Peptide Synthesis

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Efficient Solid-Phase Synthesis of Sulfotyrosine Peptides using a Sulfate Protecting-Group Strategy**

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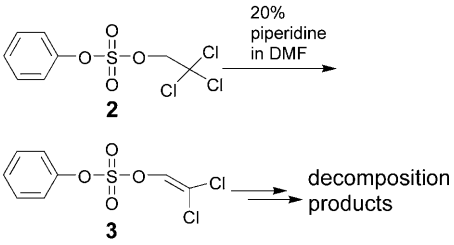
Sulfation of tyrosine residues in certain proteins has been shown to be crucial for the proper functioning of a variety of crucial biological processes, such as viral entry into cells, blood clotting, cell–cell adhesion, and progastrin processing.[1] Although it appears that many proteins bear one or more sulfotyrosine (sY) residues the precise biological roles of the majority of these proteins have not been elucidated.[1] One of the factors hindering the study of the role of sY in proteins and peptides is that sY peptides are not readily available in the quantities that are required for detailed investigations. While a number of strategies have been developed for the solid-phase synthesis of sY peptides their construction is by no means straightforward and an efficient, general approach has yet to be reported.[1,2] In the most widely employed strategy sY residues are incorporated into peptides using FmocTyr(SO_3^{-+}Na)OH (Fmoc = (9H-fluoren-9-ylmethoxy)-carbonyl).[3a] However, couplings after 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.[1,2,3b] Moreover, cleavage of the peptide from the resin and side chain deprotection is achieved under acidic conditions which can result in desulfation. To minimize desulfation, cleavage is performed using 90% aq. trifluoroacetic acid (TFA) at 0–4 °C and the highly acid labile 2-chlorotrityl (2-ClTrt) resin is preferably used. The reaction time for cleavage and deprotection need to be optimized for each peptide and even with these precautions some desulfation inevitably occurs.[1,2,3a] Additional problems with this procedure include incomplete side-chain deprotection and insufficient cleavage from the resin.[1,2] An alternative approach employing orthogonally protected tyrosine residues has drawbacks, such as multiple manipulations of the completed polymer-bound and free peptide including treating the resin-bound peptide with $SnCl_2$/PhSH/Et_3N and the use of only hydrogenolytically labile protecting groups on the side chains of the other amino acids.[3b] Given the problems associated with the above methods we embarked upon a study to develop a general and efficient approach to sY peptide synthesis.

One tactic that could potentially solve the problems mentioned above is to incorporate the sY residue(s) at the beginning of the synthesis as a protected sulfodiester(s). We recently reported the use of the trichloroethyl (TCE) group as a protecting group for sulfate esters.[4,5] This group is introduced using easily prepared $Cl_3CCH_2OSO_2Cl$ (**1**)[4] or its imidazolium derivative[5] and is readily removed under very mild reducing conditions.[4,5] Although TCE-protected sulfates are stable to a wide variety of conditions including strong acid, they are not stable to an excess of the organic bases that are commonly used to remove the Fmoc group during solid-phase peptide synthesis (SPPS), such as piperidine, morpholine or 1,8-diazabicyclo[5.4.0.]undec-7-ene (DBU) in DMF.[6] For example, ^{1}H NMR spectroscopy studies of compound **2** in 20% piperidine/[D_7]DMF revealed that it undergoes a relatively rapid elimination of HCl to give dichlorovinyl (DCV) sulfate ester **3** followed by a slower attack by piperidine on the sulfur atom of **3** and subsequent formation of decomposition products (Scheme 1).



Scheme 1. Decomposition of ester **2** in piperidine/DMF.

Although the TCE group is unstable to the usual bases that are used in Fmoc SPPS, we reasoned that if a base could be found that would not attack the sulfur atom of a DCV-protected sulfate ester yet be capable of rapid Fmoc removal then the DCV group should be employable as a sulfate protecting group during the SPPS of sY peptides. We further reasoned that such a base would have to be more sterically encumbered than piperidine yet have a basicity that was similar to piperidine. Recently, Hachmann and Lebl reported that Fmoc deprotection of FmocIle attached to chlorotrityl resin using readily available 2-methylpiperidine (2-MP)[7] occurred with a half-life that was only 1.5-times greater than that of piperidine.[8] This prompted us to determine if DCV-protected sulfate esters are stable to 2-MP and, if so, whether 2-MP could be used in place of piperidine for SPPS.

Compound **3** was used as a model ester to determine the stability of DCV-protected sulfates to base and acid. The

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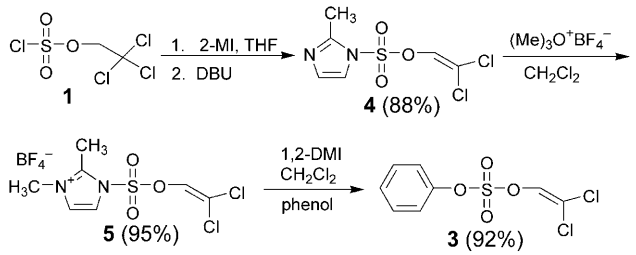
synthesis of ester **3** was achieved by first treating compound **1** with 2-methylimidazole (2-MI) followed by the addition of DBU which gave compound **4** in 88% yield (Scheme 2). Compound **4** was then subjected to Meerwein's reagent which resulted in the formation of imidazolium salt **5** in 95% yield.[9] Reaction of compound **5** with phenol in the presence of 1,2-dimethylimidazole (1,2-DMI) gave ester **3** in 92% yield.



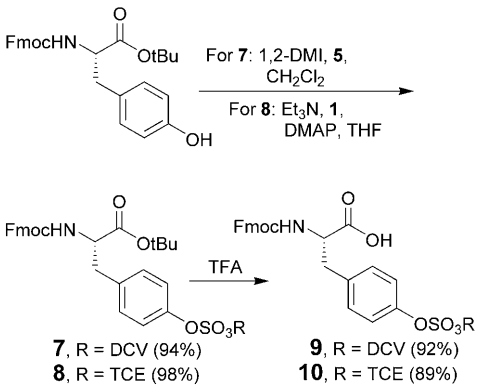
Scheme 2. Synthesis of compound **3**.

Compounds **2** and **3** were subjected to 20% 2-MP or 20% piperidine in [D_7]DMF and the mixture examined by ^{1}H NMR spectroscopy. Independently synthesized compound **3** in 20% piperidine/[D_7]DMF began to show significant decomposition after 4 h. However, in 20% 2-MP/[D_7]DMF, it exhibited little or no decomposition even after 5 days. In 20% 2-MP/[D_7]DMF, compound **2** still underwent elimination to give DCV-protected compound **3** and this was accompanied by the precipitation of the hydrochloride salt of 2-MP. However, no further decomposition occurred even after several days. We also subjected compound **3** to 98% TFA/2% triisopropylsilane (TIPS) and found no decomposition to occur even after 6 h.

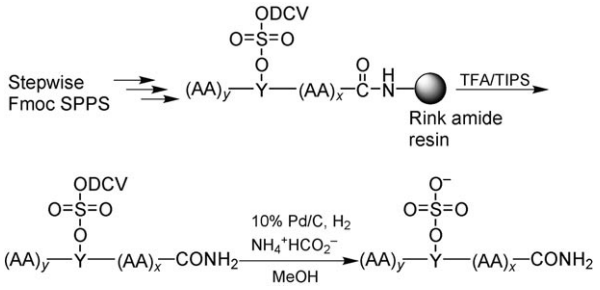
We next compared 2-MP to piperidine in SPPS by preparing the model hexapeptide $DADEYLNH_2$ (**6**)[10] using both bases. The Rink amide resin and standard SPPS methods were employed[11] except that after the coupling of each Fmoc amino acid the peptide was subjected to 3×10 min of 20% 2-MP/DMF as opposed to the standard 2×10 min method when piperidine is used. The completed peptides were cleaved from the resin using 98% TFA/2% TIPS then precipitated in ether. The HPLC chromatogram of the two crude peptides were essentially identical and consisted of mainly a large single peak which corresponded to the desired product indicating the 2-MP could be used in place of piperidine for Fmoc-based SPPS.

Since the TCE group breaks down to give the more stable DCV group in 20% 2-MP/DMF, we examined both DCV and TCE-protected amino acids **9** and **10** as building blocks. Amino acids **9** and **10** were prepared in overall yields of 86% and 87%, respectively, by reacting FmocTyrO*t*Bu[12] with reagents **5** and **1** in the presence of 1,2-DMI or Et_3N/DMAP followed by treatment with TFA (Scheme 3).[13]

The synthesis of a simple model monosulfated peptide, $DADEsYLNH_2$ (**11**),[10] was examined using the route outlined in Scheme 4. Automated SPPS was performed using the same conditions described above for the synthesis of the $DADEYLNH_2$ peptide using 2-MP as base. When building block **10** was used, the hydrochloride salt of 2-MP, which was



Scheme 3. Synthesis of protected amino acids **9** and **10**.



Scheme 4. Overview of sY peptide synthesis.

produced during each Fmoc removal, formed a precipitate though this precipitate could be removed by treatment with CH_2Cl_2. Using DCV-protected amino acid **9**, the HPLC chromatogram of the crude peptide before DCV removal showed mainly a single peak corresponding to $DADEY(SO_3DCV)LNH_2$ (**12**).[14] Using amino acid **10**, the HPLC chromatogram of the crude peptide before hydrogenolysis showed mainly two major peaks in about a 1:1 ratio corresponding to $DADEY(SO_3DCV)LNH_2$ (**12**) and $DADEY(SO_3TCE)LNH_2$ (**13**). No peak corresponding to the desulfated peptide was detected in either case.[14] To remove the DCV and TCE groups, the crude cleaved peptides were subjected to 30 wt% of 10% Pd/C, H_2 gas (balloon) and 9 equivalents of ammonium formate in MeOH at room temperature for 1 h. The HPLC chromatogram of the crude peptides consisted of mainly a single peak which corresponded to the peptide **11** and again no peak corresponding to desulfated peptide was detected.[14] When using **9** as building block, pure peptide **11** was obtained in a 71% yield. When using **10** as building block, peptide **11** was obtained in about a 45% yield but was contaminated with a small amount of an unidentified impurity that exhibited a very similar retention time to **11** which we were unable to completely remove.

To demonstrate the broad applicability of our method employing building block **9** we prepared four additional sulfated peptides. First, we prepared the trisulfated octapeptide, $AcsYEsYLDsYDFNH_2$ (**14**) which has been synthesized by others in a 27% yield using an orthogonal protecting-group strategy.[3b] This peptide corresponds to residues 5–12 of mature P-selectin glycoprotein ligand 1 (PGSL-1), which binds to P-selectin and plays an important role in the

recruitment of white blood cells to inflamed tissues.[15] The presence of sY at residues 5, 7, and 10 are important for binding to P-selectin. Using our method pure peptide **14** was obtained in a 46% yield. We also prepared the monosulfated octapeptide AcYEsYLDYDFNH$_2$ (**15**) since others reported difficulties in preparing this peptide using an orthogonal protecting-group strategy and were able to obtain this peptide in only a 5% yield.[3b] Using our usual method we obtained this peptide in a 63% yield. We also prepared the disulfated 22-mer Ac-TTPDsYGHsYDDKDTLDLNTPVDKNH$_2$ (**16**) which corresponds to residues 7–28 of C5aR, a G-protein coupled receptor which has been implicated in the pathogenesis of numerous inflammatory diseases.[16,17] This was accomplished in a 58% yield. Finally, we turned our attention to preparing a multisulfated peptide derived from the N-terminal region of chemokine receptor D6. D6 is a receptor that suppresses inflammation and tumorigenesis by scavenging extracellular pro-inflammatory CC chemokines.[18] Although it is known that D6 is sulfated in its N-terminal region, it is not known which tyrosines are sulfated.[19] The Sulfinator, a software tool that predicts tyrosine sulfation sites in protein sequences,[20] predicted that tyrosine residues 23, 24, 25, and 27 are all potential sulfation sites and it is possible that all four are sulfated. Therefore, we prepared a tetrasulfated 20-mer, Ac-DADSENSSFsYsYsYDsYLDEVAFNH$_2$ (**17**) that corresponds to residues 14–33 in D6. With its four sY residues, three of which occur consecutively, and with a total of 10 acidic residues this peptide represents a particularly stringent test of our method.[21] This synthesis was achieved in a 39% yield.

To summarize, we have described an efficient method for the synthesis of sY-bearing peptides using a novel sulfate ester protecting group and 2-MP for Fmoc removal. We anticipate that this strategy will make sY peptides and proteins more accessible and so aid in the study of this important class of biomolecules. The synthesis of yet more-complex sTyr-bearing peptides and the evaluation of this strategy with peptides containing cysteine and methionine are in progress and will be reported in due course.

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