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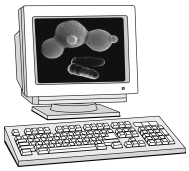
Synthesis of disulfated peptides corresponding to the *N*-terminus of chemokines receptors CXCR6 ($CXCR6_{1-20}$) and DARC ($DARC_{8-42}$) using a sulfate-protecting group strategy



Ahmed M. Ali and Scott D. Taylor*

Tyrosine sulfation is a post translational modification that occurs on integral membrane and secreted proteins, and is required for mediating crucial biological processes. Until recently the synthesis of sTyr peptides, especially those containing multiple sTyr residues, were among the most challenging peptides to prepare. We recently described an efficient strategy for Fmoc-based solid phase synthesis of sTyr peptides in which the sulfate group in the sTyr residue(s) is protected with a DCV group (FmocTyr(SO_3DCV)OH, 1). After cleavage of the peptide from the support the DCV group is removed by hydrogenolysis. Here we demonstrate that sTyr peptides containing Met or Trp residues can be prepared using our sulfate-protecting group strategy by preparing peptides corresponding to residues 1–20 of chemokine receptor CXCR6 and 8–42 of chemokine receptor DARC. Removing the DCV groups at the end of the syntheses was readily achieved, without any reduction of the indole ring in Trp, by performing the hydrogenolysis in the presence of triethylamine. These conditions were found to be particularly efficient for removing the DCV group and superiour to our original conditions using H_2, ammonium formate, Pd/C. The presence of Met was found not to interfere with the removal of the DCV group. The use of pseudoproline dipeptides and N-backbone protection with the 2,4-dimethoxybenzyl group were found to be very effective tactics for preventing aggregation and aspartimide formation during the synthesis of these peptides. We also report an alternative and more cost effective synthesis of amino acid 1.

Supporting information may be found in the online version of this article

Keywords: peptide synthesis; sulfotyrosine; protecting groups; chemokine receptors

Introduction

Tyrosine sulfation is a post translational modification that occurs on integral membrane and secreted proteins. It is believed to be widespread in nature and it has been estimated that as much as 1% of all tyrosine residues in eukaryotes are sulfated [1]. The importance of this PTM is widely appreciated since it has recently been demonstrated that tyrosine sulfation is required for some important biological processes such as viral and parasitic infection, hormone regulation, leukocyte trafficking and adhesion, blood coagulation and the immune response [2]. A variety of tools and techniques, such as bioinformatics studies, labeling studies using radioactive ^{35}S-enriched sulfate, sulfation inhibitors, anti-tyrosine antibodies and mass spectrometry are used to determine the presence, location and function of sTyr residues in proteins [2]. sTyr-containing peptides have also proven to be useful tools for studying tyrosine sulfation in proteins because they allow one to assess the function of each individual sTyr residue. Tyrosine-sulfated peptides have been used to assess the affect of tyrosine sulfation on a variety of biological processes such as chemokine binding to chemokine receptors [3–5], the binding of P-selectin glycoprotein ligand 1 to P-selectin [6], the binding of the G-protein coupled receptor C5aR to the immune invasive protein CHIPS [7], the interaction of the HIV-1 gp120 envelope glycoprotein/CD4 complex with chemokine receptor CCR5 [8] and have even been used to inhibit HIV infection *in vitro* [9].

Until very recently, the synthesis of sTyr peptides, especially those containing multiple sTyr residues, were among the most challenging peptides to prepare. Most early attempts to prepare

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Abbreviations used: *2-MP, 2-methylpiperidine; CHIPS, chemotaxis inhibitory protein of Staphylococcus aureus; DARC, Duffy antigen and receptor for chemokines; DCV, dichlorovinyl; EDT, ethanedithiol; HCTU, 2-(6-Chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate; HRMS, high resolution mass spectrometry; PvDBP, Plasmodium vivax Duffy binding protein; PTM, post-translational modification; LRMS, low resolution mass spectrometry; sTyr, sulfotyrosine; TIS, triisopropylsilane; QTOF, quadrupole time-of-flight.*

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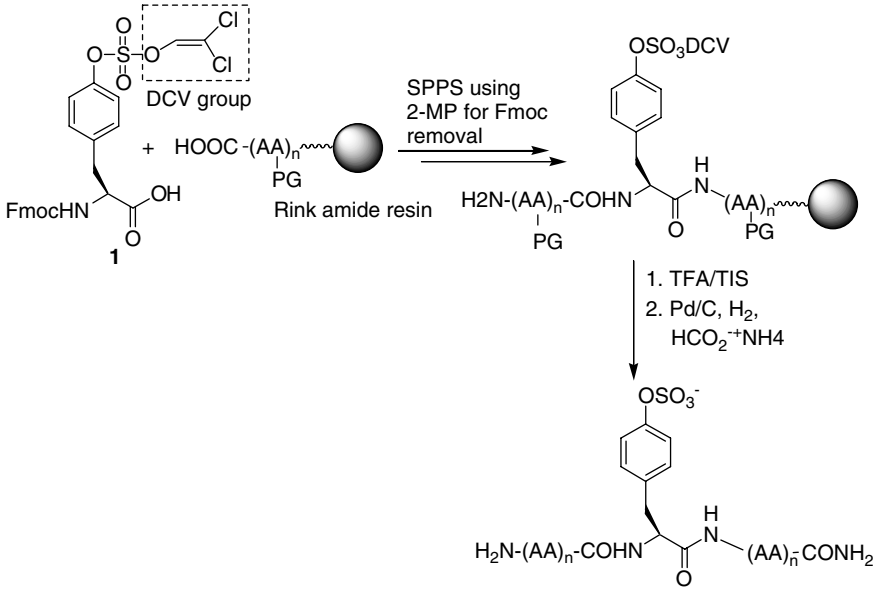

Scheme 1. Fmoc SPPS of sTyr peptides using FmocTyr(SO_3DCV)OH as building block.

these peptides used a global sulfation strategy in which the Tyr residues are sulfated, usually with a SO_3 complex, after the peptide has been constructed (For an excellent review on the synthesis of sulfated peptides see: [10]). Among the problems associated with this approach are selectivity and specificity: it is not possible to introduce sTyr residues at specific locations when more than one Tyr residue is present and the side chains of some other amino acids, such as serine, are also sulfated. Special protecting group strategies have been employed to deal with these issues; however, these strategies tend to be fairly labor intensive and the yield of sTyr peptide is, in general, quite poor [10,11]. Moreover, if the sulfation is performed when the peptide is on the resin then some desulfation occurs upon acid-promoted cleavage of the final product from the support and side chain deprotection. Perhaps the most common approach to sTyr peptides is one in which the sTyr residue is incorporated as a free sulfate monoester using Fmoc(SO_3Na)OH [10,12]. However, couplings following the incorporation of the sY residue can be sluggish and the synthesis of multiply sulfated peptides are difficult due to poor resin swelling and long coupling times are often required [11]. Moreover, as with the global sulfation strategy, acid-promoted cleavage of the peptide from the resin and side chain deprotection results in desulfation. To minimize desulfation, cleavage is performed using 90% aq. TFA at 0–4 °C. However, the reaction time for cleavage and deprotection need to be optimized for each peptide and even with these precautions some desulfation inevitably occurs [12]. Additional problems with this procedure include incomplete side chain deprotection and insufficient cleavage from the resin.

We recently introduced a new and highly effective approach to Fmoc-based solid phase synthesis of sTyr peptides that eliminates the problems associated with the methodologies mentioned earlier [13] (While this work was in progress two other reports appeared describing new approaches to Fmoc-based solid phase synthesis of sTyr peptides. One of these (see Ref. 5) uses a neopentyl group to protect the sulfate group during Fmoc solid phase peptide synthesis. The neopentyl group is removed by mild hydrolysis at the end of the synthesis. The other approach (see Ref. 7) uses a global sulfation strategy in which the tyrosine side chains that are to bear a sulfate group are initially protected with a 2-chlorotrityl group (ClTrt). The ClTrt group is removed on resin without affecting the less acid labile protecting groups used for the protection of the side chains of the other residues. The sulfate group is then introduced as a TCE-protected sulfodiester using $TCEOSO_2Cl$ (**6**) and then the peptide is cleaved from the resin and all side chain protecting groups are removed, with the exception of the TCE group, using TFA/TIS/H_2O. The resulting peptide is purified and then the TCE groups are removed using Zn/ammonium formate and the peptide purified again). In this approach the sTyr residue is introduced during the synthesis as a protected sulfodiester using FmocTyr(OSO_3DCV)OH (**1**, Scheme 1). The DCV group is used to protect the sulfate group and 2-MP is used to affect Fmoc removal. The peptide is cleaved from the resin and all side chain protective groups are removed, with the exception of the DCV protecting group, using TFA/TIS. The DCV group is removed at the end of the synthesis by hydrogenolysis using Pd/C, H_2 and ammonium formate. Using this approach we prepared some multisulfated peptides, such as a tetrasulfated 20-mer derived for the *N*-terminal region of the chemokine receptor D6 and a disulfated 20-mer derived from the *N*-terminal of the C5a-anaphylatoxin chemotactic receptor in good yield and purity [13].

Among the sTyr peptides that we wished to construct are those derived from the *N*-terminal regions of the DARC and the chemokine receptor CXCR6. DARC can serve as an erythrocyte receptor for the malaria parasite *Plasmodium vivax* [14]. *P. vivax* expresses an erythrocyte-binding antigen called *Pv*DBP [14]. The first interaction between DARC and PvDBP is a crucial step in erythrocyte invasion by the parasite and failure to complete such interaction retards the invasion. Studies using Tyr to Phe mutants and labeling studies strongly indicate that DARC is sulfated at Tyr30 and Tyr41 and Tyr41 in particular plays a crucial role in the DBP-DARC interaction and *P. vivax* infection of erythrocytes [15]. Chaudhuri *et al*., showed that a non-sulfated 35-mer *N*-terminal peptide inhibited DBP binding to Duffy positive red blood cells *in vitro* [16]. We are interested in examining the ability of sTyr peptides to inhibit DBP-DARC interactions. Chemokine receptor

CXCR6 mediates the chemotaxis and adhesion of leukocytes to soluble and membrane-anchored forms of its only known ligand CXCL16, and is an HIV-1 co-receptor. Although bioinformatics analyses predict that one Tyr residue (Tyr6) and possibly two (Tyr10) are sulfated [17], mutation of these residues to Phe and inhibition of sulfation using sodium chlorate had no effect on receptor function [18]. Sulfated peptides corresponding to the *N*-terminal region of CXCR6 may provide additional insight as to whether or not sulfation of these two Tyr residues contribute to receptor function.

Our specific synthetic targets were the disulfated peptides AcMAEHD**sY$_6$**HED**sY$_{10}$**GFSSFNDSSQNH$_2$ (**2**) which corresponds to residues 1–20 of the *N*-terminal region of CXCR6 and, AcAELSPSTENSSQLDFEDVWNSS**sY$_{30}$**GVNDSFPDGDs**Y$_{41}$**DNH$_2$ (**3**) which corresponding to residues 8–42 of the *N*-terminus region of DARC. However, unlike previous sTyr peptides that we have prepared [13] peptides **2** and **3** contain methionine (at position 1 in peptide **2**) and Trp (at position 26 in peptide **3**). This raised concerns as to whether our approach to sTyr peptides could be used to prepare these two peptides since the DCV group is removed using hydrogenolysis. Although poisoning of Pd catalysts by sulfur containing compounds is well known we anticipated that this would not be an issue using our approach since greater than stoichiometric (molar) amounts of Pd catalyst compared to Met residue(s) can be used. However, reduction of the indole ring in Trp residues has been shown to occur to give 2,3-dihydrotryptophan or even octahydrotryptophan when Trp-bearing peptides are subjected to a variety of hydrogenolysis conditions [19–21]. In this report we describe the synthesis of peptides **2** and **3** using our sulfate-protecting group strategy. Reduction of the Trp residue was avoided by removing the DCV group under basic hydrogenolysis conditions and Met did not interfere with DCV removal. The use of pseudoproline dipeptides to prevent aggregation during peptide synthesis and N-backbone protection with the 2,4-dimethoxybenzyl (Dmb) group to prevent aspartimide formation proved to be effective tactics for obtaining these peptides in good yield. We also report an alternative and more cost effective synthesis of the key amino acid **1**.

Materials and Methods

Rink amide resin, amino acids and coupling reagents used for peptide synthesis were purchased from Novabiochem Corp. (San Diego, CA, USA) and/or Advanced Chem Tech, Inc (Louisville, KY, USA). L-amino acids were used for all peptide syntheses unless stated otherwise. Reagents used for the synthesis of amino acid **1** were obtained from Aldrich Chemical Company (Oakville, ON, Canada). Pseudoproline dipeptide FmocSer(tBu)$\Psi^{Me,Me}$ProOH (**12**) and FmocAsp(O^{t}Bu)Gly(DMB)OH (**14**) were prepared according to literature procedures [22,23]. Tetrahydrofuran (THF) was distilled from sodium metal in the presence of benzophenone under argon. 2-MP was obtained from Waterstone Technology (Carmel, IN, USA) and was used without further purification. All automated SPPS was performed using the Rink amide resin and were performed on a Quartet peptide synthesizer from Protein Technologies (Tucson, AZ, USA) on a 25 μM scale. Analytical and semi-preparative RP-HPLC was achieved using Waters 600 controller equipped with a Waters 2487 detector with the detector set to 220 nm. Analytical HPLC was performed with a Vydac 218TP54 C18 column (5 μm, 4.6 mm × 250 mm) and/or Higgins PROTO 200 C18 column (5 μm, 4.6 mm × 250 mm) using a 1.0 ml/min flow rate. Semi-preparative HPLC was conducted on Vydac 218TP1022 C18 column (10 μm, 22 mm × 250 mm) using an 8.0 ml/min flow rate. Flash chromatography was performed using silica gel 60 Å (234–400 mesh) obtained from Silicycle (Laval, Quebec, Canada). Chemical shifts (δ) for ^{1}H NMR spectra run in $CDCl_3$ are reported in ppm relative to the internal standard tetramethylsilane. For ^{13}C NMR spectra run in $CDCl_3$ chemical shifts are reported in ppm relative to the $CDCl_3$ (δ 77.0 for central peak). Electron impact mass spectra were acquired with a JEOL HX110 double focusing mass spectrometer. Positive and negative ion electrospray (ESI) experiments were performed with a Waters/Micromass QTOF Ultima Global mass spectrometer. 1 : 1 CH_3CN/H_2O + 0.2% formic acid is used as a solvent for positive ion spectra and 1 : 1 CH_3CN/H_2O + 0.5% ammonium hydroxide was used as solvent for negative ion spectra.

tert-Butyl N$^\alpha$-[*tert*-butoxylcarbonyl]-L-Tyrosine Dichlorovinyl Sulfate, 8

To a solution of **5** (6.0 g, 17 mmol, 1.0 Eq) in dry THF (24 ml) at 0 °C was added reagent **6** [24] (15.7 g, 53 mmol, 3.0 Eq) followed by a solution of DMAP (2.1 g, 17 mmol, 1 Eq) and Et_3N (4.9 ml, 34 mmol, 2.0 Eq) in dry THF (48 ml). The reaction was allowed to warm to room temperature then stirred overnight and filtered. The filtrate was diluted with EtOAc (200 ml) and the resulting solution was washed with phosphate buffer (pH = 7.2, 2 × 100 ml) and brine (2 × 100 ml) then dried ($MgSO_4$), filtered concentrated by rotary evaporation. The residue was dissolved in THF (72 ml) and 1 Eq of DBU (2.5 ml, 17 mmol) was added at 1-h intervals over 4 h for a total of 5 Eq DBU. After 6 h the reaction mixture was filtered and the filtrate was diluted with EtOAc (2 × 100 ml). This solution was washed with phosphate buffer (pH = 7.2, 2 × 100 ml), and brine (2×100 ml) then dried ($MgSO_4$), filtered and concentrated by rotary evaporation. The residue was purified by flash chromatography using ethylacetate: *n*-hexane (15 : 85) to yield 8.0 g of **8** as pale yellow glassy semisolid (yield 88%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.25–7.18 (m, 4H, H_{Tyr}), 7.12 (s, 1H, H_{DCV}), 5.02 (d, J = 7.3 Hz, 1H, NH_{Tyr}), 4.43–4.40 (m, 1H, CH_{Tyr}), 3.06–3.04 (m, 2H, CH_{2-Tyr}), 1.39 (s, 9H, $H_{tert-but}$), 1.36 (s, 9H, $H_{tert-but}$); ^{13}C NMR (75 MHz, $CDCl_3$): δ 170.5, 154.9, 148.8, 137.1, 133.6, 131.2, 120.8, 117.20, 82.2, 79.7, 54.7, 37.9, 28.2, 27.8; HRMS (ESI$^+$): calculated for $C_{20}H_{28}Cl_2NO_8S$ $(M+H)^+$ 512.0913, found 512.0917.

N$^\alpha$-[(Fluoren-9-yl)methoxylcarbonyl]-L-Tyrosine Dchlorovinyl Sulfate, 1

Compound **8** (6.0 g, 11 mmol, 1.0 Eq) was dissolved in TFA (23 ml) and the mixture was stirred at room temperature for 2 h then concentrated by rotary evaporation. This process was repeated using the same quantity of TFA. After rotary evaporation a third portion of TFA (23 ml) was added and the mixture was stirred overnight. The mixture was concentrated by rotary evaporation and the residue was suspended in $CHCl_3$ (50 ml) and concentrated by rotary evaporation and this process was repeated several times until a white solid formed which was dried under high vacuum. The residue was dissolved in an aqueous solution of sodium carbonate (42 ml, 3.7 g, 34 mmol, 3.0 Eq) and the mixture was cooled using an ice bath. A solution of Fmoc–OSu (5.9 g, 17 mmol, 1.5 Eq) in dioxane (42 ml) was added and the reaction was allowed to warm to roomtemperature then stirred overnight. The reaction mixture was acidified using 1 M HCl (to pH = 2), extracted with EtOAc (3 × 100 ml), dried ($MgSO_4$), filtered and concentrated by rotary evaporation. The residue was subjected to flash chromatography

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(100% CH_2Cl_2 to 5% MeOH in CH_2Cl_2) which gave pure **1** as a white foam 5.7 g (85%). ^{1}H NMR and ^{13}C NMR data were identical to those previously reported for this compound [13]. The enantiopurity of **1** was determined by constructing diastereomeric dipeptides AcY(SO_3DCV)$A_{(DL)}NH_2$, AcY(SO_3DCV)$A_{(L)}NH_2$, and analyzing them by HPLC and ^{1}H-NMR as previously described [13]. See the Supporting Information for the HPLC chromatograms and ^{1}H-NMR spectra of the diastereomeric peptides.

General Procedure for the Solid Phase Synthesis of Peptides 2 and 3

Automated SPPS was used. Fmoc amino acids used for peptide assembly were protected at their side chains with a *tert*-butyl group for Asp, Gly, Thr, Ser, a trityl group for Asn, Gln and His, and a Boc group for Lys. For peptide **2**, Ser13 and Ser 14 were incorporated using pseudoproline dipeptide FmocSer(tBu)$\Psi^{Me,Me}$ProOH (**12**). For peptide **3**, Asp38 and Glu39 were incorporated as dipeptide FmocAsp(O^{t}Bu)Gly(DMB)OH (**14**) and residues Ser28 and Ser29 and Ser17 and Ser18 were incorporated as pseudoproline dipeptide **12.** The Rink amide resin was swollen in DMF for 30 min prior to the attachment of the first amino acid. All couplings were performed using 5-Eq amino acid in DMF and using Cl–HOBt (5 Eq)/HCTU (5 Eq)/DIPEA (5 Eq) unless stated otherwise. The DIPEA was added as a separate solution to the reaction mixture. Double couplings (2 × 20 min) were used throughout unless stated otherwise. For the synthesis of peptide **2**, Glu8, Asp9 and pseudoproline dipeptide **12** were incorporated using 4-Eq amino acid or dipeptide and HOAt (4 Eq)/HATU (4 Eq)/DIPEA (4 Eq) as coupling agents (double couplings, 2 × 45 min). For the synthesis of peptide **3**, dipeptide **14** and pseudoproline dipeptide **12** were incorporated using 4 Eq amino acid or dipeptide and HOAt (4 Eq)/HATU (4 Eq)/DIPEA (4 Eq) as coupling agents (double couplings, 2 × 45 min). To conserve on expensive dipeptides **12** and **14**, these two dipeptides were added manually to the reaction mixture rather than automatically using the machine. A capping step using a 2 : 1 : 3 solution of pyridine : acetic anhydride : DMF was performed (1 × 10 min) after the incorporation of each amino acid. Fmoc deprotections were performed using 2-MP (3 × 10 min). After each coupling and Fmoc deprotection the resin was washed with DMF (6 × 30 s). After each capping the resin was washed with dichloromethane (6 × 30 s) and DMF (6 × 30 s). After coupling and Fmoc deprotection of the last amino acid, the peptide was acylated by subjecting it to a 2 : 1 : 3 solution of pyridine : acetic anhydride : DMF for 60 min. The resin was washed with DMF (3 × 10 min), dichloromethane (3 × 10 min) and then dried. The peptides were cleaved from the resin by subjecting the resin to TFA : TIS : H_2O : EDT (92.5 : 2.5 : 2.5 : 2.5, 2.5 ml) for 2.5 h then filtered. The resin was subjected to the cleavage cocktail again (2.5 ml) for 5 min and filtered again. The combined filtrates were concentrated to half volume under reduced pressure. The material was transferred to a 50 ml centrifuge tube and diethyl ether was added which resulted in the precipitation of the peptides. The mixture was cooled in a dry ace-acetone bath for 30 min then centrifuged (5000 rpm, −4 °C). The supernatant was decanted and acetonitrile was added to the resulting pellet until the pellet dissolved. The solution was transferred to a round bottom flask and concentrated to dryness. The residue was dissolved or suspended in water and lyophilized. The crude peptides **11** and **13** were analyzed by analytical RP-HPLC eluting with a linear gradient of 5 : 95 CH_3CN : H_2O (0.1% TFA) to 95 : 5 CH_3CN : H_2O (0.1% TFA) over 60 min (λ = 220 nm) (See Figures 1B and 4C). The DCV groups in crude peptides **11** and **13** were removed by dissolving them (10 mg) in H_2O (1 ml) containing Et_3N (11 Eq for peptide **2** and 17 Eq for peptide **3** (1 Eq of Et_3N per acidic amino acid plus 2 Eq per DCV group) and the resulting solution was diluted with HPLC grade methanol (1 ml). $Pd(OH)_2$ (20% w/w, 5 mg) was added and the mixture was stirred at roomtemparature under hydrogen gas (balloon pressure) overnight. The mixture was transferred to a microcentrifuge tube and centrifuged. The solution was decanted and the residue resuspended in 0.5 ml of methanol and centrifuged and decanted again. The combined supernatants were purified using semi-preparative RP-HPLC.

Peptide **2** was eluted from the semi-preparative HPLC column using CH_3CN/20 mM ammonium acetate as eluent and a linear gradient of 10–15% CH_3CN over 30 min. This gave 3.8 mg (41% based on resin loading) of peptide **2** as a flocculent white powder after repeated lyophilizations until a constant weight was obtained. This material was determined to be approximately 97% pure by analytical RP-HPLC (linear gradient of 10 : 90, CH_3CN: 20 mM ammonium acetate to 15 : 85 CH_3CN : 20 mM ammonium acetate over 30 min, t_R = 25.92 min (see Figure 3). LRMS (ESI$^-$): calculated for $C_{103}H_{134}N_{27}O_{45}S_3$ $(M-H)^{-1}$ 2564.8195, found 2564.5745.

Peptide **3** was eluted from the semi-preparative HPLC column using CH_3CN/20 mM ammonium acetate as eluent and a linear gradient from 15% to 20% CH_3CN over 60 min, (t_R = 15.8 min). This gave 2.9 mg (32% yield based on resin loading) of peptide **3** as a flocculent white powder after repeated lyophilizations until a constant weight was obtained. This material was determined to be approximately 98% pure by analytical RP-HPLC (linear gradient of 15 : 85 CH_3CN : 20 mM ammonium acetate to 20 : 80 CH_3CN : 20 mM ammonium acetate over 60 min (t_R = 22.46 min) (see Figure 6). LRMS (ESI$^-$): calculated for $C_{169}H_{234}N_{41}O_{74}S_2$ $(M-H)^{-1}$ 4085.5255, found 4085.2175.

Results and Discussion

Key to our approach to sTyr peptides is the use of amino acid **1**. Our original approach to **1** involved reacting FmocTyrO*t*Bu with reagent **4** (Scheme 2) to introduce the DCV-protected sulfate group [13]. Although this works very well, it necessitates the prior preparation of reagent **4**. Reagent **4** is readily prepared in excellent yield but requires the use of trimethyloxonium tetrafluoroborate a relatively expensive methylating agent [13]. We wished to develop a more direct route to compound **1** that did not involve the use of reagent **4**. Toward this end amino acid **5** was reacted with readily available trichloroethylsulfurylchloride (**6**) [24] (Scheme 3) in the presence of DMAP/Et_3N to give amino acid **7**. After an aqueous workup, crude **7** was reacted with DBU which gave DCV-protected amino acid **8** in an 88% yield. Removal of the Boc and *t*-butyl groups in **8** using TFA followed by reaction with Fmoc–OSu gave compound **1** in an 85% yield. The enantiopurity of compound **1** prepared in this manner was found to be >98% as determined by the synthesis of diastereomeric peptides followed by HPLC analysis (see the Supporting Information).

Before embarking on a synthesis of peptides **1** and **2** it was necessary to develop conditions for removal of the DCV group that would not result in the reduction of the indole ring in Trp residues. We initially examined conditions that have been used to remove trichloroethyl-based protecting groups from amines and phosphates. Treatment of hexapeptide Ac-Asp-Ala-Asp-Glu-Tyr(SO_3DCV)-L-NH_2 (**9**) [13] with zinc/pyridine/acetylacetone [25], tributylphospine [26], or tetra-*n*-butylammonium fluoride [27], all failed to give Ac-Asp-Ala-Asp-Glu-Tyr(SO_3^-)-L-NH_2 (**10**). Zn(Cu) in

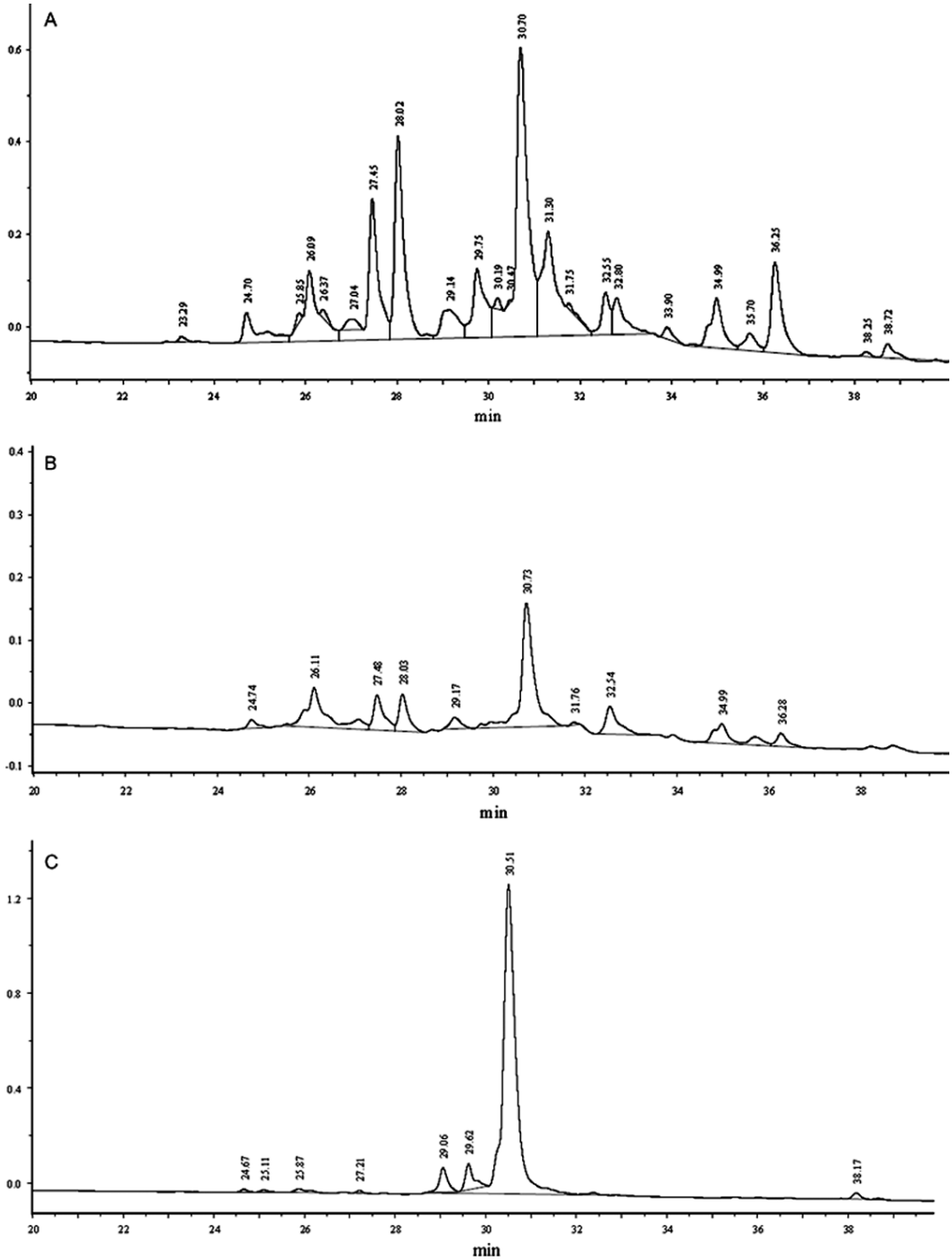

Figure 1. Analytical RP-HPLC chromatograms of the crude products obtained after cleavage from the resin and side chain deprotection from attempts to prepare peptide **2**. Chromatogram (A) using HBTU/HOBt as coupling reagents (1 × 1.5 h coupling time). Chromatogram (B) Glu8 and Asp9 were incorporated using HATU/HOAt and double couplings (2 × 45 min). The remainder of the amino acids where incorporated using HCTU/Cl–HOBt and double couplings (2 × 45 min). Chromatogram (C) Glu8, Asp9, and pseudoproline dipeptide **12** were incorporated using HATU/HOAt and double couplings (2 × 45 min). The remainder of the amino acids where incorporated using HCTU/Cl–HOBt and double couplings (2 × 45 min). See Materials and methods for details.

DMF [28] (entry 3) successfully removed the DCV group; however, the reaction turned deep brown upon completion with the formation of heavy precipitate which we anticipated would be problematic when applied to peptides. Attempts to remove the DCV group by hydrolysis in buffers ranging from pH 5 to 9 at 40 °C were also unsuccessful. Subsequent to these studies we became aware of a report by Medzihradszky-Schweiger who reported that hydrogenolysis of carbobenzyloxy (Cbz) protecting groups in Trp-bearing peptides using H_2 and 10% P/C (MeOH as solvent) did not result in the reduction of the indole ring when the hydrogenolysis was performed in the presence of bases such as Et_3N [29] (It is also worthy of note that Medzihradszky-Schweiger reported that poisoning of the Pd catalyst by sulfur-containing amino acids did not occur when the hydrogenolysis was performed

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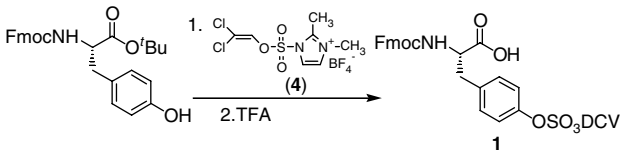


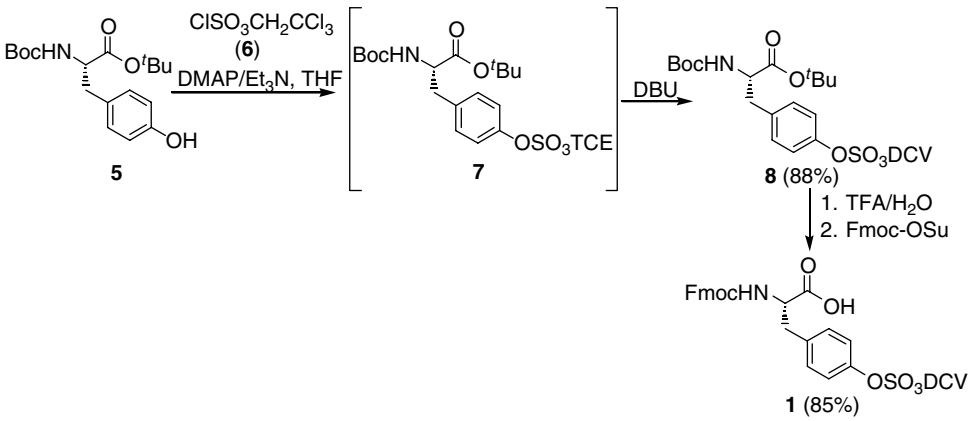
Scheme 2. Synthesis of FmocTyr(SO_3DCV)OH (**1**) using reagent **4**.

in the presence of bases such as Et_3N). This prompted us to examine whether the DCV group could be removed under basic hydrogenolysis conditions. Peptide **9** was suspended in water and 5 Eq of triethylamine was added (1 Eq per acidic group in the peptide plus one extra Eq) followed by the addition of 30–50 wt% of 10% Pd/C or $Pd(OH)_2$. Hydrogenolysis of the mixture using H_2 (balloon pressure) for 16 h (overnight) gave the desired peptide **10** in essentially quantitative yields (To confirm that no hydrogenation of the indole ring in Trp would occur when subjected to our basic hydrogenolysis conditions, we prepared dipeptide Ac-Trp-Ala-NH_2 and subjected it to 50% wt% of $Pd(OH)_2$, H_2 (balloon pressure), 2 Eq Et_3N, in H_2O at roomtemperature for 16 h. No reduction of the indole ring occurred as determined by ^{1}H-NMR. See the Supporting Information for details).

In our previous report on the synthesis of sTyr peptides, we used 95% TFA/5% TIS to cleave the peptides from the support and remove other side chain protecting groups [13]. However, for Met-bearing peptides it is usually essential that scavengers such as thiols or disulfides like EDT or thioanisole are present because these reagents help prevent the oxidation of Met residues. To determine if the DCV group is stable to cleavage cocktails containing EDT or thioanisole we subjected Ac-Asp-Ala-Asp-Glu-Tyr(SO_3DCV)-L-NH_2 (**9**) to commonly used cleavage cocktails containing these scavengers such as TFA : TIS : EDT (95 : 2.5 : 2.5), TFA : TIS : EDT : H_2O (92.5 : 2.5 : 2.5 : 2.5), TFA : TIS : thioanisole (95 : 2.5 : 2.5) and TFA : TIS : Thioanisole : H_2O (92.5 : 2.5 : 2.5 : 2.5) for 2 h at roomtemperature. No peptide resulting from loss of the DCV group was detected (using analytical HPLC) indicating that the DCV group is stable to these cleavage cocktails.

Having established conditions for removal of the DCV group that would be compatible with Trp residues and stable to sulfur-containing scavenging cocktails we embarked on the synthesis of peptides **2** and **3**. Our first attempt to synthesize peptide **2** employed our usual conditions [13] using automated SPSS with the Rink amide resin (0.71 mmol/g), 5 Eq Fmoc amino acid, HBTU/HOBt as coupling reagents (1 × 1.5 h coupling time) and using 2-MP for Fmoc removal (3 × 10 min) followed by a capping step. After the assembly of the peptide chain the peptide cleavage from the resin was achieved using TFA : TIPS : H_2O : EDT (92.5 : 2.5 : 2.5 : 2.5). However, the HPLC chromatogram of the crude material obtained after cleavage from the support showed many peaks (Figure 1A). The mass spectrum of the crude mixture indicated that the desired DCV-protected peptide AcMAEHDY(SO_3DCV)HEAY(SO_3DCV)GFSSFNDSSQNH$_2$ (**11**) was present and this corresponded to the largest peak in the HPLC chromatogram with a retention time of 30.7 min. Two other significant peaks in the mass spectrum were identified as having resulted from two truncated *acetylated* peptides corresponding to the residues 9–20 and 10–20. An attempt to improve the synthesis of **11** was made by using the apparently more potent coupling agent HCTU and performing double couplings (2 × 45 min). However, this did not result in an improvement as the HPLC chromatogram of the crude peptide was very similar to our first attempt. Since two truncated peptides corresponding to residues 9–20 and 10–20 were obtained in our previous syntheses, in our third attempt we incorporated Glu8 and Asp9 using HATU/HOAt which has been reported to be a highly efficient coupling agent [30] The remainder of the amino acids where incorporated using HCTU/Cl–HOBt. Although the HPLC trace of the crude mixture showed some improvement many other peaks were still present (Figure 1B).

One problem commonly encountered in SPPS is poor solvation of the growing peptide chain which stems from aggregation of hydrophobic residues or protecting groups and/or the formation of secondary structures such as β-sheet formation. Such aggregation leaves a limited number of free amino groups available for coupling resulting in poor coupling yields. One approach to prevent this phenomenon is to use the so-called "pseudoprolines" in which the hydroxyl group of a serine or threonine residue is reversibly bound through an alkyl bridge to the nitrogen atom of the amide backbone (Figure 2) [31–33]. Pseudoproline residues induce a kink in the peptide backbone which disrupts intermolecular or intramolecular aggregations commonly experienced during peptide synthesis. Consequently, the use of pseudoproline often results in an improvement in yield. The pseudoprolines are converted back to serine or threonine during the acid conditions used for cleaving the peptide from the support and removing side chain protecting groups. To determine if this was an issue



Scheme 3. Synthesis of FmocTyr(SO_3DCV)OH (**1**) from amino acid **5**.

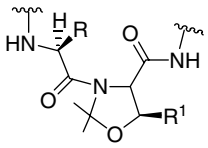


Figure 2. Incorporating pseudoproline ($\Psi^{Me,Me}$ Pro shown) into a peptide induces a kink in the peptide backbone. R═H (if derived from serine) or R═CH_3 (if derived from threonine).

with the synthesis of peptide **2** we decided to examine this tactic as a means of increasing the quality of crude peptide **11**. There are four possible sites within peptide **2** where pseudoproline can be incorporated: Ser13, Ser14, Ser18, and Ser19. Because the latter two are near the *C*-terminus, we anticipated that there would be little benefit to replace either of them with pseudoproline. Pseudoprolines are incorporated into peptides as dipeptides: Fmoc-AA-ΨPro-OH. FmocSer(tBu)$\Psi^{Me,Me}$ProOH (**12**) is a known compound and is commercially available. Therefore, we elected to replace Ser14 with $\Psi^{Me,Me}$Pro. A considerable improvement in the quality of crude **11** was obtained when we used dipeptide **12** and the conditions described above during our third preparation of **2** as indicated by the HPLC trace of the crude product which showed a single major peak corresponding to our target compound (Figure 1C) as well as a few other minor peaks. The DCV groups in crude **11** were removed by subjecting it to 50% w/w $Pd(OH)_2$ under H_2 atmosphere (balloon pressure) in water/methanol (1 : 1) containing 11 Eq Et_3N and stirring it overnight. After semi-preparative RP-HPLC purification peptide **2** was obtained in a very respectable 41% overall yield in approximately 97% purity as determined by analytical RP-HPLC (Figure 3).

Because peptide **3** contains two Ser–Ser sequences at positions 17 and 18 and 28 and 29 our initial strategy for the synthesis of peptide **3** employed HATU/HOAt to incorporate pseudoproline dipeptide **12** at positions 17 and 18 and 28 and 29 while incorporating all other amino acids using HCTU/Cl–HOBt. Double coupling (2 × 20 min) and capping steps were also employed. Fmoc deprotection and cleavage from the support were done using our usual protocols (using 2-MP for Fmoc removal (3 × 10 min) and using TFA : TIPS : H_2O : EDT (92.5 : 2.5 : 2.5 : 2.5) for 2 h for cleavage from the support). Employing this strategy and after the cleavage from the support, the crude mixture exhibited two major peaks at t_R = 23.7 and 26.3 min along with a variety of minor peaks in the HPLC chromatogram (Figure 4A). Upon MS analysis of the two major peaks we found that the peak at t_R = 23.7 min corresponded to the desired DCV-protected precursor to peptide **3**, AcAELSPSTENS$_{17}$S$_{18}$QLDFEDVWNS$_{28}$S$_{29}$**Y(SO$_3$DCV)** GVNDSFPDGD**Y(SO$_3$DCV)**D-NH_2 (**13**), while the peak at t_R = 26.3 differed from **13** by just 18 mass units suggesting loss of a water molecule. We reasoned that the peak at t_R = 26.3 was due to aspartimide formation at Asp38 and Gly39 because Asp–Gly sequences are known to be very susceptible to this side reaction during Fmoc SPPS (Scheme 4) [34]. When this reaction does occur during Fmoc-based SPPS peptide synthesis some or all of the resulting aspartimide often reacts with piperdine (if piperidine is used for Fmoc removal) to give tertiary amides. The aspartimide can also be hydrolyzed to the α-Asp and β-Asp peptides. If aspartimide formation is indeed occurring during the synthesis of peptide **13** then reaction of the aspartimide with 2-MP is not occurring because the mass spectrum of the mixture did not indicate that any products resulting from this reactions were formed. This suggests that 2-MP is too sterically hindered to attack the imide.

Although there are a variety of ways to avoid aspartimide formation perhaps the most effective way is N-backbone protection with the Hmb group or the Dmb group [34,35]. Consequently, we decided to incorporate residues Asp38 and Gly39 as the protected dipeptide FmocAsp(O^{t}Bu)Gly(DMB)OH (**14**, Figure 5). Peptide synthesis was performed using the same conditions as our previous attempt except dipeptide **14** was substituted for Asp38 and Gly39. The HPLC trace of the crude product after cleavage from the resin indicated little or no aspartimide formation as indicated by the absence of a significant peak at t_R = 23.7 min (Figure 4B). Removal of the DCV groups was achieved by subjecting crude **13** to 50% w/w of $Pd(OH)_2$ in presence of Et_3N (17 Eq), and H_2 (balloon pressure) in water : methanol (1 : 1) for 24 h. No peaks corresponding to peptide **3** containing a reduced Trp residue were evident in the mass spectrum of the crude material. After semi-preparative RP-HPLC purification peptide **3** was obtained in a 32% overall yield in approximately 98% purity as determined by analytical RP-HPLC (Figure 6).

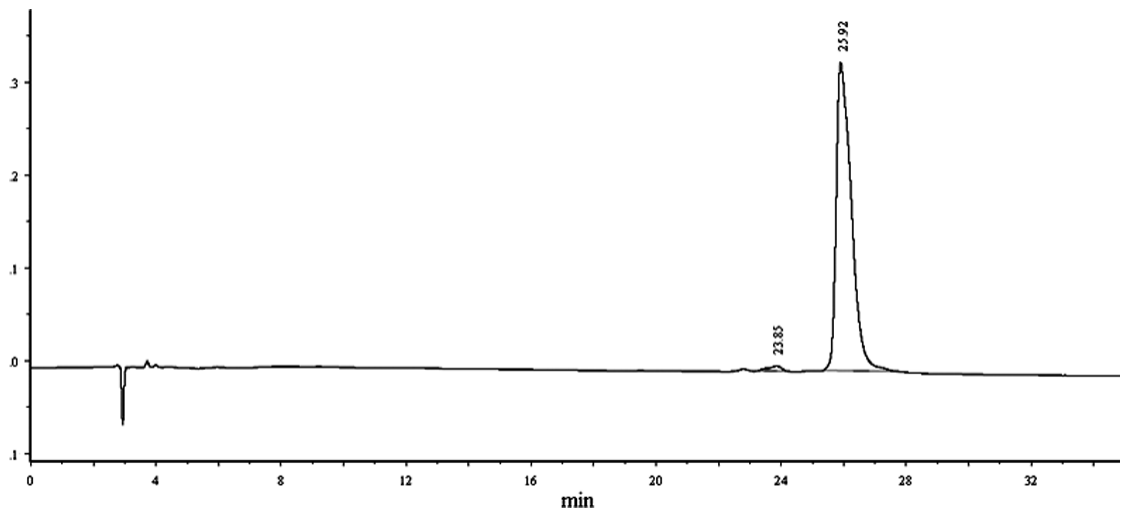


Figure 3. Analytical RP-HPLC chromatogram of peptide 2 after RP-HPLC purification. See Materials and methods for details.

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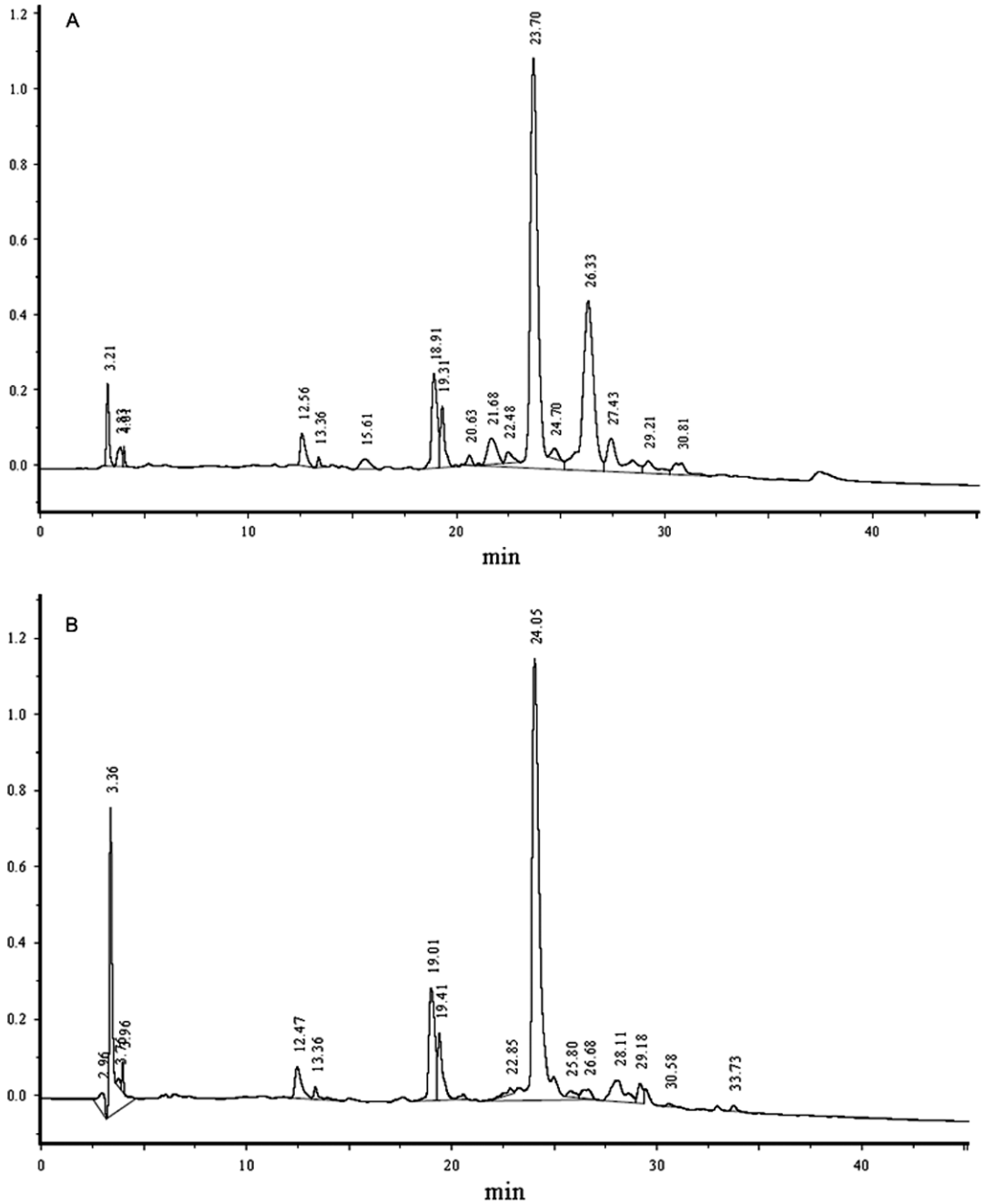
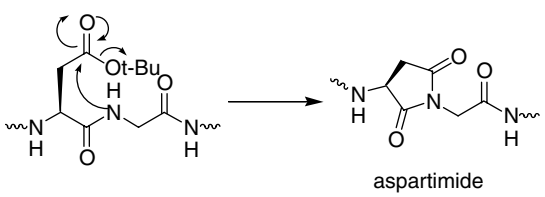


Figure 4. Analytical RP-HPLC chromatograms of the crude products obtained after cleavage from the resin and side chain deprotection from attempts to prepare peptide **3**. Chromatogram (A) pseudoproline dipeptide **12** was incorporated using HATU/HOAt and double couplings (2 × 45 min). The remainder of the amino acids where incorporated using HCTU/Cl–HOBt and double couplings (2 × 20 min). Chromatogram (B) Dipeptide **14** and pseudoproline dipeptide **12** were incorporated using HATU/HOAt and double couplings (2 × 45 min). The remainder of the amino acids were incorporated using HCTU/Cl–HOBt and double couplings (2 × 45 min). See Materials and methods for details.



Scheme 4. Aspartimide formation at an Asp-Gly sequence during peptide synthesis.

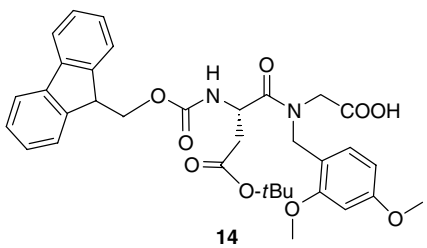


Figure 5. Structure of FmocAsp(O^{t}Bu)Gly(DMB)OH (**14**).

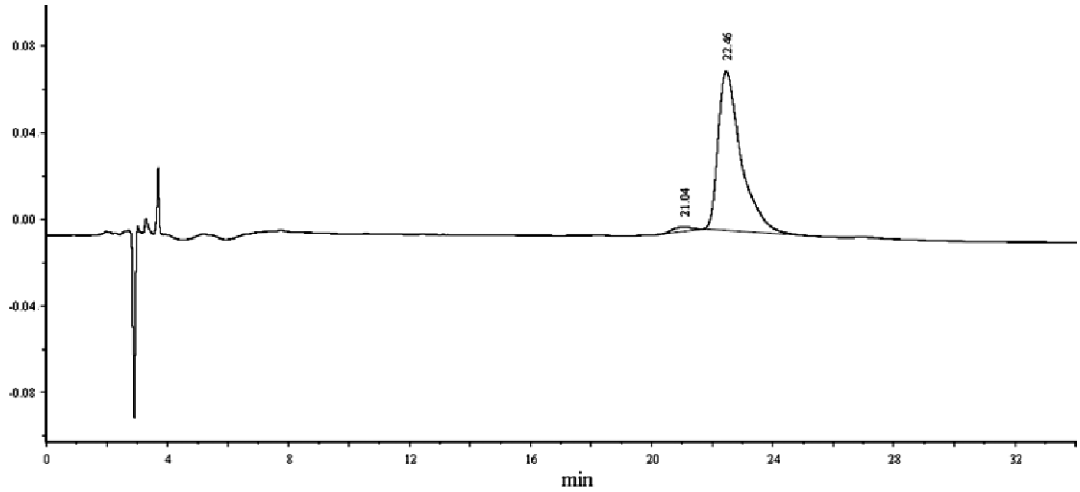


Figure 6. Analytical RP-HPLC chromatogram of peptide **3** after RP-HPLC purification. See Materials and methods for details.

Conclusions

We have shown that sTyr peptides containing Met or Trp residues can be readily prepared using our sulfate-protecting group strategy. This was demonstrated by preparing a peptide corresponding to residues 1–20 of CXCR6 (containing Met at position 1) and a 35-mer peptide corresponding to residues 8–42 in DARC at (containing a Trp residue at position 26). Removing the DCV groups at the end of the syntheses was readily achieved by hydrogenolysis without any reduction of Trp by performing the hydrogenolysis in the presence of triethylamine. Indeed, we have found these conditions to be particularly efficient for removing the DCV group and superiour to our original conditions using H_2, ammonium formate, Pd/C. The presence of Met was found not to interfere with the removal of the DCV group. The use of pseudoproline dipeptides and N-backbone protection with the Dmb group were found to be effective tactics for preventing aggregation and aspartimide formation during the synthesis of these peptides. We also found that the key amino acid **1** can be readily prepared in excellent yield from amino acid **5** and without the use reagent **4**.

Key to the success of our approach to sTyr peptides is the use of 2-MP for Fmoc removal. Although this may at first appear to be a disadvantage of our methodology we have not found this to be the case. As pointed out by Hachmann and Lebl [36] piperidine is a controlled substance and so can be a nuisance especially for peptide synthesis companies that deal with large quantities of piperidine. This prompted Hachmann and Lebl to examine other bases such as 2-MP, 3-, and 4-methylpiperidine for Fmoc removal [36]. 2-MP is not a controlled substance and is not subjected to the restrictions and regulations that piperidine is subject to [36]. The cost of 2-MP is comparable to that of piperidine. Finally, in this and our previous report [13] we have used Fmoc deprotection times (3×10 min) that are 1.5 times longer than that commonly employed for Fmoc removal (2×10 min) because Hachmann and Lebl reported that Fmoc deprotection of FmocIle attached to chlorotrityl resin using 2-MP occurred with a half-life that was 1.5 times greater than that of piperidine [36]. We have recently performed studies on the synthesis of a 12-residue peptide (AcISDRDY(SO_3DCV)MGWMDF-NH_2) derived from CCK using either 2-MP or piperidine and Fmoc deprotection times of 3 min + 11 min. The HPLC chromatograms of the crude peptides were identical suggesting that deprotection times of 3 min + 11 min can be used with 2-MP (Ali, Taylor, unpublished results). Interestingly, when piperidine was used no peptides resulting from attack of piperidine on the sulfur atom of the DCV-protected sTyr residue were detected. In any case, we suggest here that 2-MP is a very practical and cost effective alternative to piperidine for SPPS.

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Supporting information

Supporting information may be found in the online version of this article.

References

1 Baeuerle PA, Huttner WB. Tyrosine suflation of yolk proteins 1, 2 and 3 in Drosophila melanogaster. *J. Biol. Chem.* 1985; **260**: 6434–6439.
2 Stone MJ, Chuang S, Hou X, Shoham M, Zhu JZ. Tyrosine sulfation: an increasingly recognized post-translational modification of secreted proteins. *New Biotechnol.* 2009; **25**: 299–317.
3 Veldkamp CT, Seibert C, Peterson FC, Sakmar TP, Volkman BF. Recognition of a CXCR4 sulfotyrosine by the chemokine stromal cell-derived factor-1alpha (SDF-1alpha/CXCL12). *J. Mol. Biol.* 2006; **359**: 1400–1409.
4 Duma L, Häussinger D, Rogowski M, Lusso P, Grzesiek S. Recognition of RANTES by extracellular parts of the CCR5 receptor. *J. Mol. Biol.* 2007; **365**: 1063–10755.
5 Simpson LS, Zhu JZ, Widlanski TS, Stone MJ. Regulation of chemokine recognition by site-specific tyrosine sulfation of receptor peptides. *Chem. Biol.* 2009; **16**: 153–161.
6 Leppänen A, White SP, Helin J, McEver RP, Cummings RD. Binding of glycosulfopeptides to P-selectin requires stereospecific contributions of individual tyrosine sulfate and sugar residues. *J. Biol. Chem.* 2000; **275**: 39569–39578.
7 Bunschoten A, Kruijtzer JA, Ippel JH, de Haas CJ, Van Strijp JA, Kemmink J, Liskamp RM. A general sequence independent solid phase method for the site specific synthesis of multiple sulfated-tyrosine containing peptides. *Chem. Commun. (Camb)* 2009; 2999–3001.

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8 Cormier EG, Persuh M, Thompson DA, Lin SW, Sakmar TP, Olson WC, Dragic T. Specific interaction of CCR5 amino-terminal domain peptides containing sulfotyrosines with HIV-1 envelope glycoprotein gp120. *Proc. Natl. Acad. Sci. U.S.A.* 2000; **97**: 5762–5767.
9 Farzan M, Vasilieva N, Schnitzler CE, Chung S, Robinson J, Gerard NP, Gerard C, Choe H, Sodroski J. A tyrosine-sulfated peptide based on the N terminus of CCR5 interacts with a CD4-enhanced epitope of the HIV-1 gp120 envelope glycoprotein and inhibits HIV-1 entry. *J. Biol. Chem.* 2000; **275**: 33516–33521.
10 Musiol H-J, Escherich A, Moroder L. Synthesis of sulfated peptides. In *Houben-Weyl, Methods of Organic Synthesis*, E22B Goodman M (ed.). Thieme Stuttgart: New York, 2003; 425–453.
11 Young T, Kiessling LL. A strategy for the synthesis of sulfated peptides. *Angew. Chem. Int. Ed.* 2002; **41**: 3449–3451.
12 Kitagawa K, Aida C, Fujiwara H, Yagami T, Futaki S, Kogire M, Ida J, Inoue K. Facile solid-phase synthesis of sulfated tyrosine-containing peptides: total synthesis of human big gastrin-II and cholecystokinin (CCK)-39. *J. Org. Chem.* 2001; **66**: 1–10.
13 Ali AM, Taylor SD. Efficient solid-phase synthesis of sulfotyrosine peptides using a sulfate protecting-group strategy. *Angew. Chem. Int. Ed.* 2009; **48**: 2024–2026.
14 Chitnis CE, Sharma A. Targeting the *Plasmodium vivax* Duffy-binding protein. *Trends Parasitol.* 2008; **24**: 29–34.
15 Choe H, Moore MJ, Owens CM, Wright PL, Vasilieva N, Li W, Singh AP, Shakri R, Chitnis CE, Farzan M. Sulfated tyrosines mediate association of chemokines and *Plasmodium vivax* Duffy binding protein with the Duffy antigen/receptor for chemokines (DARC). *Mol. Microbiol.* 2005; **55**: 1413–1422.
16 Chitnis CE, Chaudhuri A, Horuk R, Pogo AO, Miller LH. The domain on the Duffy blood group antigen for binding *Plasmodium vivax* and *P-knowlesi* malarial parasites to erythrocytes. *J. Exp. Med.* 1996; **184**: 1531–1536.
17 Liu J, Louie S, Hsu W, Yu KM, Nicholas HB Jr, Rosenquist GL. Tyrosine sulfation is prevalent in human chemokine receptors important in lung disease. *Am. J. Respir. Cell. Mol. Biol.* 2008; **38**: 738–743.
18 Petit SJ, Chayen NE, Pease JE. Site-directed mutagenesis of the chemokine receptor CXCR6 suggests a novel paradigm for interactions with the ligand CXCL16. *Eur. J. Immunol.* 2008; **38**: 2337–2350.
19 Kikugawa Y, Kashimura M. Catalytic transfer hydrogenation of indoles to indolines in formic acid. *Synthesis* 1982; 785–787.
20 Mery J, Calas B. Tryptophan reduction and histidine racemization during deprotection by catalytic transfer hydrogenation of an analog of the luteinizing hormone releasing factor. *Int. J. Pept. Protein Res.* 1988; **31**: 412–419.
21 Kikugawa Y, Kashimura M. Side reaction during deprotection of tryptophan derivatives by catalytic transfer hydrogenation in formic acid. *Chem. Pharm. Bull.* 1982; **30**: 3386–3387.
22 Ammann T, Goetzoe S, Thern B, Welz S, Wolter KJ. Pseudo proline dipeptides. USA Patent US2008004451 (A1), 2008.
23 Zahariev S, Guarnaccia C, Pongor CI, Quaroni L, Eemažar M, Pongor S. Synthesis of 'difficult' peptides free of aspartimide and related products, using peptoid methodology. *Tetrahedron Lett.* 2006; **47**: 4121–4124.
24 Ingram LJ, Desoky A, Ali AM, Taylor SD. *O*- and *N*-Sulfations of carbohydrates using sulfuryl Imidazolium salts. *J. Org. Chem.* 2009; **74**: 6479–6485.
25 Adamiak R, Biala E. Nucleoside 3′-phosphotriesters as key intermediates for the oligoribonucleotide synthesis. IV. New method for removal of 2,2,2-trichloroethyl group and P NMR as a new tool for analysis of deblocking of intemucleotide phosphate protecting groups1. *Nucleic Acids Res.* 1977; **4**: 2321–2329.
26 Letsinger RL, Groody EP, Lander N, Tanaka T. Some developments in the phosphitetriester method for synthesis of oligonucleotides. *Tetrahedron* 1984; **40**: 137–143.
27 Ogilvie KK, Beaucage SL, Entwistle DW. A facile method for the removal of phosphate protecting groups in nucleotide synthesis. *Tetrahedron Lett.* 1976; **17**: 1255–1256.
28 Heuer M, Hohgardt K, Heinemann F, Kuhne H, Dietrich W, Grzelak D, Muller D, Welzel P, Markus A, VanHeijenoort Y. Structural analogs of the antibiotic moenomycin A with a D-glucose-derived unit F. *Tetrahedron* 1994; **50**: 2029–2046.
29 Medzihradszky-Schweiger H. Promoted hydrogenolysis of carbobenxoxyamino acids in the presence of organic bases. *Acta Chim. Acad. Sci. Hung.* 1973; **76**: 437–440.
30 Carpino LA. 1-Hydroxy-7-azabenzotriazole. An efficient peptide coupling additive. *J. Am. Chem. Soc.* 1993; **115**: 4397–4398.
31 Haack T, Mutter M. Serine derived oxazolidines as secondary structure disrupting, solubilizing building blocks in peptide synthesis. *Tetrahedron Lett.* 1992; **33**: 1589–1592.
32 Wöhr T, Mutter M. Pseudo-prolines in peptide synthesis: direct insertion of serine and threonine derived oxazolidines in dipeptides. *Tetrahedron Lett.* 1995; **36**: 3847–3848.
33 Wohr T, Wahl F, Nefzi A, Rohwedder B, Sato T, Sun XC, Mutter M. Pseudo-prolines as a solubilizing, structure-disrupting protection technique in peptide synthesis. *J. Am. Chem. Soc.* 1996; **118**: 9218–9227.
34 Mergler M, Dick F, Sax B, Weiler P, Vorherr T. The aspartimide problem in Fmoc-based SPPS. Part I. *J. Pept. Sci.* 2003; **9**: 36–46.
35 Lauer JL, Fields CG, Fields GB. Sequence dependence of aspartimide formation during 9-fluorenylmethoxycarbonyl solid-phase peptide synthesis. *Lett. Pept. Sci.* 1995; **1**: 197–205.
36 Hachmann J, Lebl MJ. Alternative to piperidine in Fmoc solid-phase synthesis. *J. Comb. Chem.* 2006; **8**: 149.

