Research Article

Journal of PeptideScience

Received: 4 August 2008 Revised: 24 November 2008 Accepted: 25 November 2008 Published online in Wiley Interscience: 2 February 2009

(www.interscience.com) DOI 10.1002/psc.1111

Investigation of the synthetic route to pepstatin analogues by SPPS using *O*-protected and *O*-unprotected statine as building blocks

Cosimo D. Cadicamo,[a] Vivian Asante,[a] Morhaf Abu Ammar,[a] Claudia Borelli,[b] Hans C. Korting[b] and Beate Koksch[a]*

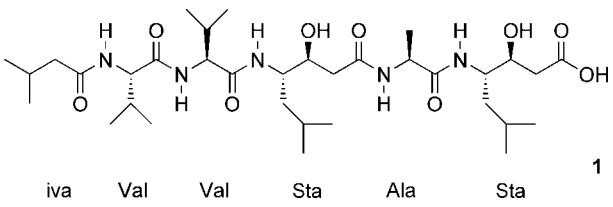
The synthetic route to pepstatin derivatives by a solid phase peptide synthesis using either *O*-protected or *O*-unprotected statine as a building block has been investigated. Statine was prepared according to a modified literature procedure, whereas protection of its 3-hydroxyl moiety using *tert*-butyldimethylsilylchloride (TBSCl) provided the novel *O*-TBS-protected statine building block. The *O*-*tert*-butyldimethylsilyl (TBS)-protected statine approach provides an improved synthetic strategy for the preparation of statine-containing peptides as demonstrated by the synthesis of the pepstatin analogue iva-Val-*Leu*-Sta-Ala-Sta.

Keywords: pepstatin A; O-silylation; SPPS; N-Fmoc-O-TBS-statine

Introduction

Pepstatin A (**1**), a pentapeptide isolated from *Actinomycetes* by Umezawa *et al*. [1], is a nonselective inhibitor of aspartic proteinases. This class of proteolytic enzymes includes human and nonhuman proteins that are associated with widespread pathological conditions like hypertension (renin), acquired immune deficiency syndrome (AIDS, HIV protease), cancer (cathepsin D), and peptic ulcer disease (pepsin) [2]. Aspartic proteinases also play a major role in amyloid disease, malaria and fungal infections [2]. The inhibitory activity of pepstatin A is achieved by its central β-hydroxy γ-amino acid statine [(*3S*, *4S*)-3-hydroxy-4-amino-6-methylheptanoic acid]. This amino acid hydrogen bonds to the two catalytic aspartate residues, which are conserved in all aspartic proteinases, by virtue of its noncleavable bond –(CHOH-CH_2)– that mimics the transition state of amide bond hydrolysis. Crystallographic studies of pepstatin A and pepstatin derivatives in complex with various members of the aspartic proteinase family such as pepsin from *Rhizopus chinensis* [3] and penicillopepsin [4] have revealed an additional role for the (3*S*)-hydroxyl group of statine [5]. This hydroxyl moiety occupies a site that is usually taken up by the catalytic water molecule that is hydrogen-bonded to the catalytic aspartate side chains in the native enzyme. Such displacement of water produces a favorable increase in entropy, which contributes to the strength of inhibitor binding. For this reason, pepstatin has been termed as a collected-substrate inhibitor [5].

Although pepstatin has not been applied as a therapeutic agent for aspartic proteinase-associated diseases, by virtue of its lack of selectivity and unfavorable pharmacokinetic properties, the discovery and characterization of this inhibitor is of paramount importance. Rational modification of pepstatin's structure represents a starting point for the design of novel selective and potent aspartic proteinase inhibitors. Therefore, the chemical synthesis of numerous pepstatin derivatives (see for example [6–11]), as well as synthetic routes to statine and analogs thereof (see for example [12–16]), have been reported in the literature over the past few decades. Although the presence of a free hydroxyl moiety could lead to competitive side reactions during amino acid couplings, e.g. *O*-acylation, the syntheses of pepstatin analogs via a SPPS methodology have been carried out, to our knowledge, using statine only in its *O*-unprotected form. A comparative study involving the preparation of pepstatin derivatives using either *O*-unprotected or *O*-protected statine approaches, however, has never been addressed. We report herein these two synthetic strategies as applied to one pepstatin analogue. Our aim was to determine the effect of the protection of the hydroxyl group of statine on SPPS efficiency for the preparation of statine-containing peptides. Statine was prepared according to a modified literature procedure [12], whereas protection of its 3-hydroxyl moiety using *tert*-butyldimethylsilylchloride (TBSCl) provided the novel *O*-*tert*-butyldimethylsilyl (TBS)-protected statine building block.



1

* *Correspondence to: Beate Koksch, Department of Organic Chemistry, FU Berlin, Takustraße 3, D-14195 Berlin, Germany. E-mail: koksch@chemie.fu-berlin.de*

a *Department of Organic Chemistry, FU Berlin, Takustraße 3, D-14195 Berlin, Germany*

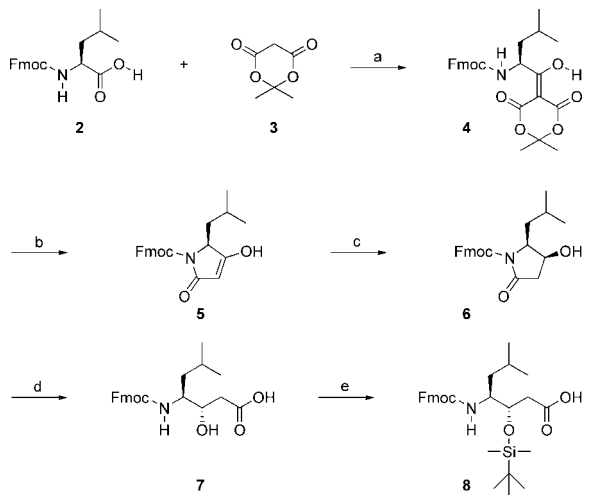
b *Departement of Dermatology and Allergy, Ludwig Maximilian University of Munich, Frauenlobstraße 9-11, D-80337 Munich, Germany*

Journal of PeptideScience

Results and Discussion

Preparation of (3*S*, 4*S*)-Fmoc-statine as its *O*-protected and *O*-unprotected Forms

Synthesis of the target amino acids, outlined in Scheme 1, was performed according to a methodology reported by Jouin *et al*. [12] with slight modifications. The key step in this approach is the stereoselective reduction of tetramic acid **5,** which generates exclusively the *pro-S* C-3 hydroxyl group of statine. In the first step, *N*,*N*-diisopropylcarbodiimide (DIC)-mediated condensation of Fmoc-L-leucine (**2**) with Meldrum's acid (**3**) in the presence of 4-*N*,*N*-dimethylaminopyridine (DMAP) afforded the adduct **4**, which was carried forward without purification. In fact, as described by Jouin and coworkers as well as by other authors [15], all attempts to purify this compound by column chromatography were unsuccessful. Therefore, tetramic acid **5** was generated by refluxing a solution of crude **4** in ethyl acetate for 45 min. Although this product is amenable to purification by silica gel chromatography, it was carried forward without purification. Stereoselective reduction of the crude intermediate **5** with sodium borohydride under acidic conditions affords (4*S*, 5*S*)-4-hydroxy-5-isobutyl-(9*H*-fluoren-9-ylmethyloxycarbonyl)-pyrrolidin-2-one (**6**) in moderate yield (~40% over three steps) after purification. It is interesting to note that stereoselectivity is determined by the presence of the *N*-protecting group of the tetramic acid; a mixture of epimers at the C-4 alcohol is obtained when the reduction is carried out on *N*-unsubstituted tetramic acids [17]. The acidic hydrolysis of **6** using an aqueous hydrochloric acid solution in dioxane under refluxing conditions for 3 h affords (3*S*, 4*S*)-Fmoc-statine **7** in high yield (80%). In the final step, the hydroxyl group of **7** was protected as its TBS ether. Such a protecting group has already been proven to be suitable for Fmoc-based peptide synthesis [18]. The use of TBS as a protecting group is advantageous as *O*-silylation normally occurs smoothly, affording the desired compound in high yield. Furthermore, it can be removed easily during cleavage of the peptide from the resin with TFA. *O*-Silylation was carried out in a concentrated DMF solution using TBSCl in the presence of imidazole and a catalytic amount of DMAP. Purification by flash chromatography afforded the novel *N*,*O*-protected statine (**8**) in high yield (86%) as an oily material which became a white solid after co-evaporation with cyclohexane.



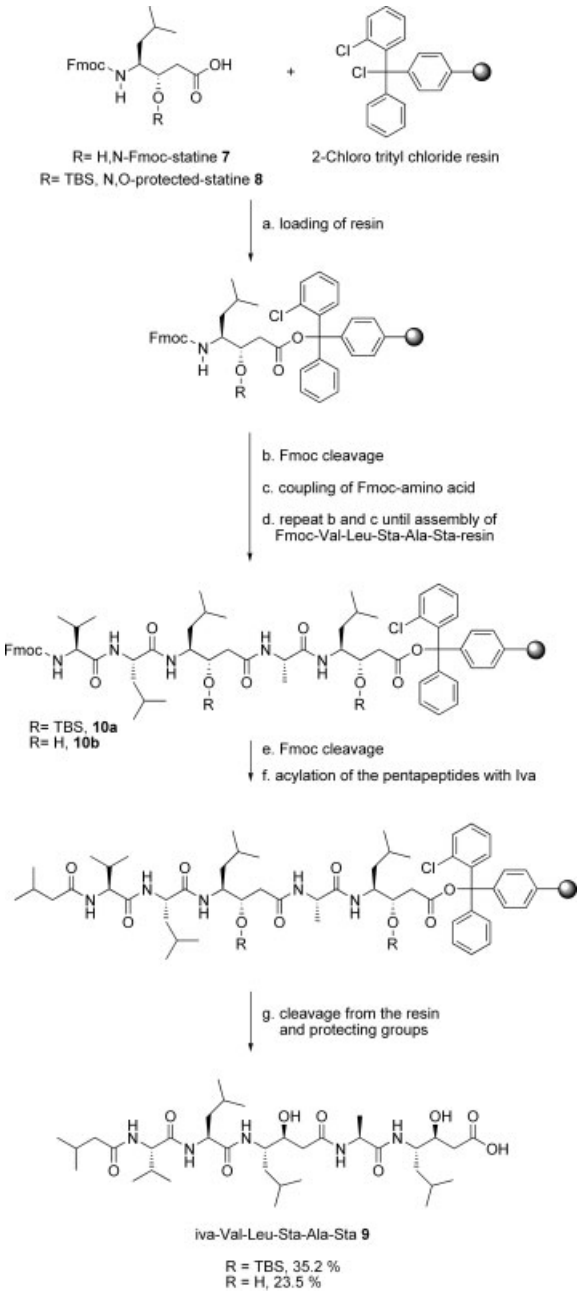
Scheme 1. Reagents and conditions: (a) DIC, DMAP, DCM, 3h; (b) EtOAc, reflux, 45 min; (c) $NaBH_4$, DCM/AcOH (9 : 1), 5h (~40% yield over three steps); (d) 1 M HCl, dioxane, 3 h (80%); (e) TBSCl, imidazole, DMAP (cat.), DMF, 24 h (86%).

Preparation of the Pepstatin Analogue iva-Val-*Leu*-Sta-Ala-Sta (9) Using Either *O*-protected or *O*-unprotected Statine

In order to compare the difference between the *O*-protected and *O*-unprotected statine building blocks in terms of efficiency of SPPS and peptide purification, we envisaged preparation of the pepstatin analogue iva-Val-*Leu*-Sta-Ala-Sta (**9**). This peptide was previously prepared in our laboratory and tested regarding its activity in inhibiting the secreted aspartic proteinases (SAP) of the human pathogen *Candida albicans* (unpublished work). Parallel syntheses of peptide **9** were performed by Fmoc-based SPPS methodology using 2-chlorotrityl chloride resin as the solid support (Scheme 2). After loading the resin with either statine **7** or **8**, the pentapeptides **10a** and **10b** were constructed automatically by sequential Fmoc-deprotection, with piperidine in DMF, and coupling of the amino acids using standard activating reagents (TBTU/HOBt) in the presence of DIPEA. The final step, acylation of the *N*-terminus, was performed manually using isovaleryl chloride and DIPEA as the base. Peptide **9** was then released from the resin by treatment with TFA and purified by semipreparative reverse phase HPLC, and finally characterized by ESI mass spectrometry.

The yields of peptide **9** from the *O*-protected and *O*-unprotected statine approaches were 42.3 and 37.1%, respectively. This small difference in yields was further investigated by analytical HPLC of the crude peptides prior to purification (Figure 1). Although the HPLC traces of the crude products appear similar, full integration of all peaks present in both HPLC chromatograms provides a quantitative description. Crude peptide **9** obtained from the *O*-unprotected statine approach (Figure 1(a)) accounts for 71.2% of all integrated peak area while that obtained from the *O*-protected statine approach accounts for 75.6% (Figure 1(b)). The major by-products that are common to both approaches were identified by ESI mass spectrometry. The peak at 22.6 min shows a molecular mass of $m/z = 700.5$ which corresponds to the $[M+H]^+$ of our target molecule. This compound could be the result of racemization that occurred during amino acid coupling. The peak at 23.2 min shows the presence of two compounds with masses of $m/z = 700.5$ and $m/z = 543.4$. The latter mass corresponds to the tetrapeptide iva-Val-Leu-Ala-Sta, which is formed as a consequence of incomplete coupling of the central statine. The by-products represented by the peaks at 18.8, 21.2 and 29.4 min indicated a mass of $m/z = 671.5$, $m/z = 644.4$ and $m/z = 796.4$, respectively. Unfortunately, no unambiguous assignments can be made for these impurities. A further mass analysis was carried out for the peak at 27.3 min which is the major noncommon side product present just in the *O*-unprotected statine strategy (Figure 1(a)). ESI mass analysis gave a mass of $m/z = 799.5$, which disappointingly could not be assigned.

Although both approaches produced fewer similar impurities, it is evident that the use of *O*-unprotected statine as a building block gives rise to a more numerous and more abundant by-products resulting in a lower yield of peptide **9**. The syntheses were carried out twice using both approaches, and the final outcome was reproducible. This result demonstrates that although statine-based peptides can be successfully obtained using *O*-unprotected statine, the use of *O*-protected statine as a building block provides an improved synthetic route, which is especially important



Scheme 2. SPPS methodology for the preparation of the pepstatin analogue **9** using either *O*-TBS-protected and *O*-unprotected statine building blocks.

when considering longer sequences containing multiple statine residues.

Conclusion

The synthesis of a pepstatin analogue has been accomplished via two strategies, in which either *O*-TBS protected statine or *O*-unprotected statine were used as building blocks. Both routes produced the target peptide in reasonable yields, but the *O*-protected statine strategy proved to be a more efficient approach as the pure pepstatin analogue was obtained with fewer side products and in a higher overall yield. On the basis of the results

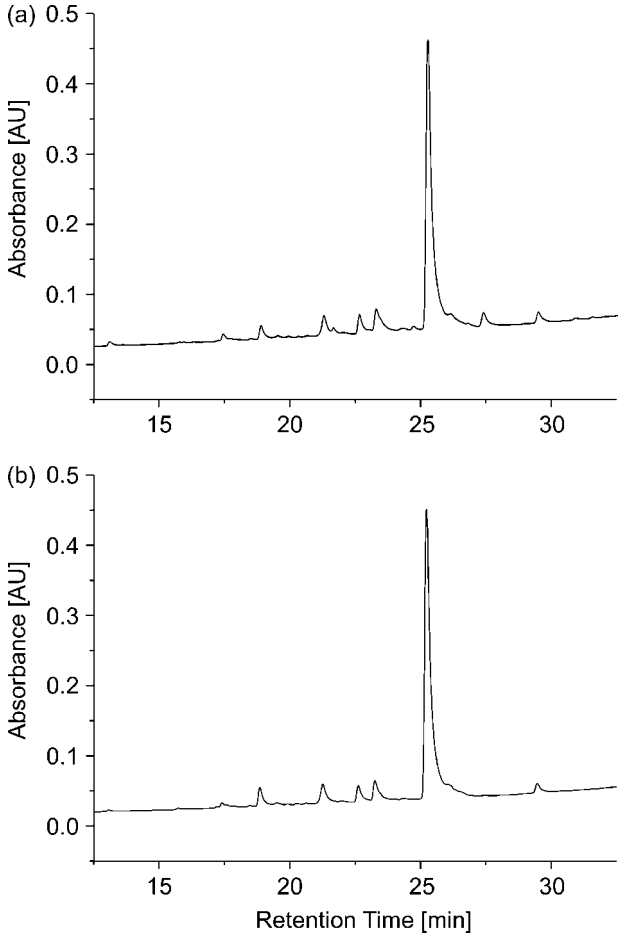


Figure 1. HPLC analyses of the crude peptide iva-Val-*Leu*-Sta-Ala-Sta prepared via (a) *O*-unprotected and (b) *O*-TBS-protected statine approaches.

reported here, we expect that the use of *N*-Fmoc-*O*-TBS-statine as a building block for the synthesis of statine-containing peptides will be especially important for the preparation of peptides with longer amino acid sequences and/or a greater statine content.

Experimental

Materials and Methods

All reagents of synthetic grade were used as supplied. 2-Chlorotritylchloride resin (1.55 mmol/g) was purchased from Iris Biotech GmbH (Marktredwitz, Germany). 2-(1*H*-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU), 1-hydroxybenzotriazole (HOBt) and all amino acids with the exception of statine were purchased from Gerhardt (Wolfhagen, Germany). Dimethylformamide (DMF), acetonitrile [(ACN), HPLC grade], *N*,*N*-DIPEA), *N*,*N*-diisopropylcarbodiimide (DIC), trifluoroacetic acid (TFA), triisopropylsilane (TIS), piperidine (99% extra pure), isovaleryl chloride, Meldrum's acid, TBSCl and *N*,*N*-DMAP were purchased from Acros Organics (Geel, Belgium). HPLC grade TFA (Uvasol) was purchased from Merck (Darmstadt, Germany). Acetic anhydride (99%, Acros) was distilled prior to use. DCM was dried over calcium hydride. Deionized water for HPLC was prepared using the MilliQ-AdvantageA10-System (Millipore). Air- and moisture-sensitive reactions were carried out under inert atmosphere using oven-dried glassware (>100 °C). Reaction progress

was monitored by TLC performed using Merck silica gel 60 F_{254} plates. Compounds were detected by either UV or by the use of an appropriate staining agent. Column chromatography was performed using Sigma Aldrich (Munich, Germany) Kieselgel 60 silica gel (230–400-nm mesh).

NMR Spectroscopy

All NMR spectra were measured using a *Bruker* AC 250 spectrometer operating at 250 MHz for ^{1}H and 63 MHz for ^{13}C, and a *JEOL* ECP 500 operating at 500 MHz for ^{1}H and 125 MHz for ^{13}C. All chemical shifts (δ) are reported in parts per million (ppm) and are quoted relative to the residual proton peak of either $CDCl_3$ or d_6-DMSO. Spectral coupling patterns are designated as follows; s: singlet; d: doublet; dd: doublet of doublets; ddd: doublet of doublet of doublets; t: triplet; q: quartet; m: multiplet and br: broad signal.

Mass Spectroscopy

ESI-TOF mass spectrometry was carried out on an Agilent 6210 ESI-TOF, Agilent Technologies, Santa Clara, CA, USA; electron impact mass spectrometry (EI-MS) and **e**lectron impact high-resolution mass spectrometry (EI-HRMS) measurements were carried out on a Varian MAT 711.

Analytical HPLC

Analytical reverse-phase HPLC was carried out on a LaChrom-HPLC L-7000 interface (Merck) equipped with two HPLC pumps L-7100, a diode array continuous flow detector L-7450 and an autosampler L-7200 with 100-µl sample loop. The following column was used: Capell C8(2) column (5-µm particle size, 300-Å pore size, 250×4.60-mm inner diameter, Shiseido, Japan). The gradient elution was performed with solutions A and B, where A was 100% H_2O, with 0.1% TFA (v/v) and B was 100% ACN, with 0.1% TFA (v/v). The gradient used was as follows: Linear gradient from 95% A–5% B to 20% A–80% B over 30 min.

Preparative HPLC

Crude peptides were purified by reverse-phase HPLC on a Smartline Manager 5000 System (Knauer GmbH, Berlin, Germany) equipped with two smartline pumps 1000 and a UV detector 2500 on a LUNA C8(2) column (10-µm particle size, 300-Å pore size, 250 × 21.20-mm inner diameter, Phenomenex, USA). Peptides were dissolved in 10 ml of 50% ACN, 50% H_2O with 0.1% TFA (v/v/v) and multiply injected into the HPLC. Purification was performed by means of a Linear A and B gradient, where solvent A was 100% H_2O with 0.1% TFA (v/v/v) and solvent B was 100% ACN with 0.1% TFA (v/v/v). The gradient applied to isolate the pure peptides was: linear gradient from 95% A–5% B to 40% A–60% B over 30 min. The flow rate was 20 ml/min and absorbance was recorded at 230 nm.

(2′*S*)-5-{[1-hydroxy-4-methyl-2-(9*H*-fluoren-9-ylmethyloxycarbonyl)-amino]-pentylidene}-2,2-dimethyl-1,3-dioxane-4,6-dione (4)

To a solution of *N*-Fmoc-Leucine (3.53 g, 10 mmol) in DCM (50 ml), Meldrum's acid (1.59 g, 11 mmol, 1.1 eq) and DMAP (1.83 g, 20 mmol, 1.5 eq) were added. DIC (1.9 ml, 12 mmol, 1.2 eq) was subsequently added drop-wise, and the reaction was stirred for 3 h at room temperature. The reaction mixture was filtered and a solution of potassium hydrogen sulphate (5 g in 50 ml H_2O) was added to the filtrate with vigorous stirring. After 5 min, the organic phase was separated and washed first with brine and then with water. Finally, the organic phase was dried over sodium sulphate and the solvent removed under vacuum. Crude product **4** was carried forward without further purification.

(5*S*)-4-Hydroxy-5-isobutyl-1-(9*H*-fluoren-9-ylmethyloxycarbonyl)-pyrrol-2(5*H*)-one (5)

Crude product **4** was dissolved in ethyl acetate (50 ml) and the solution was heated at 70 °C for 45 min. Afterwards, the solvent was evaporated and the product dried under high vacuum. Crude product **5** was carried forward without further purification.

(4*S*, 5*S*)-4-Hydroxy-5-isobutyl-1-(9*H*-fluoren-9-ylmethyloxycarbonyl)-pyrrolidin-2-one (6)

Crude product **5** was dissolved in a mixture of DCM and acetic acid (50 : 5 ml) and cooled to 0 °C in an ice bath. Sodium borohydride (0.7 g, 10 mmol) was then added over a period of 30 min. After stirring for 5 h at 0 °C, the mixture was first washed with brine and then with water. The organic phase was dried over sodium sulphate and the solvent removed under vacuum. Purification of the crude product over silica gel using a mixture of hexane/ethyl acetate as the eluent (50 : 50) afforded **6** as a white foam (1.52 g, 40.1%); *m*/z (EI+) 379.1 $[M + H]^+$; ^{1}H-NMR (500 MHz; $CDCl_3$): δ = 0.89 [6 H, dd, $(C\underline{H}_3)_2$–CH], 1.44–1.50 [1 H, m, $(CH_3)_2$–CH–CH–H], 1.67–1.80 [2 H, m, $(CH_3)_2$–C$\underline{H}$, $(CH_3)_2$–CH–C$\underline{H}$—H], 2.36 (1 H, br, OH), 2.64 (1 H, dd, 3-CH–H), 2.74 (1 H, dd, 3-C$\underline{H}$–H), 4.16–4.21 (1 H, m, 5-CH), 4.30 (1 H, t, fluorenyl C$\underline{H}$–CH_2O), 4.49–4.59 (3 H, m, 4-CH, fluorenyl CH–C$\underline{H}_2$O), 7.33 and 7.41 (4 H, 2 pseudo t, 4× fluorenyl CH), 7.71–7.78 (4 H, m, 4× fluorenyl CH); ^{13}C-NMR (63 MHz; $CDCl_3$): δ = 21.9, 23.0 ($\underline{C}H_3)_2$–CH, 24.8 $(CH_3)_2$–$\underline{C}$H, 36.6 [$\underline{C}H_2CH(CH_3)_2$], 40.2 ($\underline{C}H_2$–CO–N), 46.5 (fluorenyl $\underline{C}$H–CH_2), 60.0 $(CH_3)_2$–CH–CH_2–$\underline{C}$H), 65.2 (CH–OH), 68.3 (fluorenyl CH–$\underline{C}H_2$O), 119.8, 125.0, 127.1, 127.7 (8× fluorenyl CH), 141.1, 143.2 (4× fluorenyl quartenary C), 151.6 (N–CO–O), 171.8 (COOH).

(3*S*, 4*S*)-3-Hydroxy-4-(9*H*-fluoren-9-ylmethyloxycarbonyl)-amino-6-methylheptanoic acid (7)

To a solution of **6** (1.20 g, 3.16 mmol) in dioxane (15 ml), HCl (1 M, 3.00 ml, 3 mmol) was added and the mixture was heated to reflux for 3 h. The mixture was then cooled to room temperature and the solvent evaporated. Ethyl acetate (50 ml) and brine (30 ml) were added and, after separation, the organic phase was washed with brine again. The organic phase was dried over sodium sulphate and the solvent removed under vacuum. Purification of the crude product over silica gel using a mixture of ethyl acetate/hexane (80 : 20) and 0.3% acetic acid as the eluent afforded an oil which yielded **7** as a white solid after precipitation from an ether/hexane solution (1.00 g, 80%); *m*/z (EI+) 398.2 $[M + H]^+$; ^{1}H-NMR (500 MHz; d_6-DMSO): δ = 0.85 [6 H, dd, $(C\underline{H}_3)_2$—CH], 1.21–1.39 [2 H, m, $(CH_3)_2$CH–C$\underline{H}_2$], 1.50–1.61 [1 H, m, $(CH_3)_2$—C$\underline{H}$], 2.15 (1 H, dd, HC$\underline{H}$–COOH), 2.37 (1H, dd, $\underline{H}$CH–COOH), 3.54–3.61 [1 H, m, $(CH_3)_2$CHCH$_2$—C$\underline{H}$], 3.84–3.91 (1 H, m, C$\underline{H}$–OH), 4.21 (1 H, t, fluorenyl C$\underline{H}$–CH_2O), 4.25–4.35 (2 H, m, fluorenyl CH–C$\underline{H}_2$O), 6.94 (1 H, d, NH), 7.31 (2 H, dd, 2× fluorenyl CH), 7.41 (2 H, pseudo t, 2× fluorenyl CH), 7.71 (2 H, pseudo t, 2× fluorenyl CH), 7.88 (2 H, d, 2× fluorenyl CH); ^{13}C-NMR (63 MHz; d_6-DMSO): δ = 21.7, 23.3 ($\underline{C}H_3)_2$–CH, 24.3 $(CH_3)_2$–$\underline{C}$H, 38.2 ($\underline{C}H_2$–COOH), 38.6

$(CH_3)_2CH-\underline{C}H_2$, 46.8 (fluorenyl $\underline{C}H-CH_2O$), 52.6 $(CH_3)_2CHCH_2-\underline{C}H$, 65.0 (fluorenyl $CH-\underline{C}H_2O$), 69.1 (CH–OH), 120.0, 125.1, 126.9, 127.5 (8× fluorenyl CH), 140.1, 143.7, 142.8 (4× fluorenyl quartenary C), 156.0 (N–CO–O), 173.0 (COOH).

(3*S*, 4*S*)-3-*tert*-butyldimethylsilanoyloxy-4-(9*H*-fluoren-9-ylmethyloxycarbonyl)-amino-6-methylheptanoic acid (8)

7 (1.02 g, 2.57 mmol) was dissolved in DMF (4.00 ml) under inert atmosphere. After adding imidazole (1.02 g, 14.98 mmol, 6 eq), TBSCl (1.14 g, 7.56 mmol, 3 eq) and a catalytic amount of DMAP, the reaction mixture was stirred for 5 h at room temperature. Imidazole (1.02 g, 14.98 mmol, 6 eq) and TBSCl (1.14 g, 7.56 mmol, 3 eq) were then added again and the reaction was stirred for 24 h. Methanol (9.70 ml/1.1 mmol) was added and the mixture stirred for another hour. This mixture was diluted with 25% citric acid (20 ml) and extracted with ethyl acetate (3× 20 ml). The combined extracts were washed with water and brine and finally dried over sodium sulphate. Evaporation of the solvent gave a viscous oil, which was purified over silica gel using a mixture of ethyl acetate/hexane (70 : 30) and 0.1% acetic acid as the eluent. Co-evaporation with cyclohexane of the oily residue obtained afforded final product **8** as a white solid (1.14 g, 86%); *m*/z (EI-HRMS) calcd for $C_{25}H_{32}NO_5Si$ $[M-C_4H_9]^+$ 454.2050, found 454.2066; ^{1}H-NMR (500 MHz; d_6-DMSO): δ = 0.01 (3 H, s, Si–$C\underline{H}_3$), 0.08 (3 H, s, Si–$C\underline{H}_3$), 0.76–0.91 [15 H, m, $(C\underline{H}_3)_2-CH$, $C(C\underline{H}_3)_3$], 1.22–1.32 [2 H, m, $(CH_3)_2-CH-C\underline{H}_2$], 1.50–1.60 [1 H, m, $(CH_3)_2-\underline{C}H$], 2.10 (1 H, dd, $HC\underline{H}-COOH$), 2.46 (1 H, d, $\underline{H}CH-COOH$), 3.55–3.65 [1 H, m, $(CH_3)_2CHCH_2-\underline{C}H$], 4.02–4.11 [1 H, m, $C\underline{H}-O-Si-(CH_3)_2$], 4.21 (1 H, t, fluorenyl $C\underline{H}-CH_2O$), 4.25–4.40 (2 H, m, fluorenyl $CH-C\underline{H}_2O$), 7.18 (1 H, d, NH), 7.32 (2 H, ddd, 2× fluorenyl CH), 7.41 (2 H, pseudo t, 2× fluorenyl CH), 7.68 (2 H, pseudo t, 2× fluorenyl CH), 7.88 (2 H, d, 2× fluorenyl CH), 12.17 (1 H, br, COOH); ^{13}C-NMR (125 MHz; d_6-DMSO): δ = −5.1, −4.8 $(CH_3)_2Si$, 17.5 $[\underline{C}(CH_3)_3]$, 21.3, 23.5 $(\underline{C}H_3)_2-CH$, 24.3 $(CH_3)_2-\underline{C}H$, 25.6 $[C(\underline{C}H_3)_3]$, 35.5 $(CH_3)_2-CH-\underline{C}H_2$, 37.1 ($\underline{C}H_2-COOH$), 46.8 (fluorenyl $\underline{C}H-CH_2O$), 52.6 $(CH_3)_2-CH-CH_2-\underline{C}H$, 65.0 (fluorenyl $CH-\underline{C}H_2O$), 71.0 $[\underline{C}H-OSi-(CH_3)_2]$, 120.0, 125.0, 126.9, 127.5 (8× fluorenyl CH), 140.6, 140.7, 143.7, 143.8 (4× fluorenyl quartenary C), 155.8 (N–CO–O), 173.1 (COOH).

Peptide Synthesis

The pepstatin analogue was synthesized using a SyroXP-I peptide synthesizer (MultiSynTech GmbH, Witten, Germany) on a 0.05-mmol scale using 2-Cl-tritylchloride resin and Fmoc-protected amino acids.

Preparation of the Statine Loaded Resins

2-Cl-tritylchloride resin (268.5 mg) was preswelled in DCM (3 ml) in a syringe with a sinter for 10 min. After swelling, the solvent was removed. Statine (0.1 mmol) and DIPEA (75 µl, 0.43 mmol) were dissolved in dry DCM (4 ml) and this solution was added to the resin. The mixture was shaken for 3 h. After removing the solvent, any free 2-Cl-tritylchloride linkers were capped by treatment of the resin with a solution of DCM/MeOH/DIPEA (17 : 2 : 1; v/v/v) (3× 6 ml) for 15 min, and subsequently with a solution of DMF/DIPEA/acetic anhydride (8 : 1 : 1; v/v/v) (2× 5 ml) for 20 min. The resin was then washed with DMF (2× 3 ml) and DCM (6× 3 ml) and finally dried under vacuum. The loading and loading efficiency for the *O*-protected statine were 0.36 mmol/g and 90%, respectively, using the method described by Markus Gude and coworkers [19]; for the *O*-unprotected statine the values were determined to be 0.34 mmol/g and 85%.

Fmoc-cleavage

Fmoc deprotection was performed twice (10 min each) after each coupling step, using first a solution of 40% piperidine in DMF (2 ml) and secondly, 20% piperidine in DMF (2 ml).

Coupling of the Amino Acids

All amino acids were coupled following preactivation to the corresponding HOBt ester using a DMF solution of HOBt/TBTU (2 ml) in the presence of 4 eq of DIPEA in *N*-methylpyrrolidone (NMP, 0.4 ml). Each coupling was performed twice for 30 min using a fourfold excess of the amino acid and activating reagents. In the case of statine, the coupling was performed manually using a threefold excess in the first coupling and one equivalent in second.

Acylation of the *N*-terminal Group

Acylation of the amino terminal group was performed manually prior to cleavage from the resin with TFA. Isovaleryl chloride (2 eq) was dissolved in DMF (6 ml) in the presence of DIPEA (6 eq). This solution was added to the resin and the mixture shaken for 15 min. This operation was repeated and the resin was washed first with DMF (3× 3 ml) and finally with DCM (6× 3 ml).

Cleavage of the Resin and Protecting Groups

A solution of TFA/TIS/H_2O (95 : 3 : 2 v/v/v) (3 ml) was added to the resin and the mixture stirred for 90 min. After the resin was filtered out, ice-cold diethyl ether was added to the solution containing the peptide and the mixture stored in the freezer. The resulting precipitate was isolated by centrifugation and dried under vacuum.

Purification of Peptide 9

Crude peptides were purified by semipreparative HPLC as described above. Pooled fractions containing the pure peptide were concentrated on a rotary evaporator until ACN and TFA had been removed. The peptides were partially dissolved by addition of acetic acid (∼10%) and the solution was subsequently lyophilized;

O-unprotected statine strategy: iva-Val-*Leu*-Sta-Ala-Sta: 13 mg, 37.1%; *m*/z (ESI+) 700.5 $[M + H]^+$

O-protected statine strategy: iva-Val-*Leu*-Sta-Ala-Sta: 14.8 mg, 42.3%; *m*/z (ESI+) 700.5 $[M + H]^+$

Acknowledgements

Financial support by the Research Commission of Freie Universität of Berlin is kindly acknowledged.

References

1. Umezawa H, Aoyagi T, Morishima H, Matsuzaki M, Hamada M. Pepstatin, a new pepsin inhibitor produced by actinomycetes. *J. Antibiot. (Tokyo)* 1970; **23**: 259–262.

2. Cooper JB. Aspartic proteinases in disease: A structural perspective. *Curr. Drug Targets* 2002; **3**: 155–173, references cited therein.
3. Bott R, Subramanian E, Davies DR. Three-dimensional structure of the complex of the *Rhizopus chinensis* carboxyl proteinase and pepstatin at 2.5-Åresolution. *Biochemistry* 1982; **21**: 6956–6962.
4. James MN, Sielecki A, Salituro F, Rich DH, Hofmann T. Conformational flexibility in the active sites of aspartyl proteinases revealed by a pepstatin fragment binding to penicillopepsin. *Proc. Natl. Acad. Sci. U.S.A.* 1982; **79**: 6137–6141.
5. Rich DH. Pepstatin-derived inhibitors of aspartic proteinases. A close look at an apparent transition-state analogue inhibitor. *J. Med. Chem.* 1985; **28**: 263–273.
6. Pesenti C, Arnone A, Bellosta S, Bravo P, Canavesi M, Corradi E, Frigerio M, Meille SV, Monetti M, Panzeri W, Viani F, Venturini R, Zanda M. Total synthesis of a pepstatin analog incorporating two trifluoromethyl hydroxymethylene isosteres (Tfm-GABOB) and evaluation of Tfm-GABOB containing peptides as inhibitors of HIV-1 protease an MMP-9. *Tetrahedron* 2001; **57**: 6511–6522.
7. Pichová I, Pavlicková L, Dostál J, Dolejsi E, Hrusková-Heidingsfeldová O, Weber J, Ruml T, Soucek M. Secreted aspartic proteases of *Candida albicans, Candida tropicalis, Candida parapsilosis* and *Candida lusitaniae. Inhibition with peptidomimetic inhibitors*. *Eur. J. Biochem.* 2001; **268**: 2669–2677.
8. McConnell RM, Frizzell D, Camp A, Evans A, Jones W, Cagle C. New pepstatin analogues: Synthesis and pepsin inhibition. *J. Med. Chem.* 1991; **34**: 2298–2300.
9. Maibaum J, Rich DH. Synthesis of the novel π-(benzyloxymethyl)-protected histidine analogue of statine. Inhibition of penicillopepsin by pepstatin-derived peptides containing different statine side-chain derivatives. *J. Med. Chem.* 1989; **32**: 1571–1576.
10. Boger J, Payne LS, Perlow DS, Lohr NS, Poe M, Blaine EH, Ulm EH, Schorn TW, LaMont BI, Lin TY, Kawai M, Rich DH, Veber DF. Renin inhibitors. Syntheses of subnanomolar competitive, transition-state analogue inhibitors containing novel analogue of statine. *J. Med. Chem.* 1985; **28**: 1779–1790.
11. Rich DH, Sun ETO, Ulm E. Synthesis of analogues of the carboxyl protease inhibitor pepstatin. Effect of structure on inhibition of pepstin and renin. *J. Med. Chem.* 1980; **23**: 27–33.
12. Jouin P, Castro B, Nisato D. Stereospecific synthesis of *N*-protected statine and its analogues *via* chiral tetramic acid. *J. Chem. Soc., Perkin Trans. 1* 1987; 1177–1782.
13. Reddy GV, Rao GV, Iyengar DS. A novel Wittig reaction of oxazolidinones: Stereospesific synthesis of *N*-Boc-(3*S*, 4*S*)-statine and *N*-Boc-(3*S*, 4*S*)-AHPPA. *Tetrahedron Lett.* 1999; **40**: 775–776.
14. Rich DH, Sun ET, Boparai AS. Synthesis of (3*S*, 4*S*)-4-amino-3-hydroxy-6-methylheptanoic acid derivatives. Analysis of diastereomeric purity. *J. Org. Chem.* 1978; **43**: 3624–3626.
15. Fehrentz JA, Bourdel E, Califano JC, Chaloin O, Devin C, Garrouste P, Lima-Leite AC, Llinares M, Rieunier F, Vizavonna J, Winternitz F, Loffet A, Martinez J. Synthesis of chiral urethane *N*-alkoxycarbonyl tetramic acids from urethane *N*-carboxyanhydrides (UNCAs). *Tetrahedron Lett.* 1994; **35**: 1557–1560.
16. Rittle KE, Homnick CF, Ponticello GS, Evans BE. A synthesis of statine utilizing an oxidative route to chiral α-amino aldehydes. *J. Org. Chem.* 1982; **47**: 3016–3018.
17. Galetotti N, Poncet J, Chiche L, Jouin P. Diastereofacial selectivity in reduction of chiral tetramic acids. *J. Org. Chem.* 1993; **58**: 5370–5376.
18. Ghosh AK, Shin D, Downs D, Koelsch G, Lin X, Ermolieff J, Tang J. Design of potent inhibitors for human brain memapsin 2 (β-Secretase). *J. Am. Chem. Soc.* 2000; **122**: 3522–3523.
19. Gude M, Ryf J, White PD. An accurate method for the quantitation of Fmoc-derivatized solid phase supports. *Lett. Pept. Sci.* 2002; **9**: 203–206.

