# Preparation of novel *O*-sulfated amino acid building blocks with improved acid stability for Fmoc-based solid-phase peptide synthesis

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***Received (in Cambridge, UK) 13th August 2001, Accepted 18th January 2002***
***First published as an Advance Article on the web 5th February 2002***

The chemical synthesis and use of *O*-sulfated hydroxy amino acids in solid-phase peptide synthesis has long been a difficult and delicate task for peptide chemists due to the intrinsic acid lability of the *O*-sulfate linkage. In this report, a significantly improved method for the introduction and acid-stabilization of sulfate groups onto serine, threonine, and hydroxyproline residues is described. In all three cases, the optimal sulfation conditions were found to be 5 equivalents of sulfur trioxide–*N,N*-dimethylformamide ($SO_3$·DMF) complex in DMF under anhydrous conditions. The addition of tetrabutylammonium (TBA) counter-ions during work-up served as a powerful *O*-sulfate stabilization agent. The preparation of Fmoc-Ser($SO_3^-N^+Bu_4$)-OH **3**, Fmoc-Thr($SO_3^-N^+Bu_4$)-OH **4** and Fmoc-Hyp($SO_3^-N^+Bu_4$)-OH **6** building blocks gave stable pure products with good solubilities in organic solvents in reproducible, high yields. Importantly, the tetrabutylammonium salts of *O*-sulfated hydroxy amino acids minimized the desulfation during fluoren-9-ylmethoxycarbonyl (Fmoc)-based peptide synthesis, TFA cleavage, and reversed-phase HPLC purification. Stability experiments with 95% TFA at room temperature showed that for all three derivatives desulfation was less than 5% after standard times for peptide deprotection and resin-cleavage times. In contrast to previous approaches that usually involve the use of sodium and barium salts, the synthesis and mass spectrometric analysis of sulfated amino acids and sulfate peptides was much improved by the presence of tetrabutylammonium salts.

## Introduction

The post-translational modification of peptides and proteins is an important biological process. A prerequisite for the study of such molecules by chemical synthesis is the availability of efficient and general methods for their effective preparation and purification.[1–8] In contrast to routine solid-phase peptide synthesis, the synthesis of post-translationally modified peptides, such as glycosylated, phosphorylated, and sulfated peptides, is considerable more challenging.[9–11] We examined the general preparation of sulfated serine, threonine and hydroxyproline derivatives for use in peptide synthesis because sulfated amino acid residues may be useful molecular probes. Although many synthetic studies have been performed on naturally occurring tyrosine-sulfated peptides, a general and simple synthetic strategy with wide applicability has not been established.

In general, the acid lability of the *O*-sulfate linkage creates a problem for the preparation of sulfated peptides with Fmoc-based solid-phase peptide synthesis (SPPS)[12] techniques. Although the synthesis of several tyrosine-sulfated peptides including cholecystokinin (CCK) analogues has been reported,[13] they require strict conditions, such as TFA cleavages at 0 °C and HPLC analysis at pH 6–7. In fact, Kitagawa *et al.* have recently reported[11] that Fmoc-Tyr($SO_3Na$)-OH derivatives undergo approximately 60% desulfation within 2 h at 18 °C. Although aliphatic sulfate esters may show different stability, it will be advantageous to stabilize the *O*-sulfate linkage during standard synthesis and purification conditions.

It has been predicted that sulfated amino acids can be stabilized by forming conjugate acid–base pairs or ion pairs with cations other than protons, sodium and barium.[14] Unfortunately, in our hands, we found that the formation of sodium and barium salts of sulfated amino acids, Fmoc-Aa($SO_3X$)-OH (Aa = Ser, Thr; X = Na, Ba), did not adequately improve their stability during HPLC purification, and severely hampered mass analysis. The use of protecting groups for the synthesis of sulfated carbohydrates[15] appears promising but such methods are not compatible with conventional solid-phase peptide synthesis.

Interestingly, it has been observed that ionic interactions between arginine (Arg) residues and sulfated amino acids within sulfated peptides form more-acid-stable salt bridge structures.[16] Moreover, the usefulness of tetrabutylammonium (TBA) salt formation in the synthesis of sulfated-tyrosine peptides have been recently reported to improve the stability of the *O*-sulfate groups towards mild acid resin-cleavage conditions (*i.e.*, acetic acid–dichloromethane 1 : 9) and reduce fragmentation during mass analysis.[17] However, this work did not investigate the applicability of TBA salts in the preparation of sulfated amino acid derivatives or its usefulness with standard side-chain deprotection and resin-cleavage conditions during Fmoc-SPPS (95% TFA).

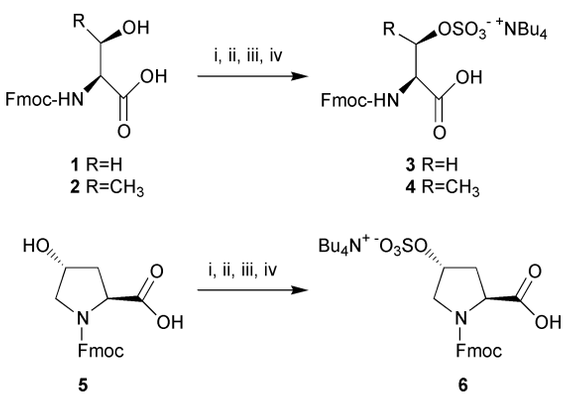
In this article, the simple and reliable preparation of novel sulfated hydroxy amino acids as stable building blocks and their use in solid-phase peptide synthesis is reported. More specifically, the preparation and stabilization of $N^{\alpha}$-Fmoc-protected sulfated derivatives of serine, threonine and hydroxyproline compatible with Fmoc-SPPS is described. The stability of the *O*-sulfate linkage in these building blocks, Fmoc-Aa-($SO_3^-N^+Bu_4$)-OH (Aa = Ser, Thr, Hyp), and also in resin-bound peptides to standard side-chain deprotection and resin-cleavage conditions (95% TFA at rt for 2–2.5 h) was investigated.

 DOI: 10.1039/b107320f

## Results and discussion

Several procedures have been reported for the *O*-sulfation of tyrosine residues, including $SO_3$·DMF and $SO_3$·pyridine complexes.[18] However, after the sulfation reaction a practical stabilization method for the strongly acid-labile sulfate group is necessary. Previously, barium and sodium salts have been used for this purpose; however, the preparation of these salts is prone to irreproducibility and is difficult to scale up.[19] The recent introduction of the TBA salt as a stabilization agent appears to be a promising solution to these problems. The usefulness of Fmoc-Tyr($SO_3^-N^+Bu_4$)-OH has recently been shown by the solid-phase synthesis of sulfated peptides containing two sulfated tyrosine residues.[17]

The present study describes the synthesis of novel sulfated amino acids (serine, threonine and hydroxyproline) as building blocks for Fmoc-SPPS. The lability of the *O*-sulfate linkage has been circumvented by the formation of the TBA salt on the sulfate group. The stability was shown to exist, under solid-phase-cleavage conditions, such as 95% TFA at room temperature. More specifically, the sulfated building blocks, Fmoc-Ser($SO_3^-N^+Bu_4$)-OH **3**, Fmoc-Thr($SO_3^-N^+Bu_4$)-OH **4** and Fmoc-Hyp($SO_3^-N^+Bu_4$)-OH **6**, were prepared from hydroxy amino acids **1**, **2** and **5** respectively, and synthesized by direct sulfation with DMF·$SO_3$ complex in DMF under anhydrous conditions for 2 hours at room temperature. The formation of TBA salts was required in all cases immediately after the sulfation reaction to stabilize the product. The TBA salt was formed at alkaline pH and, after acidification to pH 5, the product was extracted into chloroform. Following concentration and subsequent lyophilization from 1,4-dioxane–$H_2O$ (1 : 1) the expected products were obtained in high yield (Scheme 1).




**Scheme 1** *Reagents and conditions*: i) $SO_3$·DMF complex in DMF; ii) $Bu_4NHSO_4$, sat. aqueous $NaHCO_3$, 0 °C; iii) citric acid (pH 5); iv) extraction with chloroform.

High-performance liquid chromatography (HPLC)-based retention-time analysis of the crude reaction product clearly showed that the sulfation reactions were quantitative (Fig. 1). Compounds **3**, **4** and **6** were characterized by nuclear magnetic resonance (NMR) spectroscopy and electrospray mass spectrometry (ES-MS). The introduction of an electronegative *O*-sulfate group resulted in a downfield shift for the neighbouring proton, of 2 to 6 ppm for the adjacent carbon in the $^{13}C$ spectra, and from 0.2 to 0.6 ppm in the $^1H$ spectra. At the position of the substitution, for the building blocks **3**, **4** and **6**, the $^{13}C$ chemical shifts increased by about 7 ppm and $^1H$ chemical shifts increased by about 0.6 ppm upon sulfation.

The stabilities of these protected sulfated amino acids were investigated under the acidic conditions that typically are employed during Fmoc-SPPS. Compounds **3**, **4** and **6** were stirred in 95% TFA–$H_2O$ at room temperature, and small aliquots of each solution were periodically withdrawn to determine the degree of desulfation by HPLC (Fig. 2). All three derivatives experienced less than 5% desulfation at room temperature after 2–2.5 h, sufficient time for the removal of most of the commonly employed protecting groups in Fmoc-SPPS and cleavage from the solid support. From these results it was concluded that the above TBA-stabilized sulfated building blocks were compatible with 95% TFA and could be used in the synthesis of sulfated peptides.

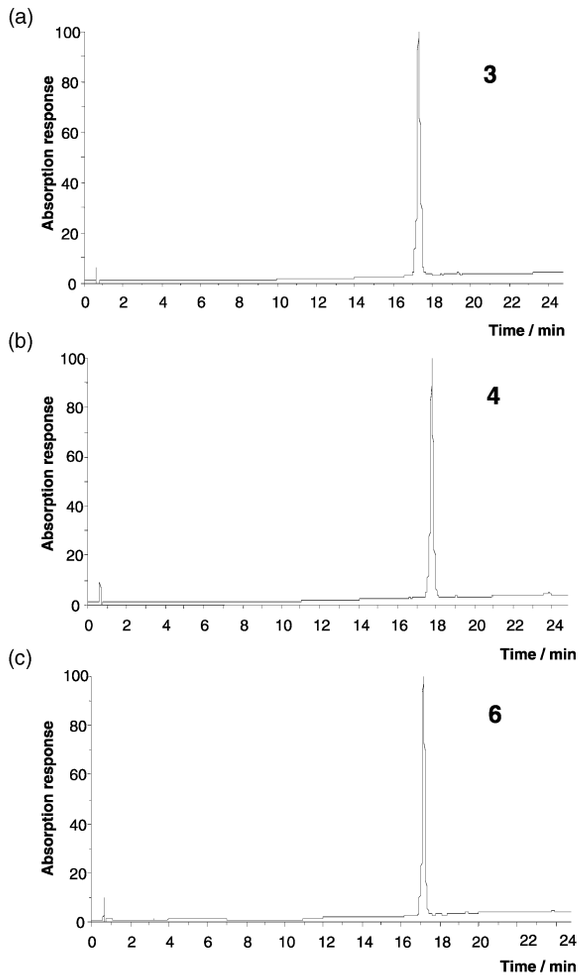



**Fig. 1** HPLC chromatograms of crude sulfated building blocks (a) **3**, (b) **4** and (c) **6**. Column: RCM C-18 (8 × 200 mm). Elution gradient: From 0–80% B (0.1% TFA in 10% aqueous acetonitrile) in 25 min at a flow rate of 1 ml $min^{-1}$. Detection: 215 and 280 nm.

Indeed, the three sulfated building blocks, **3**, **4** and **6**, were cleanly incorporated into model peptides without difficulty. The general synthetic strategy for Ser($SO_3^-N^+Bu_4$)-, Thr($SO_3^-N^+Bu_4$)- and Hyp($SO_3^-N^+Bu_4$)-containing model peptides **7**, **8** and **9** (Scheme 2) were as follows: (i) the peptides were directly assembled on Rink amide 4-methylbenzhydrylamine (MBHA) resin using Fmoc-SPPS; (ii) **3**, **4** and **6** building blocks were introduced using standard 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) coupling chemistry;[20] (iii) the protected sulfated peptide-resins were treated with 95% aqueous TFA for an appropriate period of time (2–2.5 h) for side-chain deprotection and resin cleavage. The products **7**, **8** and **9** were then analyzed by reversed-phase HPLC (Fig. 3) and had the expected masses as determined by ES-MS.

### Conclusions

A simple and reliable synthetic method for the preparation of tetrabutylammonium-stabilized sulfated serine, threonine and hydroxyproline amino acids as stable building blocks for use in Fmoc solid-phase peptide synthesis has been developed. These novel sulfated derivatives were compatible with Fmoc-SPPS

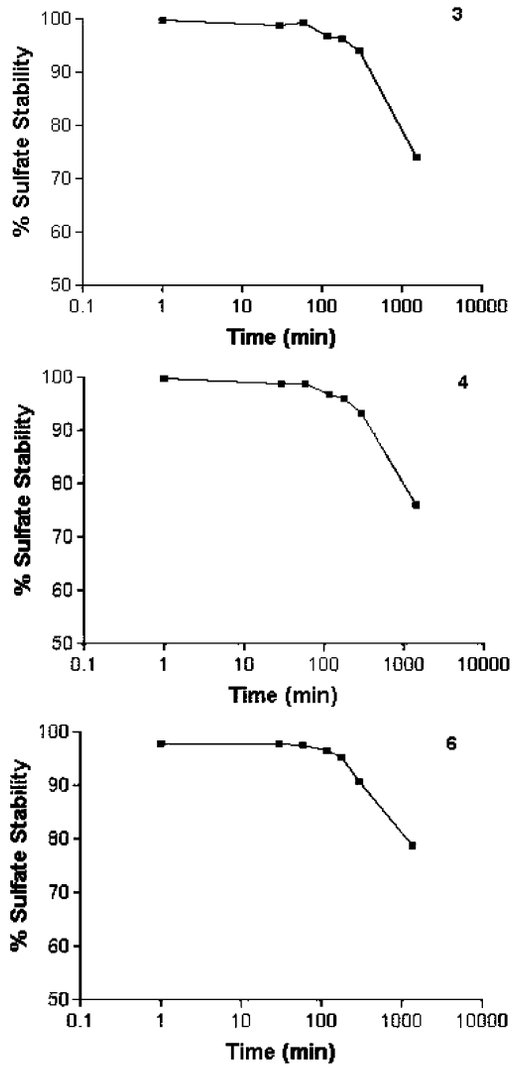



**Fig. 2** Rates of desulfation for building blocks **3**, **4** and **6** in 95% TFA–$H_2O$ at room temperature.

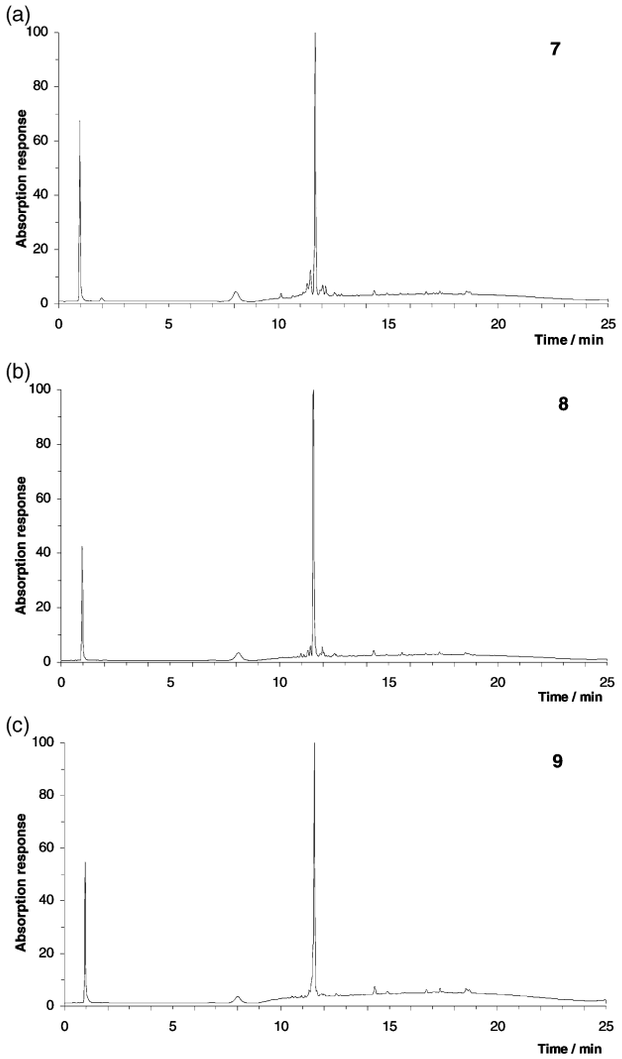


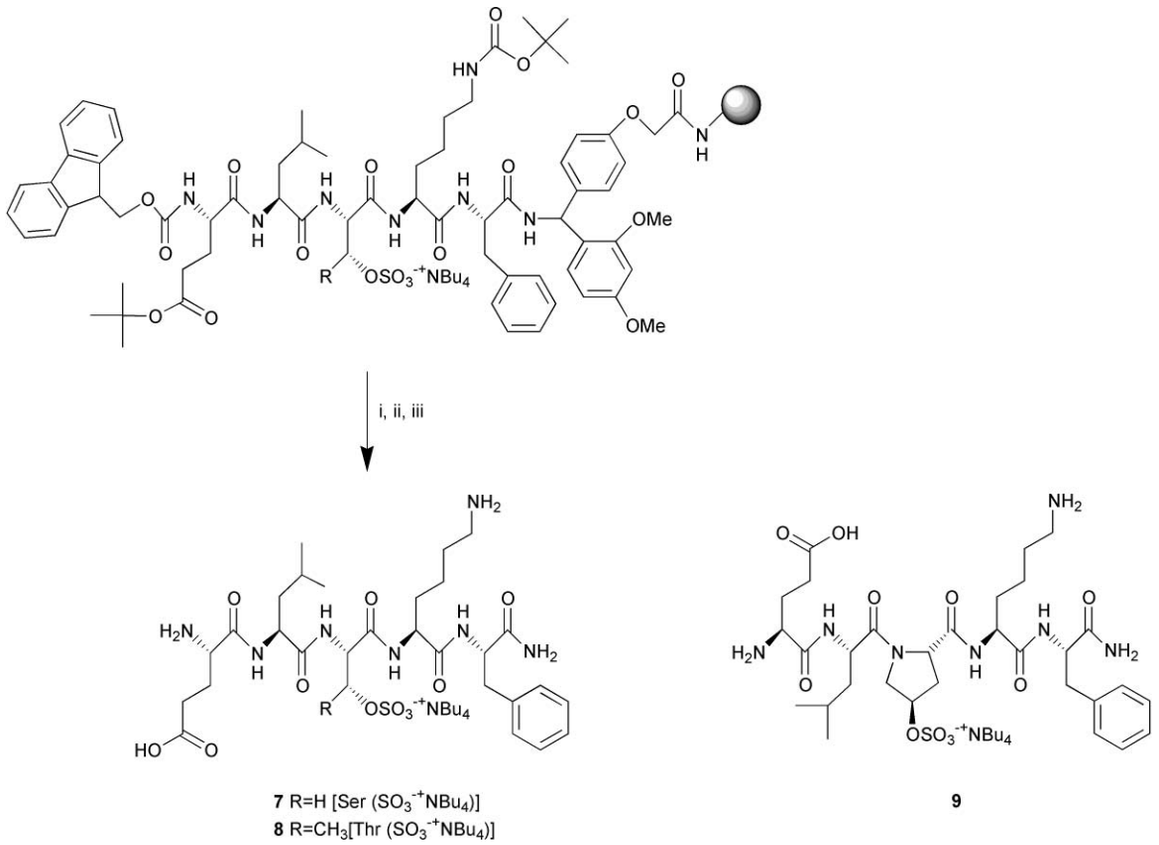

**Fig. 3** HPLC of crude peptides containing sulfated building blocks (a) **7**, (b) **8** and (c) **9**. Column: RCM C-18 (8 × 200 mm). Elution gradient: From 0–80% B (0.1% TFA in 10% aqueous acetonitrile) in 25 min at a flow rate of 1 ml $min^{-1}$. Detection: 215 and 280 nm.

and 95% TFA treatment for 2 h at room temperature and enabled the preparation of relatively pure sulfated peptides. In contrast to previous approaches that usually involved the use of sodium and barium salts, the mass spectrometric analysis of sulfated amino acids and sulfated peptides was much improved and simplified by the presence of TBA salts. An added advantage was that TBA-stabilized sulfated peptides could be readily purified by conventional reversed-phase HPLC conditions employing 0.1% TFA-containing buffer systems.

## Experimental

### Materials and methods

All solvents were used without further purification unless otherwise indicated. DMF was distilled under reduced pressure and stored over activated 4 Å molecular sieves. Rink amide MBHA resin [4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxyacetamido-norleucyl-MBHA resin] loading: 0.49 mmol $g^{-1}$ was obtained from NovaBiochem (Läufelfingen, Switzerland). The polymer matrix, MBHA (4-methylbenzhydrylamine resin), is copoly(styrene–1% divinylbenzene), 100–200 mesh. Suitably protected amino acids ($N^{\alpha}$-Fmoc-Aa-OPfp and $N^{\alpha}$-Fmoc-Aa-OH) were purchased from Bachem (Bubendorf, Switzerland) and NovaBiochem (Läufelfingen, Switzerland). Fmoc-Ser-OH, Fmoc-Thr-OH, and Fmoc-Hyp-OH were dried by co-evaporation with 1,4-dioxane and storage over $P_2O_5$ *in vacuo*. Sulfur trioxide·DMF was dried *in vacuo* over $P_2O_5$. The substitution of the resins was determined by spectrophotometric analysis at 290 nm of the dibenzofulvene–piperidine adduct formed upon deprotection of the amino terminal, using a Perkin-Elmer Lambda 7 UV/vis spectrophotometer. ES mass spectra were recorded with a VG-Quattro instrument from Fisons. Solid-phase peptide-coupling reactions were monitored using the Kaiser test.[21] Reaction mixtures were dried with anhydrous $Na_2SO_4$ and then concentrated *in vacuo*. NMR spectra were recorded on a Varian UNITY INOVA 500 MHz or a Bruker DRX-600 MHz spectrometer. The $^1H$ and $^{13}C$ resonances were assigned by $^1H$, $^{13}C$, $^1H$–$^1H$ COSY, and HSQC experiments. Chemical shifts of $^1H$ and $^{13}C$ are given in ppm and referenced to MeOD ($\delta_H$ 3.34 and $\delta_C$ 49.0); *J*-values are given in Hz. Analytical reversed-phase HPLC separations were performed using an analytical Zorbax (5 μm; 0.46 × 5 cm) C-18 column with flow rates of 1 ml $min^{-1}$. Detection was at 215 nm with a Shimadzu UV absorbance detector. Solvent system A: 0.1% TFA in water; solvent system B: 0.1% TFA in 10% aqueous acetonitrile. ICR-MS was performed at the MS facility of The University of Southern Denmark, Odense on a 4.7 Tesla Ultima Fourier Transform 337 nm MALDI mass spectrometer from Ionspec, Irvine, CA using DHB matrix peak [M − $H_2O$ + H] *m*/*z* 273.0393 as an internal reference.




**Scheme 2** *Reagents and conditions*: i) 20% Piperidine in DMF; ii) 95% TFA–$H_2O$, 2 h, rt; then 95% AcOH–$H_2O$ extraction, $H_2O$; iii) evaporation with toluene and drying. Compound **9** was obtained by the same procedure, when sulfated hydroxyproline was incorporated into the peptide chain.

### General procedure for building-blocks sulfation and TBA salt formation

Dried amino acids, **1** (1.760 g, 5.37 mmol), **2** (2 g, 5.85 mmol) and **5** (2 g, 5.66 mmol), were dissolved in DMF (25 ml) under a nitrogen atmosphere and DMF·$SO_3$ complex (4 eq.) was added in one portion. The reaction mixture was stirred for 2 h at room temperature, the solvent was evaporated off *in vacuo*, and the residue was treated with cooled saturated aq. sodium hydrogen carbonate (50 ml) at 0 °C and tetrabutylammonium hydrogen sulfate (2 eq.). The solution was slightly acidified with 10% aq. citric acid at 0 °C (25 ml, pH 5) and extracted four times with chloroform. After removal of the solvent, and lyophilization of the residue from 1,4-dioxane–water (1 : 1), compounds **3** and **4** were obtained as stable amorphous solids (**3**, 3.9 g, 87%), (**4**, 4.18 g, 84%) and compound **6** as a stable syrup (**6**, 4.1 g, 89%). The identity of the products was established by NMR spectroscopy, ICR-MS and ES-MS.

**Fmoc-Ser($SO_3^-N^+Bu_4$)-OH 3.** $^1$H NMR (500 MHz; MeOD) $\delta_H$ 7.78–7.28 (4H, m, Fmoc Ar*H*), 4.43 (1H, m, C*H*$^{\alpha}$), 4.35 (1H, m, C$H_2^{\beta}$), 4.32 (1H, m, C$H_2^{\beta}$), 4.29–4.19 (3H, m, Fmoc C$H_2$ and Fmoc C*H*), 3.19 (8H, m, TBA salt C$H_2^{\alpha}$), 1.62 (8H, m, TBA salt C$H_2^{\beta}$), 1.39 (8H, sextet, *J* 7.4, TBA salt C$H_2^{\gamma}$) and 0.99 (12 H, t, *J* 7.3, TBA salt C$H_3$); $^{13}$C NMR (500 MHz; MeOD) $\delta_C$ 173.0 (*C*$O_2$H), 158.7 (Fmoc *C*O), 145.1 (2), 142.5 (2) (4 × Ar*C*), 128.8 (2), 128.2 (2), 126.4 (2), 120.9 (2) (8 × Fmoc Ar*C*H), 68.2 (*C*$^{\beta}$ and Fmoc *C*$H_2$), 59.5 (4 × TBA salt *C*$^{\alpha}$), 55.7 (*C*$^{\alpha}$), 48.1 (Fmoc *C*H), 24.7 (4 × TBA salt *C*$^{\beta}$), 20.6 (4 × TBA salt *C*$^{\gamma}$) and 13.9 (4 × TBA salt *C*$H_3$). High-resolution ICR-MS: *m*/*z* Calc. for $[M + m + H]^{2+}$ $C_{34}H_{52}N_2O_8S/2$: 445.8180; Found: *m*/*z*: 445.8212. ES-MS negative-ion mode, mass Calc.: 406.4 $[M - H]^-$; mass Found: 406 $[M - H]^-$; IR (KBr) $\nu_{max}$ 1077m, 1250s, 1266, 1720s cm$^{-1}$.

**Fmoc-Thr($SO_3^-N^+Bu_4$)-OH 4.** $^1$H NMR (600 MHz; MeOD) $\delta_H$ 7.79–7.30 (4H, m, Fmoc Ar*H*), 4.89 (1H, m, C$H_2^{\beta}$), 4.29 (2H, m, Fmoc C$H_2$), 4.23 (1H, m, C*H*$^{\alpha}$), 4.22 (1H, m, Fmoc C*H*), 3.19 (8H, m, TBA salt C$H_2^{\alpha}$), 1.62 (8H, m, TBA salt C$H_2^{\beta}$), 1.39 (8H, m, C$H_3$ and TBA salt C$H_2^{\gamma}$) and 0.99 (12 H, t, *J* 7.3, TBA salt C$H_3$); $^{13}$C NMR (600 MHz; MeOD) $\delta_C$ 173.0 (*C*$O_2$H), 158.7 (Fmoc *C*O), 145.1 (2), 142.5 (2) (4 × Ar*C*), 128.8 (2), 128.2 (2), 126.4 (2), 120.9 (2) (8 × Fmoc Ar*C*H), 75.4 (*C*$^{\beta}$), 68.3 (Fmoc *C*$H_2$), 60.5 (*C*$^{\alpha}$), 59.5 (4 × TBA salt *C*$^{\alpha}$), 48.3 (Fmoc *C*H), 24.7 (4 × TBA salt *C*$^{\beta}$), 20.6 (4 × TBA salt *C*$^{\gamma}$), 18.5 (*C*$H_3$) and 13.9 (4 × TBA salt *C*$H_3$). High-resolution ICR-MS: *m*/*z* Calc. for $[M + m + H]^{2+}$ $C_{35}H_{54}N_2O_8S/2$: 452.8258; Found: *m*/*z*: 452.8254. ES-MS negative-ion mode, mass Calc.: 420.42 $[M - H]^-$; mass Found: 420.1 $[M - H]^-$; IR (KBr) $\nu_{max}$ 1039m, 1270s, 1720s cm$^{-1}$.

**Fmoc-Hyp($SO_3^-N^+Bu_4$)-OH 6.** Due to the rotational barrier of the carbimide bond between the hydroxyproline and the Fmoc group, hydroxyproline derivatives appeared as mixtures of *cis* and *trans* isomers (1 : 1) in NMR spectroscopy.

*Isomer 1*. $^1$H NMR (600 MHz; MeOD) $\delta_H$ 7.79–7.29 (4H, m, Fmoc Ar*H*), 5.02 (1H, m, C*H*$^{\gamma}$), 4.40 (1H, t, *J* 7.9, C*H*$^{\alpha}$), 4.3 (2H, m, Fmoc C$H_2$), 4.18 (1H, m, Fmoc C*H*), 4.02 (1H, d, $J_{H\delta-H\delta}$ 11.9, C$H_2^{\delta}$), 3.79 (1H, dd, $J_{H\delta-H\delta}$ 11.9, $J_{H\delta-H\gamma}$ 4.4, C$H_2^{\delta}$), 3.19 (8H, m, TBA salt C$H_2^{\alpha}$), 2.66 (1H, m, C$H_2^{\beta}$), 2.24 (1H, m, C$H_2^{\beta}$), 1.62 (8H, m, TBA salt C$H_2^{\beta}$), 1.38 (8H, sextet, *J* 7.4, TBA salt C$H_2^{\gamma}$) and 0.99 (12 H, t, *J* 7.3, TBA salt C$H_3$); $^{13}$C NMR (600 MHz; MeOD) $\delta_C$ 176.4 (*C*$O_2$H), 156.5 (Fmoc *C*O), 145.1 (2), 142.5 (2) (4 × Ar*C*), 128.8 (2), 128.3 (2), 126.4 (2), 120.9 (2) (8 × Fmoc Ar*C*H), 77.3 (C$^{\gamma}$), 69.1 (Fmoc *C*$H_2$), 59.5 (4 × TBA salt *C*$^{\alpha}$), 59.3 (*C*$^{\alpha}$), 53.8 (*C*$^{\delta}$), 48.1 (Fmoc *C*H), 37.5 (*C*$^{\beta}$), 24.7 (4 × TBA salt *C*$^{\beta}$), 20.6 (4 × TBA salt *C*$^{\gamma}$) and 13.9 (4 × TBA salt *C*$H_3$).

*Isomer 2*. $^1$H NMR (600 MHz; MeOD) $\delta_H$ 7.79–7.29 (4H, m, Fmoc Ar*H*), 4.99 (1H, m, C*H*$^{\gamma}$), 4.50 (1H, t, *J* 7.9, C*H*$^{\alpha}$), 4.3 (2H, m, Fmoc C$H_2$), 4.18 (1H, m, Fmoc C*H*), 3.92 (1H, d, $J_{H\delta-H\delta}$ 11.8, C$H_2^{\delta}$), 3.72 (1H, dd, $J_{H\delta-H\delta}$ 11.8, $J_{H\delta-H\gamma}$ 4.4, C$H_2^{\delta}$), 3.19 (8H, m, TBA salt C$H_2^{\alpha}$), 2.74 (1H, m, C$H_2^{\beta}$), 2.28 (1H, m,

$CH_2^{\beta}$), 1.62 (8H, m, TBA salt $CH_2^{\beta}$), 1.38 (8H, sextet, *J* 7.4, TBA salt $CH_2^{\gamma}$) and 0.99 (12 H, t, *J* 7.3, TBA salt $CH_3$); $^{13}$C NMR (600 MHz; MeOD) $\delta_C$ 176.4 ($CO_2H$), 156.5 (Fmoc *C*O), 145.1 (2), 142.5 (2) (4 × Ar*C*), 128.8 (2), 128.3 (2), 126.4 (2), 120.9 (2) (8 × Fmoc Ar*C*H), 77.3 ($C^{\gamma}$), 69.1 (Fmoc $CH_2$), 59.5 (4 × TBA salt $C^{\alpha}$), 59.3 ($C^{\alpha}$), 53.8 ($C^{\delta}$), 48.1 (Fmoc *C*H), 37.5 ($C^{\beta}$), 24.7 (4 × TBA salt $C^{\beta}$), 20.6 (4 × TBA salt $C^{\gamma}$) and 13.9 (4 × TBA salt $CH_3$). High-resolution ICR-MS: *m*/*z* Calc. for $[M + m + H]^{2+}$ $C_{36}H_{54}N_2O_8S/2$: 458.8258; Found: *m*/*z* 458.8214. ES-MS negative-ion mode, mass Calc.: 432.43 $[M - H]^-$; mass Found: 432.0 $[M - H]^-$; IR (neat) $\nu_{max}$ 1038m, 1121, 1231, 1254s, 1704 $cm^{-1}$.

### Fmoc-based solid-phase peptide synthesis (general procedures)

Solid-phase synthesis was carried out using the syringe technique. The amino-resin was washed with $CH_2Cl_2$ (10×) and dried under high vacuum for at least 24 h before use. Side-chain-protecting groups that were used in the synthesis were $^t$Bu for Glu and Boc for Lys. Fmoc groups for $N^{\alpha}$-protection were cleaved by treatment with 20% piperidine in DMF (2 min) followed by a second treatment with the same reagent for 15 min. After the Fmoc cleavage, the peptide-resin was washed with DMF (×6). The next residue was then incorporated with the Fmoc–TBTU–NEM (4-ethylmorpholine) coupling protocol[20] [Fmoc-amino acid (3 eq.), TBTU (2.9 eq.), and NEM (4 eq.)] or the Fmoc–OPfp ester methodology [Fmoc-amino acid pentafluorophenyl ester (3 eq.), and Dhbt-OH (1 eq.)]. Reaction completion was determined using the Kaiser test.[21] The side-chain protection groups were removed and the peptides were cleaved from the resin using 95% TFA initially for 10 min and then for 2 h (both solutions were collected). The resin was then washed successively with 95% aqueous acetic acid (4 × 5 min) and $H_2O$ (6 × 2 min) and the combined solvents were evaporated with toluene (3×) and dried *in vacuo*. The crude peptides were purified by HPLC. The structures were confirmed by Edman degradation sequence analysis where the sulfated residue appeared as a trace amount of the non-sulfated amino acid. 1D-$^1$H-NMR data of Ser($SO_3^-$)-containing peptide **7** confirmed the optical purity of the peptide.

### Sulfate stability test

The sulfated building blocks (2 mg) were dissolved in 2 ml of 95% TFA and left at rt. After 1 min, 30 min, 1 h, 2 h, 3 h, 6 h and 24 h 50 μl aliquots were taken, dried *in vacuo* and diluted with 70% $CNCH_3$–$H_2O$ (100 μl). They were evaluated by RP-HPLC using a 0–80% B gradient over a period of the C-18 25 min on column. Desulfation percentages were determined by HPLC peak integration of sulfated and non-sulfated building blocks. Retention times: Fmoc-Ser($SO_3^-N^+Bu_4$)-OH 17.2 min, Fmoc-Ser-OH 18.1 min, Fmoc-Thr($SO_3^-N^+Bu_4$)-OH 18 min, Fmoc-Thr-OH 19 min, Fmoc-Hyp($SO_3^-N^+Bu_4$)-OH 17.2 min and Fmoc-Hyp-OH 18 min.

## Acknowledgements

This work was performed under the SPOCC program supported by The Danish National Research Foundation. Dr Charlotte Gotfredsen is thanked for many helpful discussions about NMR data. Technician Bent Ole Petersen is acknowledged for recording the 500 and 600 MHz NMR spectra. Dr Anita Jansson is acknowledged for measuring ES-MS spectra.

## References

1 V. J. Hruby, F. A. Al-Obeidi and W. Kazmierski, *Biochem. J.*, 1990, **268**, 249; V. J. Hruby and B. M. Pettit, Conformation Biological Activity Relationships for Receptor-Selective, Conformationally Constrained Opioid Peptides, in *Computer-Aided Drug Design, Method and Application*, eds. T. J. Perun and C. L. Propst, Marcel Dekker, New York, 1989, p. 405.
2 J.-L. Fauchere, *Adv. Drug Res.*, 1986, **15**, 29.
3 D. J. Ward, *Peptide Pharmaceuticals. Approaches to the Design of Novel Drugs*, ed. D. J. Ward, Open University Press, Buckingham, 1991.
4 W. F. DeGrado, *Adv. Protein Chem.*, 1988, **39**, 51.
5 C. Toniolo, *Int. J. Pept. Protein Res.*, 1990, **35**, 287.
6 M. Goodman and S. Ro, Peptidomimetics for drug design, in *Medicinal Chemistry and Drug Design. Principles of Drug Discovery*, ed. M. E. Wolff, John Wiley & Sons, New York, 5th edn., 1994, vol. 1, p. 803.
7 H. Kessler, A. Haupt and M. Will, Design of Conformationally Restricted Cyclopeptides for the Inhibition of Cholate Uptake of Hepatocytes, in *Computer-Aided Drug Design, Method and Application*, eds. T. J. Perun and C. L. Propst, Marcel Dekker, New York, 1989, p. 461.
8 R. S. Struthers, A. T. Hagler and J. Rivier, Design of Peptide Analogs. Theoretical Simulation of Conformation, Energetics and Dynamics, in *Conformationally Directed Drug Design*, eds. J. A. Vida and M. Gordon, ACS Symposium Series 251. American Chemical Society, Washington DC. 1984, p. 23.
9 K. M. Halkes, P. M. St. Hilaire, A. M. Jansson, C. H. Gotfredsen and M. Meldal, *J. Chem. Soc., Perkin Trans. 1*, 2000, 2127.
10 Y. Higashimoto, S. Saito, X. H. Tong, A. Hong, K. Sakaguchi, E. Appella and C. W. Anderson, *J. Biol. Chem.*, 2000, **275**, 23199.
11 K. Kitagawa, C. Aida, H. Fujiwara, T. Yagami, S. Futaki, M. Kogire, J. Ida and K. Inoue, *J. Org. Chem.*, 2001, **66**, 1.
12 E. Atherton and R. C. Sheppard, *Solid-Phase Peptide Synthesis: A Practical Approach*, eds. D. Rickwood and B. D. Hames, Oxford University Press, Oxford, 1989.
13 Solution-phase: (*a*) L. Moroder and E. Wünsch, in *Natural Product Chemistry*, ed. Attar-ur-Rahman, Springer-Verlag, Berlin, 1986, 255; and references cited therein; (*b*) M. A. Ondetti, J. Pluscec, E. F. Sabo, J. T. Sheehan and N. Williams, *J. Am. Chem. Soc.*, 1970, **92**, 195; (*c*) Y. Kurano, T. Kimura and S. Sakakibara, *J. Chem. Soc., Chem. Commun.*, 1987, 323; (*d*) N. Fujii, S. Futaki, S. Funakoshi, K. Akaji, H. Morimoto, R. Doi, K. Inoue, M. Kogire, S. Sumi, M. Yun, T. Tobe, M. Aono, M. Matsuda, H. Narusawa, M. Moriga and H. Yajima, *Chem. Pharm. Bull.*, 1988, **36**, 3281. Solid-phase; (*e*) B. Penke and J. Rivier, *J. Org. Chem.*, 1987, **52**, 1197; (*f*) B. Penke and L. Ngyerges, *J. Pept. Res.*, 1991, **4**, 289; (*g*) S. Futaki, T. Taike, T. Akita and K. Kitagawa, *Tetrahedron*, 1992, **48**, 8899; (*h*) K. Kitagawa, S. Futaki, T. Yagami, S. Sumi and K. Inoue, *Int. J. Pept. Protein Res.*, 1994, **43**, 190; (*i*) Y. Han, S. L. Bontems, P. Hegyes, M. C. Munson, C. A. Minor, S. Kates, F. Albericio and G. Barany, *J. Org. Chem.*, 1996, **61**, 6326.
14 T. Yagami, K. Kitagawa, C. Aida, H. Fujiwara and S. Futaki, *J. Pept. Res.*, 2000, **56**, 239.
15 A. D. Proud, J. C. Prodger and S. L. Flitsch, *Tetrahedron Lett.*, 1997, **38**, 7243.
16 P. D. Schnier, W. D. Price, R. A. Jockusch and E. R. Williams, *J. Am. Chem. Soc.*, 1996, **118**, 7178; T. Wyttenbach, G. von Helden and M. T. Bowers, *J. Am. Chem. Soc.*, 1996, **118**, 8355; A. R. Dongré, J. L. Jones, Á. Somogyi and V. H. Wysocki, *J. Am. Chem. Soc.*, 1996, **118**, 8365; G. Tsaprailis, H. Nair, Á. Somogyi, V. H. Wysocki, W. Zhong, J. H. Futrell, S. G. Summerfield and S. J. Gaskel, *J. Am. Chem. Soc.*, 1999, **121**, 5142.
17 M. Ueki, S. Watanabe, R. Yamanaka, M. Ohta and O. Okunaka, *Biopolymers*, 1999, **51**, 117.
18 Ref. 13*e*, See also: S. Futaki, T. Taike, T. Yagami, T. Ogawa, T. Akita and K. Kitagawa, *J. Chem. Soc., Perkin Trans. 1*, 1990, 1739.
19 T. Yagami, S. Shiwa, S. Futaki and K. Kitagawa, *Chem. Pharm. Bull.*, 1993, **41**, 376; E. Wuensch, L. Moroder, L. Wilschowitz, W. Goehring, W. Scharf and J. D. Gardner, *Hoppe-Seyler's Z. Physiol. Chem.*, 1981, **362**, 143.
20 R. Knorr, A. Trzeciak, W. Bannwarth and D. Gillessen, *Tetrahedron Lett.*, 1989, **30**, 1927.
21 E. Kaiser, R. L. Colescott, C. D. Bossinger and P. I. Cook, *Anal. Biochem.*, 1970, **34**, 595.