography, columns packed with silica gel 60 were used. The semipreparative HPLC was performed on a LDC analytical apparatus equipped with a Vydac Protein and Peptide C_{18} column, with the flow rate of 12 ml/min, and UV detection at 220 nm. The linear gradients were applied with the solvent systems as those for the analytical HPLC. The synthesis and characterization of the derivatives TosOH × HmS(Ipr)–OH and TosOH × HmS(Ipr)–OMe were described earlier [12]. The fluorides of proteinaceous amino acids [16] and of MeA were prepared according to the procedure in the literature [17].

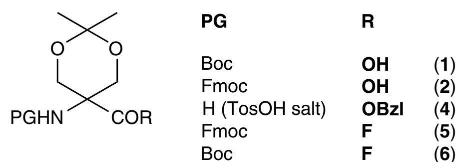

Figure 1 The protected derivatives of the *O*,*O*-isopropylidene α-hydroxymethylserine used in this study.

HmS(Ipr) Derivatives

(1) Boc-HmS(Ipr)-OH ($C_{12}H_{21}NO_6$ MW. 275.3). To a solution of TosOH × HmS(Ipr)–OH (9.044 g, 26 mmol) in 1 N NaOH (100 ml), a solution of di-*t*-butyl dicarbonate (17.00 g, 100 mmol) in dioxane (100 ml) was added dropwise with vigorous stirring in two equal portions (with a 4-h interval). After 24 h, dioxane was evaporated and the washed (2 × ether) aqueous layer was acidified with 1 N $KHSO_4$ and extracted three times with AcOEt. The crude product, after drying and evaporation, was recrystallized from the AcOEt/hexane mixture.

Yield 6.133 g (86%); m.p. 166–169 °C (dec.); TLC: 0.86 (I), 0.50 (II), 0.74 (III); HPLC: purity 100%, t_R = 11.28 min (20–50% B). ^{1}H NMR ($CDCl_3$): 1.46, 1.48 (2s, 15H, Ipr and Boc); 4.04 (d, 2H, J_{AB} = 12.5 Hz, H_β HmS); 4.21 (d, 2H, J_{AB} = 12.5 Hz, H_β HmS); 5.53 (bs, 1H, NH).

(2) Fmoc-HmS(Ipr)-OH ($C_{22}H_{23}NO_6$ MW. 397.4). To a suspension of TosOH × HmS(Ipr)–OH (3.474 g, 10 mmol) in dioxane (20 ml) a 1 N K_2CO_3 (40 ml) was added, and then, after cooling to 0 °C the solution of fluorenylmethyl chloroformate (5.18 g, 20 mmol) in dioxane (40 ml) was added dropwise in two equal portions (with a 4-h interval). The reaction mixture was allowed to warm up to RT overnight. Then dioxane was evaporated and the diluted aqueous layer was washed (2 × ether), cooled to 4 °C and acidified with a precooled 1 N $KHSO_4$. The product was extracted (three times) with AcOEt. The residue, obtained after drying and evaporation of the organic layer was recrystallized from AcOEt/hexane. Yield 3.412 g (86%); m.p. 177–179 °C (dec.); TLC: 0.76 (II); HPLC: purity 99%, t_R = 13.23 min (40–70% B). ^{1}H NMR ($CDCl_3$): 1.50 (s, 6H, Ipr); 2.82 (b, COOH); 4.13 (d, 2H, J_{AB} = 12 Hz, H_β HmS); 4.20 (d, 2H, J_{AB} = 12 Hz, H_β HmS); 4.26 (t, 1H, J = 7 Hz, Fmoc CH); 4.42 (d, 2H, J = 7 Hz, Fmoc CH_2); 5.80 (bs, 1H, NH); 7.29–7.78 (m, 8H, Fmoc arom.).

(3) Fmoc-HmS(Ipr)-OBzl ($C_{29}H_{29}NO_6$ MW. 487.5). To a solution of compound **2** (2.388 g, 6 mmol) in MeOH (30 ml) the aqueous solution of $CsHCO_3$ (1.183 g in 6 ml, 6.1 mmol) was added. After 15 min, the solvents were removed *in vacuo*, the residue was dissolved in anhydrous DMF (20 ml), and benzyl bromide (0.715 ml, 6.1 mmol) was added with the intense stirring. After 6 h, the cesium bromide was filtered off, and the residue obtained after evaporation of the filtrate was dissolved in AcOEt and washed with saturated $KHCO_3$, 1 N $KHSO_4$ and brine. The crude product obtained after drying and evaporation of the organic layer was recrystallized from AcOEt/hexane. Yield 2.310 g (79%); m.p. 140–142 °C; TLC: 0.79 (V), 0.49 (VI); HPLC: purity 99.1%, t_R = 12.19 min (60–90% B). ^{1}H NMR ($CDCl_3$): 1.44 (s, 3H, Ipr); 1.47 (s, 3H, Ipr); 3.95–4.37 (m, 7H, H_β HmS and Fmoc $CHCH_2$); 5.14 (s, 2H, Bzl CH_2); 5.67 (bs, 1H, NH); 7.27 (bs, 5H, Bzl arom.); 7.31–7.77 (m, 8H, Fmoc arom.).

(4) TosOH × HmS(Ipr)-OBzl ($C_{14}H_{19}NO_4 \times C_7H_8O_3S$ MW. 437.5). To a solution of compound **3** (2.64 g, 5.41 mmol) in DCM (16 ml) piperidine (4 ml) was added, and stirring

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was continued for 10 min with the TLC control. The residue obtained after the evaporation was subjected to chromatography (AcOEt/heptane 10:1). The fractions containing H-HmS(Ipr)-OBzl (R_F 0.50) were collected, evaporated, and the amino acid ester was redissolved in ether (20 ml) and cooled to 0 °C. The precooled ethereal solution of TosOH monohydrate (1.03 g, 5.41 mmol) was added with stirring. The precipitated crude salt (2.12 g, 89%) was then recrystallized from MeOH/ether. Yield 1.745 g (74%); m.p. 152–153 °C; TLC: 0.14 (II), 0.63 (IV); HPLC: purity 100% (except TosOH) $t_R = 9.43$ min (20–50% B), $t_R = 16.51$ min (10–40% B). ^1H NMR ($CDCl_3$): 1.40 (s, 3H, Ipr); 1.43 (s, 3H, Ipr); 2.32 (s, 3H, TosOH CH_3); 4.23 (s, 4H, H_β HmS); 5.18 (s, 2H, Bzl CH_2); 7.07 (d, 2H, $J_{AX} = 8$ Hz, TosOH arom.); 7.29 (bs, 5H, Bzl arom.); 7.72 (d, 2H, $J_{AX} = 6$ Hz, TosOH arom.); 8.75 (b, NH_3).

(5) Fmoc-HmS(Ipr)-F ($C_{22}H_{22}FNO_5$ MW. 399.4). To a suspension of compound **2** (0.796 g, 2 mmol) in DCM (5 ml) pyridine (0.162 ml, 2 mmol) and then cyanuric fluoride (0.170 ml, 2 mmol) were added with stirring. After 3 h at RT, DCM (10 ml) and the ice-cold water (10 ml) were added and the resulting layers were separated. The aqueous layer was extracted with DCM and the combined organic phases, after drying and evaporation, gave the residue, which crystallized upon trituration with hexane. Yield 0.674 g (84%); m.p. 147–149 °C; TLC: 0.73 (V), 0.53 (VI) (as methyl ester); HPLC: purity 99%, $t_R = 20.44$ min (40–70% B) (as methyl ester). ^1H NMR ($CDCl_3$): 1.45 (s, 3H, Ipr); 1.48 (s, 3H, Ipr); 3.95–4.27 (m, 5H, H_β HmS and Fmoc CH); 4.48 (d, 2H, $J = 7$ Hz, Fmoc CH_2); 5.66 (bs, 1H, NH); 7.28–7.77 (m, 8H, Fmoc arom.). ^{19}F NMR: 26.57 ppm with $CFCl_3$ as the internal standard.

(6) Boc-HmS(Ipr)-F ($C_{12}H_{20}FNO_5$ MW. 277.3). A suspension of compound **1** (1.102 g, 4 mmol) in DCM (10 ml) was cooled to −15 °C. Then pyridine (0.325 ml, 4 mmol) and cyanuric fluoride (0.340 ml, 4 mmol) were added with stirring. After 1 h the reaction mixture was worked up as described for compound **5**. The product crystallized upon rotary evaporation of its DCM/hexane solution. Yield 0.752 g (68%); m.p. 110–113 °C (dec.); TLC: 0.59 (IV), 0.09 (VI) (as methyl ester); HPLC: purity 99%, $t_R = 17.98$ min (20–50% B) (as methyl ester). ^1H NMR ($CDCl_3$): 1.44, 1.46, 1.47 (3s, 15H, Boc and Ipr); 3.81–4.36 (m, 4H, H_β HmS); 5.40 (b, 1H, NH). ^{19}F NMR: 25.28 ppm with $CFCl_3$ as the internal standard.

Examples of Coupling

PyBroP method. A carboxyl component (0.5 mmol); TosOH × HmS(Ipr)–OMe (0.5 mmol); PyBroP (0.6 mmol); DMAP (0.3 mmol) and diisopropylethylamine (DIEA) (1.2 mmol) were stirred in DCM (2 ml) for 1 h. The reaction mixture diluted with DCM (10 ml) was then washed with 1 N $KHSO_4$, 1 N $NaHCO_3$ and brine. The crude product, after drying and evaporation of the organic layer, was purified by flash chromatography.

HATU method. A carboxyl component (0.5 mmol); TosOH × HmS(Ipr)–OMe (0.5 mmol); HATU (0.55 mmol) and DIEA (1.5 mmol) were stirred in DCM (2 ml) for 1 h. After the usual workup (see above method), the crude product was either recrystallized or purified by chromatography.

Fluoride method. To a solution of an amino component (0.5 mmol) and $NaHCO_3$ (1 mmol) in water (5 ml) a solution of *N*-protected amino acid fluoride (0.55 mmol) in DCM (5 ml) was added over 1 min with vigorous stirring. After 1 h, the organic layer was separated and workedup as usual, and the crude product was either recrystallized or purified by chromatography.

(7) Boc-Ala-HmS(Ipr)-OMe ($C_{16}H_{28}N_2O_7$ MW. 360.4). m.p. 151–153 °C; $[\alpha]_D = -42.70°$ ($c = 0.37$ MeOH); TLC: 0.74 (IV), 0.27 (VI); HPLC: purity 100%, $t_R = 9.92$ min (30–60% B). ^1H NMR ($CDCl_3$): 1.37 (d, 3H, $J = 7$ Hz, H_β Ala); 1.42, 1.46, 1.48 (3s, 15H, Boc, Ipr); 3.73 (s, 3H, OCH_3); 3.88–3.97, 4.17–4.24 (m, 5H, H_β HmS and H_α Ala); 4.99 (b, 1H, NH Ala); 6.95 (bs, 1H, NH HmS). FAB-MS (m/z): 361 $(MH)^+$.

PyBroP – Boc–Ala–OH was used; chromatography (AcOEt/hexane 1:1) yielded 0.118 g (66%) of the title dipeptide. *HATU* – Boc–Ala–OH was used; the recrystallization (AcOEt/hexane) yielded 0.145 g (81%) of the dipeptide **7**. *Fluoride* – Boc–Ala–F was used; recrystallization (AcOEt/hexane) yielded 0.121 g (67%) of compound **7**.

(8) Boc-MeA-HmS(Ipr)-OMe ($C_{17}H_{30}N_2O_7$ MW. 374.4). m.p. 133–134 °C; TLC: 0.59 (IV), 0.54 (V), 0.33 (VI); HPLC: purity 100%, $t_R = 12.21$ min (30–60% B). ^1H NMR ($CDCl_3$): 1.41, 1.46, 1.51 (3s, 21H, Boc, Ipr and H_β MeA); 3.72 (s, 3H, OCH_3); 3.91 (d, 2H, $J_{AX} = 12$ Hz, H_β HmS); 4.23 (d, 2H, $J_{AX} = 12$ Hz, H_β HmS); 4.88 (bs, 1H, NH MeA); 7.31 (bs, 1H, NH HmS). FAB-MS (m/z): 375 $(MH)^+$.

PyBroP – Boc–MeA–OH was used; chromatography (AcOEt/heptane 1:1) yielded 0.060 g (32%) of the title dipeptide. *HATU* – Boc–MeA–OH was used; chromatography (AcOEt/heptane 1:1) yielded 0.110 g (59%) of compound **8**.

(9) Boc-HmS(Ipr)-HmS(Ipr)-OMe ($C_{20}H_{34}N_2O_9$ MW. 446.5). m.p. 167–168 °C; TLC: 0.74 (IV), 0.46 (VI), 0.26 (VII); HPLC: purity 100%, $t_R = 11.335$ min (40–70% B), $t_R = 17.78$ min (30–60% B). ^1H NMR ($CDCl_3$): 1.43, 1.48, 1.52 (3s, 21H, Boc and Ipr groups); 3.74 (s, 3H, OCH_3); 3.83 (d, 2H, $J_{AX} = 12$ Hz, H_β HmS-2); 3.96 (d, 2H, $J_{AB} = 12$ Hz, H_β HmS-1); 4.22 (d, 2H, $J_{AB} = 12$ Hz, H_β HmS-1); 4.66 (d, 2H, $J_{AX} = 12$ Hz, H_β HmS-2); 5.79 (bs, 1H, NH HmS-1); 8.16 (bs, 1H, NH HmS-2). FAB-MS (m/z): 447 $(MH)^+$, 469 $(MNa)^+$.

PyBroP – compound **1** was used; chromatography (AcOEt/heptane 1:2) yielded 0.130 g (58%) of the title homodipeptide. *HATU* – compound **1** was used; chromatography (AcOEt/heptane 1:1) yielded 0.200 g (90%) of the dipeptide **9**. *Fluoride* – compound **6** was used; the recrystallization (AcOEt/heptane) yielded 0.165 g (74%) of the product **9**.

(10) Fmoc-MeA-HmS(Ipr)-OMe ($C_{27}H_{32}N_2O_7$ MW. 496.5). Fmoc-MeA-F was used; after the chromatography with AcOEt/heptane (1:1), 0.090 g (36%) of the title dipeptide was obtained. Another experiment with doubled concentration of reactants as well as the reaction time (2 h) gave, after chromatography, the product in 61% yield (0.152 g). TLC: 0.26 (VI); HPLC: purity 99.3%, $t_R = 13.16$ min (45–75% B). ^1H NMR ($CDCl_3$): 1.38, 1.46, 1.56 (3s, 12H, Ipr and H_β MeA); 3.72 (s, 3H, OCH_3); 3.90–4.43 (m, 7H, H_β HmS and Fmoc $CHCH_2$); 5.39 (bs, 1H, NH MeA); 7.15 (bs, 1H, NH HmS); 7.28–7.78 (m, 8H, Fmoc arom.). FAB-MS (m/z): 497 $(MH)^+$, 519 $(MNa)^+$.

(11) Fmoc-HmS(Ipr)-HmS(Ipr)-OBzl ($C_{36}H_{40}N_2O_9$ MW. 644.7). Compounds **4** and**5** were coupled according to the fluoride method. The crude product was recrystallized from DCM/hexane. Yield 0.251 g (78%); m.p. 180–181 °C; TLC:

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0.57 (VI); HPLC: purity 98.4%, $t_R = 14.45$ min (60–90% B). ^{1}H NMR ($CDCl_3$): 1.41 (s, 3H, Ipr); 1.46 (s, 3H, Ipr); 1.51 (s, 3H, Ipr); 1.54 (s, 3H, Ipr); 3.73 (d, 2H, $J_{AX} = 12$ Hz, H_β HmS-2); 4.00 (d, 2H, $J_{AB} = 12$ Hz, H_β HmS-1); 4.19–4.25 (m, 3H, H_β HmS–1 and Fmoc CH); 4.36 (d, 2H, $J = 7$ Hz, Fmoc CH_2); 4.66 (d, 2H, $J_{AX} = 12$ Hz, H_β HmS-2); 5.17 (s, 2H, Bzl CH_2); 6.09 (bs, 1H, NH HmS-1); 7.34 (bs, 5H, Bzl arom.); 7.28–7.78 (m, 8H, Fmoc arom.); 8.19 (bs, 1H, NH HmS-2).

(12) Fmoc-Ala-HmS(Ipr)-OBzl ($C_{32}H_{34}N_2O_7$ MW. 558.6). Compound **4** and Fmoc-Ala-F were coupled according to the fluoride method. The crude product was recrystallized from AcOEt/hexane. Yield 0.222 g (80%); m.p. 140–141 °C; $[\alpha_D] = -21.99°$ ($c = 0.35$ MeOH); TLC: 0.28 (VI), 0.13 (VII); HPLC: purity 99.5%, $t_R = 9.76$ min (60–90% B), R_t 12.552 min (55–85% B). ^{1}H NMR ($CDCl_3$): 1.36 (d, 3H, $J = 7$ Hz, H_β Ala); 1.39 (s, 3H, Ipr); 1.45 (s, 3H, Ipr); 3.92–4.42 (m, 8H, H_β HmS, H_α Ala and Fmoc $CHCH_2$); 5.14 (s, 2H, Bzl CH_2); 5.31 (bd, 1H, $J = 6.5$ Hz, NH Ala); 6.83 (bs, 1H, NH HmS); 7.31 (bs, 5H, Bzl arom.); 7.28–7.78 (m, 8H, Fmoc arom.).

(13) Fmoc-Leu-HmS(Ipr)-OBzl ($C_{35}H_{40}N_2O_7$ MW.600.7). Compound **4** and Fmoc-Leu-F were coupled according to the fluoride method (2 mmole scale). The crude product was recrystallized from DCM/hexane. Yield 0.971 g (81%); m.p. 138–140 °C; $[\alpha_D] = -29.04°$ ($c = 0.63$ MeOH); TLC: 0.59 (VI), 0.40 (VII); HPLC: purity 99.3%, $t_R = 14.56$ min (60–90% B). ^{1}H NMR ($CDCl_3$): 0.91–0.94 (m, 6H, H_δ Leu); 1.39 (s, 3H, Ipr); 1.45 (s, 3H, Ipr); 1.60–1.71 (m, 3H, H_β and H_γ Leu); 3.91–4.48 (m, 8H, H_β HmS, H_α Leu and Fmoc $CHCH_2$); 4.97 (d, 1H, $J_{AB} = 12$ Hz; Bzl CH); 5.12 (bd, 1H, $J = 8.5$ Hz, NH Leu); 5.15 (d, 1H, $J_{AB} = 12$ Hz; Bzl CH); 6.79 (bs, 1H, NH HmS); 7.32 (bs, 5H, Bzl arom.); 7.28–7.78 (m, 8H, Fmoc arom.).

Synthesis of Tripeptides by (2 + 1) Coupling

(14) Fmoc-Phe-Leu-OH ($C_{30}H_{32}N_2O_5$ MW. 500.6). The title dipeptide was prepared using TBTU for the coupling of Fmoc-Phe-OH and TosOH × Leu–OBzl. The benzyl ester was cleaved by hydrogenolysis (at 50 psi on a Parr Apparatus for 4 h). m.p. 151–153 °C; $[\alpha]_D = -28.69°$ ($c = 1$ MeOH); TLC: 0.53 (II); HPLC: purity 99%, R_t 13.141 min (50–80% B), $t_R = 7.92$ min (60–90% B). ^{1}H NMR (DMSO-d_6): 0.82 (d, 3H, $J = 5.5$ Hz, H_δ Leu); 0.88 (d, 3H, $J = 5.5$ Hz, H_δ Leu); 1.45–1.70 (m, 3H, H_β and H_γ Leu); 2.68–2.97 (m, 2H, H_β Phe); 4.11–4.37 (m, 5H, H_α Leu, H_α Phe and Fmoc $CHCH_2$); 7.16–7.48 (m, 9H, Phe arom., Fmoc arom. and NH Phe); 7.55–7.87 (m, 5H, Fmoc arom.); 8.44 (bd, 1H, $J = 7$ Hz, NH Leu).

(15) Boc-HmS(Ipr)-Ala-OH ($C_{15}H_{26}N_2O_7$ MW. 346.4)

Synthesis of the dipeptide Boc-HmS(Ipr)-Ala-OBzl. Compound **1** (0.552 g, 2 mmol), TosOH × Ala–OBzl (0.703 g, 2 mmol), TBTU (0.707 g, 2.2 mmol) and DIEA (1.03 ml, 6 mmol) were stirred in DCM (5 ml) overnight. After the usual workup the residue was purified by chromatography (AcOEt/heptane 1 : 2) to give the protected dipeptide in a 71% yield (0.620 g).

The C-deprotection. A solution of Boc-HmS(Ipr)-Ala-OBzl (0.437 g, 1 mmol) in MeOH (20 ml) was hydrogenolyzed (4 h) in the presence of 10% Pd/charcoal catalyst (50 mg). After the filtration and evaporation, the residue was recrystallized from AcOEt/hexane. Yield 0.325 g (94%); m.p. 110–114 °C; $[\alpha]_D = -12.85°$ ($c = 0.35$ MeOH); TLC: 0.60 (I), 0.50 (II); HPLC: purity 100%, $t_R = 6.82$ min (30–60% B), $t_R = 12.18$ min (20–50% B). ^{1}H NMR (DMSO-d_6): 1.24 (d, 3H, $J = 7$ Hz, H_β Ala); 1.32, 1.34, 1.36 (3s, 15H, Boc and Ipr); 3.86 (d, 2H, $J_{AB} = 12$ Hz, H_β HmS); 4.02 (d, 2H, $J_{AB} = 12$ Hz, H_β HmS); 4.24 (qt, 1H, $J = 7$ Hz, H_α Ala); 6.90 (bs, 1H, NH HmS); 7.70 (bd, 1H, $J = 6.5$ Hz, NH Ala).

(16) Fmoc-Phe-Leu-HmS(Ipr)-OBzl ($C_{44}H_{49}N_3O_8$ MW. 747.9). To a cooled (−15 °C) solution of the dipeptide **14** (0.252 g, 0.5 mmol) and NMM (0.056 ml, 0.5 mmol) in DCM (1 ml) IBCF (0.066 ml, 0.5 mmol) was added and stirring at −15 °C was continued for 15 min. Then, the precooled solution of compound **4** (0.220 g, 0.5 mmol) and NMM (0.056 ml, 0.5 mmol) in DCM (2 ml) was added and the reaction mixture was stirred overnight allowing it to warm up to RT slowly. After the usual workup the crude product was purified by chromatography (AcOEt/heptane 2 : 3) to give the title compound as an amorphous solid. Yield 0.316 g (84%); $[\alpha]_D = -25.49°$ ($c = 0.40$ MeOH); TLC: 0.39 (V); 0.25 (VIII); HPLC: purity 99.6%, $t_R = 13.01$ min (65–95% B), $t_R = 16.76$ min (60–90% B). ^{1}H NMR ($CDCl_3$): 0.83 (s, 3H, H_δ Leu); 0.86 (s, 3H, H_δ Leu); 1.43 (s, 3H, Ipr); 1.46 (s, 3H, Ipr); 1.51–1.61 (m, 3H, H_β and H_γ Leu); 3.00–3.08 (m, 2H, H_β Phe); 3.92–3.98 (m, 2H, H_β HmS); 4.15–4.25 (m, 3H, H_β HmS and Fmoc CH); 4.30–4.48 (m, 4H, H_α Leu, H_α Phe and Fmoc CH_2); 5.12 (s, 2H, Bzl CH_2); 5.23 (bd, 1H, $J = 6$ Hz, NH Phe); 6.11 (b, 1H, NH Leu); 6.91 (bs, 1H, NH HmS); 7.16–7.78 (m, 18H, Phe arom., Bzl arom. and Fmoc arom.). FAB-MS (m/z): 690 $(M\text{-butyl})^+$.

The *HATU* method yielded 90% of the mixture of diastereoisomers Fmoc-Phe-L-Leu-HmS(Ipr)-OBzl and Fmoc-Phe-D-Leu-HmS(Ipr)-OBzl with the approximate ratio of 55 : 45, as judged by ^{1}H and ^{13}C NMR. The optical rotation – $[\alpha]_D = -2.90°$ ($c = 0.825$ MeOH) differed from that for the pure compound **16**. The HPLC analysis of the *N*-deprotected (with 20% piperidine in DMF) sample obtained by the HATU method revealed the presence of two epimers. The identical analysis of a sample obtained by the IBCF method indicated the single epimer.

(17) Boc-HmS(Ipr)-Ala-HmS(Ipr)-OBzl ($C_{29}H_{43}N_3O_{10}$ MW. 593.7). Compounds **15** (0.174 g, 0.5 mmol) and **4** (0.219 g, 0.5 mmol) were subjected to the identical procedure as described in the synthesis of compound **16.** The crude product was purified by chromatography with AcOEt/heptanes (1 : 2) to give an amorphous solid. Yield 0.219 g (74%); $[\alpha]_D = -11.99°$ ($c = 0.25$ MeOH); TLC: 0.73 (IV), 0.20 (VI), 0.15 (VII); HPLC: purity 99%, $t_R = 10.45$ min (50–80% B), $t_R = 16.43$ min (40–70% B). ^{1}H NMR ($CDCl_3$): 1.35, 1.38, 1.43, 1.45 (4s, 24H, Boc, Ipr groups and H_β Ala); 3.91–4.00 (m, 4H, H_β HmS); 4.17–4.42 (m, 4H, H_β HmS); 4.53 (q*t*, 1H, $J = 7$ Hz, H_α Ala); 5.11 (d, 1H, $J_{AB} = 12.5$ Hz, Bzl CH); 5.16 (d, 1H, $J_{AB} = 12.5$ Hz, Bzl CH); 5.56 (bs, 1H, NH HmS-1); 7.12 (bs, 1H, NH HmS-3); 7.35 (bs, 5H, Bzl arom.); 7.46 (bd, 1H, $J = 7.5$ Hz, NH Ala). FAB-MS (m/z): 594 $(MH)^+$, 616 $(MNa)^+$.

The HPLC analysis of the hydrolyzate (6 N HCl, 24 h, 110 °C) derivatized with the Marfey's reagent [18] revealed the 2.5% of D-Ala formed during the coupling reaction.

Cyclizations to Diketopiperazines

(18) cyclo-(Ala-HmS(Ipr)) ($C_{10}H_{16}N_2O_4$ MW. 228.2). To a solution of compound **12** (0.280 g, 0.5 mmol) in DCM (2 ml), piperidine (0.5 ml) was added with stirring. After 5 min,

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precipitation was observed. After 30 min, solids were filtered off, thoroughly washed with DCM and dried. Yield 0.096 g (84%); m.p. 293–295 °C (dec.); TLC: 0.76 (III), 0.33 (IV); HPLC: purity 99%, $t_R = 4.25$ min (15–30%B), $t_R = 12.72$ min (0–30% B). ^{1}H NMR ($CDCl_3/CF_3COOD$ 10 : 1): 1.60 (d, 3H, $J = 7$ Hz, H_β Ala); 2.30 (s, 6H, Ipr); 3.93 (dd, $J_{AB} = 12$ Hz, $J = 3$ Hz, H_β HmS); 4.07 (dd, $J_{AB} = 12$ Hz, $J = 7$ Hz, H_β HmS); 4.37 (q, 1H, $J = 7$ Hz, H_α Ala). FAB-MS (m/z): 228 $(M)^+$.

(19) cyclo-(Leu-HmS(Ipr)) ($C_{13}H_{22}N_2O_4$ MW.270.3). Compound **13** (0.301 g; 0.5 mmol) was subjected to the identical procedure as described in the synthesis of **18**. Yield 0.100 g (74%); m.p. 290 °C (dec.); TLC: 0.56 (IV); 0.10 (V); HPLC: purity 98%, $t_R = 4.84$ min (30–60% B), $t_R = 8.54$ min (20–50% B). ^{1}H NMR ($CDCl_3/CF_3COOD$ 10 : 1): 0.97 (d, 3H, $J = 7$ Hz, H_δ Leu); 1.00 (d, 3H, $J = 7$ Hz, H_δ Leu); 1.72–1.96 (m, 3H, H_β and H_γ Leu); 2.30 (s, 6H, Ipr); 3.94 (dd, $J_{AB} = 12$ Hz, $J = 3$ Hz, H_β HmS); 4.07 (dd, $J_{AB} = 12$, 7 Hz, H_β HmS); 4.26 (dd, 1H, $J = 9$, 3.5 Hz, H_α Leu). FAB-MS (m/z): 270 $(M)^+$, 271 $(MH)^+$, 309 $(MK)^+$.

(20) cyclo-(Ala-HmS) ($C_7H_{12}N_2O_4$ MW. 188.2). To a solution of compound **25A** (57 mg, 0.11 mmol) in DMF (2 ml), piperidine (0.5 ml) was added. After 30 min, the reaction mixture was evaporated *in vacuo* and the residue was partitioned between chloroform and water. The organic layer was then washed twice with water and the combined aqueous layers were lyophilized. Yield 19 mg (90%); TLC: 0.33 (I); HPLC: purity 97%, $t_R = 3.14$ min (0–30% B). ^{1}H NMR (DMSO-d_6): 1.28 (d, 3H, $J = 7$ Hz, H_β Ala); 3.24–3.57 (m, 4H, H_β HmS); 3.84 (q, 1H, $J = 7$ Hz, H_α Ala); 4.95 (t, 1H, $J = 6$ Hz, OH); 5.00 (t, 1H, $J = 6$ Hz, OH); 7.50 (s, 1H, NH HmS); 8.01 (b, 1H, NH Ala). FAB-MS (m/z): 189 $(MH)^+$, 211 $(MNa)^+$, 227 $(MK)^+$.

(21) Cyclo-(Leu-HmS) ($C_{10}H_{12}N_2O_4$ MW. 230.3). Compound **26A** (63 mg, 0.11 mmol) was subjected to the identical procedure as described in the synthesis of compound **20**. Yield 23 mg (92%); TLC: 0.46 (I); HPLC: purity 97%, $t_R = 9.07$ min (0–30% B). ^{1}H NMR (DMSO-d_6): 0.83 (d, 3H, $J = 5.5$ Hz, H_δ Leu); 0.85 (d, 3H, $J = 5.5$ Hz, H_δ Leu); 1.53–1.68 m, 2H, H_β Leu); 1.77–1.94 (m, 1H, H_γ Leu); 3.21–3.56 (m, 4H, H_β HmS); 3.70–3.76 (m, 1H, H_α Leu); 4.96 (bt, 2H, $J = 5$ Hz, OH); 7.50 (s, 1H, NH HmS); 8.03 (b, 1H, NH Leu). FAB-MS (m/z): 231 $(MH)^+$, 253 $(MNa)^+$, 269 $(MK)^+$, as in Figure 2.

Isopropylidene Removal and the Accompanying N → O-acyl Shift

(22) Ac-HmS(Ipr)-NHMe ($C_{10}H_{18}N_2O_5$ MW. 230.3). *Synthesis of Ac-HmS(Ipr)-OH.* To a solution of TosOH × HmS(Ipr)–OH (1.315 g, 3.78 mmol) and DMAP (20 mg) in pyridine (4 ml), the acetic anhydride (4 ml) was added dropwise and stirring was continued overnight. The residue obtained after evaporation was worked up analogously as in the synthesis of compound **1**. The crude product was recrystallized from the mixture of AcOEt/MeOH/hexane to yield 0.388 g of the desired derivative (47%).

Coupling with methylamine. Ac-HmS(Ipr)-OH (0.218 g, 1 mmol), TBTU (0.354 g, 1.1 mmol) and DIEA (0.354 ml, 2 mmol) were stirred in DCM (2 ml) for 1 h. Then, methylamine (1 ml of 33% w/w solution in ethanol, *ca* 8 mmol) was added and the reaction mixture was stirred for another

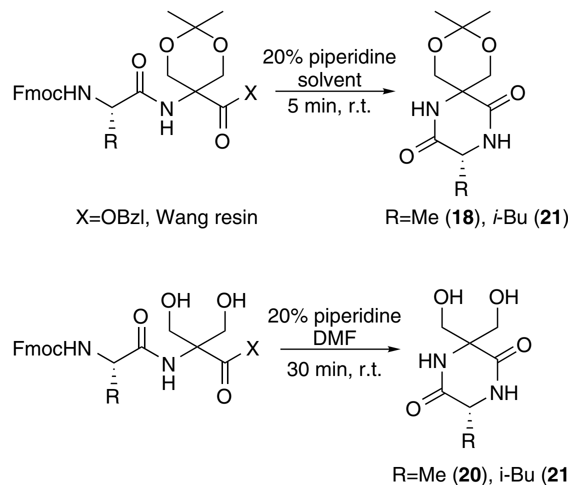

Figure 2 Cyclization of the dipeptides with the *C*-terminal HmS(Ipr) and HmS residues to the corresponding diketopiperazines.

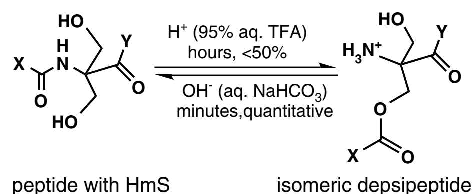

Figure 3 The $N \rightarrow O$-acyl shift observed for the α-hydroxy methylserine peptides. **X** and **Y** represent *N*- and *C*-terminus of the molecule respectively.

4 h. After the evaporation the mixture was subjected directly to flash chromatography ($CHCl_3$/MeOH 40 : 1). The fractions containing product (R_F 0.19) were evaporated and the resulting residue was recrystallized from $CHCl_3$/hexane. Yield 0.170 g (74%); m.p. 145–146 °C; HPLC: purity 100%, $t_R = 7.64$ min (5–30% B). ^{1}H NMR ($CDCl_3$): 1.47 (s, 3H, Ipr); 1.66 (s, 3H, Ipr); 2.03 (s, 3H, Ac); 2.89 (d, 2H, $J = 5$ Hz, Me); 3.72 (d, 2H, $J_{AX} = 12.5$ Hz, H_β HmS); 4.78 (d, 2H, $J_{AX} = 12.5$ Hz, H_β HmS); 6.68 (bs, 1H, NH HmS); 7.34 (b, 1H, NH).

(23) Ac-HmS-NHMe ($C_7H_{14}N_2O_4$ MW. 190.2). Compound **22** (23 mg, 0.1 mmol) was treated with the 95% aq. TFA (2 ml) for 5 min. After the evaporation, the residue was redissolved in water (8 ml) and the saturated $NaHCO_3$ solution (200 µl) was added to convert the 28% (as determined by HPLC) of the HmS(*mono*-Ac)-NHMe **(24)** back to the desired compound **23**. After evaporation, HPLC purification (0–30% B) and lyophilization, a glassy material was obtained. Yield: 17 mg (89%); HPLC: purity 99.4%, $t_R = 3.21$ min (0–30% B). ^{1}H NMR (D_2O with DSS standard): 2.04 (s, 3H, Ac); 2.72 (s, 3H, Me); 3.82 (d, 2H, $J_{AB} = 12.5$ Hz, H_β HmS); 3.92 (d, 2H, $J_{AB} = 12.5$ Hz, H_β HmS). ^{13}C NMR (D_2O with DSS standard): 25.02 (CH_3 Ac); 28.53 (CH_3 methylamide); 63.48 (C_β HmS); 67.08 (C_α HmS); 175.83 (amide CO); 176.94 (amide CO). FAB-MS (m/z): 191 (MH^+), 213 (MNa^+), 229 (MK^+).

(24) HmS(mono-Ac)-NHMe (as TFA salt) ($C_7H_{14}N_2O_4$ x $C_2HF_3O_2$ MW. 304.2). Compound **22** was deprotected with the 95% aq. TFA for 1 h. The mixture of compounds **23** and

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24 was formed in the 40 : 58 ratio, as determined by HPLC. Those compounds were separated by semipreparative HPLC (for conditions – see compound **23**). The later eluting peak was identified to be the product of $N \rightarrow O$-acyl rearrangement.

HPLC: purity 99%, $t_R = 3.75$ min (0–30% B). ^{1}H NMR (D_2O with DSS standard): 2.13 (s, 3H, Ac); 2.80 (s, 3H, Me); 3.92 (d, 1H, $J_{AX} = 12.5$ Hz, H_β HmS CH_2OH); 4.00 (d, 1H, $J_{AB} = 12.5$ Hz, H_β HmS CH_2OAc); 4.42 (d, 1H, $J_{AB} = 12.5$ Hz, H_β HmS CH_2OAc); 4.54 (d, 1H, $J_{AX} = 12.5$ Hz, H_β HmS CH_2OH). ^{13}C NMR (D_2O with DSS standard): 22.53 (CH_3 Ac); 28.97 (CH_3 methylamide); 63.51 (C_β HmS in CH_2OH); 65.52 (C_β HmS in CH_2OAc); 66.61 (C_α HmS); 119.04 (q, $^1J_{CF} = 292$ Hz, TFA CO); 165.9 (q, $^2J_{CF} = 35$ Hz, CF_3); 170.27 (ester CO); 175.41 (amide CO). FAB-MS (m/z): 191 (MH^+), 213 (MNa^+), 229 (MK^+) calcd for $C_7H_{14}N_2O_4$ 190.

(25) Deprotection of Fmoc-Ala-HmS(Ipr)-OBzl

(25A) Fmoc-Ala-HmS-OBzl ($C_{29}H_{30}N_2O_7$ MW. 518.6). Compound **12** (0.172 g, 0.3 mmol) was treated with the 95% aq. TFA (2 ml) for 5 min. Then the reaction mixture was diluted with AcOEt and washed three times with saturated $NaHCO_3$ solution, then with 1 N $KHSO_4$, and finally with brine. After the drying and evaporation of the organic layer, the crude product was recrystallized from AcOEt/hexane. Yield 0.127 g (82%); m.p. 137–139 °C; $[\alpha_D] = -3.61°$ ($c = 0.47$ DMF); TLC: 0.51 (IV), 0.06 (V); HPLC: purity 97.8%, $t_R = 10.18$ min (50–80% B). ^{1}H NMR (DMSO-d_6): 1.27 (d, 3H, $J = 7$ Hz, H_β Ala); 3.79–3.88, 4.15–4.32 (m, 8H, H_β HmS, H_α Ala and Fmoc $CHCH_2$); 4.72 (bt, 1H, $J = 6$ Hz, OH); 4.80 (bt, 1H, $J = 6$ Hz, OH); 5.11 (s, 2H, Bzl CH_2); 7.29–7.45 (m, 10H, Bzl and Fmoc arom. and NH Ala); 7.71–7.74 (m, 2H, Fmoc arom.); 7.85 (s, 1H, NH HmS); 7.89 (d, 2H, $J = 8$ Hz, Fmoc arom). FAB-MS (m/z): 519 $(MH)^+$, 541 $(MNa)^+$, 557 $(MK)^+$.

(25B) HmS(mono-FmocAla)-OBzl (as TFA salt). When compound **12** was treated with the 95% aq. TFA for a prolonged period (48 h) the depsipeptidic product was formed in 52% as determined by HPLC. This compound was separated by semipreparative HPLC (50–80% B). TLC: 0.37 (IV); HPLC: $t_R = 6.47$ min (50–80% B). FAB-MS (m/z): 519 $(MH)^+$, 541 $(MNa)^+$, 557 $(MK)^+$. The ^{13}C NMR and ^{1}H NMR spectra revealed the presence of two diastereoisomers in a 45 : 55 ratio. The depsipeptide was converted to Fmoc-Ala-HmS-OBzl **(25A)** by a brief treatment with 0.1 N $NaHCO_3$ in the 50% aq. acetonitrile.

(26) Deprotection of Fmoc-Leu-HmS(Ipr)-OBzl

(26A) Fmoc-Leu-HmS-OBzl ($C_{32}H_{36}N_2O_7$ MW. 560.6). Compound **13** (0.200 g, 0.33 mmol) was subjected to the identical procedure as described in the synthesis of **25A**. The crude product was recrystallized from AcOEt/MeOH/hexane.

Yield 0.143 (77%); m.p. 177–178 °C; $[\alpha_D] = -15.20°$ ($c =$ 0.48 DMF); TLC: 0.70 (IV), 0.16 (V); HPLC: purity 99.8%, $t_R = 8.15$ min (60–90% B). ^{1}H NMR (DMSO-d_6): 0.86 (d, 3H, $J = 6$ Hz, H_δ Leu); 0.88 (d, 3H, $J = 6$ Hz, H_δ Leu); 1.46–1.69 (m, 3H, H_β and H_γ Leu); 3.79–3.90, 4.11–4.40 (m, 8H, H_β HmS, H_α Leu and Fmoc $CHCH_2$); 4.70 (bt, 1H, $J = 6$ Hz, OH); 4.81 (bt, 1H, $J = 6$ Hz, OH); 5.09 (s, 2H, Bzl CH_2); 7.28–7.48 (m, 10H, Bzl and Fmoc arom. and NH Leu); 7.72 (d, 2H, $J = 8$ Hz, Fmoc arom.); 7.87 (s, 1H, NH HmS); 7.89 (d, 2H, $J = 8$ Hz, Fmoc arom.). FAB-MS (m/z): 561 $(MH)^+$, 583 $(MNa)^+$, 599 $(MK)^+$.

(26B) HmS(mono-FmocLeu)-OBzl (as TFA salt). When compound **13** was treated with the 95% aq. TFA for a prolonged period (24 h), the depsipeptidic product was formed (36% as determined by HPLC). That compound was separated by semipreparative HPLC (60–90% B). TLC: 0.44 (III); HPLC: $t_R = 5.82$ min (60–90% B). FAB-MS (m/z): 561 $(MH)^+$, 583 $(MNa)^+$, 599 $(MK)^+$. The ^{13}C NMR spectrum revealed the presence of two diastereoisomers in an almost 1 : 1 ratio. The depsipeptide was converted to Fmoc-Leu-HmS-OBzl **(26B)** by a brief treatment with 0.1 N $NaHCO_3$ in the 50% aq. acetonitrile.

Solid-phase Synthesis

(27) Bz-Phe-HmS-Asp-Lys-OH (× TFA) ($C_{30}H_{39}N_5O_{10} \times C_2HF_3O_2$ FW. 743.7). **Resin loading:** Wang resin (0.89 mmol OH/g) was acylated twice (2 × 30 min) with Fmoc-Lys(Boc)-F (3 equiv., conc. 0.2 M) in the presence of 40% pyridine in DCM [15]. The unreacted hydroxyl groups were capped with the Ac_2O/pyridine/DCM (1 : 2 : 3) mixture for 30 min. The resin loading was determined as 0.66 mmol/g. The 2-chlorotrityl chloride resin was loaded with Fmoc-Lys(Boc)-OH according to the procedure in the literature [19], which resulted with the loading of 0.46 mmol/g.

Peptide assembly: In the coupling reactions performed on Wang resin, the mixture of Fmoc-AA-OH (3 equiv.), HATU (3 equiv.) and DIEA (3 equiv.) in DCM was added. In the synthesis applying 2-chlorotrityl chloride resin, double the amount of DIEA (6 equiv.) in DMF was added. Couplings were repeated, and to accomplish the maximal acylation of the HmS(Ipr) amino group the prolonged reaction times (24 and 2 h for the repeated coupling) were applied. Each Fmoc-removal was carried out by a single (15 min) treatment with the 20% piperidine in DMF. The assembled N-deprotected tetrapeptide was benzoylated while attached to the resin with the 20 equiv. of Bz–Cl in 30% pyridine/DCM for 30 min.

Peptide cleavage and purification: Cleavage from Wang resin was accomplished by treatment with 95% aq. TFA for 4 h. Cleavage from the 2-chlorotrityl resin was performed with the AcOH/TFE/DCM (1 : 1 : 8) mixture [20] for 30 min. The crude products were purified by HPLC (20–50% B). Yields (0.1 mmol scale) are corrected for the actual loading of the resin.

1. Synthesis on Wang resin. The crude product (yield 57 mg–83%) was a mixture of peptide/depsipeptide (63 : 37).

Peptide Bz-Phe-HmS-Asp-Lys-OH (× TFA) – 26 mg (38%); HPLC: $t_R = 15.70$ min (10–40% B); FAB-MS (m/z): 630 $(MH)^+$, 652 $(MNa)^+$, 668 $(MK)^+$, calcd for $C_{30}H_{39}N_5O_{10}$ 629.

Depsipeptide HmS(*mono*-BzPhe)-Asp-Lys-OH (× 2TFA) (FW. 857.7): 20 mg (25%); HPLC: $t_R = 14.27$ min (10–40% B); FAB-MS (m/z): 630 $(MH)^+$, calcd for $C_{30}H_{39}N_5O_{10}$ 629. The depsipeptide was converted on the HPLC scale to the parent peptide by a brief treatment with a 0.1 N $NaHCO_3$ solution.

2. Synthesis on the 2-chlorotrityl chloride resin. The cleavage yielded the side-chain-protected tetrapeptide Bz-Phe-HmS(Ipr)-Asp(Bu^t)-Lys(Boc)-OH. Yield 60 mg (72%); TLC:

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0.38 (II); HPLC: purity 86%, $t_R = 13.94$ min (50–80% B). FAB-MS (m/z): 826 $(MH)^+$, 848 $(MNa)^+$, 864 $(MK)^+$, calcd for $C_{42}H_{59}N_5O_{12}$ 825.

The cleaved compound was deprotected under the controlled conditions (using the HPLC monitoring) with the 95% aq. TFA for 30 min. After the purification the title tetrapeptide was obtained in 83% yield (overall for cleavage and deprotection was 60%).

(28) Fmoc-HmS$_4$-OH ($C_{31}H_{40}N_4O_{15}$ MW. 708.7). The procedure, as per the literature, [20] was used to attach Fmoc-HmS(Ipr)-OH **(2)** to the 2-chlorotrityl chloride resin (1.4 mmol Cl/g), which resulted with loading of 0.52 mmol/g. The evaluation of the crude mixture after cleavage was performed by TLC and HPLC analysis, using the standard samples of Fmoc-[HmS(Ipr)]$_2$-OH and Fmoc-[HmS(Ipr)]$_3$-OH, prepared in the separate experiments. After the acidic deprotection (95% aq. TFA for 15 min) the tetrapeptide **30** was isolated by HPLC (30–60% B).

1. Fluoride method. Double couplings (2 × 1 h) were applied, using 3 equiv. of Fmoc-HmS(Ipr)-F in the presence of 3 equiv. of DIEA in DMF (conc. 0.2 M). The composition of the mixture (30 mg; crude 36%) after cleavage: Fmoc-[HmS(Ipr)]$_4$-OH [TLC: 0.38 (II); HPLC: $t_R = 13.52$ min (60–90% B)] – 52%, Fmoc-[HmS(Ipr)]$_3$-OH (R_F 0.49; t_R = 11.40 min) – 36%, Fmoc-[HmS(Ipr)]$_2$–OH (R_F 0.56; t_R = 9.33 min) – 9%.

Yield: 4 mg (6%). For the analytical data – see the HATU method.

2. HATU method. Double couplings (2 × 1 h) were applied, using 3 equiv. of Fmoc-HmS(Ipr)-OH/HATU/DIEA mixture in a 1 : 1 : 2 molar ratio (in DMF, conc. 0.2 M). The composition of the mixture (53 mg, crude 62%) after cleavage: Fmoc-[HmS(Ipr)]$_4$-OH – 77%, Fmoc-[HmS(Ipr)]$_3$-OH – 15%, Fmoc-[HmS(Ipr)]$_2$-OH – 4%.

Yield: 23 mg (32%). TLC: 0.24 (I); HPLC: $t_R = 10.12$ min (20–50% B); ^{1}H NMR (DMSO-d_6): 3.67–3.85 (m, 17H, H_β HmS and Fmoc CH); 4.31–4.71 (b, OH and Fmoc CH_2); 7.35–7.50 (m, 6H, Fmoc arom. and NH); 7.64 (bs, 1H, NH); 7.79–7.96 (m, 5H, Fmoc arom. and NH). FAB-MS (m/z): 709 $(MH)^+$, 731 $(MNa)^+$, 747 $(MK)^+$.

RESULTS AND DISCUSSION

The HmS(Ipr) Derivatives

In our previous paper [13,14], we described the usefulness and limitations of the Z/Ipr/OMe protective group combination for the orthogonal protection of α-hydroxymethylserine. However, the key intermediate TosOH × HmS(Ipr)-OH provides an easy access to other derivatives of the O,O-protected α-hydroxymethylserine. Compound Boc-HmS(Ipr)-OH **(1)** is readily available in 86% yield by the acylation with di-t-butyl dicarbonate. This result clearly contrasts with the low (25–30%), hardly reproducible yield of the three-day preparation of Boc-HmS-OH [8]. The other O,O-protected derivative Boc-HmS(MOM)$_2$-OH was obtained by the Japanese group [9] in a three-step synthesis in 33% overall yield starting from HmS. Our investigation of the relative stability of the Boc and Ipr protective groups against several acidolytic agents revealed, as expected, the higher liability of the O,O-isopropylidene protection. This fact precludes the use of the Boc strategy for the elongation of the peptide from the C-terminus. Therefore, the application of the Boc-HmS(Ipr)-OH derivative is limited for the preparation of peptides with the N-terminal HmS (see tripeptide **17**). On the other hand, the Fmoc/Ipr combination offers the possibility of the orthogonal deprotection. The derivative Fmoc-HmS(Ipr)-OH **(2)** was obtained in 86% yield by acylation with 9-fluorenylmethyl chloroformate. The results of our earlier syntheses with the O,O-unprotected HmS ranged from 40 to 50% of Fmoc-HmS-OH [12]. The C-protecting benzyl ester was introduced via cesium salt [21] of compound **2**, furnishing the fully protected compound Fmoc-HmS(Ipr)-OBzl **(3)** in 79% yield. The Fmoc-removal accomplished by using 20% piperidine in DCM expectedly did not affect the Ipr group and yielded the O,O-protected benzyl ester HmS(Ipr)-OBzl (see experiment **4**). Also as expected, the Fmoc group was stable against the acidolytic conditions (95% aq. TFA) applied for the removal of the Ipr protection (e.g. experiments **25–26**).

The N-urethane-protected amino acid fluorides developed by Carpino *et al.* [16] are regarded as highly activated derivatives suitable for the assembly of sterically hindered sequences [17]. We have employed this known method for the synthesis of Fmoc-HmS(Ipr)-F **(5)** and Boc-HmS(Ipr)-F **(6)** [15]. Both compounds were obtained in crystalline, stable form in good yields (84 and 68% for **5** and **6**, respectively).

The solid-supported version of the C-protection namely, the attachment of the Fmoc-protected HmS(Ipr) residue to Wang, and the 2-chlorotrityl chloride resin [20], has already been described [15].

Incorporation of the HmS(Ipr) into a Peptide Chain

Our earlier experiments [13,14] showed that TBTU was efficient to couple the HmS(Ipr) residue as the carboxyl component, whereas it gave mediocre yields when the amino group of the HmS(Ipr) was acylated. In order to find more efficient methods for coupling HmS(Ipr) as the amino component we compared PyBroP [22], fluorides [16] and HATU [23] in the synthesis of the dipeptides with the C-terminal HmS(Ipr) residue.

HATU outperformed the other activation methods tested in this study. In particular, the high yield (90%) in the synthesis of the homodipeptide **9** is noteworthy (Table 1). The assembly of the MeA-HmS(Ipr) sequence seems to be particularly demanding, even with the use of Fmoc-MeA-F. This derivative proved its efficiency in a high-yielding (>90%) synthesis of homodipeptide Fmoc-MeA$_2$-OMe [24]. Our result indicates lower

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effectiveness of the fluoride method in the acylation of the HmS(Ipr) amino group, as compared with coupling involving the prototypical $C^{\alpha,\alpha}$- disubstituted glycine MeA.

In order to find the most effective coupling method for the solid-phase incorporation of the sterically hindered HmS(Ipr) residue, we compared the acid fluoride method and HATU for the assembly of a homotetrapeptide. The 2-chlorotrityl chloride resin was chosen as the solid support in order to preserve the Ipr protection [15], which should facilitate the analytical and preparative separation of peptides differing by a single amino acid unit. We applied double couplings (2×1 h) to each of the compared methods. The crude mixtures obtained after the cleavage were analyzed by TLC and HPLC using the standard samples of sequences with deletions. Both methods worked well in the first coupling, but for the longer sequence the HATU was more efficient. As a result, the crude mixture obtained by fluoride activation **(28A)** contained only 52% of the protected tetrapeptide Fmoc-[HmS(Ipr)]$_4$-OH, along with 36% of the tri- and 9% of the dipeptide. This contrasts with the higher crude yield and purity obtained by the HATU method **(28B)** – the tetrapeptide (77% by HPLC) was accompanied by smaller amount of incomplete sequences (15 and 4% of tri–and dipeptide, respectively). The overall yield of the deprotected homotetrapeptide Fmoc-HmS$_4$-OH **(33)** (Table 1) obtained with the use of HATU was reasonable (32%), as compared to the poor result when using the acid fluoride method (6%). Our results indicate that synthesis of HmS(Ipr) homosequence is more demanding than the assembly of the adjacent MeA units. Indeed, the use of the acid fluoride was successful in the solid-phase synthesis of MeA homotetrapeptide, giving nearly quantitative coupling yields [17]. In conclusion, for incorporation of the

Table 1 Methods applied for the ‘difficult’ coupling with the HmS(Ipr) residue

Peptide	PyBroP (%)	PG-AA-F (%)	HATU (%)
Boc-Ala-HmS(Ipr)-OMe **(7)**	66	67	81
Boc-MeA-HmS(Ipr)-OMe **(8)**	32	a	59
Boc-HmS(Ipr)-HmS(Ipr)-OMe **(9)**	58	74	90
Fmoc-MeA-HmS(Ipr)-OMe **(10)**	—	36	—
Fmoc-HmS$_4$-OH **(28)**[b]	—	6[c]	32[c]

[a] Compound Boc-MeA-F (not previously described in literature) could not be obtained; the synthesis of the dipeptide **10** was performed as a complementary experiment.
[b] Compound **28** was synthesized applying the 2-chlorotrityl chloride resin as the solid support.
[c] Yields after peptide cleavage, removal of the Ipr protection and HPLC purification.

third and further consecutive HmS(Ipr) residues, the HATU coupling should be repeated three times and the reaction time extended to 24 h.

Side Reactions Accompanying Peptide Elongation

We encountered difficulties in the attempted stepwise peptide $C \rightarrow N$ elongation from the *C*-terminal HmS(Ipr) residue.

During the *N*-deprotection of the dipeptides Fmoc-AA-HmS(Ipr)-OBzl [AA: Ala **(12)**, Leu **(13)**] carried out under the usual conditions, a fast cyclization to the corresponding diketopiperazines (**18** and **19**) occurred (see Figure 2). Interestingly, also the corresponding dipeptides (**25A** and **26A**) containing the acyclic, *O,O*-unprotected HmS underwent spontaneous cyclization as a consequence of the *N*-deprotection. It is known, that diketopiperazine formation can be substantial with residues that can form the *cis* peptide bond, e.g. *N*-methylamino acids, Pro, Gly or D-amino acids in either the first or second position of the ($C \rightarrow N$) synthesis [25]. Whether a peptide with the $C^{\alpha,\alpha}$-disubstituted glycine such as HmS, may exist in a *cis–trans* equilibrium is an open question.

The undesired cyclization precludes the stepwise ($C \rightarrow N$) synthesis. Thus, aiming to prepare tripeptides bearing the *C*-terminal HmS, we have examined the fragment $(2 + 1)$ condensation.

When HATU was applied in the synthesis of Fmoc-Phe-Leu-HmS(Ipr)-OBzl **(16),** the resulting product contained a substantial amount (45%) of the D-Leu epimer. Coupling conditions affected the chirality of the activated Leu residue, which could be detected by the ^{1}H and ^{13}C NMR spectroscopy. Although those epimers could not be chromatographically resolved, the HPLC analysis of the *N*-deprotected samples revealed the presence of the two isomers. In another preparation of the tripeptide **16,** we tried the mixed anhydride method using IBCF [26]. The spectroscopic examination of the product furnished in that experiment did not reveal the undesired D-Leu epimer. This observation was confirmed by the HPLC analysis of the *N*-deprotected sample, which indicated a single compound. Therefore, we were encouraged to apply the mixed anhydride method for the fragment coupling leading to Boc-HmS(Ipr)-Ala-HmS(Ipr)-OBzl **(17)**. We obtained 74% of the product contaminated with only 2.5% of the D-Ala epimer, as determined by the HPLC analysis of hydrolyzate derivatized according to Marfey’s method [18].

It is likely, that in the coupling experiments described above, the use of HATU at room temperature leads to “overactivation”, allowing undesired epimerization at the *C*-terminus to compete with the desired amide bond formation. Similar findings regarding significant (up to 75%) epimerization occurred during coupling of tetrapeptidic fragments with aromatic (unreactive) amines have just recently been described [27]. We

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did not attempt to optimize HATU conditions in terms of temperature or choice of base, hoping that milder (performed at −20 °C), mixed anhydride activation might strike the balance between the rate of coupling *versus* epimerization. In the fragment coupling involving the HmS(Ipr) amino group the mixed anhydride method affected the optical purity of the activated residue only to a small extent, and therefore this method should be recommended.

The solid-phase synthesis of peptides with a *C*-terminal HmS residue is troublesome by using the Wang resin, due to the premature cleavage of the dipeptides, while the use of 2-chlorotrityl chloride resin as a solid support eliminates the diketopiperazine formation, which is described in detail in our earlier publication [15].

Isopropylidene Removal and the Acompanying N → O-acyl Shift

The preferred reagent for the *O,O*-deprotection of the HmS(Ipr) peptides is the 95% aq. TFA. This was documented by the succesful preparation of the Z-(HmS)$_n$-OMe ($n = 1-3$) series of homopeptides in solution [12]. However, during the prolonged acidic exposure of a peptide with the *O,O*-unprotected HmS an undesired process may occur. The $N \rightarrow O$-acyl shift [28] resulting in the formation of an isomeric depsipeptide was observed for certain peptides with the HmS preceded by a proteinaceous α-amino acid (Figure 3).

The extent of migration depends mainly on the nature and the steric bulk of the neighboring acyl segment. As an example, acidolysis of the model compound Ac-HmS(Ipr)-NHMe **(22)** gave rise to 28% (as determined by HPLC) of HmS(mono-Ac)-NHMe **(24)** in 5 min, while the dipeptides Fmoc-AA-HmS(Ipr)-OBzl could be smoothly deprotected under identical conditions (see experiments **25–26**). The corresponding depsipeptides HmS(mono-FmocAA)-OBzl were detected in substantial amounts only after longer acidolysis – 52% of **25B** (AA = Ala) and 36% of **26B** (AA = Leu) after 24 or 48 h, respectively. The $N \rightarrow O$-acyl shift creates a new center of chirality at the α-carbon of HmS, but the isolated depsipeptides **25B** and **26B** appeared in the HPLC analysis as single peaks. Only closer spectroscopic examination revealed the presence of the two diastereoisomers in an almost 1 : 1 ratio. All isolated depsipeptides were quantitatively transformed into isomeric, desired peptides under mild basic treatment with diluted $NaHCO_3$, which is a well-known method to reverse the $N \rightarrow O$-acyl shift [28].

The acidolytic peptide cleavage after the solid-phase synthesis could also be endangered by the undesired $N \rightarrow O$-acyl migration. When Wang resin was applied as a solid support in the synthesis of the tetrapeptide Bz-Phe-HmS-Asp-Lys-OH the isomeric depsipeptide HmS(mono-BzPhe)-Asp-Lys-OH **(27A)** was formed in substantial amount (37% by HPLC) during the 4 h peptide cleavage by means of 95% aq. TFA. The isolated side product was characterized by FAB-MS and showed the identical molecular mass peaks as the parent peptide. The depsipeptide was transformed on the HPLC scale into the corresponding desired peptides by a brief treatment with diluted $NaHCO_3$ solution. The presence of the Ipr protective group in the cleaved peptide synthesized by the 2-chlorotrityl chloride resin methodology [15] is advantageous for the post-cleavage transformation, like the side-chain deprotection. This allows for the minimization of the undesired $N \rightarrow O$-acyl shift, which was demonstrated in the synthesis of the tetrapeptide Bz-Phe-HmS-Asp-Lys-OH (see experiment **27B**). After the peptide assembly on the solid support the tetrapeptide Bz-Phe-HmS(Ipr)-Asp(^{t}Bu)-Lys(Boc)-OH was cleaved from the solid support. The final deprotection was optimized thanks to careful HPLC control, avoiding the prolonged acidic treatment and the extensive rearrangement. As a result, the depsipeptide formation was minimized (<5%) and the final product was obtained in a better overall yield as compared to the synthesis on Wang resin. Concluding, in order to diminish the extent of the $N \rightarrow O$-acyl shift the deprotection time should be reduced to minimum.

CONCLUSIONS

HATU proved to be the most efficient coupling reagent for the stepwise incorporation of the HmS(Ipr) residue into a peptide chain both in solution and in solid-phase synthesis. The dipeptides with the *C*-terminal HmS(Ipr) and HmS residue have distinguished tendency for cyclization to diketopiperazines, which precludes their $C \rightarrow N$ elongation. For the fragment $(2 + 1)$ coupling with the HmS(Ipr) amino group the mixed anhydride method should be preferred in order to reduce the risk of epimerization that highly affects the HATU activation method. Another side reaction endangering certain peptides with the *O,O*-unprotected HmS is the $N \rightarrow O$-acyl shift occurring during the prolonged exposure to TFA. However, the products of the rearrangement may be converted back to the desired peptides by a brief treatment with $NaHCO_3$ solution. The possibility of the Ipr removal in a separate step offered by the use of the 2-chlorotrityl chloride resin allowed us to minimize the undesired migration in the solid-phase synthesis of the HmS-derived peptides.

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