Synthesis of Sulfotyrosine Bearing Peptides and Analogues

by

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A thesis
presented to the University of Waterloo
in fulfillment of the
thesis requirement for the degree of
Doctor of Philosophy
in
Chemistry

Waterloo, Ontario, Canada, 2010

AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

Sulfation of tyrosine residues is a post-translational modification that occurs on many secretory as well as transmembrane proteins. This modification is believed to be essential for numerous biological processes. However, one of the factors hindering the study of the significance of sulfotyrosine (sTyr) within a protein is the absence of a general method that enables the synthesis of sTyr peptides in satisfactory yields and purity.

A general approach to the synthesis of sTyr-bearing peptides was developed in which the sTyr residue is incorporated into the peptides with the sulfate group protected. For the implementation of this general approach a new protecting group for sulfates, namely, the dichlorovinyl (DCV) group was developed. This was accomplished by conducting a careful analysis of the reaction of a trichloroethyl (TCE)-protected sulfate ester with piperidine and 2-methylpiperidine (2-MP). A unique sulfuryl imidazolium reagent, compound **2.22**, was also developed that enabled the ready synthesis of DCV-protected sulfates. This reagent was used to prepare the amino acid building block FmocTyr(SO_3DCV)OH (**2.23**). An alternative and more economical synthesis of FmocTyr(SO_3DCV)OH (**2.23**) was also developed that did not require reagent **2.22**. Fmoc-based solid phase peptide synthesis (SPPS) was used to incorporate **2.23** into peptides using 2-MP for Fmoc removal. After cleavage of the peptide from the support, the DCV group was removed by hydrogenolysis to give sTyr peptides in good yield and purity. Using this approach a variety of sTyr peptides were prepared including a tetrasulfated 20-mer corresponding to residues 14-33 in chemokine receptor D6 and a disulfated 35-mer corresponding to residues 8-42 of the *N*-terminus region of the chemokine receptor DARC and this is the largest multisulfated sTyr–bearing peptide made to

date. It was also demonstrated that the incorporation of an important non-hydrolyzable sTyr analog, 4-(sulfonomethyl)phenylalanine (Smp), into peptides can be accomplished in good yield by protecting the sulfonate residue with a TCE group during SPPS. This approach was shown to be superior to the previously reported method where the sulfonate group is unprotected. Finally, a number of sulfotyrosine bearing peptides were synthesized and tested as protein tyrosine phosphatase-1B (PTP1B) inhibitors

Acknowledgements

First, I am grateful to God Almighty for giving me the soul support to continue my graduate studies and to complete this dissertation with all kind of pressure and difficulties I faced throughout my doctoral studies.

I would like to express the deepest appreciation to my Supervisor, Professor Scott D. Taylor, who has the attitude and the substance of a perfect supervisor: he continually and convincingly conveyed a spirit of adventure in regard to research and scholarship, and an excitement in regard to teaching. Without his guidance and persistent help this dissertation would not have been possible.

I also thank my advisory committee, Professor Michael J. Chong, Professor Gary Dmitrienko and Professor France-Isabelle Auzanneau for their valuable guidance and generous help during my PhD study. I have benefited a lot by taking Professor Chong's and Professor Auzanneau's courses. I also would like to thank Jan Venne for all her help with NMR use and a particular thanks to Richard Smith for runing the mass analysis for my compounds and for the fruitful discussions.

I would like to thank all my current and former lab mates at the Taylor lab (2006-2010). I had the pleasure of working with many great people throughout the years within the lab and the department of chemistry. I specially would like to thank Yong Liu a former student in Taylor lab for his generous support during my first period in the lab. I am eternally grateful to Laura Ingram for her help during my first days in the lab and for her countless supportive advice about living in Canada. I would like to thank my Egyptian lab-mate Ahmed Desoky for his good company during these years. I wish to express my special appreciation to Waseem El-Huneidi and Kamal Mroue who help me the most throughout my stay here in Waterloo and provided me with friendship and support I will never forget.

I am grateful to the Ministry of Higher Education, Egypt for the financial support during my PhD study. I am thankful to all my professors at Faculty of Pharmacy, Assiut University for the foundation they gave me without which I would not manage to continue in Waterloo.

Last but not least my lovely people. Special grateful to my parents who give me all the love and support I need throughout my life and work so hard during their lives to make me a better person. My warm thanks to my soul mate my wife Rania for her love, patience and support. During the last four years we were exposed to a lot of pressure and challenges and without our tight relation and Rania's support and wisdom we would not make it through. THANKS RANIA.

To My lovely;

Wife Rania

Sons, Marwan and Yaseen

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List of Schemes

Glu-Phe(p-$CH_2SO_3^-$)-Glu-Phe(p-$CH_2SO_3^-$)-Leu-Asp-NH_2 (**3.60**, t_R=15.76 min) prepared from unprotected amino acid **3.16**

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List of Abbreviations

AcOH	Acetic acid
1-Ada	1-adamantyl
ADP	Adenosine 5'-diphosphate
APS	Adenosine 5'-phosphosulfate
ATP	Adenosine 5'-triphosphate
Bn	Benzyl
Boc	*tert*-butoxycarbonyl
BOP	Benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphonium Hexafluorophosphate
BrZ	2-bromobenzyloxycarbonyl
^{t}Bu	*tert*-butyl
CCK	Cholecystokinin
ClZ	2-Chlorobenzyloxycarbonyl
2-Cl-Trt	2-chlorotrityl
cHx	Cyclohexyl
DARC	Duffy Antigen Receptor for chemokines
DBL	Duffy-Binding Like domain
DBU	1,8-Diazabicyclo[5.4.0]undec-7-ene
DCC	Dicyclohexylcarbodiimide
DCM	Dichloromethane
DCV	Dichlorovinyl
DCV-SIS	DCV-sulfuryl imidazolium salt
DDVP	Dichlorvos or 2,2-dichlorovinyl dimethyl phosphate
DFMP	Difluoromethylenephosphonyl
F_2Pmp	Difluorphosphonomethylphenylalanine
DiFMUP	6,8-difluoro-4-methylumbelliferyl phosphate
DIPC or DIPCDI	Diisopropylcarbodiimide
DIPEA	Diisopropylethylamine
DMAP	4-Dimethylaminopyridine
Dmb	2,4-Dimethoxybenzyl
DMF	Dimethylformamide
1,2-DMI	1,2-Dimethylimidazole
DMS	Dimethylsulfide
DNA	Deoxyribonucleic acid
DNP	2,4-Dinitrophenol
EDT	Ethanedithiol
ESMS	electrospray mass spectrometry
FC	Flash chromatography

Fmoc	Fluorenylmethyloxycarbonyl
For	Formyl
GPCRs	G-protein coupled receptors
HATU	*O*-(1H-7-azabenzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate
HBTU	*O*-(1H-benzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate
HCTU	*O*-(6-Chloro-1H-benzotriazole-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate
HIV	Human immunodeficiency virus
Hmb	2-Hydroxyl 4-methoxy benzyl
HOAt	[1,2,3]Triazolo[4,5-b]pyridin-1-ol
HOBt	Benzotriazol-1-ol
HOCt	6-Chloro-benzotriazol-1-ol
HOOBT	Hydroxy-3,4-dihydro-4-oxo-1,2,3-benzotriazine
HPLC	High performance liquid chromatography
IL	Interleukin
IR	Insuline receptor
IRS	Insuline receptor substrate
MCP-1 (CCL2)	Monocyte chemotactic protein-1
MGSA (CXCL1)	Melanoma growth stimulatory activity
2-MI	2-Methylimidazole
2-MP	2-Methylpiperidine
Nle	Norleucine
NMP	N-Methyl-2-pyrrolidone
Ompe	3-methylpent-3-yl
Pac	Phenacyl
PAP (3’,5’-ADP)	Adenosine 3’,5’-diphosphate
PAPS	Adenosine 3’-phosphate 5’-phosphosulfate
PAS	Pyridinium acetylsulfate
Pbf	Pentamethyl-dihydrobenzofuran-5-sulfonyl
PhA	Phenoxyacetyl
PTKs	protein tyrosine kinases
PTPs	protein tyrosine phosphatases
PTP-1B	protein tyrosine phosphatase-1B
PSGL-1	P-Selectin Glycoprotein Ligand-1
PTM	Post-translation modification
pTyr	Phospho-tyrosine
*Pv*DBP	*Plasmodium vivax* Duffy Binding Protein
RANTES (CCL-5)	Regulated upon Activation, Normal T-cell Expressed, and Secreted
RV	Rotary evaporator
SIS	Sulfuryl imidazolium salt
Smp	4-(sulfonomethyl)phenylalanine

SPPS	Solid phase peptide synthesis
sTyr	Sulfo-tyrosine
TCE	2,2,2-Trichloroethyl
TBAF	tetra-*n*-butylammonium fluoride
TBAI	tetra-*n*-butylammonium iodide
TFA	Trifluoroacetic acid
TFE	Trifluoroethanol
THF	Tetrahydrofuran
TIPS (TIS)	Triisopropylsilane
Tos	Tosyl
Tmb	2,4,6-Trimethoxybenzyl
TNF	Tumor necrosis factor
TPSTs	Tyrosylprotein sulfotransferases
Troc	2,2,2-Trichloroethoxycarbonyl
WSCI	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide.HCl
Z (CBz)	Benzyloxycarbonyl

Chapter 1

Tyrosine Sulfation as a Post-Translational Modification and the Synthesis of Sulfotyrosine-Bearing Peptides

1.1 Tyrosine sulfation as a post translational modification.

Living cells can produce a wide variety of peptides and proteins. The amino acid composition, number and sequence of a peptide or protein are initially determined by the genomic sequences of DNA. However, cells can broaden the diversity of the synthesized protein via modifications which target the fully assembled protein or peptide chains in a process known as post-translation modification (PTM).[1, 2] In PTMs the assembled protein is varied either by cleaving or splicing the protein amide bond at certain position(s) or by adding new organic or inorganic moieties to one or more amino acids on the protein chain.[1] Perhaps the most famous example of a PTM involving a cleavage process is insulin which is first translated as a single chain prohormone but is then converted to a disulfide bridged two-chain active form by the aid of specific proteases.[3] The number of PTMs involving the covalent addition of organic or inorganic moieties is vast. PTMs such as glycosylation, phosphorylation, sulfation, acetylation, methylation, ubiquitination, and hydroxylation are the most common PTMs of this type. This thesis is concerned with sulfation as a PTM.

Although phosphorylation is perhaps the most studied PTM, modification by adding a sulfate group instead of a phosphate group has not been as extensively studied. Throughout the rest of this chapter we will discuss in detail how sulfotyrosine (sTyr or sY) containing

peptides/proteins are synthesized *in vivo*, the biological significance of these peptides/protein and, finally, we will describe in detail the available methods to obtain these peptides/proteins synthetically.

O-Sulfation of tyrosine residues in proteins is a PTM that is believed to occur in all eukaryotes on secreted and membrane-spanning proteins.[4] It has been suggested that up to 1% of all tyrosine residues of the overall protein content in an organism are sulfated which makes tyrosine sulfation an important post-translation modification.[5] Tyrosine sulfation is catalyzed by members of the sulfotransferase family of enzymes called **t**yrosyl**p**rotein **s**ulfo**t**ransferases (TPSTs). TPSTs catalyze the transfer of a sulfate group from the sulfate donor adenosine 3'-phosphate 5'-phosphosulfate (PAPS) to the phenolic group of a tyrosine residue(s) within the fully assembled peptide or protein to generate the sulfotyrosine bearing peptide along with adenosine 3',5'-diphosphate (PAP) (**Figure 1.1**).[6]

To date two TPST isozymes have been identified, namely TPST-1 and TPST-2.[6-9] The two isozymes show 63% sequence identity with each other but there is considerably higher sequence identity (> 90%) for each of these isozymes between mammalian species.[6] This high degree of sequence conservation between mammalian species suggests the functional importance of these differences between the two isozymes.[10] *In vitro* studies have shown that the two isozymes have overlapping but not identical substrate specificities.[8] The physiological significance of the existence of the two TPST isozymes is not well understood.[6,8]

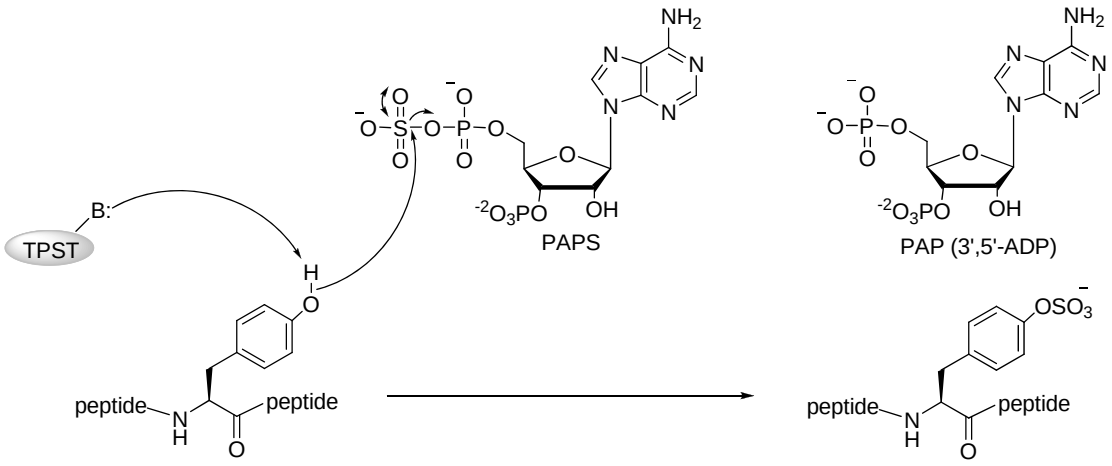


Figure 1.1. TPST catalyzed protein tyrosine sulfation.

Protein sulfation occurs in the *trans*-Golgi network. The TPSTs are transmembrane proteins with a short N-terminal cytoplasmic domain, a single transmembrane domain and a luminal catalytic domain. Such architecture requires both unsulfated substrate together with PAPS to be presented in the lumen of the *trans*-Golgi network.[6]

PAPS is synthesized in the cytosol by a bifunctional enzyme called PAPS synthase which has a sulfurylase domain and a kinase domain. It uses two molecules of ATP and one molecule of inorganic sulfate which enters the cells via a sulfate transporter. One molecule of ATP reacts with inorganic sulfate to form adenosine 5'-phosphosulfate (APS) at the ATP sulfurylase domain. 3'-phosphorylation of APS using the second ATP molecule to give PAPS occurs in the kinase domain (**Figure 1.2**).[6]

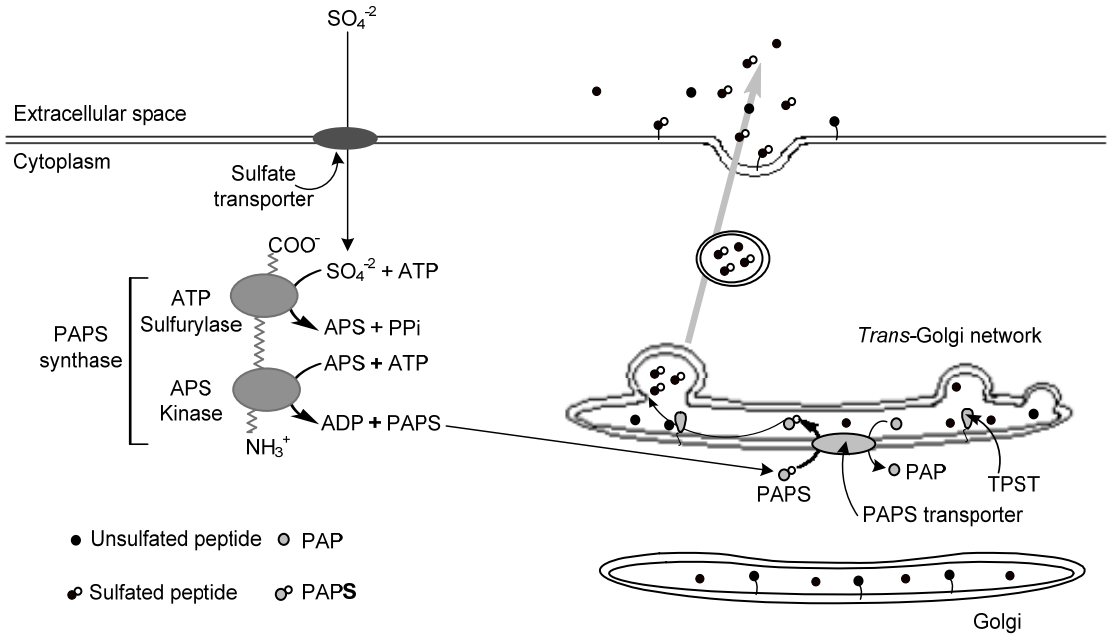


Figure 1.2. Schematic representation of process of tyrosine sulfation in *trans*-Golgi network (adapted from Moore[6])

There does not appear to be a specific sequence that determines which tyrosyl residue within a protein transiting the secretory compartment is to be sulfated. However, studies conducted on synthetic peptides have shown that the presence of the acidic residues aspartate or glutamate in the vicinity of the sulfated tyrosine is required.[11-13] Besides acidic residues, small amino acid residues are usually located 1-2 residue(s) upstream of the tyrosyl moiety which is believed to facilitate binding of substrates to TPSTs.[11, 14] For the same reason, sulfation sites are usually well separated from both disulfide bonds and N-linked glycosylation sites.[13]

1.2 Biological significance of tyrosine sulfation.

It is now believed that the main role of tyrosine sulfation is to modulate protein-protein interactions.[15-17] Although many proteins are tyrosine sulfated, how essential the sTyr residue(s) to the biological function of these proteins is, in most cases, not known. Nevertheless, the role of tyrosine sulfation in some proteins has been elucidated to some degree. In the following pages we will discuss some of these proteins as well as proteins that have been shown to contain sTyr but the function of the sTyr residue is unknown.

1.2.1 Tyrosine sulfation of P-Selectin Glycoprotein Ligand-1 (PSGL-1).

One of the most important steps in the process of inflammation is the recruitment of leukocytes into the site of inflammation which is derived by an intercellular communication cascade between cell adhesion molecules extended from the cell membranes of both endothelium cells of various blood vessels and leukocytes (**Figure 1.3**).[15]

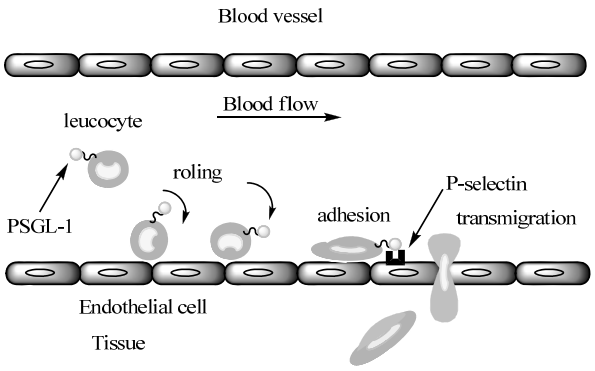
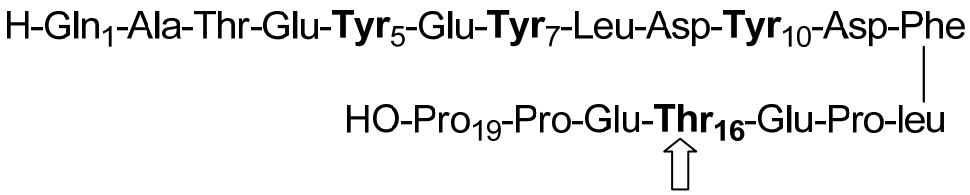


Figure 1.3. Schematic representation of process of leukocytes rolling, adhesion and transmigration into the site of inflammation.

At the site of inflammation, chemokines are produced as signalling molecules by different cells in response to different inflammatory stimuli like bacterial/virus infections

and/or physical damage. Chemokines stimulate endothelial cells of venules at this particular site to express P-selectin and E-selectin on their luminal surface.[15] The natural ligand of P-selectin is P-selectin glycoprotein ligand-1 (PSGL-1) which is expressed on the surface of leukocytes as disulfide-linked homodimers and post-translationally modified by *O*-glycosylation and tyrosine sulfation.[18] Studies have shown that only the first 19 amino acid residues within the *N*-terminal region, which has an *O*-glycan attached to threonine 16 and has three sulfotyrosine residues at positions 5, 7 and 10, are required for optimum binding to P-selectin.[19] In fact, it has been demonstrated that just this N-terminal portion (**1.1**, **Figure 1.4**), in its glycosylated and sulfated form, binds as efficiently to P-Selectin as the fully dimeric PSGL-1.[20] Furthermore, inhibition of sulfation at these particular sites using chlorate, an inhibitor of tyrosine sulfation, or site directed mutagenesis of tyrosine residues into phenylalanine dramatically affects the binding affinity to P-selectin. [21, 22] All three sTyr residues contribute to high affinity binding to P-selectin.[20, 23] From all the above one can conclude that finding an efficient method for the synthesis of the truncated PSGL-1 or its analogs and elucidating the details of its binding to P-selectin will pave the way for the discovery of novel anti-inflammatory drugs.



1.1

Figure 1.4. The N-terminal domain (residues 1-19) of mature PSGL-1 showing the sulfation sites at 5, 7 and 10 residues as well as glycosylation site at residue 16 (indicated by arrow).

1.2.2 Tyrosine sulfation of chemokine receptors.

Chemokines are a group of functionally related, small, soluble proteins (~ 8-14 kDa). They are named chemokines because of chemoattractant properties. There are about 40 chemokines currently known. A considerable number of basic amino acids as well as several cysteine residues (usually four) are characteristic features of these proteins. Chemokines have a high expression level and so they are produced in large amounts in order to create a concentration gradient to attract the migrating cells (chemotactic cytokines).[24,25] Chemokines are classified into four chemokine subfamilies according to the number and spacing of the conserved cysteine residues. The two largest subfamilies are the CC chemokines (for example CCL5), where the first two cysteines appear consecutively and, the CXC chemokines (for example CXCL16) where the first two cysteines are intervened by another amino acid residue X (where X is any amino acid residue except proline). The other two subfamilies, which are relatively new and small compared to the first two subfamilies, are the XC subfamily, which contains only one conserved cysteine (for example XCL1) and the CX_3C chemokines (for example CX_3CL1) which contains three amino acids spacing the first two conserved cysteines. Each subfamiliy contains many members and the members within one subfamily resemble each other more than the members of other families.[24] Functionally, chemokines can be divided into two types; "homeostatic chemokines" which manage leukocyte homing and lymphocyte recirculation under normal conditions and "inflammatory chemokines" which are produced in response to inflammatory stimuli like TNF (tumor necrosis factor) and responsible for recruitment of leukocytes to sites of inflammation.[26] During the last few years chemokines have earned more attention due to

several findings showing that chemokines are involved in diseases such as autoimmune diseases and infectious diseases (e.g. HIV/AIDS).[24]

Chemokines bind to a group of G-protein coupled receptors named chemokine receptors. There are 19 known chemokine receptors. Typically the same receptor binds multiple chemokines in a subfamily restricted manner.[25] Chemokine receptors are usually classified and named according to the classification of their ligands. For example CCR5 is the receptor for CCL5 chemokines.[27] Chemokine receptors are seven transmembrane domain receptors with an acidic *N*-terminal extracellular domain and a serine/threonine-rich intracellular C-terminal domain. The *N*-terminal domain is linked through disulfide bonds to a second loop while the first and third loops are connected through another one. Chemokine receptors are mainly expressed on the surface of leukocytes and their binding to their ligand induces the expression of other surface receptors called integrins. The latter receptors bind firmly to endothelial cells and such binding promotes morphological changes in leukocytes which induces the migration of leukocytes through endothelial cells into the underlying tissue.[10] Interestingly, the *N*-terminal of all known chemokines receptors contain sTyr residues as confirmed by experiment or as predicted through bioinformatic analyses.[10] In the following pages we will discuss some of these sulfated chemokine receptors and we will emphasize the importance of the presence of the sulfate group(s) for proper functioning of the receptors as well as present some examples where the role of the sTyr residue(s) is not known.

1.2.2.1 CCR5 and CXCR4

Although the main role of chemokines receptors is to facilitate leukocyte trafficking some chemokine receptors can themselves facilitate infection. Examples of such receptors are CCR5 and CXCR4. It has been reported that CCR5 and CXCR4 are crucial for the entry of HIV-1 into the target cells in that the HIV-1 gp120 envelope glycoprotein uses CCR5 and CXCR4 as co-receptors besides CD4 to mediate the virus-membrane fusion and hence virus entry. [28, 29] To emphasize the importance of CCR5 to the process of viral entry it was found that certain chemokine proteins, CCR5 inhibitors and anti-CCR5 antibodies inhibit HIV-1 infections.[30] Indeed, Maraviroc®, which is a CCR5 antagonist, has been approved for clinical use in treatment-experienced patients.[31]

H-Met$_1$-Asp-**Tyr**$_3$-Gln-Val-Ser-Ser-Pro-Ile-**Tyr**$_{10}$-Asp-Ile-Asn

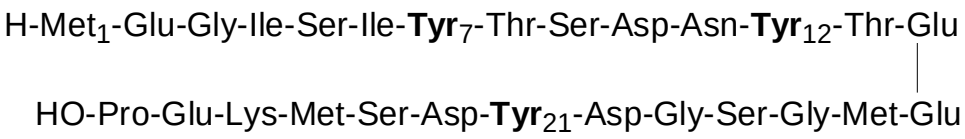
HO-Cys-Pro-Glu-Ser-Thr-**Tyr**$_{15}$-**Tyr**$_{14}$

1.2

Figure 1.5. Amino acid sequence (residue 1-20) of the N-terminal region of CCR5. Sulfation sites are in bold.

The *N*-terminal region and the second extracellular loop are the domains of CCR5 that are responsible for binding of CCR5 with gp120. The *N*-terminal of CCR5 (**1.2**, **Figure 1.5**) has four tyrosine residues at positions 3, 10, 14 and 15 which are vulnerable to sulfation. To underscore the importance of the presence of these sulfate groups it was found that chlorate decreases the binding affinity of CCR5 to the gp120/CD4 complex without affecting the rate of expression in cells transfected with CCR5.[32] Furthermore, site directed mutagenesis of the sTyr moieties to phenylalanine greatly reduces the binding efficiency and

hence the entry ability of HIV.[32] Last but not least, the presence of the sTyr residues are critical for binding to the gp120/CD4 complex *in vitro*.[33] Interestingly, Siebert *et al.* showed that the *in vitro* sulfation of peptide sequences corresponding to the N-terminal of CCR5 using TPST-1 and TPST-2 enzymes occurs in a nonrandom and sequential manner.[34] The authors demonstrated that the sulfation of tyrosine residues at position 14 or 15 occurs first followed by position 10 then finally at position 3. Moreover, there is more than one report showing that sulfation at positions 10 and 14 is sufficient to induce the binding between CCR5 and gp120/CD4 complex and absence of a sulfate group at these positions greatly decreases such binding.[33, 35] Why sulfation also occurs at positions 3 and 15 is not known.



1.3

Figure 1.6. Amino acid sequence (residue 1-27) of N-terminal region of CXCR4. Possible sulfation sites are in bold.

Surprisingly, although the N-terminal domain of the CXCR4 (**1.3**, **Figure 1.6)** contains at least three possible sites for sulfation the presence of a sulfate group(s) does not seem to play a major role of binding of CXCR4 to gp120/CD4 complex.[36] The role of tyrosine sulfation in CXCR4 is not yet known.

1.2.2.2 Chemokine Decoy receptors (D6 and DARC).

Chemokine decoy receptors are an atypical family of chemokine receptors differing from other signaling chemokine receptors in that they bind to a wide range of chemokines and more importantly, such binding does not induce any further signaling machinery.[37] The lack of signaling ability is due to the absence of a DRYLAIV motif (or simply DRY motif) in the second intracellular loop of these atypical chemokine receptors. The DRY motif is responsible for coupling chemokine binding to the triggering of intracellular events. Because this unique class of chemokine receptors lacks such a motif they bind their cognate ligands without triggering any biological response (**Figure 1.7**).[37, 38] When a chemokine binds to these receptors, it is either transported through the cytoplasm and expelled from the other side of the cell in a process known as transcytosis or it is degraded in the cytoplasm.[25] It is believed that the main biological role of these "silent" receptors is to compete with signaling receptors for excess chemokines and thus act as negative modulators of inflammation.[25] The decoy receptor family includes DARC (Duffy Antigen Receptor for chemokines), D6, CCX and CKR receptors.[25] We will discuss the first two receptors in detail.

DARC is a promiscuous decoy receptor that binds to 11 pro-inflammatory chemokines from CC and CXC family but not with homeostatic chemokines.[39, 40] DARC is expressed under normal conditions in erythrocytes and endothelial cell of postcapillary venules and veins of lymph nodes, skin, kidney, lung, brain, thyroid and spleen.[41] It was reported that there is high rate of DARC expression in the inflamed tissues such as the rheumatoid joint synovium, psoriatic skin and pathological conditions involving skin and kidney.[39, 42]

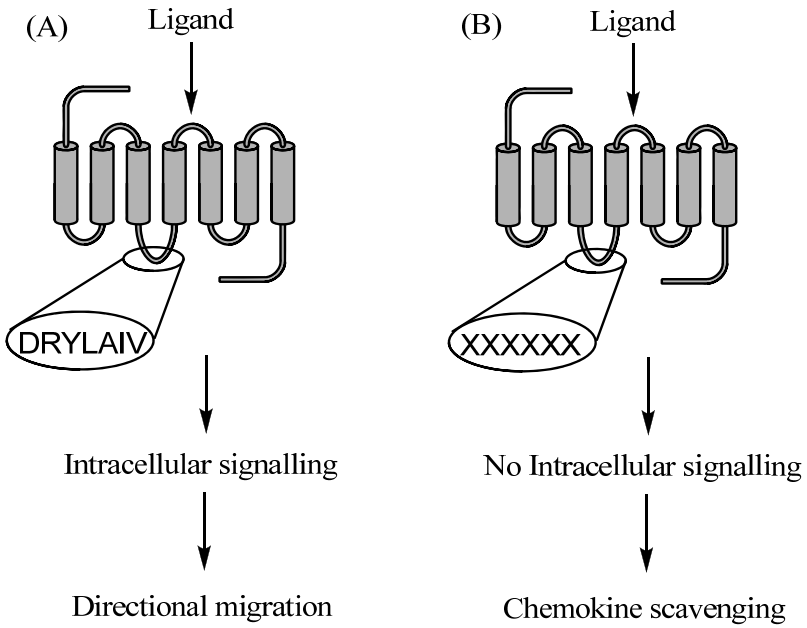
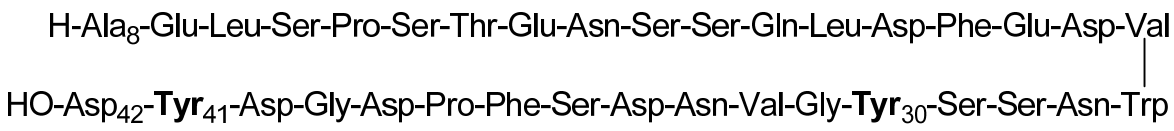


Figure 1.7. Typical (A) and atypical chemokine receptors (B) signaling cascades.

It is believed that the presence of DARC on erythrocytes regulates the concentration of circulating chemokines by acting as sink or reservoir for chemokines. This belief is confirmed by the observation that DARC-null mice rapidly eliminate the chemokines from circulation. [39, 43] On the other hand, in the venular endothelial cells DARC acts as negative modulator of inflammatory process through scavenging the excess chemokines by binding to them.[44]

In addition to its chemokine-binding ability, DARC receptor can serve as erythrocyte receptor for the malaria parasite *Plasmodium vivax*.[45] It was found that the erythrocyte invading stage of *Plasmodium vivax* expresses an erythrocyte-binding antigen called *Plasmodium vivax* **D**uffy **B**inding **P**rotein (*Pv*DBP). The N-terminal of *Pv*DBP contains a

highly-conserved cysteine-rich domain called the **D**uffy-**B**inding **L**ike (DBL) domain.[46, 47] The first interaction between DARC and DBL is a crucial step in erythrocyte invasion by the parasite and failure to complete such interaction retards the invasion. In order to demonstrate the importance of such interaction in the consequence of parasite invasion, Chaudhuri *et al.* showed that a 35-mer *N*-terminal peptide of DARC (non-sulfated) or even smaller peptides can prevent DBL binding to Duffy positive red blood cell. Moreover, antibodies against *N*-terminus of DARC interfere with the erythrocyte invasion by DARC-dependent *Plasmodium yoelii*. [48] All these results highlight the importance of DARC-DBL interaction for parasite invasion and open a new avenue for treating this condition.



1.4

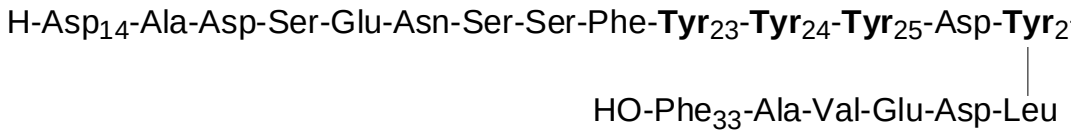
Figure 1.8. Amino acid sequence (residue 8-42) of N-terminal region of DARC. Sulfation sites are in bold.

The *N*-terminal region of human DARC (**1.4**, **Figure 1.8**) contains two sTyr residues at positions 30 and 41.[49] sTyr-41 plays a crucial role in promoting DARC-DBL interactions as indicated by total loss of binding ability on mutating Tyr-41 to Phe.[49] Interestingly, the binding affinity to different chemokines depends on the sulfation pattern of the DARC N-terminus. Where alteration of Tyr-41 to Phe-41 interferes with binding of chemokines MCP-1, RANTES and MGSA with DARC, such alteration does not affect the IL8 chemokine. On

the other hand, the Y30F modification affected IL8 chemokine binding to DARC but not the binding of other chemokines.[49] Thus, it seems that both tyrosine moieties should be sulfated for full activity. However, the full significance of the sulfate moieties and their effect on binding to different chemokines and/or *Pv*DBL has yet to be fully elucidated.

Most often the role of sTyr in chemokine receptors is not known. An example of this is D6. D6 is a chemokine decoy receptor which binds a broad range of inflammatory CC chemokines but does not recognize homeostatic CC chemokines or any other chemokine subfamilies such as CXC chemokines.[50] D6 is expressed at low levels by the circulating leukocytes but it is selectively expressed in the high levels at endothelial cells of the skin, gut, lungs and placenta.[51] As a member of decoy chemokine receptors, D6 does not facilitate any conventional intracellular signaling and is not involved in transcytosis. It is generally believed that D6 competes with the typical chemokine receptors for inflammatory CC chemokines. After binding chemokines, the ligand-receptor vesicle is mobilized intracellularly, the chemokines are rapidly dissociated and degraded during vesicle acidification leaving D6 for recycling.[52-54] Furthermore, the *in vivo* studies using D6-deficient mice support the chemokine scavenging role of D6 where the D6 deficient mice showed an exacerbated inflammatory response. The exacerbated effect was due to a high level of chemokine aggregation which could be prevented by the pretreatment with inflammatory CC chemokine-antibodies.[55, 56] The chemokine scavenging action of D6 was demonstrated in placenta where D6 functions to reduce the amount of inflammatory CC chemokines passed to the fetus as shown by an increase in inflammation-induced fetal loss in D6-deficient pregnant mice.[57] Besides its action as a chemokine scavenger, Neil *et al.*

demonstrated that D6 is expressed on primary astrocytes and is used by gp120 of dual tropic isolate of HIV-1 and HIV-2 as a coreceptor in a manner similar to CCR5 and CXCR4.[58]



1.5

Figure 1.9. Amino acid sequence (residue 14-33) of N-terminal region of D6. Possible sulfation sites are in bold.

The N-terminal domain of D6 has been shown to be sulfated as demonstrated by a metabolic labeling experiment.[59] This region (**1.5**, **Figure 1.9**) contains four Tyr residues all of which are possible sulfation sites. However, it is not known which Tyr residues are sulfated and how important sTyr is for chemokine binding and for HIV entry.[59] Clearly the role of sulfation of D6 merits further investigation.

1.2.2.3 CXCR6

Another example where the role of sTyr in a chemokine receptors is not known is CXCR6. CXCR6 is a member of CXC family of chemokine receptors which has a unique character of being one of the few chemokine receptors which binds only one ligand, namely CXCL16.[25] CXCL16 itself is an unusual chemokine being expressed in soluble as well as membrane bound forms where the latter can act as a receptor itself for scavenging oxidized lipoproteins and bacteria besides facilitating the tight adhesion of cells expressing CXCR6 such as T cells. On the other hand, soluble CXCL16 acts as a chemoattractant for $CXCR6^+$ T cells.[60-62] Both CXCR6 and its ligand are coexpressed within the atherosclerotic lesion and

they are believed to be involved in the pathogenesis of atherosclerosis.[63] Furthermore, it was found that CXCR6 also plays a role as co-receptor for the entry of HIV-1 onto macrophages and T-cells.[64]

H-Met$_1$-Ala-Glu-His-Asp-**Tyr**$_6$-His-Glu-Asp-**Tyr**$_{10}$-Gly-Phe-Ser-Ser-Phe
HO-Gln-Ser-Ser-Asp-Asn

1.6

Figure 1.10. Amino acid sequence (residue 1-20) of N-terminal region of CXCR6. Sulfation sites are in bold.

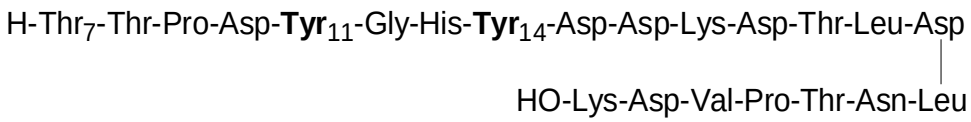
As with most chemokine receptors the N-terminal region of CXCR6 (**1.6**, **Figure 1.10**) is post-translationally modified through tyrosine sulfation and glycosylation. But interestingly, Petite *et al.* showed that the presence of acidic residues in general is not crucial for binding of CXCL16 to its receptor. Instead, they proposed a model for binding of soluble as well as membrane-bound forms of CXCL16 to CXCR6 which is completely different from the established model of chemokine-receptor binding where sTyr plays crucial role.[65] Such a model opens up a wide array of questions about why these tyrosine residues are sulfated and why CXCR6 has a binding profile which differs from its chemokines mates.

1.2.3 Tyrosine sulfation of other G-protein coupled receptors.

Besides chemokine receptors there are a number of other GPCRs for which the presence of a sTyr residue(s) is crucial for its functions. For example, C5a-anaphylatoxin chemotactic receptor (C5aR), C3a-anaphylatoxin chemotactic receptor (C3aR), Type 1 Sphingosine 1-phosphate receptor ($S1P_1R$), Follicle-stimulating hormone receptor (FSHR), Luteinising hormone receptor (LHR)/Chorionic gonadotropin receptor (CGR) and Thyroid-

stimulating hormone receptor (TSHR) are GPCRs in which the extracellular domain contains sulfotyrosine residues as demonstrated by ^{35}S-sulfate metabolic labeling experiments.[10] The presence of these residues creates a negative charge on the N-terminal domain of these receptors which is crucial for the initial docking of theirs hormonal ligands. Not surprising, mutagenesis of these particular sites results in decreased binding affinity. Of this class of receptors, C5aR and its ligand C5a have received a lot of attention because their involvement in numerous inflammatory diseases.[10]

C5a is one of the most potent inflammatory peptides producing a wide-range of effects depending on the cell type it acts upon like, phagocytosis, degranulation, H_2O_2 production, granule enzyme release, delay or enhancement of apoptosis, chemokine and cytokine production and chemotaxis.[66] C5aR and C5L2 are the natural receptors of C5a. The binding of C5a to the former is believed to be fundamental for the development of several disease states for example, rheumatoid arthritis, psoriasis and acute respiratory distress syndrome.[66] The *N*-terminal domain of C5aR contains two sTyr residues (residues 11 and 14) as indicated by labeling experiments.[67] The presence of these sulfated residues is crucial for the binding of C5aR to its ligand as indicated by site directed mutagenesis experiment.[67] On the other hand, sulfated peptide sequence corresponding to residues 7-28 but not its unsulfated analogue (**1.7**, **Figure 1.11**) was able to block the binding of C5a to it receptor.[67]



1.7

Figure 1.11. Amino acid sequence (residue 7-28) of the N-terminal region of C5aR. Sulfation sites are in bold.

Recently, Soliris® (eculizumab) a monoclonal antibody targeting C5a was approved by the FDA (USA) for treating paroxysmal nocturnal hemoglobinuria (PNH) and so prevent hemolysis.[68] Moreover, due to the widespread involvement of C5a-C5aR in many inflammatory conditions it has been suggested that C5aR is a potential target for developing anti-inflammatory drugs for treating conditions such as rheumatoid arthritis.[66]

1.3 Synthesis of sulfotyrosine containing peptides.

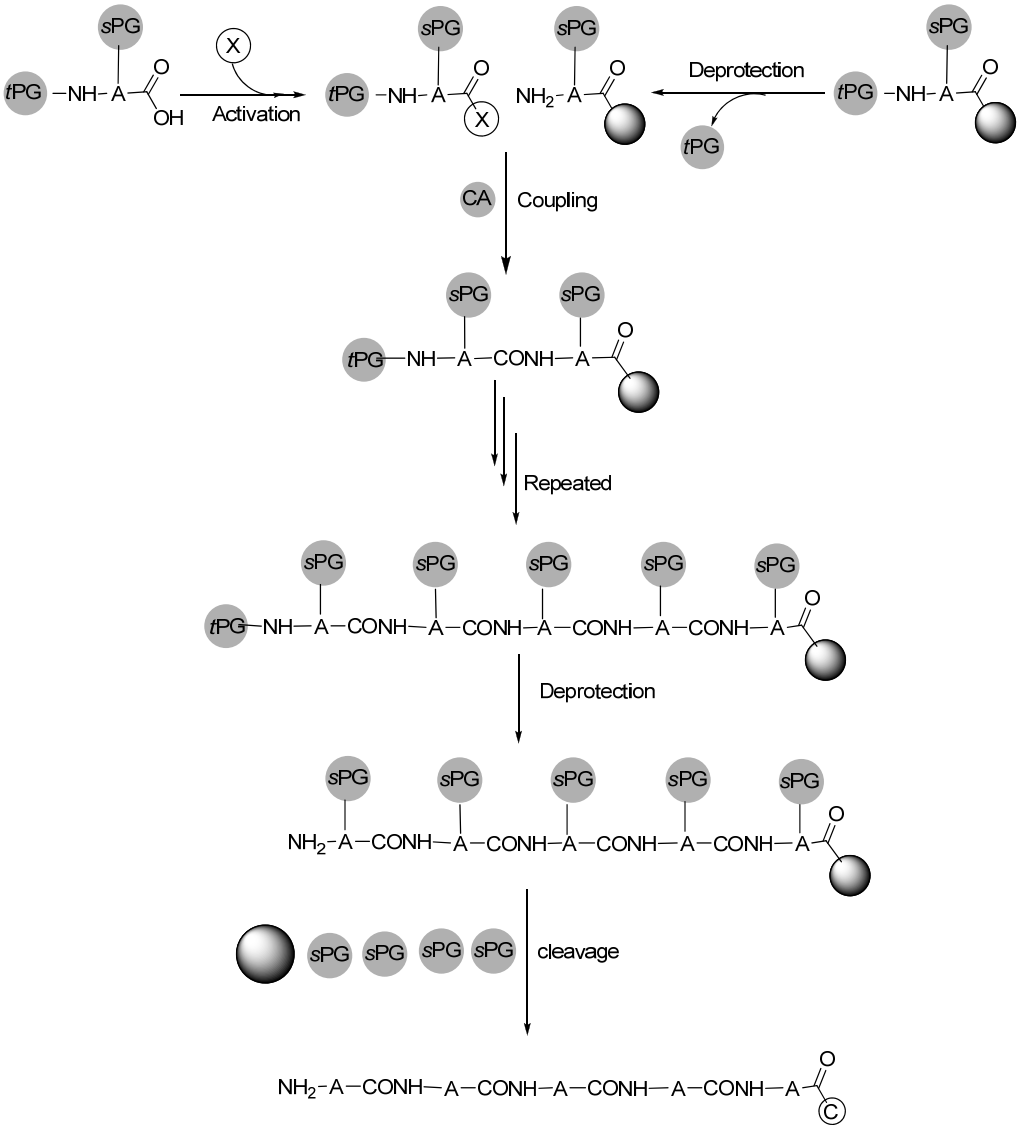
1.3.1 Peptide synthesis and the chemistry of sulfate monoesters

The study of biological systems has been greatly enhanced by the ready availability of synthetic peptides. Synthetic peptides are also very important in the pharmaceutical industry. This is mainly because many pharmaceutical targets are receptors and enzymes whose ligands are peptides or other proteins. Synthetic peptides corresponding to sequences within the bound peptides or proteins are used as lead structures for drug development. Peptides themselves are also used as drugs though this is less common due to bioavailability issues with peptidyl drugs.[69] Since many enzymes and receptors specifically recognize a sequence within peptides or proteins that has been post-translationally modified, the synthesis of peptides bearing modified amino acids is also very important. For some PTMs, such as tyrosine phosphorylation, the corresponding synthetic peptides (peptides bearing phosphotyrosine) are readily prepared. However, sometimes the synthesis of peptides

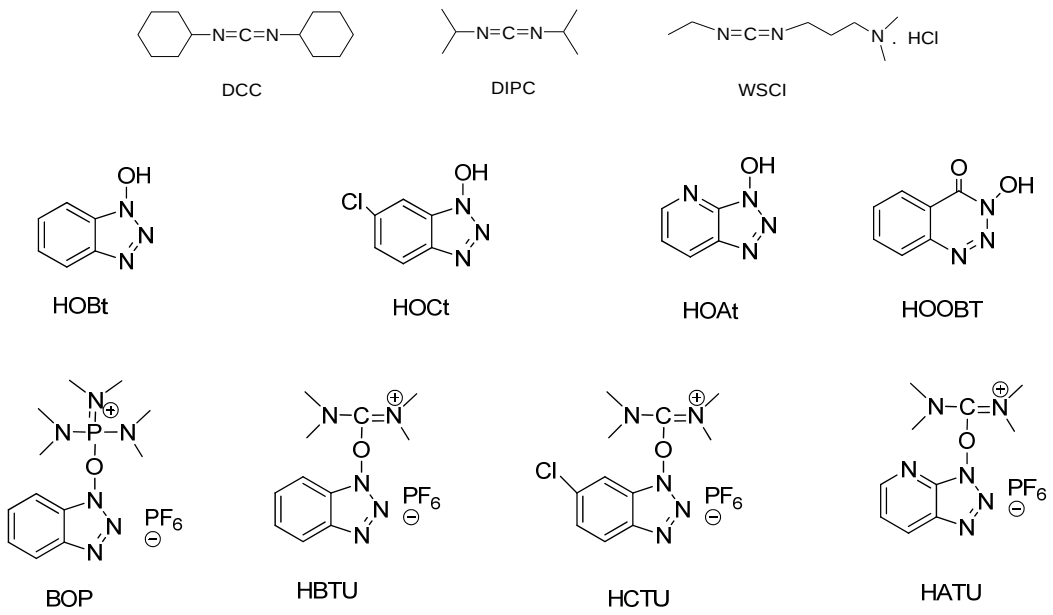
bearing modified amino acids can be very challenging. This has been the case for sTyr-bearing peptides. Indeed, one of the factors that has, to a certain extent, limited the study of tyrosine sulfation in proteins has been the lack of a general and effective method for readily preparing sTyr-bearing peptides in quantities necessary for detailed studies. It is this issue, the synthesis of sTyr-bearing peptides, that is the primary focus of this thesis.

In order to explain why the synthesis of sTyr peptides has been difficult some background on the synthesis of peptides in general and the chemistry of sulfate monoesters is required. Peptides can be prepared "in solution" using standard synthetic organic chemistry techniques or they can be prepared using a process known as solid phase peptide synthesis (SPPS). We will focus our attention on SPPS since the vast majority of peptides today are prepared using that approach.[70, 71] SPPS is based on the sequential addition of protected amino acids (α-amino group and side chains are protected) to the N-terminus of a side chain-protected peptide that has been attached to an insoluble polymer (such as cross-linked polystyrene) via its C-terminus carboxyl group (**Scheme 1.1**). There are two types of SPPS: Boc/Bn SPPS, where the acid [usually trifluoroacetic acid (TFA)]-labile Boc (*tert*-butoxycarbonyl) group is used for temporary N^{α}-protection and most of side chains are protected with HF-labile groups [such as benzyl (Bn) or other HF-labile groups], or Fmoc/^{t}Bu SPPS, where the base-labile Fmoc (Fluorenylmethyloxycarbonyl) group is used for temporary N^{α}-protection and most of side chains are protected as *tert*-butyl ethers or esters or some other group that is removed using TFA (**Scheme 1.1**). After removing the temporary N^{α}-protecting group, using TFA in case of Boc SPPS, or piperidine in case of Fmoc SPPS, the incoming amino acid is coupled to the growing peptide chain using one of a variety of

coupling agents or a preactivated amino acid is used (**Scheme 1.2**). After each step the excess reagents are removed by filtration. At the end of the synthesis, all the side chain protecting groups are removed and the peptide is detached from the resin in one step through treatment with HF when using Boc chemistry or TFA when using Fmoc chemistry. During side chain deprotection and cleavage of the peptide from the support it is common to add reagents, such as thiols, to the HF or TFA which can scavenge reactive cations that are generated during this process. The crude peptide is obtained by filtration and then purified using HPLC. Fmoc-based SPPS, as opposed to Boc-based SPPS, is by far the more common approach since it does not require the need for repeated treatment with TFA and, most importantly, does not require treatment with hazardous HF, which requires specialized equipment and training, to cleave the peptide from the resin. Solution phase synthesis of peptides is carried out in a similar manner except the peptides are not built on a polymer support and the C-terminus is protected usually as a benzyl ester. Unlike the synthesis of peptides in solution, SPPS does not require the purification of intermediates since the amino acids and reagents are added in excess to drive the reactions to as to close to 100% completion as possible and the excess reagents are removed by filtration. SPPS has also been automated. The 1984 Nobel Prize in chemistry was awarded to Bruce Merrifield for his invention of SPPS.

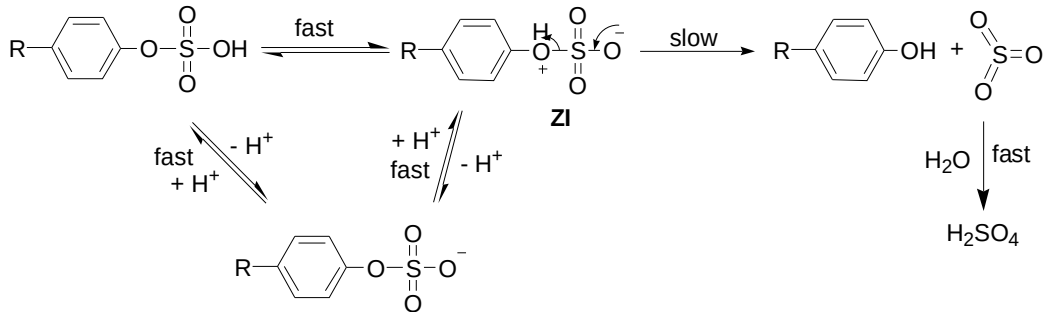


Scheme 1.1. Schematic representation of SPPS. *t*PG = temporary protecting group (Fmoc or Boc). *s*PG = semi-permanent protecting group. CA= coupling agent



Scheme 1.2. Common coupling agents used in SPPS.

Sulfate monophenylesters are acid labile. Several studies have suggested that they hydrolyze in acid via an A-1 type mechanism as shown in **Scheme 1.3**.[72-75] Dissociation of the zwitterionic intermediate (ZI) is the rate limiting step in the hydrolysis.



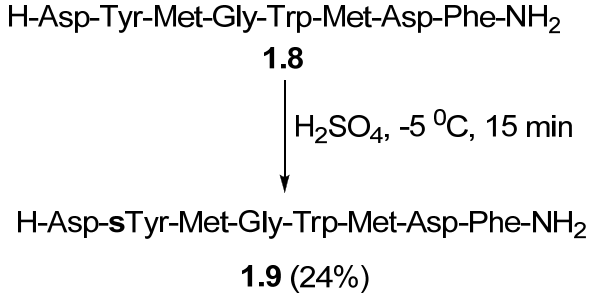
Scheme 1.3. Suggested mechanism for acid-catalyzed hydrolysis of arylmonosulfates in acidic solution.

It is the acid lability of sulfate monoesters that makes the synthesis of sTyr peptides a considerable challenge since acid treatment of the peptide, either during chain elongation (when using Boc chemistry either in solution or during solid phase synthesis) or during cleave of the peptide from the support (using Boc or Fmoc chemistry) is a necessary component of peptide synthesis. Consequently, unless special precautions are taken, loss of the sulfate group will occur during the synthesis of sTyr bearing peptides. In the following section we will outline the various approaches that have appeared in the literature previous to our studies for constructing sTyr-bearing peptides.

1.3.2 The global sulfation approach to the synthesis of sTyr peptides.

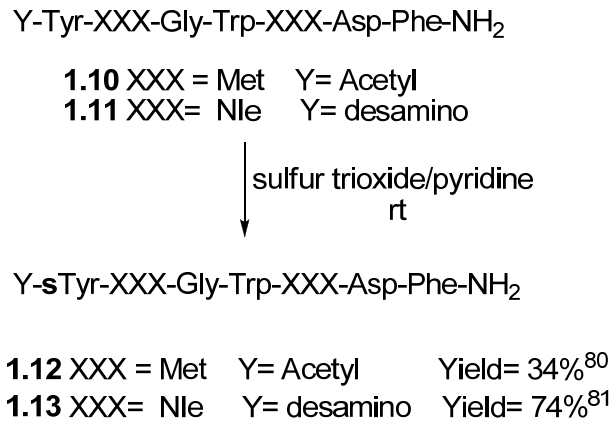
In the global sulfation approach the fully extended peptide chain is assembled first using solution or solid phase methods then the tyrosine moieties are sulfated using a sulfating agent. Any protecting groups on the side chains of the other amino acids are then removed and the peptide is cleaved from the support (if doing SPPS). Several considerations should be taken into account on applying the global sulfation approach. First, the sulfating agent should be of appropriate sulfating power to give the intended tyrosine sulfated compound in good yield without any other side reactions occurring on the tyrosyl moiety itself or other amino acid side chains. Second, the side chain protecting groups on the other amino acids must withstand the synthesis and sulfating conditions yet are removed under conditions which will not affect the sTyr residue. Third, if using SPPS, then the conditions for removal of the peptide from the support must not result in desulfation.

A variety of sulfating agents have been used in the global sulfation strategy. Simple neat concentrated sulfuric acid was among the first. When using sulfuric acid the hydroxyl and thiol groups of other amino acids should be protected otherwise they will be quantitatively sulfated and the reaction should be run at low temperature (-5 ^{o}C) and for short period of time otherwise an appreciable degree of 3-sulfonation of tyrosyl moieties occurs. On the other hand, running the reaction under these attenuated conditions usually gives low yields. For example Ondetti *et al.* used concentrated sulfuric acid to sulfate CCK-(cholecystokinin) octapeptide **1.8** after constructing it using Boc/Bn solution phase chemistry (**Scheme 1.4**).[76] Although, the reaction was conducted at -5 ^{o}C for 15 min a considerable degree of tyrosine sulfonation took place and the yield of the sulfation step to give peptide **1.9** was only 24%. In order to enhance the sulfation reaction using sulfuric acid, a mixture of DCC and [S^{35}]-sulfuric acid was used to effect the radiolabelling of unprotected CCK-8; however the labeled CCK-8 was obtained in a moderate yield (40 % for the sulfation step only) even in presence of four-fold molar excess of sulfuric acid and 40-fold molar excess of DCC. Moreover, other side chains were found to have reacted with the sulfating agent.[77]



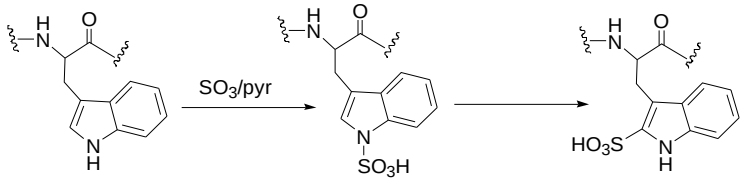
Scheme 1.4. Sulfation of CCK-8 using concentrated sulfuric acid.

Sulfur trioxide complexes with pyridine, dimethylformamide or triethylamine are reported to effectively react with tyrosine residues to give sTyr without any appreciable sulfonation even at high temperatures.[78] On using these sulfating agents, full protection strategies are required since sulfation of hydroxyl groups, thiol groups, amino, guanidine and primary amido occurs besides sulfation of phenol groups.[78] Furthermore, methionine residues may be harmed when exposed to these sulfating agents for long periods of time as found by Beacham *et al.* during the solution phase synthesis of a decapeptide derived from human gastrin and using sulfur trioxide/pyridine in aqueous medium as sulfating agent.[79] Since homoserine was detected amongst the products it is believed that the sulfur of methionine reacted with sulfur trioxide/pyridine to form the corresponding sulfonium derivative which decomposed to give homoserine.[79] This was further supported by the fact that replacing methionine residues in CCK-heptapeptide for norleucine (Nle) resulted in a dramatic increase in the obtained yield under almost identical reaction conditions (**Scheme 1.5**).[80, 81]



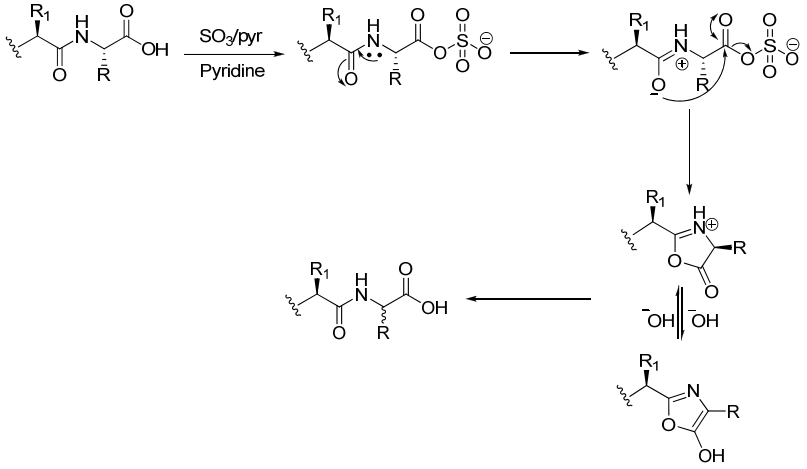
Scheme 1.5. Sulfation of CCK-heptapeptide and its norleucine analogue.

Tryptophan residues may also be modified upon exposure to sulfur trioxide/pyridine through 2-sulfonation (**Scheme 1.6**).[82] Fortunately, this side reaction can be prevented by protecting the indole ring with a formyl group which in turn can removed with short treatment with piperidine/DMF.[82]



Scheme 1.6. Sulfonation of tryptophan residues upon treatment with sulfur trioxide/pyridine.

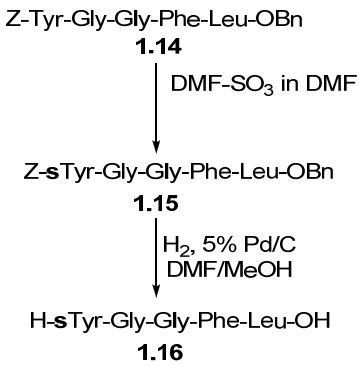
Epimerization (up to 20% occurred) at the C-terminal residue can also occur when using sulfur trioxide/pyridine and using pyridine/DMF 1:1 as solvent for peptide sulfation but only if the C-terminus is left unprotected. The epimerization is proposed to occur through oxazol-5(4H)-one formation as shown in **Scheme 1.7**. Luckily, such racemization is greatly inhibited by shifting to sulfur trioxide/DMF and using DMF as solvent.[83]



Scheme 1.7. Mechanism of C-terminus epimerization of hirudine on treatment with sulfur trioxide/pyridine.

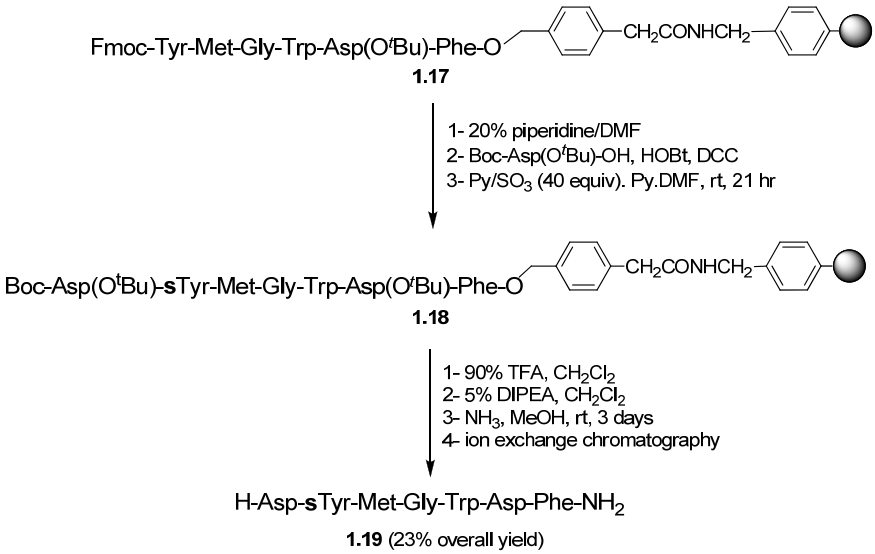
Sulfur trioxide/DMF complex is a stronger sulfating agent than sulfur trioxide/pyridine complex.[84] Furthermore, Futaki and his coworkers reported that using sulfur trioxide/DMF complex eases the purification of the final product since the water solubility of sulfur trioxide/DMF complex is better than that of the pyridine complex.[84] Sulfur trioxide/Et_3N has also been used but is less reactive than sulfur trioxide/DMF.[85] Pyridinium acetylsulfate (PAS) has also been used as a sulfating agent for sTyr peptide synthesis. As the sulfation reaction is conducted in acidic medium, AcOH or TFA, sulfation of amino, guanidino, imidazole, indole groups is suppressed. Serine residues must be protected. On the other hand, under these conditions, sulfonation of tyrosine moieties can occur, albeit to a small extent. The main drawback of pyridinium acetylsulfate is its reduced reactivity and so sometimes quantitative sulfation is hard to obtain even with using a large excess of sulfating agent and long reaction times.[86]

As mentioned earlier, one of the problems with the global sulfation approach is selectivity. Sulfation often occurs at other residues and so these residues must be protected and their protecting groups must be removed at the end of the synthesis without affecting the sTyr residue. One way of getting around this problem is to use protecting groups on the side chains that can be cleaved with non-acidic reagents and performing the synthesis in solution or, when using SPPS, employing a linker to the resin that is extremely acid labile. Futaki *et al.* employed side chain protecting groups that were removed by hydrogenolysis for the synthesis of Leu-enkephalin sulfate (**1.16**, **Scheme 1.8**).[84]



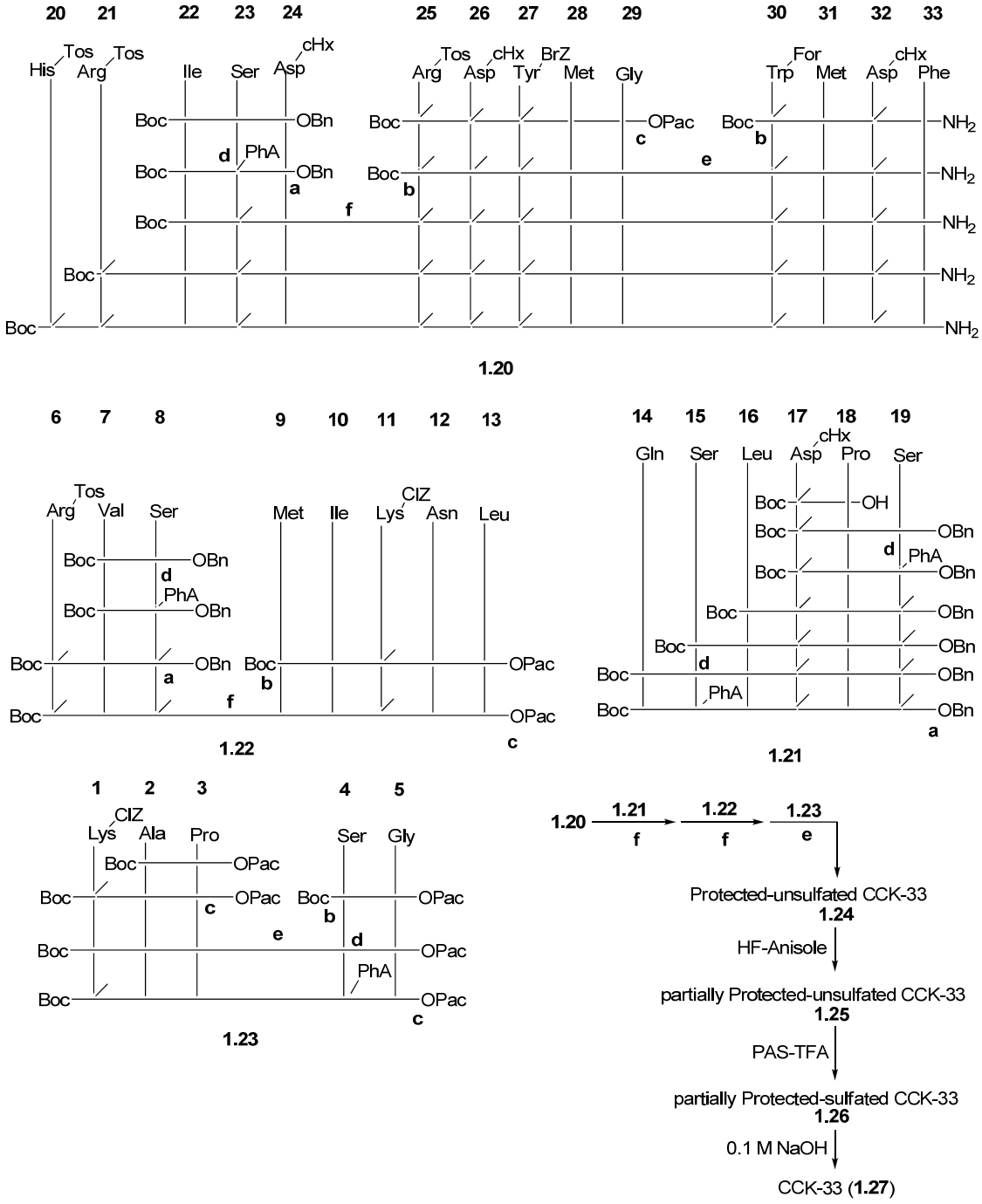
Scheme 1.8. Synthesis of Leu-enkephalin sulfate **1.16**.

Rosamond *et al.* used PAM resin to construct appropriately protected CCK-8 following Fmoc-SPPS.[87] After on-resin sulfation using sulfur trioxide/pyridine, the acid labile side chain protecting groups were removed under attenuated acidic conditions without affecting the peptide benzyl linkage to the PAM resin. The linkage was cleaved upon ammonolysis for 3 days to give the desired compound in 23% yield (**Scheme 1.9**).[87]



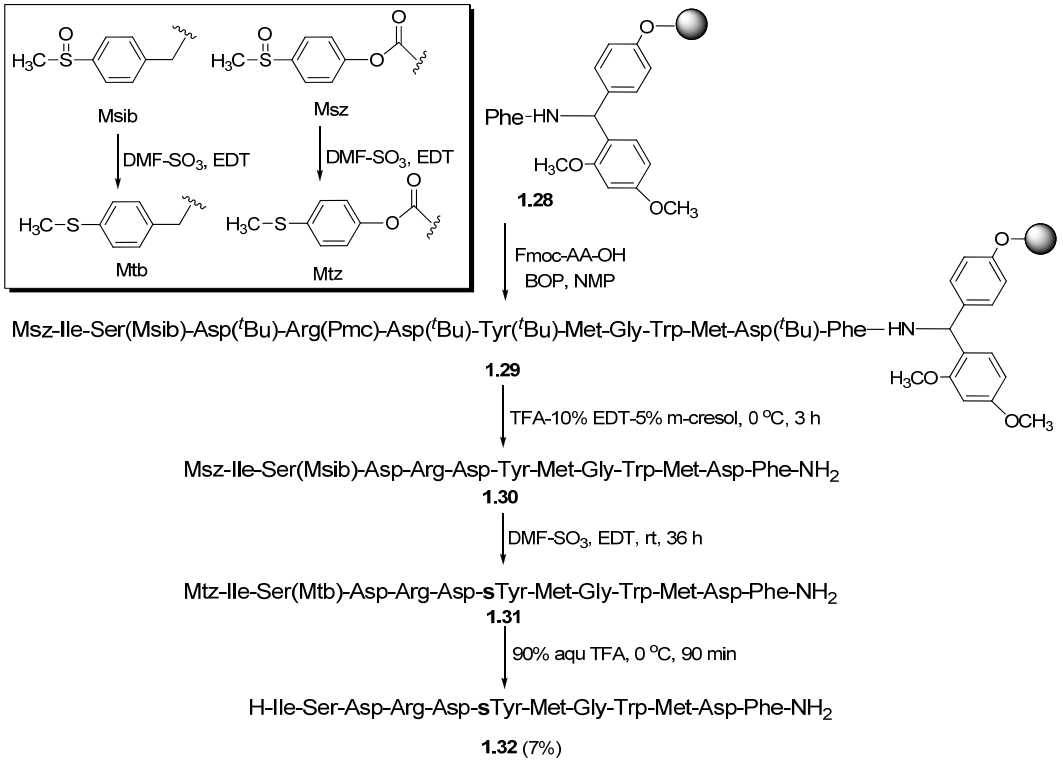
Scheme 1.9. Solid phase synthesis of CCK-8 followed by on resin sulfation and deprotection.

An interesting example of the global sulfation approach that employs an unconventional side chain protecting group has been reported by Kurano and coworkers for the synthesis of CCK-33 as outlined in Scheme 1.10.[88] Four segments were constructed and then coupled together to assemble the final peptide using solution phase Boc chemistry and applying water soluble-carbodiimide WSCI/HOBT and WSCI/ Hydroxy- 3,4-dihydro-4-oxo-1,2,3-benzotriazine (HOOBt) for coupling. All of the serine residues were protected with phenoxyacetyl (PhA) groups which are stable to hydrogen fluoride. After constructing the full length peptide chain, all of the protecting groups within the peptide chain were removed using HF except the PhA groups and the formyl group of tryptophan. The tyrosine residue was sulfated with PAS. Deprotection of the serine residues and deformylation of tryptophan under basic conditions gave the final product in 5% yield. The low yield was attributed to methionine oxidation and sulfonation of tryptophan by PAS.[78]



Scheme 1.10. Synthesis of CCK-3. WSCI-HOBT method used as coupling agent. **a**, catalytic hydrogenolysis; **b**, TFA; **c**, Zn-acetic acid; **d**, phenoxyacetic anhydride; **e**, WSCI-HOBT; **f**, WSCI-HOOBT. Pac= phenacyl

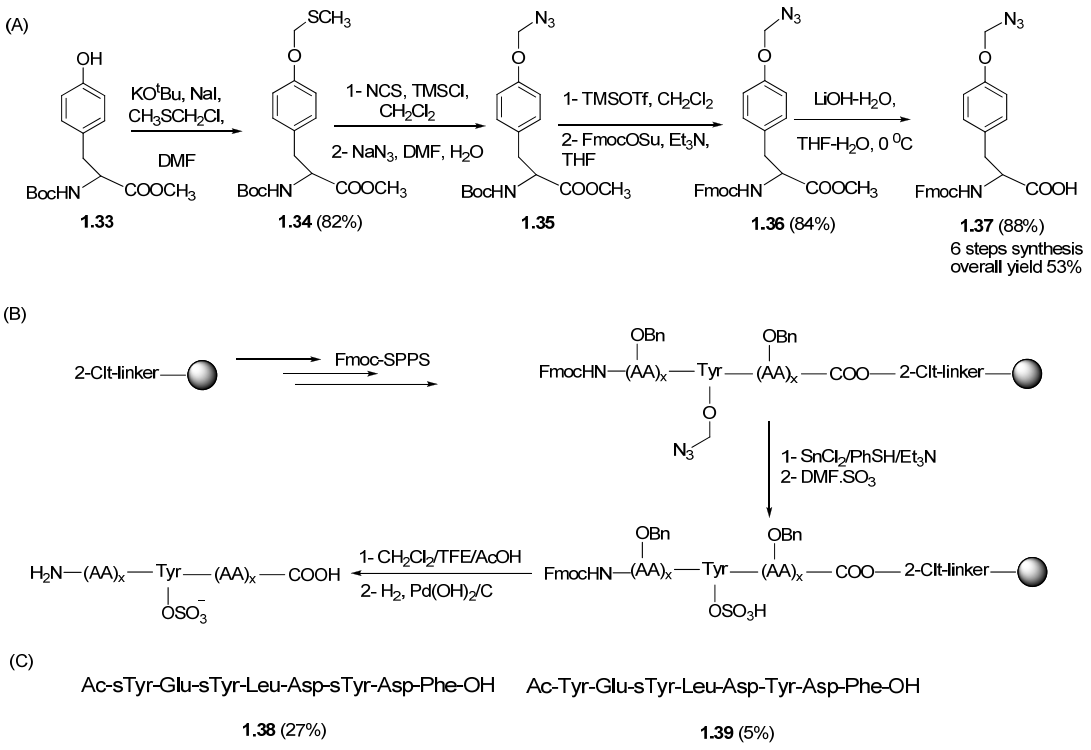
Another interesting example of the global sulfation approach that employs an unconventional side chain protecting group has been reported by Futaki *et al.* (Scheme 1.11). These workers used the acid stable *P*-(methylsulfinyl)benzyl (Msib) group to protect hydroxyl groups and *P*-(methylsulfinyl)benzyloxy carbonyl (Msz) to protect the amino groups.[89] These protecting groups allowed them to apply Fmoc SPPS using the usual TFA-labile protecting groups for the other amino acid side chains. After deprotection and cleavage of the peptide from the support under acidic conditions, sulfation was conducted using SO_3/DMF-ethanedithiol (EDT) which caused concomitant reduction of both Msib and Msz to acid labile Mtb and Mtz, respectively (**Scheme 1.11**).[89] Mtb and Mtz were subsequently removed under mild acidic conditions to yield the sulfated peptides. Unfortunately, the overall yield was only 7% and furthermore, this approach is generally time consuming and its applicability in the synthesis of larger peptides was not examined.[90]



Scheme 1.11. Synthesis of CCK-12 using Msib and Msz protecting groups

Young and Kiessling have used non acid-labile protecting groups and the global sulfation strategy to construct sTyr peptides.[91] The azidomethyl group (Azm) was used to protect the tyrosine residues that would ultimately be sulfated and benzyl protecting groups were used for the side chains of other amino acids (**Scheme 1.12A**). The Azm-protected tyrosine moiety was incorporated into the growing peptide chain using regular Fmoc-SPPS and using the highly acid labile chlorotrityl resin (**Scheme 1.12B**). Tin chloride was used to remove the Azm groups followed by on-resin sulfation. Cleavage of the peptide from the resin was achieved using trifluoroethanol (TFE)-acetic acid (AcOH) to yield the sulfated peptides and then the rest of the protecting groups on the side chain of the other amino acids were removed by hydrogenolysis. Unfortunately, the application of this method to the

synthesis of trisulfated octapeptide **1.38** which corresponds to residues 5-12 of PSGL-1 and its monosulfated analogue **1.39** proceeded in low yields of 27% and 5%, respectively (**Scheme 1.12C**). There are several drawbacks to this procedure such as the use of harsh conditions for Azm removal, the use of only hydrogenolytically-labile protecting groups on the other amino acid side chains and the synthesis of Fmoc-protected, Azm-protected tyrosine required a tedious multi-step synthesis and proceeded with somewhat low overall yield (**Scheme 1.12A**).



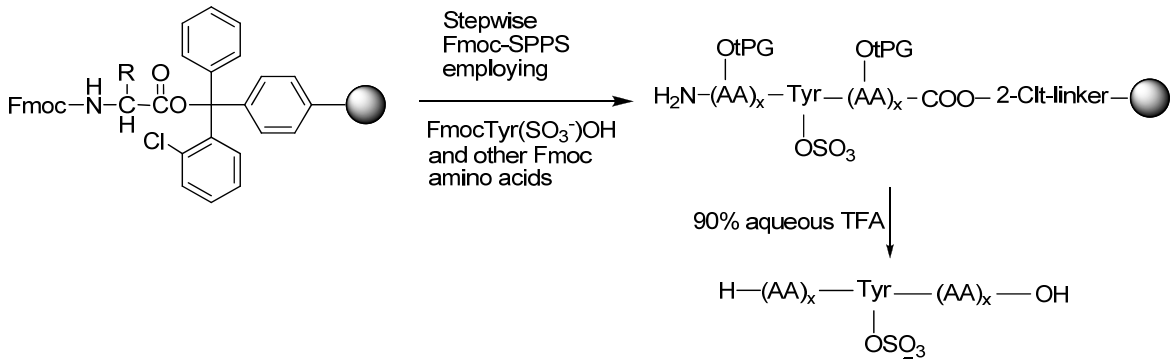
Scheme 1.12. Synthesis of residues 5-12 of PSGL-1 using the Azm-protecting group.

1.3.3 The sTyr building block strategy for the synthesis of sulfated peptides.

This strategy involves the incorporation of N^{α}-protected sulfotyrosine into the growing peptide chain. In principle, this procedure is more straightforward than global sulfation since it allows the chemoselective control of the sulfation sites and minimizes the deleterious effects of sulfating agents on other residues. However, the major obstacle th the application of this procedure is the intrinsic acid lability of the sulfate group. Although small sulfated peptides have been synthesized applying solution phase peptide synthesis utilizing Z-Tyr($SO_3Ba_{1/2}$)$OBa_{1/2}$[92] or Boc-Tyr($SO_3Ba_{1/2}$)$OBa_{1/2}$[93], these methods are not applicable to larger or more complex peptides. Concerning the SPPS of large complex peptides, the use of the Boc/Bn method is highly troublesome because the repeated acid treatments employed during peptide chain growth as well as the highly acidic conditions employed for the final cleavage lead to extensive sulfate hydrolysis. On the other hand, Fmoc-solid phase peptide synthesis is the most feasible technique because the basic conditions used to remove the Fmoc group are compatible with sulfotyrosine and the only step to be considered is the final treatment with TFA in order to deprotect all of the side chain protecting groups and cleave the peptide from the resin.

Kitagawa *et al.* used FmocTyr(SO_3Na)OH as a building block for Fmoc-SPPS to construct a number of challenging sulfated peptides.[90] Before performing their syntheses they conducted a kinetic study on the hydrolysis of the sulfate group in FmocTyr(SO_3Na)OH in TFA. They found that the formation and dissociation of the zwitterion (**Scheme 1.3**) is favored under acidic conditions and in nonpolar solvents. On the other hand, polar solvents such as TFA or TFE and salt bridging or ion pairing moieties with cationic functional groups

stabilize the zwitterionic intermediate. These effects are not translated to the transition state of the rate-determining desulfation step and so desulfation proceeds slower. Importantly, they also found that the rate of hydrolysis of the sulfate group was greatly suppressed at lower temperature and, at 0 ^{o}C, the rate of hydrolysis of *tert*-butyl protecting group of serine and pentamethyl-dihydrobenzofuran-5-sulfonyl (Pbf) protecting group of arginine occurs faster than the hydrolysis of the sulfoester. Therefore, TFA at 0 ^{o}C was selected to be the best conditions for the cleavage and protection of sulfated peptides. However, another problem that needed to be considered was the choice of the scavenger for the deprotection/cleavage cocktail. In a previous study, the same research group found that sulfur-containing scavengers such as ethanedithiol (EDT), thioanisole, or dimethylsulfide (DMS) were very detrimental to the sulfate group whereas other weaker scavengers such as water, *m*-cresol or 2-methylindole were much less harmful.[94] In this context, the use of 90-95% aqueous TFA at 0 ^{o}C was considered to be best for peptide cleavage from the resin and side chain deprotection. It has to be noted that the water concentration should be kept between 5-10% otherwise the rates of cleavage and deprotection are drastically affected. The general approach developed by Kitagawa using FmocTyr(SO_3Na)OH and other Fmoc-protected amino acids, employing the highly acid labile chlorotrityl resin and using 10% aq. TFA at 0 ^{o}C to remove the side chain protecting groups and to cleave the peptide from the support is shown in **Scheme 1.13**.[90] Using this approach Kitagawa and his coworkers were able to achieve the Fmoc-based SPPS of several challenging peptides (**1.40**-**1.46**) derived from CCK and human gastrin II (**Figure 1.12**).



Scheme 1.13. Kitagawa's approach to sTyr peptide synthesis.[90]

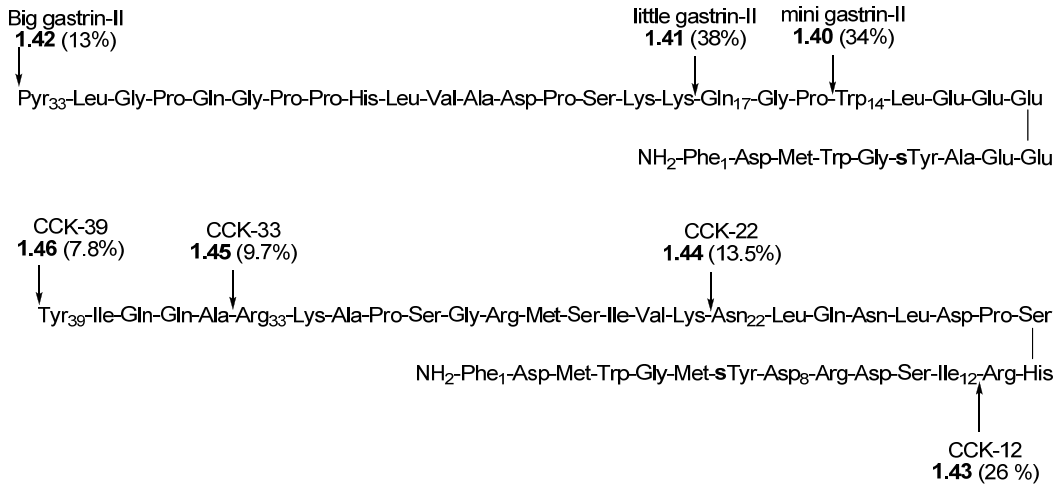


Figure 1.12. Peptides prepared by Kitagawa using the sTyr building block strategy.

The sTyr building block strategy developed by Kitagawa is currently the most widely used approach for sTyr peptide synthesis. It provides purer products with higher overall yields as compared to the global sulfation approach. The side reactions created by the action of the sulfating agents on the fully assembled peptide are avoided. However, this approach has its own drawbacks. The presence of the sTyr residue in this ionized state can render the

coupling of subsequent amino acids is difficult due to aggregation or poor swelling of the resin and so extended coupling times are sometimes required especially when more than one sTyr residue is present.[91] The use of aq. TFA can result in insufficient cleavage of the peptide from the resin and incomplete side-chain deprotection. On top of that, even under the optimized conditions, 10-15% desulfation usually occurs depending on the nature of the synthesized peptide.[90] Thus, deprotection conditions need to be optimized for every sulfated peptide.

1.3.4 Enzymatic synthesis of sulfated peptides

As mentioned in previously, sulfated peptides are synthesized *in vivo* by the action of TPST-1 and TPST-2.[7] These two isozymes are obtained by heterologous over-expression in eukaryotic cell lines and purified by affinity methods. Recently, TPST preparations were used by Seibert *et al.* to sulfate a 38 residue peptide corresponding to N-terminus of CXCR-4.[95] These studies were performed to determine the order of sulfation of CXCR-4 by these enzymes. In addition to TPSTs, arylsulfate sulfotransferase (ASST), which is a bacterial sulfotransferase obtained from *Eubacterium* A-44, has also been used to sulfate certain peptides *in vitro*. ASST uses *p*-nitrophenylsulfate as a cheap sulfate donor instead of expensive PAPS. However, the main obstacle toward the widespread application of ASST is that it does not respond to tyrosine residues adjacent to acidic residues, a property which puts most sulfated peptides out of its spectrum.[7] Although the use of enzymes for the synthesis of sTyr peptides holds considerable promise, their potential is yet to be fully developed.

1.4 Objective

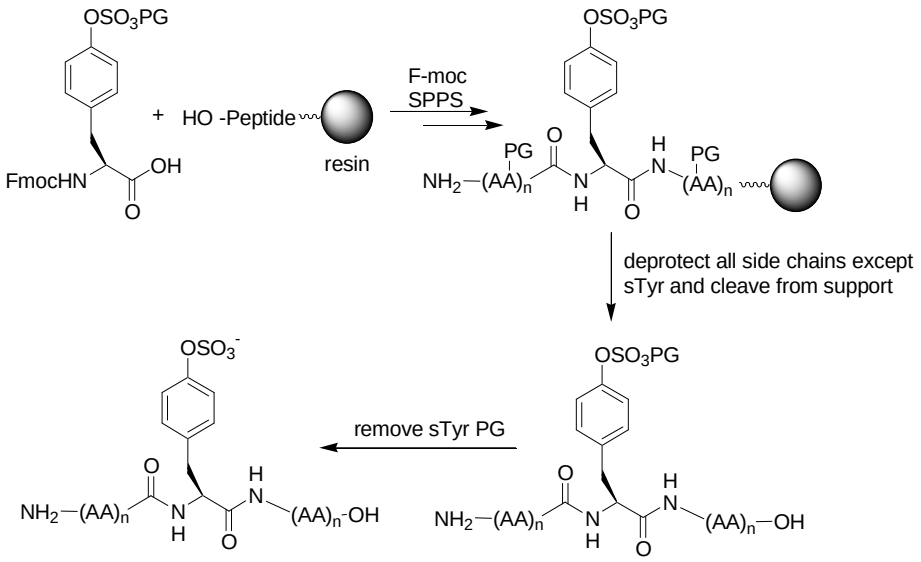
It is clear from the above discussion that no effective and general approach has been developed for the synthesis of sTyr peptides. The objective of the majority of the work presented in this thesis is to develop a general and effective method for preparing sTyr-bearing peptides.

Chapter 2

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

2.1 Introduction

It is clear from our discussion in chapter one on the synthesis of sTyr peptides that no general and effective approach has been developed for the synthesis of this class of peptides. Nevertheless, we envisioned a tactic that would eliminate all the aforementioned problems associated with the synthesis of sTyr peptides. This tactic relies on using conventional Fmoc-SPPS to incorporate the sTyr residue(s) at the beginning of the synthesis as a protected sulfodiester(s). After elongation of the peptide chain the peptide would be cleaved from the support and all other side chain protecting groups removed under the usual acidic conditions. The final step would involve removing the sulfate protecting group (**Scheme 2.1**). The success of this approach depends upon choosing a protecting group for the sulfate moiety that is able to withstand the peptide synthesis and cleavage conditions (stable to both acid and base) yet be removed under mild conditions leaving the sulfate group intact.

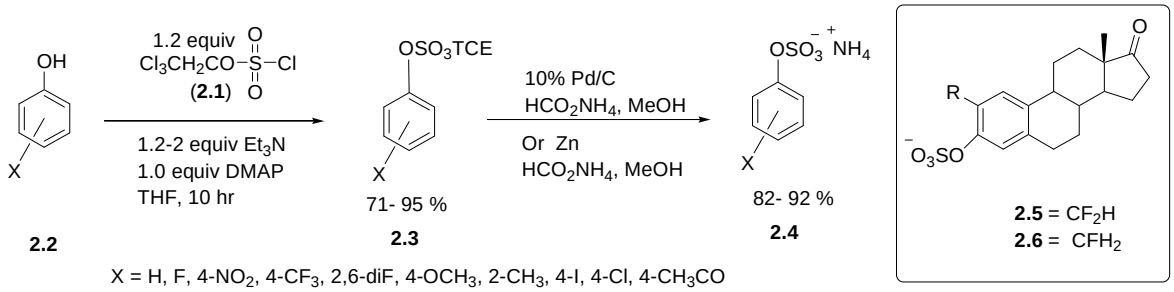


Scheme 2.1. Our proposed strategy for the synthesis of sTyr bearing peptides.

There are very few protecting groups for sulfate esters. Sulfates protected with groups such as methyl, ethyl, benzyl or t-butyl cannot be used since they are too labile to bases such as piperidine. The phenyl group has been used by Perlin and Penney to protect the sulfate group in sulfated carbohydrates.[96] However this group would clearly be inappropriate to protect the sulfate group in sTyr. Proud *et al.* introduced the 2,2,2-trifluoroethyl group (TFE) group as a protecting group for the sulfate group in sulfated carbohydrates.[97] However, its introduction required the use of trifluorodiazoethane, an unstable and dangerous reagent that is difficult to prepare, and we were concerned that sulfate esters protected with this group would be unstable to basic conditions encountered in peptide synthesis such as 20% piperidine in DMF.

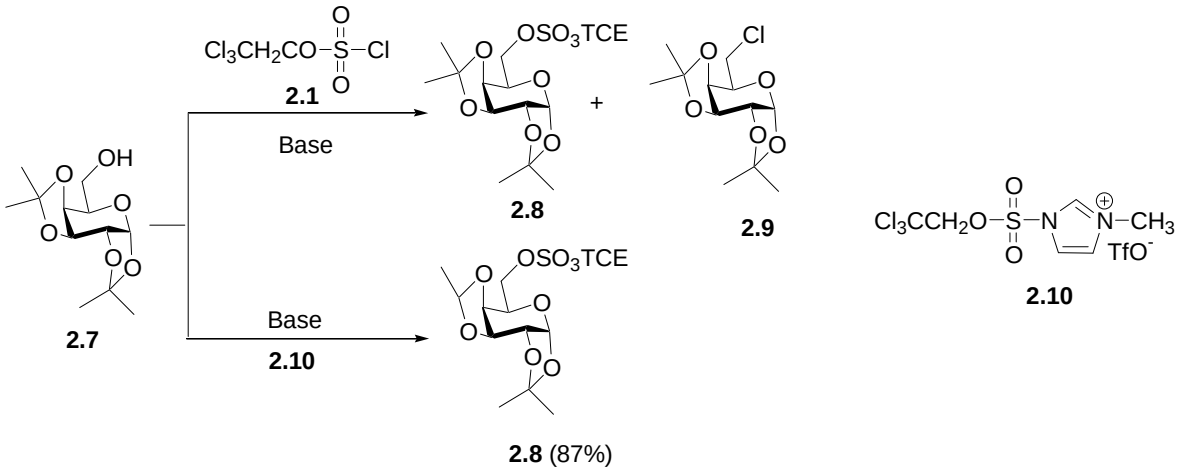
In 2004, the Taylor group introduced the 2,2,2-trichloroethyl (TCE) group as the first truly practical protecting group for sulfate esters.[98] TCE-protected sulfate esters (of type **2.3**) are prepared by subjecting phenol derivatives (of type **2.2**) to chlorosulfuric acid TCE ester

($Cl_3CCH_2OSO_2Cl$, **2.1**) in presence of a base. Deprotection is achieved under mild reducing condition utilizing catalytic Pd/C or Zn powder and excess ammonium formate to yield the desired aryl sulfates (of type **2.4**) in a high yields (**Scheme 2.2**). The presence of ammonium formate was found to be crucial in this step since it not only acted as a source of hydrogen but it also buffered the solution preventing it from becoming acidic (if just H_2 is used as the hydrogen source, the solution becomes acidic resulting in desulfation). This methodology was used to prepare estrone sulfate derivatives **2.5** and **2.6** which could not be obtained by previous sulfation methodologies (i.e. treatment of a phenol with a sulfating agent such as SO_3-pyridine).



Scheme 2.2. General scheme for using the TCE protecting group in the synthesis of aryl sulfates.

The Taylor group has also used the TCE group to protect the sulfate group in sulfated carbohydrates. However, reagent **2.1** was not very successful in introducing TCE sulfates into carbohydrates as illustrated for carbohydrate **2.7** in **Scheme 2.3**. These results prompted our group to design and prepare a unique sulfating agent, compound **2.10**, called a sulfuryl imidazolium salt (SIS) (**Scheme 2.3**).[99] Using this reagent carbohydrate **2.7** and many others could be readily sulfated in high yield.[100]



Scheme 2.3. SIS **2.10** as a sulfating agent in the preparation of TCE-protected carbohydrate sulfates.

Yong Liu, a former graduate student in the Taylor group, carried out some preliminary studies on the stability of TCE-protected aryl sulfates. He found that they are remarkably stable to acids such as TFA, TFA containing 5% of 30% HBr/AcOH, 30% HBr/AcOH, and 4 M HCl in dioxane over a period of 24 h at room temperature (rt). They are also stable to weak organic bases such as 10% Et_3N in CH_2Cl_2, 20% *N*-ethylmorpholine in CH_2Cl_2 for at least 24 h, 2 equiv of aqueous LiOH in THF at 0 °C for 30 min, and $NaBH_4$/MeOH. However, they are not stable to an excess of the organic bases that are commonly used to remove the Fmoc group during Fmoc SPPS such as 20% piperidine in DMF, 50% morpholine in DMF or excess DBU in DMF.[98] Thus, it appeared that no current sulfate protecting group was suitable for Fmoc SPPS.

2.2 Objectives

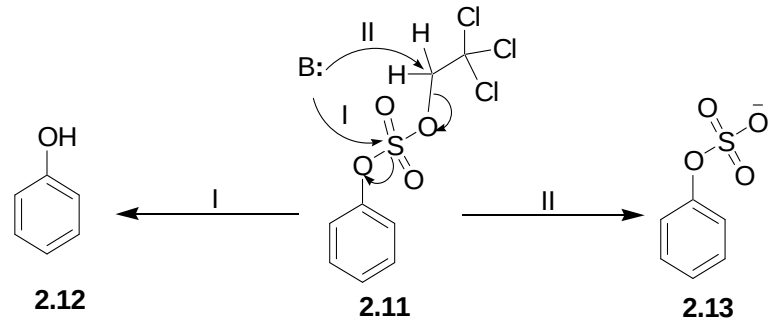
The objective of the work presented in this chapter is to develop a sulfate protecting group that has properties compatible with Fmoc SPPS and to use this protecting group to construct sTyr peptides using the strategy outline in **Scheme 2.1**.

2.3 Results and Discussion

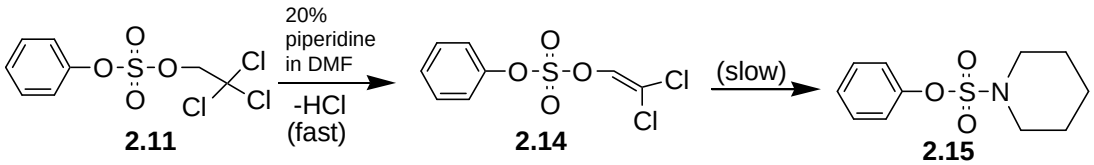
We reasoned that the design of a new sulfate protecting group that is compatible with Fmoc SPPS would be more readily achieved if we knew more about how such esters react with bases such as piperidine. We focussed our initial studies on TCE-protected sulfate esters since a detailed investigation into how TCE sulfate esters react with bases such as piperidine had not been performed.

2.3.1 Stability of TCE aryl sulfates to piperidine

Stability studies in 20% piperidine in DMF were performed using compound **2.11** as a model ester (**Scheme 2.4**). Initially, we assumed that the TCE-protected sulfate esters would react with piperidine by one of two different pathways. The piperidine would attack the sulfur atom resulting in loss of phenol (**2.12**) (the better leaving group based on pK_a's) (pathway I, **Scheme 2.4**). Alternatively, the nucleophile could attack the methylene carbon of the TCE group resulting in loss of the TCE group and the formation of phenyl sulfate (**2.13**). However, ^{1}H-NMR studies of compound **2.11** in 20% piperidine/DMF-d_7 revealed that relatively rapid elimination of HCl occurs first to give the *dichlorovinyl (DCV)* ester **2.14** (**Scheme 2.5**). The piperidinium hydrochloride formed in the reaction immediately precipitated out of solution and was isolated by filtration and identified by ^{1}H NMR. Compound **2.14** then underwent a slower reaction resulting in the formation of various byproducts such as compound **2.15**.



Scheme 2.4. Pathways of degradation of TCE phenyl sulfate in basic media.



Scheme 2.5. Decomposition of ester **2.11** in piperidine/DMF

Although the TCE group was clearly unstable to the usual bases that are used in Fmoc SPPS, it was reasoned that if a base could be found that would not attack the sulfur atom of a *dichlorovinyl (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 sTyr peptides. It was reasoned further that such a base would have to be more sterically encumbered than piperidine yet have a basicity that was similar to piperidine. With a more sterically hindered base the elimination of HCl from the TCE group would probably proceed at a rate similar to piperidine since the steric requirement for elimination reactions involving proton removal tend to be rather small while the second step of the reaction, attack on sulfur, would proceed more slowly compared to piperidine since the steric requirement for substitution reactions in general tend to be fairly large. Recently, Hachmann and Lebl

reported that Fmoc deprotection of Fmoc-Ile attached to chlorotrityl resin using readily available 2-methylpiperidine (2-MP) occurred with a half-life that was only 1.5 times greater than that of piperidine.[101] This lead to efforts to determine if DCV-protected sulfate esters are stable to 2-MP.

2.3.2 Stability studies on dichlorovinyl ester 2.14

In 20% 2-MP/DMF-d_7, compound **2.11** still underwent elimination to give DCV-protected compound **2.14** (accompanied by the precipitation of the hydrochloride salt of 2-MP) as determined by ^{1}H-NMR. However, we were pleased to find that no further decomposition of **2.14** occurred even after several days at rt. We were even able to isolate compound **2.14** from the reaction mixture and unequivocally identify it by NMR and mass spectrometry. We subjected the isolated **2.14** to 20% 2-MP/DMF-d_7, and, as expected, it exhibited little or no decomposition even after 5 days at rt. In 20% piperidine/DMF-d_7 it began to show significant decomposition after about 4 h.

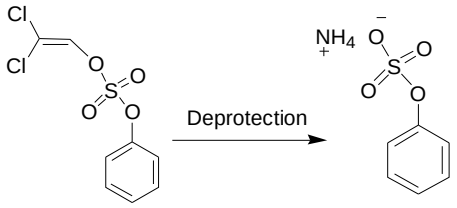
With compound **2.14** in hand we were also able to evaluate its stability in acid. We were pleased to find that compound **2.14** was stable to 98%TFA/2% triisopropylsilane (TIPS or TIS) for 6 h, conditions that have been used to remove peptides from resins and to deprotect the side chains in Fmoc-based SPPS.

2.3.3 Removal of the DCV group.

If the DCV group is to be used for SPPS, it was necessary to find a very mild method for its removal. We examined the reducing conditions that we developed to remove the TCE group; however these were not very effective (**Table 2.1**, entries 1 and 2). Using palladium

black instead of Pd/C we were able to effect some loss of the DCV group but the reaction was slow (entry 3). However, we found that using a combination of H_2 and ammonium formate as the hydrogen sources the DCV group could be removed in good yield using either Pd/C or Pd^0 (entries 4 and 5).

Table 2.1. Removal of the DCV from 2.14.



2.14 **2.13**

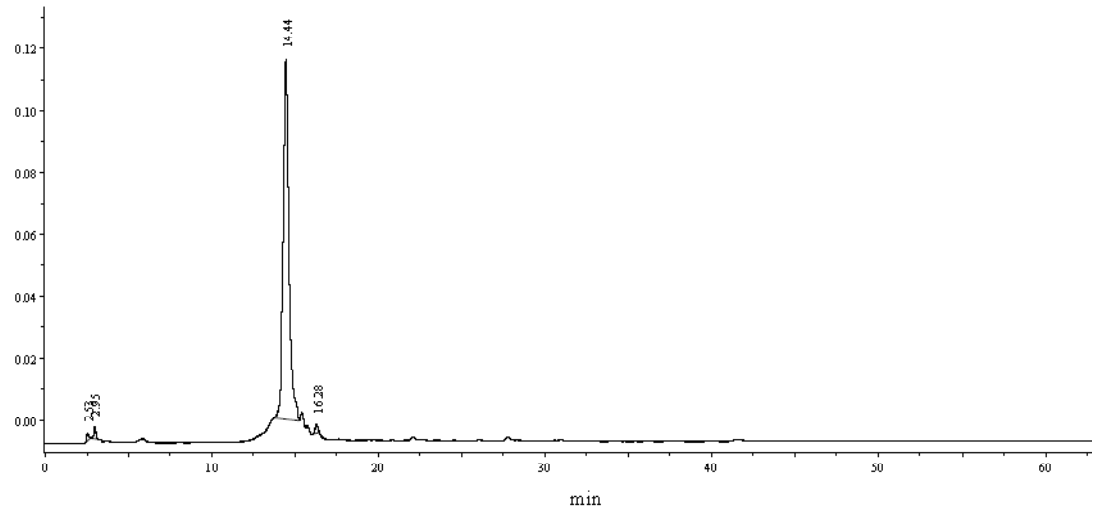
% Yield **2.13**	Equiv Zn or wt. % cat./equiv HCO_2NH_4	Reagents	Entry
starting material	6/6, 24 h then 6/6, 24 h	Zn/HCO_2NH_4[a]	1
starting material	10/6, 24 h then 10/6, 24 h	10 % $Pd/C/HCO_2NH_4$	2
Incomplete reaction	5/6, 24 h then 20/6, 24 h	Pd^0/ and 10 % Pd/C/ HCO_2NH_4	3
82%	20/6, 24 h	Pd^0/HCO_2NH_4, H_2 (1 atm)	4
85%	30/6, 24 h	10 % Pd/C /HCO_2NH_4, H_2 (1 atm)	5

2.3.4 2-MP as a base for Fmoc SPPS

Although the DCV group looked promising, it appeared that it would only be possible to use the DCV group to protect the sulfate group of sTyr during SPPS if 2-MP could be used in place of piperidine during SPPS. To determine if this was the case we compared 2-MP to piperidine in SPPS by preparing a simple model hexapeptide $DADEYLNH_2$ (**2.16**) using

both bases. Hexapeptide **2.16** corresponds to a sequence within the epidermal growth factor receptor. It was chosen as a model peptide since we expected that sulfation of the tyrosine residue would yield a peptide that would be a good inhibitor of protein tyrosine phosphatase 1B (PTP1B).[102] PTP1B is involved in the down regulation of insulin signaling and inhibitors of PTP1B are being examined as drugs for treating type II diabetes. The synthesis was conducted manually using Rink amide resin (this resin provides the final peptide product with a carboxamide terminus) and employing 4 equiv HBTU/HOBT as coupling agents in presence of diisopropylethylamine (DIPEA) and 1.5 h coupling times. After the coupling of each Fmoc amino acid the peptide was subjected to 3 x 10 min of 20% 2-MP/DMF as opposed to the standard 2 x 10 minutes protocol 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 (**Figures 2.1 A** and **B**) consisted of mainly a large single peak which corresponded to the desired product (confirmed by ESMS). However, in case of using 2-MP the crude appeared to be cleaner (**Figures 2.1 A** vs **B)**, which suggested to us that 2-MP could be used in place of piperidine for Fmoc-based SPPS.

(A)



(B)

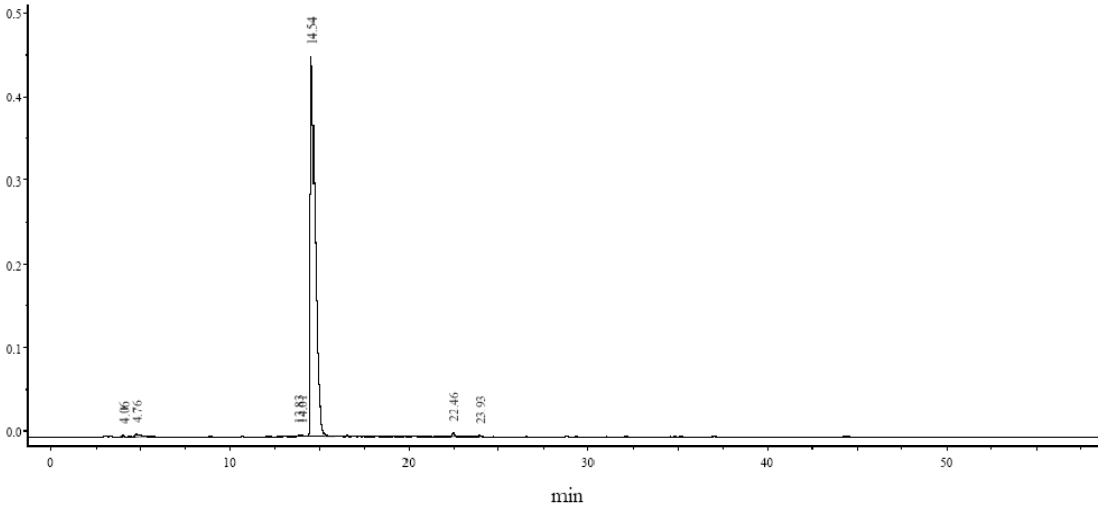
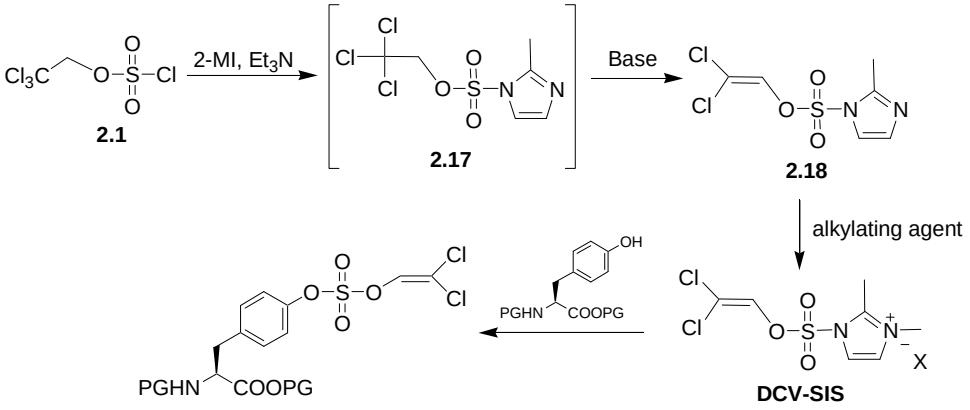


Figure 2.1. Analytical HPLC chromatograms of the DADEYLNH$_2$ peptide (**2.16**) obtained using piperidine (A) and 2-MP (B) for Fmoc removal

2.3.5 Synthesis of DCV esters of aryl sulfates

The next step was to devise a method for introducing the DCV sulfate ester group into an appropriately protected tyrosine derivative. We envisioned that the best way to prepare DCV sulfates was to design a new SIS capable of introducing the sulfate group in its DCV-protected state. The general approach is outlined in **Scheme 2.6.** $TCEOSO_2Cl$ (**2.1**) would be reacted with 2-methylimidazole (2-MI) in the presence of Et_3N to give compound **2.17**. **2.17** would be reacted in situ with an additional base to effect elimination of HCl and give the corresponding DCV sulfurylimidazole (**2.18**). Treatment of **2.18** with methyl triflate or trimethyloxonium tetrafluoroborate should give the desired DCV sulfuryl imidazolium salt (DCV-SIS). Reaction of the DCV-SIS with an N^{α}-, C^{α}-protected tyrosine derivative would give the desired DCV-protected tyrosine derivative.

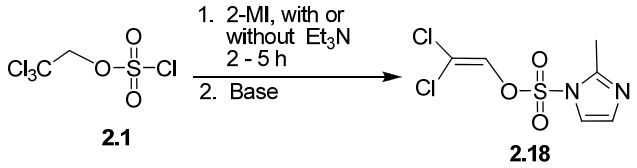


Scheme 2.6. Proposed scheme for the preparation of DCV-protected sTyr.

Compound **2.17** was prepared by reacting **2.1** with one equiv 2-MI and 2 equiv Et_3N in CH_2Cl_2, a procedure adapted from an unpublished procedure developed by Ahmed Desoky in the Taylor group. After 5 hours of stirring at room temperature, 20 equivalents of

piperidine were added (almost the same number of equiv used in the initial stability studies discussed above) and the reaction was allowed to stir overnight. After an aqueous work up using 0.1 N HCl and purification **2.18** was obtained but in poor yield (Table 2.2, entry 1). Attempts to increase the yield by increasing the number of equivalents of piperidine or running the reaction at higher temperature resulted in an increase in yield but the yields were still moderate (entries 2 and 3). It was suspected that the low yields may have been due to the aqueous workup. Thus the solvent was changed to THF which resulted in the rapid precipitation of the piperidinium hydrochloride which could be removed by filtration. The filtrate was concentrated *in vacuo* and the residue applied to a silica gel column. However, again the yield was moderate (entry 4). Decreasing the equivalents of piperidine and increasing the reaction time or changing the base to DIPEA or morpholine still resulted in low yields (entries 5-10). Fortunately, an appreciable increase in the yield was obtained using the stronger base DBU (entries 11-13). Finally, it was possible to obtain **2.18** in an 88% yield by increasing the number of equivalents of 2-MI to 3.6 and using 1.5 equivalents of DBU as the only base (besides 2-MI) and performing an aqueous. workup using phosphate buffer (entry 14).

Table 2.2. Synthesis of compound 2.18.

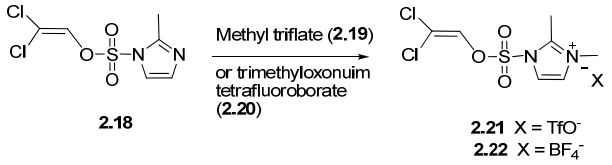


2.1 **2.18**

	Equiv 2-MI	Equiv Et_3N	Base (equiv)	Reaction conditions	% yield **2.18**
1	1	2	Piperidine (20)	CH_2Cl_2, rt, O/N, aq.work up	20
2	1	2	Piperidine (25)	CH_2Cl_2, rt, O/N, aq. work up	43
3	1	2	Piperidine (20)	CH_2Cl_2, reflux, 5 h, aq. work up	50
4	1	2	Piperidine (20)	THF, rt, O/N, no work up	55
5	1	2	Piperidine (15)	THF, rt, O/N, no work up	40
6	1	2	Piperidine (15)	THF, rt, 48 h , no work up	47
7	1	2	Piperidine, (10)	THF, rt, 4 days, no work up	46
8	1	2	Piperidine (5)	THF, rt, 4 days , no work up	48
9	1	2	Morpholine (20)	THF, rt, 7 days, no work up	20
10	1	2	DIPEA (5)	THF, rt, 7 days, no work up	10
11	1	2	DBU (1)	THF, rt, O/N, no work up	60
12	1	2	DBU (2.5)	THF, rt, O/N, aq. work up	69
13	1	0	DBU (2)	THF, rt, O/N, no work up	60
14	3.6	0	DBU (1.5)	THF, rt, 3 h, aq. work up	88

Attention was then turned to the formation of the SIS. SISs are too unstable to be chromatographed. Thus it was necessary to develop conditions that would result in the precipitation of the SIS in pure form. The reaction of **2.18** with methyl triflate (**2.19**) in different solvents gave a semisolid composed of a mixture of starting materials and product (**2.21**) and it was not possible to isolate **2.21** in pure form by precipitation (**Table 2.3**, entries 1-3). However, upon changing to trimethyloxonium tetrafluoroborate (**2.20**) it was possible to obtain the tetrafluoroborate salt **2.22** in 83% yield (entry 4). This was achieved by performing the reaction in CH_2Cl_2 for 5 h and then removing the solvent by rotary evaporation. Trituration of the **2.22** using hot THF followed by filtration gave pure **2.22**. Attempts to increase the yield even more by changing the solvent failed to give better results; indeed it appears that DCM is the optimum solvent for this reaction. Finally, it was possible to obtain SIS **2.22** in 95% yield by letting the reaction proceed for 16 h followed by the workup described above (entry 7).

Table 2.3. Synthesis of sulfating agent **2.22.**



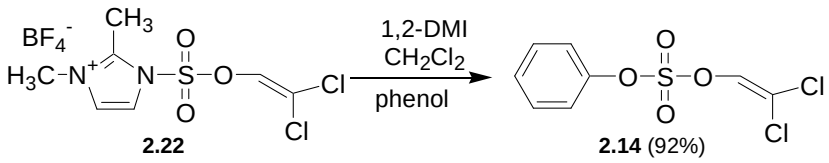
2.18

2.21 X = TfO^-
2.22 X = BF_4^-

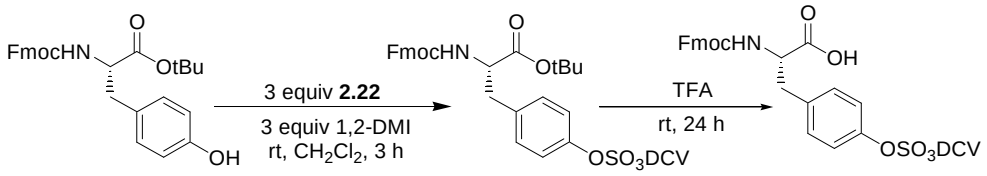
Entry	Alkylating agent	Solvent	Reaction conditions	% Yield **2.21** or **2.22**
1	**2.19**	Ether	- 20-0 ^oC, rt, 5 h	ND[a]
2	**2.19**	CH_2Cl_2	0 ^oC, 5 h	ND[a]
3	**2.19**	THF	0 ^oC, 5 h	ND[a]
4	**2.20**	CH_2Cl_2	rt, 5 h	83
5	**2.20**	THF	rt, 5 h	ND[a]
6	**2.20**	CH_3CN	rt, 5 h	ND[a]
7	**2.20**	CH_2Cl_2	rt, O/N	95

[a]ND = Not determined. Mixture of starting material and product as semisolids.

Reagent **2.22** was tested as a sulfating reagent by reacting it (3.0 equiv) with phenol in the presence 3.0 equiv of 1,2-dimethylimidazole (1,2-DMI) in methylene chloride. This gave ester **2.14** in 92% yield (**Scheme 2.7**). Encouraged by these results we then turned our attention to preparing FmocTyr(SO_3DCV)OH (**2.23**). To prepare **2.23**, FmocTyrO^{t}Bu (**2.24**)[103] was reacted with 3 equiv of **2.22** in the presence of 1,2-DMI to give DCV ester **2.25** in 94% yield (**Scheme 2.8**). After the removal of the *tert*-butyl group using TFA the desired compound **2.23** was obtained in 92% yield (an overall yield of 86%, **Scheme 2.8**).

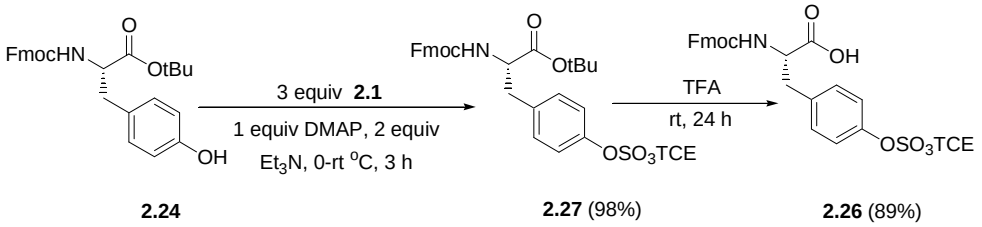


Scheme 2.7. Synthesis of DCV phenyl sulfate **2.14** using reagent **2.22**.



Scheme 2.8. Synthesis of DCV Fmoc-tyrosine sulfate building block **2.23** using reagent **2.22**.

Since the TCE group breaks down to give the more stable DCV group in 20% 2-MP/DMF, we also prepared TCE-protected amino acid **2.26** (**Scheme 2.9**). Thus, reaction of **2.24** with 3.0 equiv of **2.1** in presence of 2.0 equiv Et_3N and 1 equiv DMAP gave amino acid **2.27** in 98% yield. Removal of the *tert*-butyl group in **2.27** using TFA gave **2.26** in 87% overall yield.



Scheme 2.9. Synthesis of TCE Fmoc-tyrosine sulfate building block **2.26**.

2.3.6 Determination of the enantiomeric purity of 2.23 and 2.26

The enantiopurities of **2.23** and **2.26** were determined by comparing the HPLC chromatograms and ^{1}H-NMR's of dipeptides Ac-Y(SO_3DCV)-$A_{(L)}$-NH_2 (**2.28LL**) and Ac-Y(SO_3TCE)-$A_{(L)}$-NH_2 (**2.29LL**) to that of dipeptides Ac-Y(SO_3DCV)-$A_{(DL)}$-NH_2 (**2.28LD**/**2.28LL**) and Ac-Y(SO_3TCE)-$A_{(DL)}$-NH_2 (**2.29LD**/**29LL**). These dipeptides were prepared using the same protocol described for the preparation of hexapeptide **2.16** using 2-MP for Fmoc removal.

As expected, the diastereomeric dipeptide mixture Ac-Y(SO_3DCV)-$A_{(DL)}$-NH_2 showed two peaks in the HPLC trace and two sets of peaks in the ^{1}H-NMR spectrum corresponding to peptides **2.28LD** and **2.28LL** (**Figure 2.2** and **2.3**). Peptide Ac-Y(SO_3DCV)-$A_{(L)}$-NH_2 showed almost one peak in the HPLC trace and one set of peaks in the ^{1}H-NMR spectrum corresponding to peptide **2.28LL** (**Figures 2.4** and **2.5**). Based on peak integration, the amino acid **2.23** was obtained in > 98% ee.

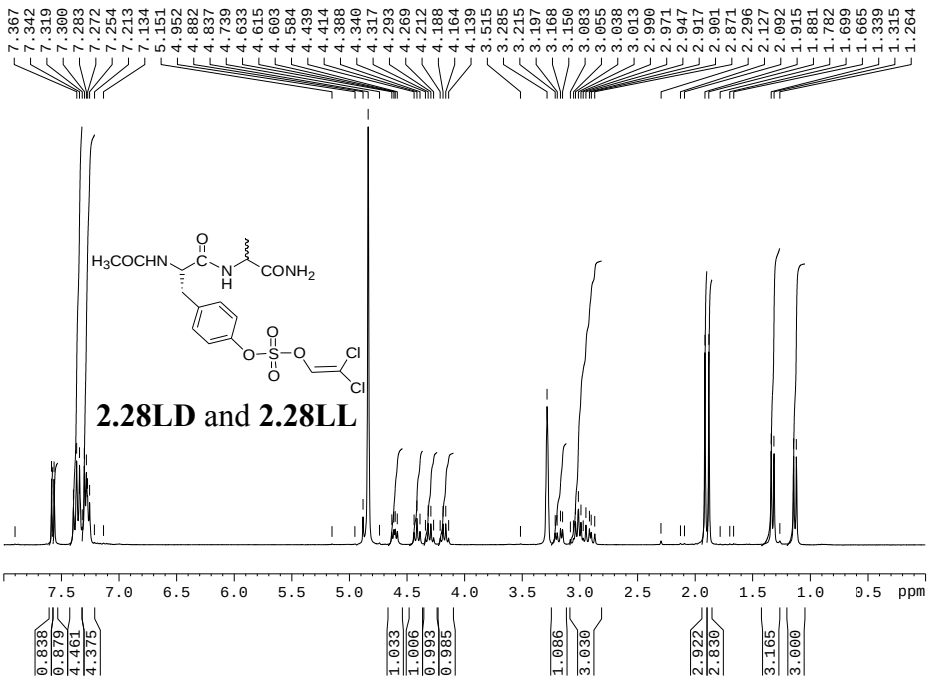


Figure 2.2. 300 MHz ^{1}H-NMR (CD_3OD) spectrum of diastereomeric dipeptides Ac-Y(SO_3DCV)-$A_{(DL)}$-NH_2 (**2.28LD** and **2.28 LL**).

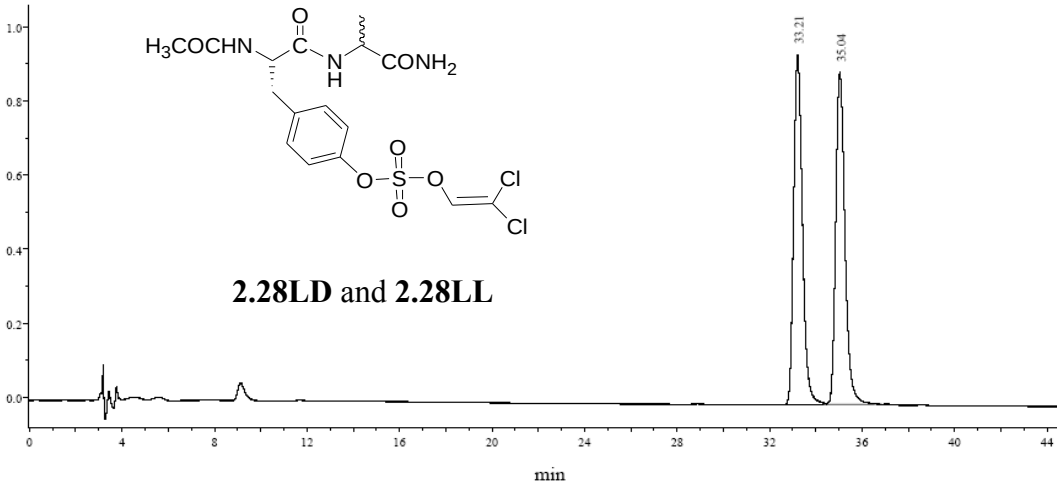


Figure 2.3. Analytical HPLC chromatogram of diastereomeric dipeptides Ac-Y(SO_3DCV)-$A_{(DL)}$-NH_2 (**2.28LD** and **2.28 LL**).

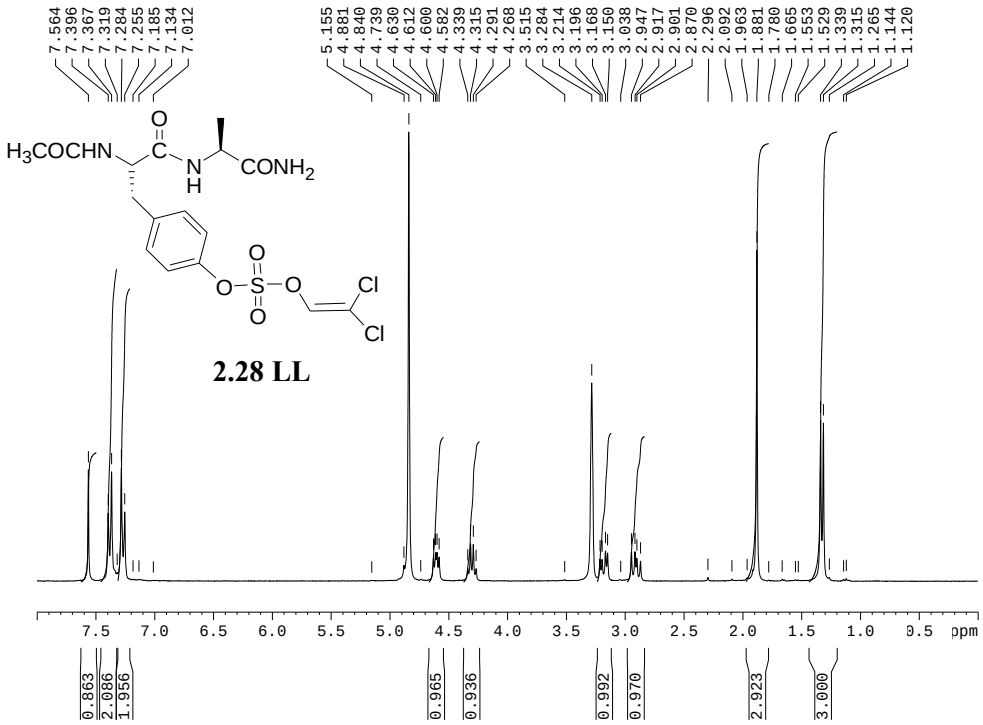


Figure 2.4. 300 MHz ^{1}H-NMR (CD_3OD) spectrum of dipeptide Ac-Y(SO_3DCV)-$A_{(L)}$-NH_2 (**2.28 LL**).

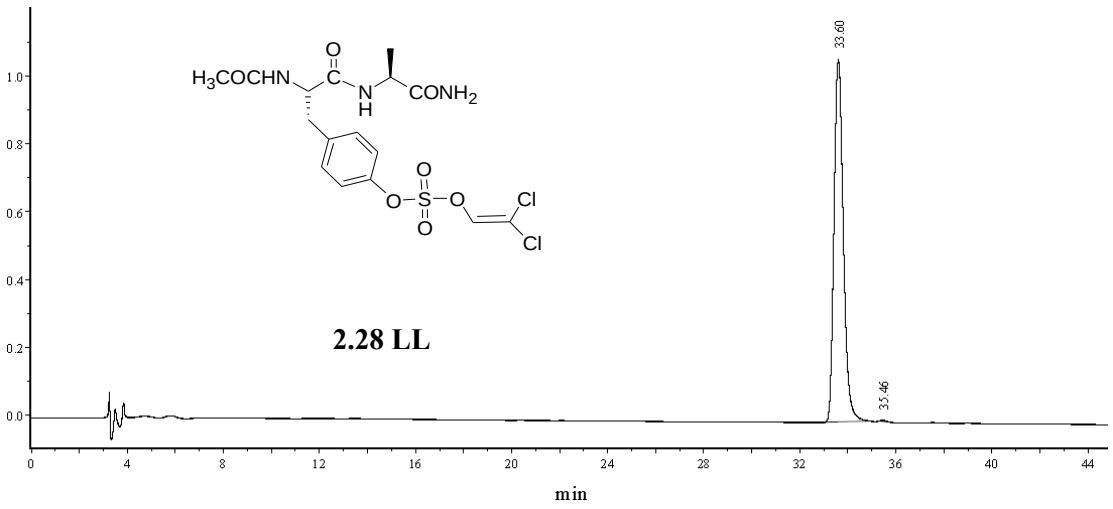


Figure 2.5. **A**nalytical HPLC chromatogram of dipeptide Ac-Y(SO_3DCV)-$A_{(L)}$-NH_2 (**2.28LL**).

Ac-Y(SO_3TCE)-$A_{(LD)}$-NH_2 showed two sets of peaks in the ^{1}H-NMR corresponding to diastereomeric dipeptides **2.29LD** and **2.29LL** (**Figure 2.6**). Small amounts of the diastereomeric dipeptides **2.28LD** and **2.28LL** were also evident (as indicated by two singlets at 7.56 and 7.58 ppm) which were formed by the action of 2-MP on the TCE protecting group during peptide synthesis. Ac-Y(SO_3TCE)-$A_{(DL)}$-NH_2 showed four peaks in its HPLC chromatogram (**Figure 2.7**). Two of these (the larger set) correspond to (**2.29LD** and **2.29LL**). The two extra smaller set of peaks correspond to **2.28LD** and **2.28LL**. The amount of **2.28LD** and **2.28LL** present in the HPLC chromatogram appears to be considerably greater than that indicated by NMR. For HPLC analysis, the detector was set to 220 nm and this *apparent* discrepancy is due to the greater absorbance of the DCV group at 220 nm in comparison to the TCE group.

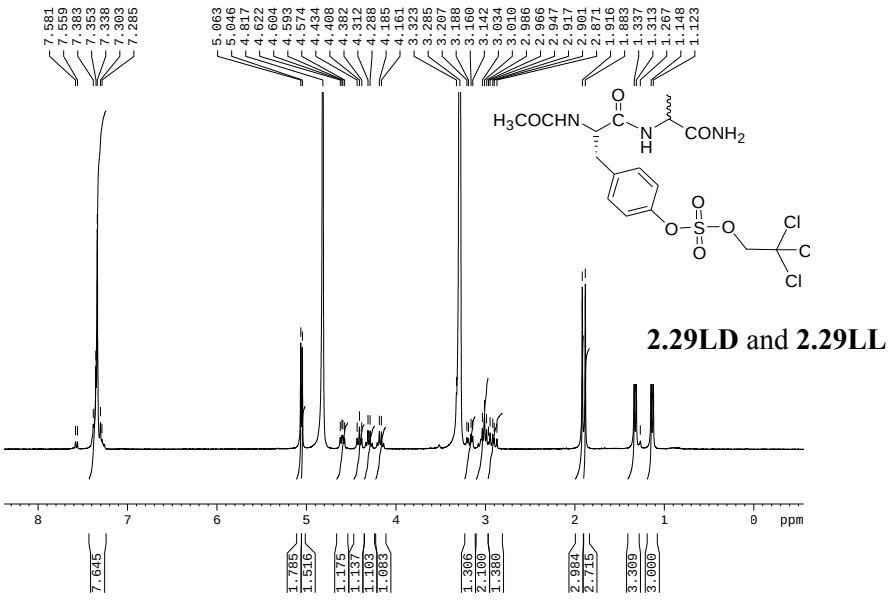


Figure 2.6. 300 MHz ^{1}H-NMR (CD_3OD) spectrum of diastereomeric dipeptide Ac-Y(SO_3TCE)-$A_{(LD)}$-NH_2 (**2.29LD and 2.29LL**). Note that a small quantity of diastereomeric dipeptides **2.28LD** and **2.28LL** (as indicated by the two singlets at 7.56 and 7.58 ppm) is also present which was formed during peptide synthesis.

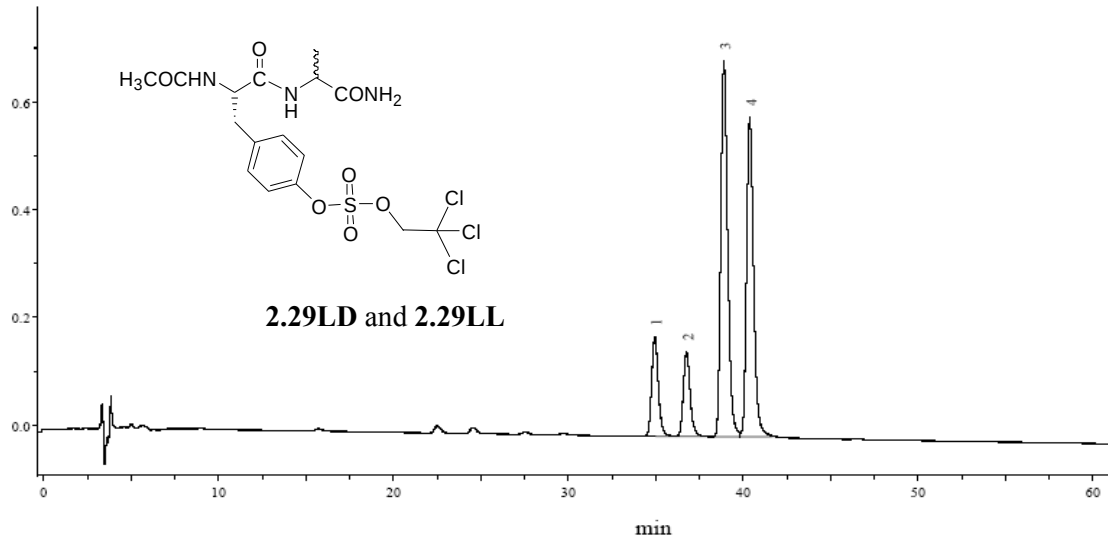


Figure 2.7. Analytical RP-HPLC chromatogram (λ = 220 nm) of diastereomeric dipeptide Ac-Y(SO_3TCE)-$A_{(LD)}$-NH_2 (**2.29LD and 2.29LL**, t_R= 38.9 and 40.4 min). Note the presence of diastereomeric dipeptides **2.28LD** and **2.28LL** formed during peptide synthesis (t_R= 34.9 and 36.8 min). In comparison with the ^{1}H-NMR of this mixture (**Figure 2.6**) the amount of **2.28LD** and **2.28LL** present appears to be considerably greater. This is due to the greater absorbance of the DCV group at 220 nm in comparison to the TCE group.

Peptide Ac-Y(SO_3TCE)-$A_{(L)}$-NH_2 showed peaks in the ^{1}H-NMR corresponding to peptide **2.29LL**. **2.28LL** was also present as indicated by the small peak at 7.56 ppm. This was again due to the action of 2-MP on the TCE group (**Figure 2.8**). No peaks corresponding to **2.29DL** or **2.28DL** were evident. The HPLC chromatogram showed two peaks, one corresponding to **2.29LL** and the other corresponding to **2.28LL** (**Figure 2.9**). No peaks corresponding to **2.29DL** or **2.28DL** were evident. These results indicate that amino acid **2.26** was obtained in > 98% ee.

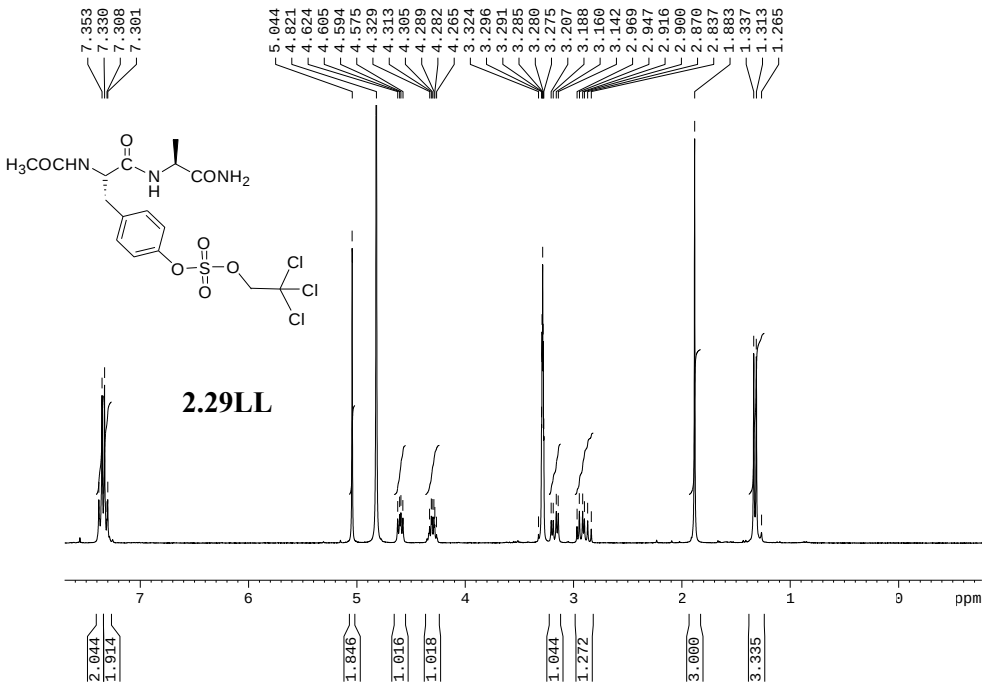


Figure 2.8. 300 MHz ^{1}H-NMR (CD_3OD) spectrum of dipeptide dipeptide Ac-Y(SO_3TCE)-$A_{(L)}$-NH_2 (**2.29LL**). A small quantity of dipeptide **2.28LL** is also present (as indicated by the small singlet at 7.56 ppm) which was formed during peptide synthesis.

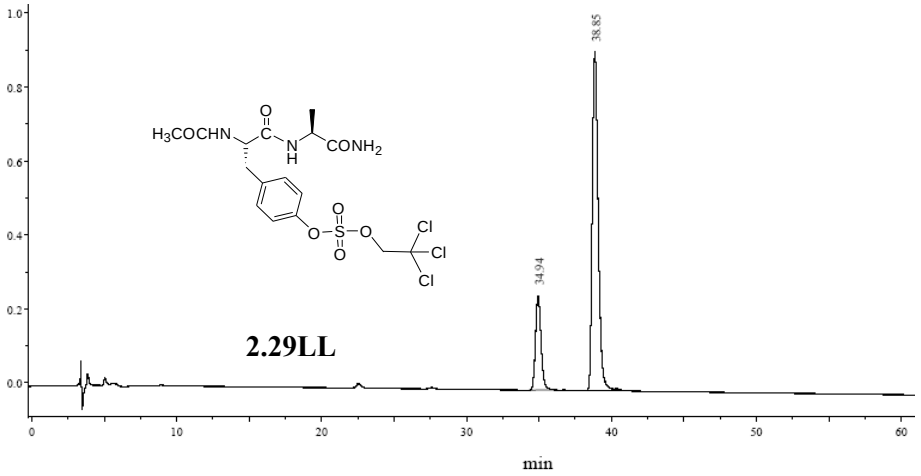


Figure 2.9. Analytical RP-HPLC chromatogram (λ = 220 nm) of dipeptide Ac-Y(SO_3TCE)-$A_{(L)}$-NH_2 (**2.29LL**, t_R = 38.9 min). Note the presence of dipeptide **2.28LL** (t_R = 34.9 min) that was formed during peptide synthesis. In comparison with the NMR of this mixture (**Figure 2.8**) the amount of **2.28LL** present appears to be considerably greater. This is due to the greater absorbance of the DCV group at 220 nm in comparison to the TCE group.

2.3.7 Synthesis of model sulfated compound DADEsYLNH$_2$ (2.30)

Both the DCV and TCE-protected amino acids **2.23** and **2.26** were examined as building blocks in the synthesis of a simple model monosulfated compound DADE**sY**LNH$_2$ **2.30** using 2-MP for Fmoc removal. Manual SPPS was performed on a Rink amide resin and using the same conditions described above for the synthesis of the DADEYLNH$_2$ (**2.16**) peptide. When TCE-protected building block **2.26** was used the hydrochloride salt of 2-MP, which was produced during each Fmoc removal, formed a precipitate although most of this precipitate was removed by treatment with CH_2Cl_2 after each Fmoc deprotection. Using DCV-protected amino acid **2.23**, the HPLC chromatogram of the crude peptide before DCV removal showed mainly a single peak corresponding to DADEY(SO_3DCV)LNH$_2$ (**2.31**, **Figure 2.10**) as confirmed by mass spectrometry analysis. Using TCE-protected amino acid **2.26**, the HPLC chromatogram of the crude peptide before hydrogenolysis showed mainly two major peaks in an *apparent* 1:1 ratio corresponding to DADEY(SO_3DCV)LNH$_2$ (**2.31**) and DADEY(SO_3TCE)LNH$_2$ (**2.32**) (**Figure 2.11**) as confirmed by mass spectrometric analysis. No peaks corresponding to the desulfated peptide or 2-MP-sulfonamide peptide (from attack of 2-MP on the sulfur of the DCV-protected sulfate group) were detected in either case. 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 equiv of ammonium formate in MeOH at rt for 1-3 h. The HPLC chromatogram of the crude peptides consisted of mainly a single peak which corresponded to peptide **2.30** and again no peak corresponding to the desulfated peptide was detected as confirmed by the coinjection with its unsulfated analogue **2.16** (**Figures 2.12** and **2.13**).

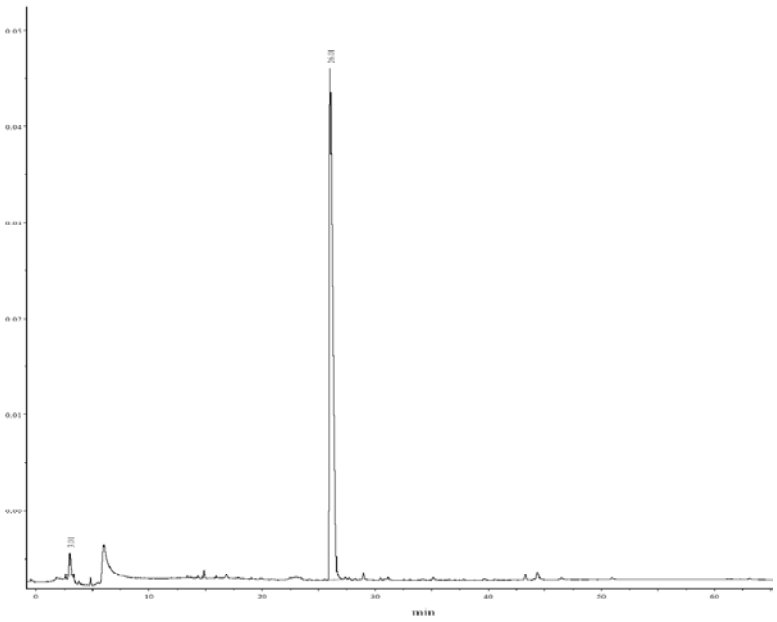


Figure 2.10. Analytical HPLC chromatogram of the crude DADEY(SO_3DCV)LNH_2 (**2.31**) obtained using amino acid **2.23**.

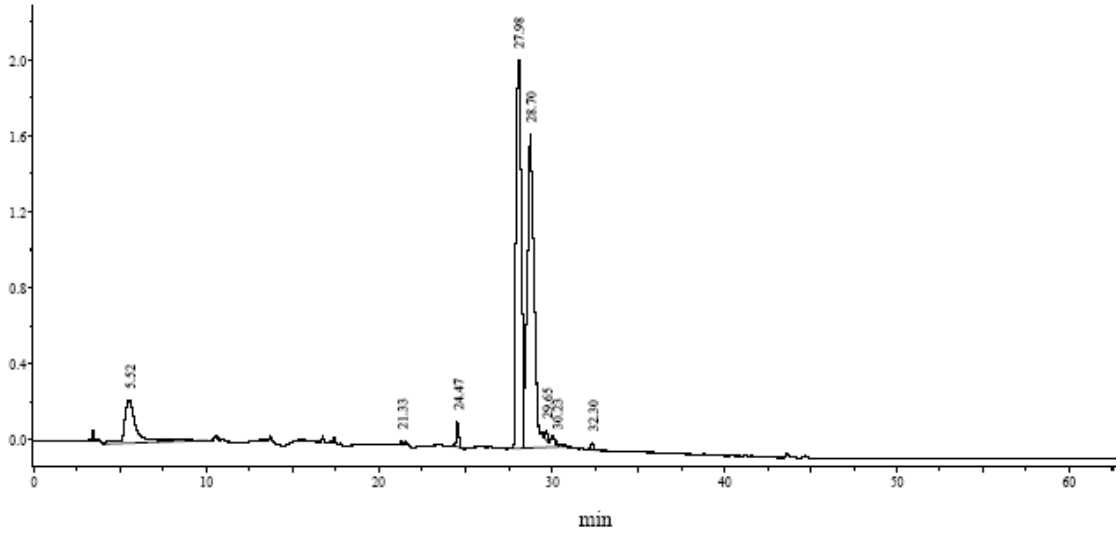


Figure 2.11. Analytical HPLC chromatogram of the crude hexapeptides DADEY(SO_3DCV)LNH_2 (**2.31**) and DADEY(SO_3TCE)LNH_2 (**2.32**) obtained using amino acid **2.26**.

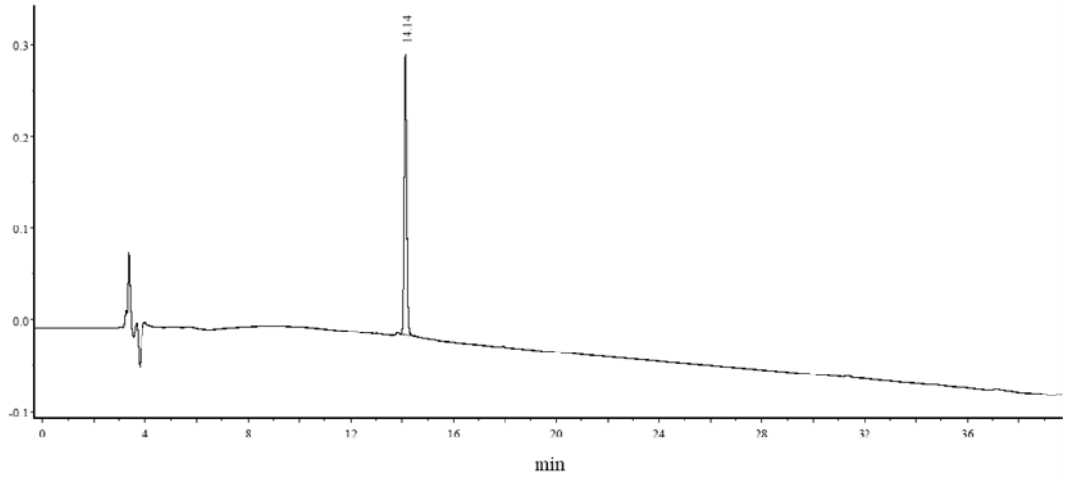


Figure 2.12. Analytical HPLC chromatogram of the hexapeptide DADEsYLNH$_2$ (**2.30**) obtained using amino acid **2.23** as building block.

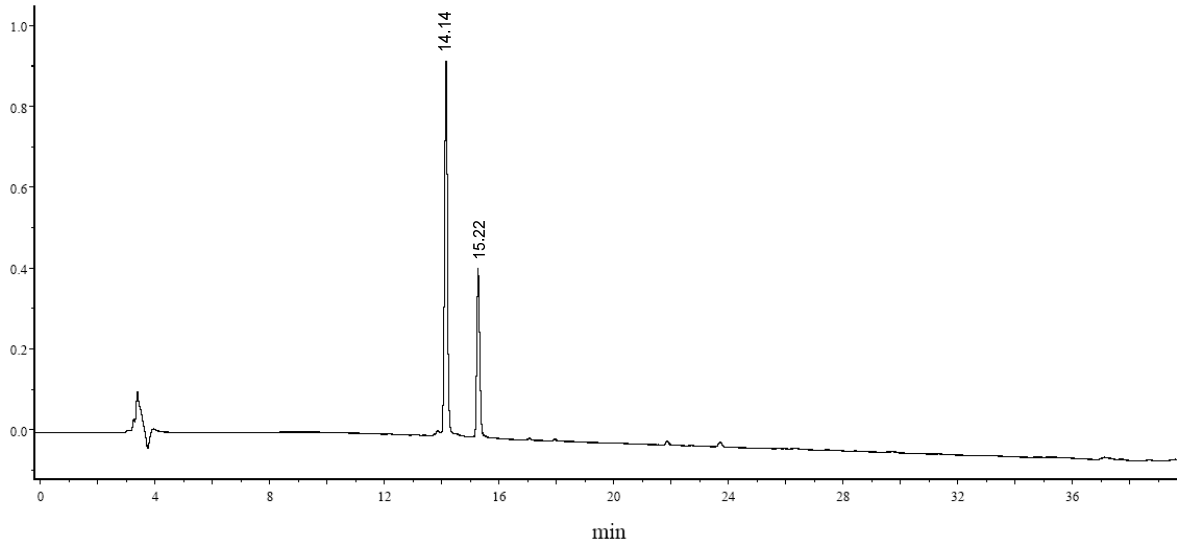


Figure 2.13. Analytical HPLC chromatogram of crude DADEsYLNH$_2$ (**2.30**) (t_R= 14.1 min) **SPIKED** with hexapeptide DADEYLNH$_2$ **2.16** (t_R= 15.2 min). This experiment further confirms that no desulfation occurred during deprotection of the sY residue in peptide **2.31**.

When **2.23** was used as the building block, pure peptide **2.30** was obtained in a 71% yield after RP-HPLC purification. When **2.26** was used as the building block, peptide **2.30** was obtained in about a 45% yield. The lower yield using **2.26** was a result of our repeated attempts to remove a small amount of an unidentified impurity that exhibited a very similar retention time to **2.30** which we were unable to completely remove. In addition to the usual techniques for analyzing peptides (mainly HPLC and electrospray mass spectrometry (ESMS)), we also found that we could readily determine if any desulfated peptide was present after purification by ^{1}H-NMR. **Figures 2.14** and **2.15** shows the ^{1}H-NMR's of the aromatic regions of peptide **2.16** (DADEYLNH$_2$) and peptide **2.30** (DADEsYLNH$_2$) respectively. It is very clear from these spectra that peptide **2.30** is not contaminated with peptide **2.16**.

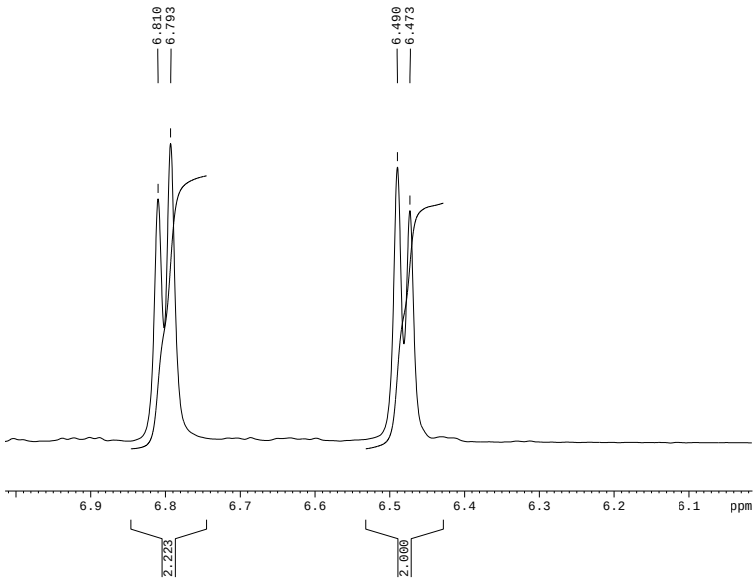


Figure 2.14. 500 MHz ^{1}H-NMR (D_2O) spectrum of the aromatic region of peptide DADEYLNH$_2$ (**2.16**).

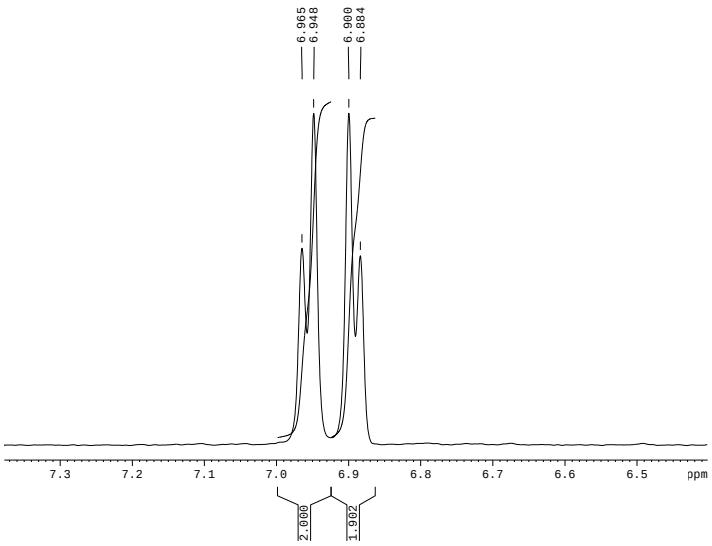


Figure 2.15. 500 MHz ^{1}H NMR (D_2O) spectrum of the aromatic region of pure DADEsYLNH$_2$ (**2.30**) obtained using amino acid **2.23**.

2.3.8 Synthesis of more complex sulfated peptides

To demonstrate the broad applicability of our methodology we prepared more complex sulfated peptides which contained various numbers of sTyr residues and a wide range of amino acid residues. Although both **2.23** (DCV-protected) and **2.26** (TCE-protected) could be used to prepare sTyr peptides, **2.23** was used for all of our future studies. One reason is because the yield of the model hexapeptide **2.30** was greater using **2.23**. Another reason has to do with the synthesis of multisulfated peptides: when using **2.26**, some of the TCE group is converted into the DCV group during peptide synthesis which means that the crude HPLC chromatograms of peptides bearing multiple sTyr residues (before TCE/DCV removal) could be very complex making it difficult to assess the quality of the peptide before TCE/DCV removal.

First, we prepared the trisulfated octapeptide, Ac**s**Y**E**s**Y**LD**s**Y**D**FNH$_2$ (**2.33**) which corresponds to residues 5-12 of mature P-selectin glycoprotein ligand 1 (PGSL-1, see chapter 1, section 1.2.1).[22] This peptide has been synthesized by others in a 27% yield using a global sulfation strategy (see chapter 1, page 36).[91] The synthesis was done using a Quartet automated peptide synthesizer from Protein Technologies at 25 μM scale (all subsequent peptides throughout this chapter were prepared using the Quartet peptide synthesizer). In general, each coupling cycle started with an Fmoc removal step (3 x 10 min) followed by a washing step then a coupling step using HBTU/HOBt in the presence of DIPEA for 1.5 h except for the first amino acid which coupled as a pentafluorophenyl ester in the presence of HOBt (applying double coupling for 1.5 h each). Cleavage from the resin was done using 5% TIPS in TFA and the peptide was precipitated with *t*-butyl methyl ether. The analytical RP-HPLC chromatogram of the DCV-protected peptide, AcY(SO_3DCV)EY(SO_3DCV)LDY(SO_3DCV)DFNH$_2$ (**2.34**), shows one major peak in addition to several minor peaks (**Figure 2.16**). Pure **2.33** was obtained in a 46% yield after removal of the three DCV groups using 50 wt% of 10% Pd/C in presence of 21 equivalent of ammonium formate under hydrogen atmosphere and purification by RP-HPLC (**Figure 2.17**).

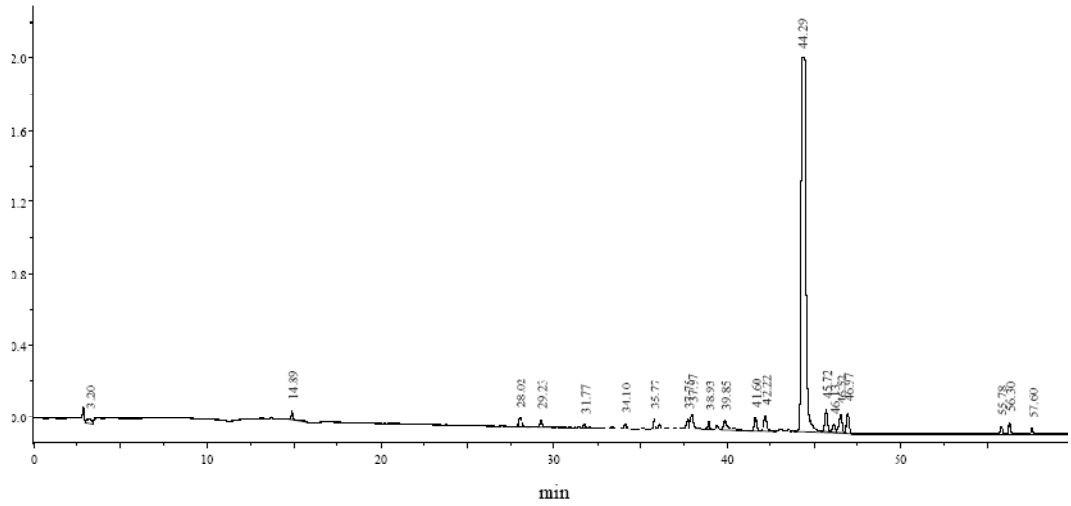


Figure 2.16. Analytical HPLC chromatogram of the crude octapeptide AcY(SO_3DCV)EY(SO_3DCV)LDY(SO_3DCV)DFNH_2 (**2.34**, t_R= 44.3 min). Note that the peak corresponding to **2.34** is off scale.

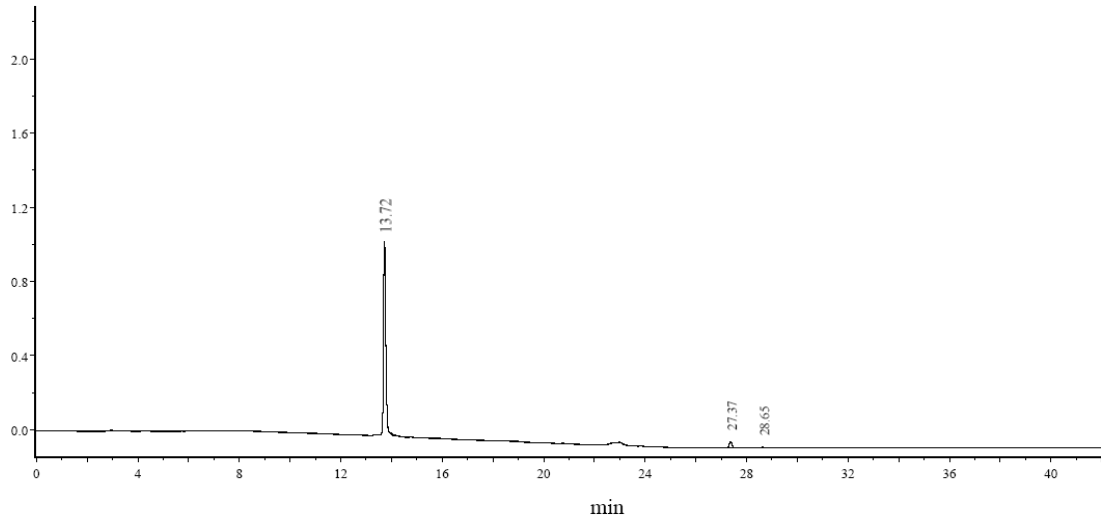


Figure 2.17. Analytical HPLC chromatogram of pure AcsYEsYLDsYDFNH_2 (**2.33**, t_R= 13.7 min).

The monosulfated octapeptide AcYE**s**YLDYDFNH$_2$ (**2.35**) was also prepared since others reported difficulties in preparing this peptide using a global sulfation strategy and were able to obtain this peptide in only a 5% yield.[91] Applying the protocol described above for the trisulfated analogue **2.33**, the DCV-protected monosulfated octapeptide AcYEY(SO$_3$DCV)LDYDFNH$_2$ (**2.36**) appeared as mainly a single peak in the analytical HPLC chromatogram after cleavage from the support and precipitation with ether (**Figure 2.18**). After hydrogenolysis using 50 wt% of 10% Pd/C in the presence of 9 equiv of ammonium formate under hydrogen atmosphere, we obtained pure peptide **2.35** in a 63% yield and the analytical HPLC chromatogram for this peptide is shown in **Figure 2.19**. This represents a dramatic increase in yield compared to a previous report of 5% as mentioned above.

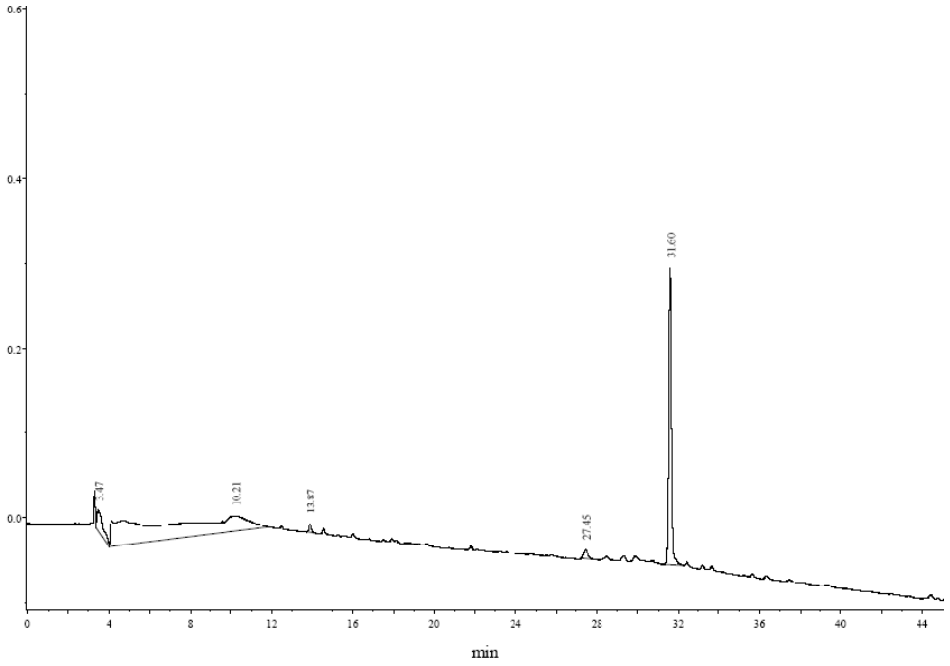


Figure 2.18. Analytical HPLC chromatogram of the crude peptide AcYEY(SO$_3$DCV)LDYDFNH$_2$ (**2.36**, t_R= 31.6 min).

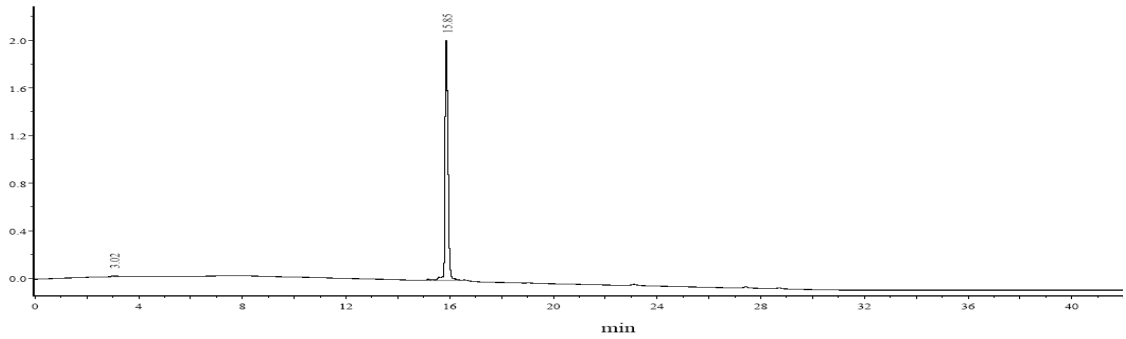


Figure 2.19. Analytical RP-HPLC chromatogram of pure AcYEsYLDYDFNH$_2$ (**2.35**, t_R= 15.9 min).

In order to demonstrate conclusively that no desulfation occurs during the synthesis steps we prepared the nonsulfated analogue AcYEYLDYDFNH$_2$ (**2.37**). The analytical HPLC chromatogram for this purified peptide is shown in **Figure 2.20**. The ^{1}H NMR's of the aromatic regions of peptides **2.33**, **2.35** and **2.37** are shown in **Figures 2.21-2.23** respectively. The aromatic tyrosyl protons for the unsulfated peptide **2.37** between 6.35 ppm to 6.80 ppm (set # 1) while the aromatic protons for the Phe residue appear between 6.9 ppm to 7.1 ppm (set #2) (**Figure 2.23**). Sulfation of one tyrosyl moiety (peptide **2.35**) resulted in a downfield shift of four protons from the first to second set (**Figure 2.22**). When the three tyrosyl moieties are sulfated (peptide **2.33**) all the aromatic protons appear above 6.7 ppm (**Figures 2.21**). These NMR experiments, in conjunction with our HPLC and MS results, strongly indicate that no desulfated peptides are contaminating peptides **2.33** and **2.35**.

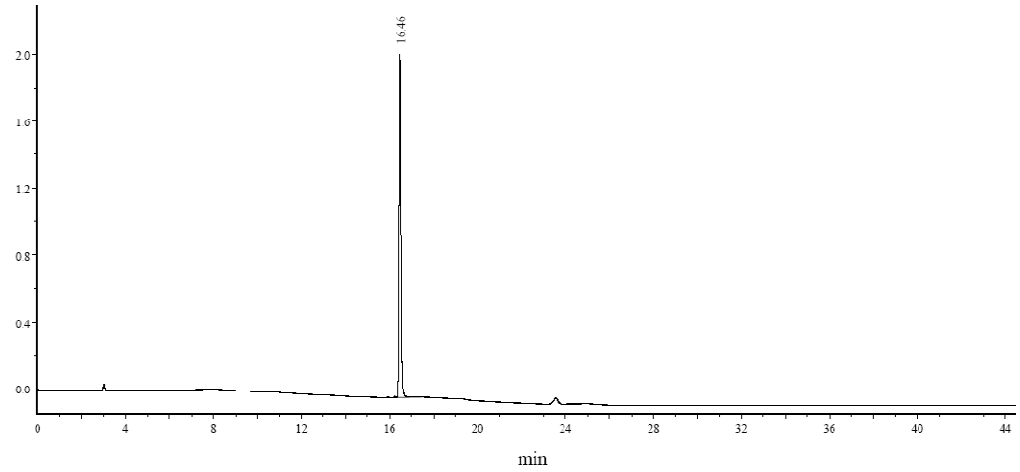


Figure 2.20. Analytical HPLC chromatogram of pure AcYEYLDYDFNH$_2$ (**2.37**, t_R= 16.5 min).

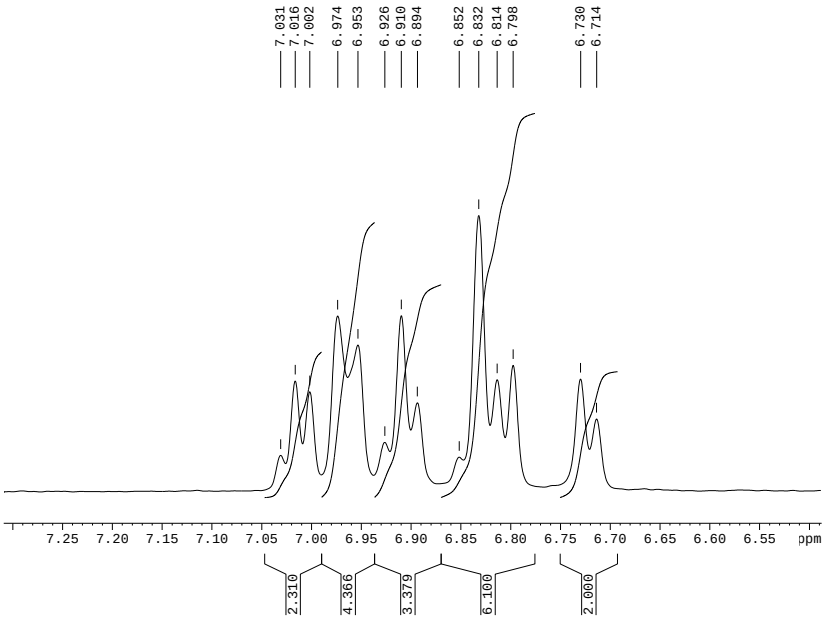


Figure 2.21. 500 MHz ^{1}H-NMR (D_2O) of the aromatic region of pure AcsYEsYLDsYDFNH$_2$ (**2.33**).

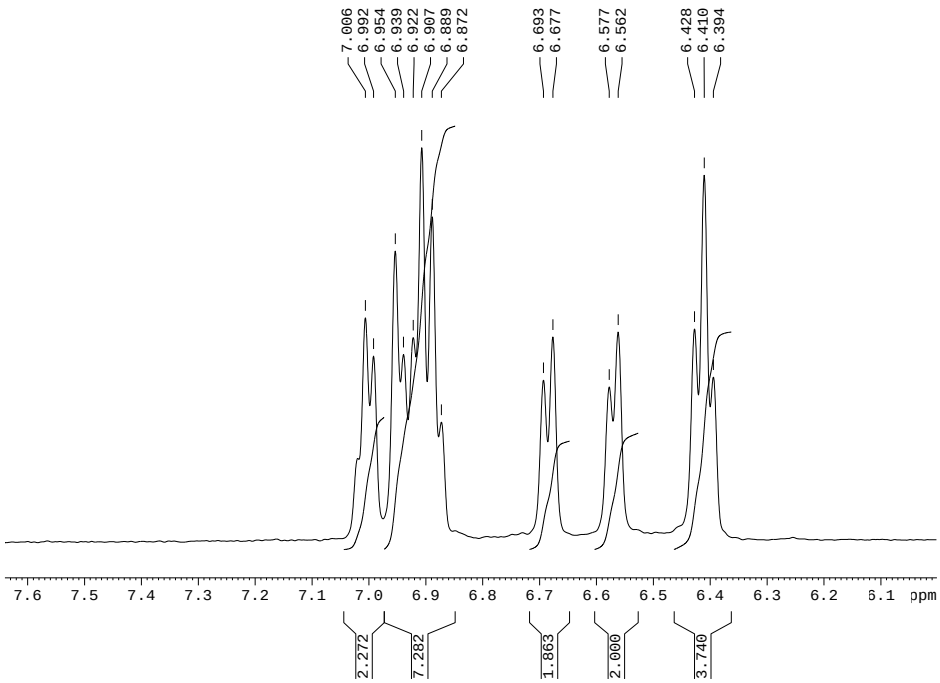


Figure 2.22. 500 MHz ^{1}H-NMR (D_2O) of the aromatic region of pure AcYEsYLDYDFNH$_2$ (**2.35**).

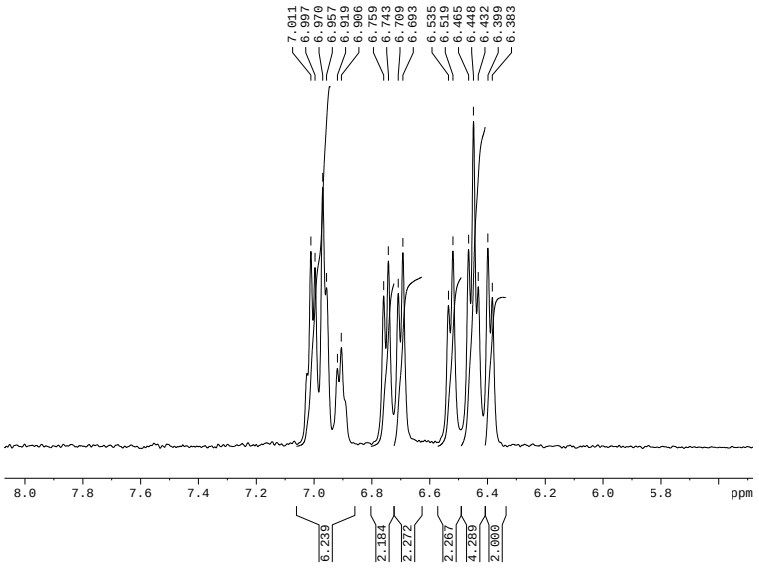


Figure 2.23. 500 MHz ^{1}H-NMR (D_2O) of the aromatic region of pure AcYEYLDYDFNH$_2$ (**2.37**).

Next the attention was turned to an even more challenging target, a multisulfated peptide derived from the *N*-terminal region of chemokine receptor D6 (see chapter 1, section section 1.2.2.2 for a discussion of D6). Although it is known that D6 is sulfated in its *N*-terminal region, it is not known which tyrosines are sulfated.[59] The Sulfinator, a software tool that predicts tyrosine sulfation sites in protein sequences,[104] 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 decided to prepare a tetrasulfated 20-mer, Ac-DADSENSSF**sY**$_{23}$**sY**$_{24}$**sY**$_{25}$D**sY**$_{27}$LDEVAFNH$_2$ (**2.38**) 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 (acidic residues are known to have a destabilizing effect on sY residues in peptides) this peptide represents a particularly stringent test of our methodology.

Our first trial to prepare **2.38** employing our usual coupling procedure (5 equiv of HBTU/HOBt, 5 equiv DIPEA and 5 equiv amino acid for 1.5 h) and using 2-MP for Fmoc removal (3 x 10 min) failed to give the desired DCV-protected peptide very cleanly as the crude analytical HPLC chromatogram showed many peaks. Repeating the synthesis with the incorporation of a capping step after each coupling cycle (treating the peptide with Ac_2O/pyridine after each coupling cycle to acetylate any remaining free amino groups), or performing double couplings together with capping did not improve the synthesis significantly. However, analysis of the major peaks in the HPLC trace of the latter trial showed the desired DCV-protected peptide AcDADSENSSF$_{(DCV)}$Y$_{23(DCV)}$Y$_{24(DCV)}$Y$_{25}$D$_{(DCV)}$Y$_{27}$LDEVAFNH$_2$ (**2.39**) along with deletion

peptides resulting from incomplete coupling between DCV-protected residue 25 and 24 or 24 and 23 (**Figure 2.24**).

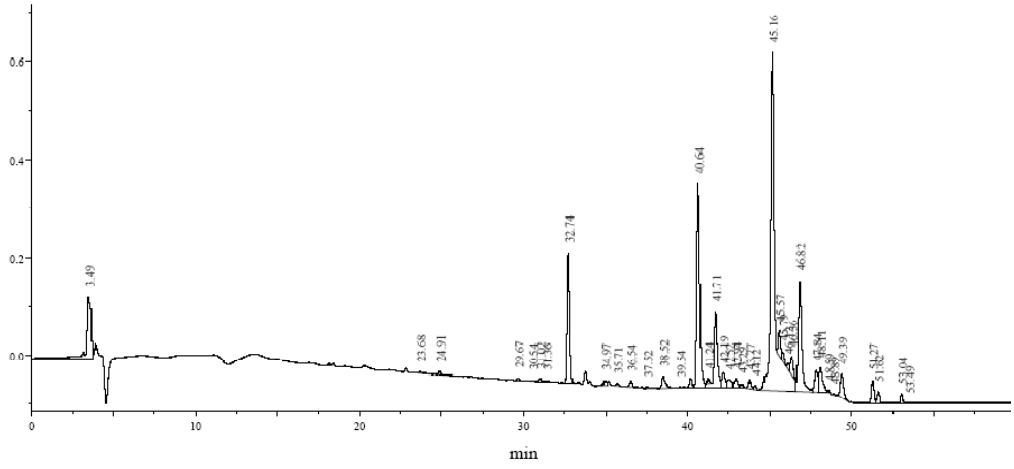


Figure 2.24. Analytical HPLC chromatogram of the mixture obtained after cleavage from the resin from the synthesis of peptide **2.39** using HBTU/HOBt as coupling agents and capping using Ac_2O/pyr. The peak at t_R = 45.2 min corresponds to $AcDADSENSSF_{(DCV)}Y_{\mathbf{23}(DCV)}Y_{\mathbf{24}(DCV)}Y_{\mathbf{25}}D_{(DCV)}Y_{\mathbf{27}}LDEVAFNH_2$ (**2.39**) which is the desired DCV-protected peptide. The peak at t_R = 40.6 min corresponds to the deletion peptide $AcDADSENSSF_{(DCV)}Y_{(DCV)}Y_{\mathbf{25}}D_{(DCV)}Y_{\mathbf{27}}LDEVAFNH_2$. The peak at t_R = 32.7 min corresponds to the deletion peptide $AcY_{25}D_{(DCV)}Y_{\mathbf{27}}LDEVAFNH_2$.

It was then decided that the coupling agent HCTU, which has been reported to be a better coupling agent than HBTU yet of almost similar price, should be used.[105] After the application of double couplings for 45 min using HCTU/HOCt followed by capping, the crude HPLC chromatogram showed a major peak corresponding to our desired peptide **2.38** (t_R= 44.88 min, **Figure 2.25**). However, there were still a significant number of other products.

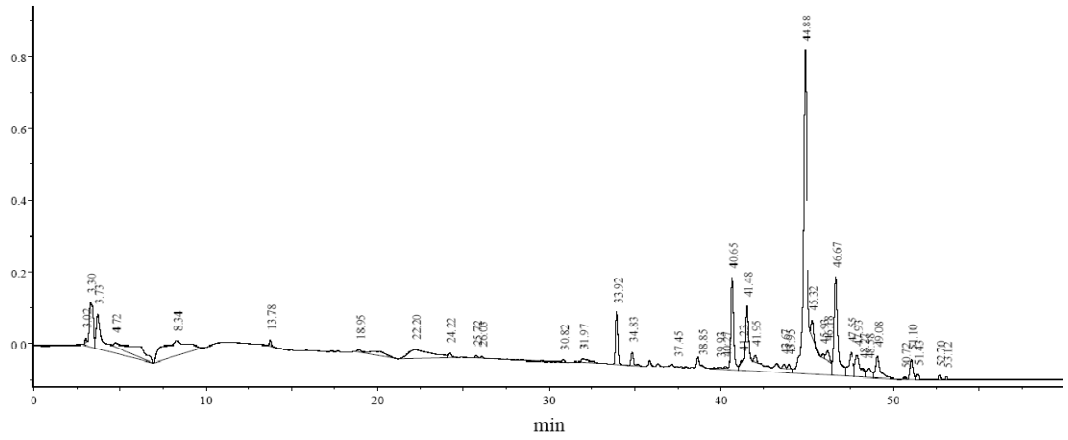


Figure 2.25. Analytical HPLC chromatogram of the mixture obtained after cleavage from the resin from synthesis of peptide **2.39** using HCTU/HOCt as coupling agents and capping using Ac_2O/pyr. The peak at t_R= 44.88 min corresponds to the desired peptide AcDADSENSSF$_{(DCV)}$Y$_{\mathbf{23}(DCV)}$Y$_{\mathbf{24}(DCV)}$Y$_{\mathbf{25}}$D$_{(DCV)}$Y$_{\mathbf{27}}$LDEVAFNH$_2$ (**2.39**).

In order to determine if the difficulties in making this peptide arose from intrinsic nature of the peptide or due to the presence of the DCV groups we prepared the unsulfated 20-mer, AcDADSENSSFY$_{23}$Y$_{24}$Y$_{25}$DY$_{27}$LDEVAFNH$_2$ (**2.40**) using the HCTU protocol mentioned above. After cleavage from the support, the crude HPLC trace showed many peaks (**Figure 2.26**) indicating that the difficulty of the synthesis was not probably stemming from the presence of DCV groups.

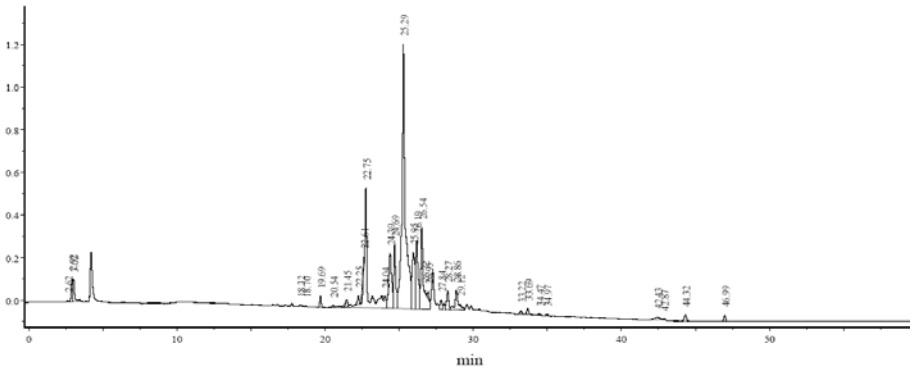


Figure 2.26. Analytical HPLC chromatogram of the mixture obtained after cleavage from the resin during the synthesis of peptide **2.40** using the HCTU/HOCt protocol.

In order to improve the synthesis of **2.38**, residues 23, 24 and 25 were incorporated using HATU/HOAt, an even stronger coupling agent.[106] This significantly improved the quality of the crude peptide **2.39** as indicated by analytical RP-HPLC (**Figure 2.27**). The deprotection of sTyr residues was achieved using 50 wt% of 10% Pd/C in the presence of 30 equiv $HCOONH_4$ under one atm. H_2 which gave the desired sulfated peptide **2.38** in a 39% yield after HPLC purification (**Figure 2.28**).

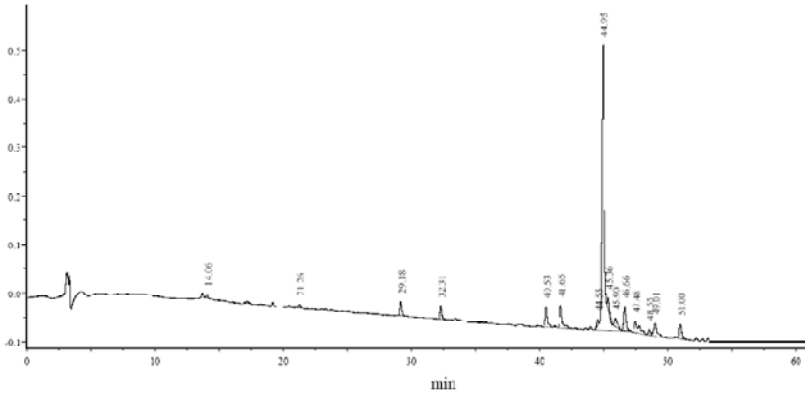


Figure 2.27. **A**nalytical HPLC chromatogram of the mixture obtained after cleavage from the resin from the synthesis of peptide **2.39** using the HCTU/HOCt-HATU/HOAt protocol and capping using Ac_2O/pyr. The peak at t_R= 44.9 min corresponds to the desired peptide $AcDADSENSSF_{(DCV)}Y_{23(DCV)}Y_{24(DCV)}Y_{25}D_{(DCV)}Y_{27}LDEVAFNH_2$ (**2.39**).

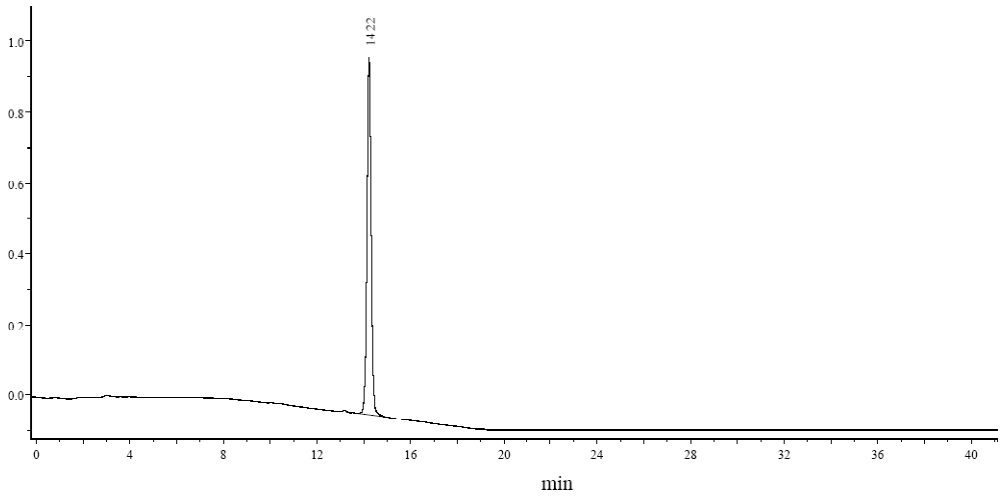


Figure 2.28. Analytical HPLC chromatogram of pure AcDADSENSSFsYsYsYDsYLDEVAFNH$_2$ (**2.38**).

Encouraged by the above successful results on the 20-mer from D6, we also prepared the disulfated 22-mer Ac-TTPDsYGHsYDDKDTLDLNTPVDKNH$_2$ (**2.41**) which corresponds to residues 7-28 of C5aR (see chapter 1, section 1.2.3 for a discussion of C5aR). The disulfated 22-mer contains two sTyr residues as well as a variety of basic and acidic amino acid residues. The synthesis was done automatically on a Quartet peptide synthesizer using Rink amide resin and applying the usual Fmoc-deprotection protocol. HCTU/HOCt in the presence of DIPEA was used for couplings. After cleavage from the support, the HPLC trace showed one major peak corresponding to the DCV-protected peptide AcTTPD$_{(DCV)}$YGH$_{(DCV)}$YDDKDTLDLNTPVDKNH$_2$ (**2.42**) as confirmed by mass spectrometry (**Figure 2.29**). Deprotection of the DCV groups was done using 50 wt% of 10% Pd/C in the presence of 19 equiv HCOONH$_4$ under a hydrogen atmosphere which gave peptide **2.41** in a 58% yield after HPLC purification (**Figure 2.30**).

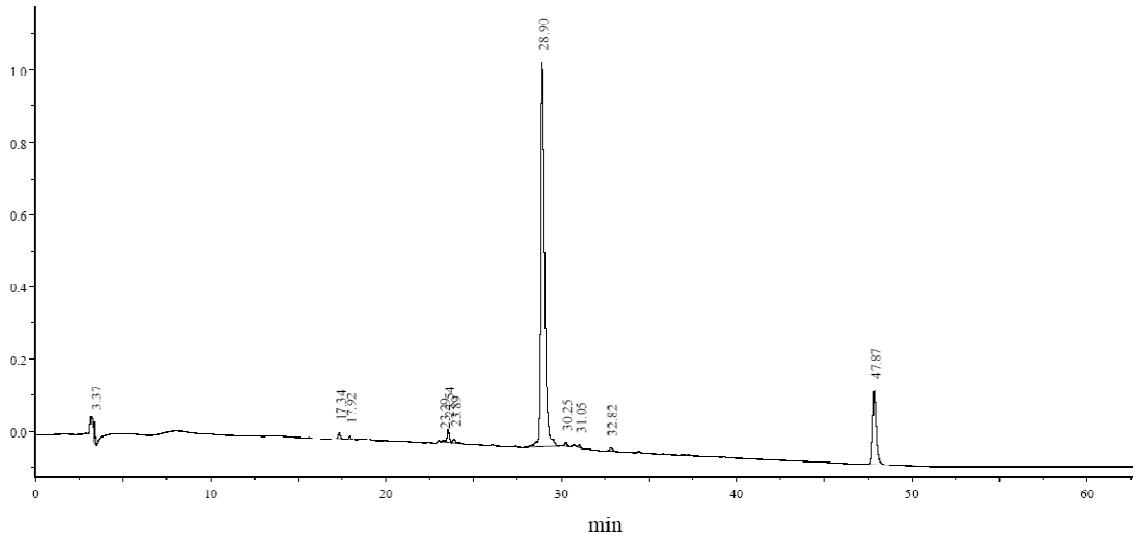


Figure 2.29. Analytical HPLC chromatogram of crude $AcTTPD_{(DCV)}YGH_{(DCV)}YDDKDTLDLNTPVDKNH_2$ (**2.42**, t_R= 28.9 min).

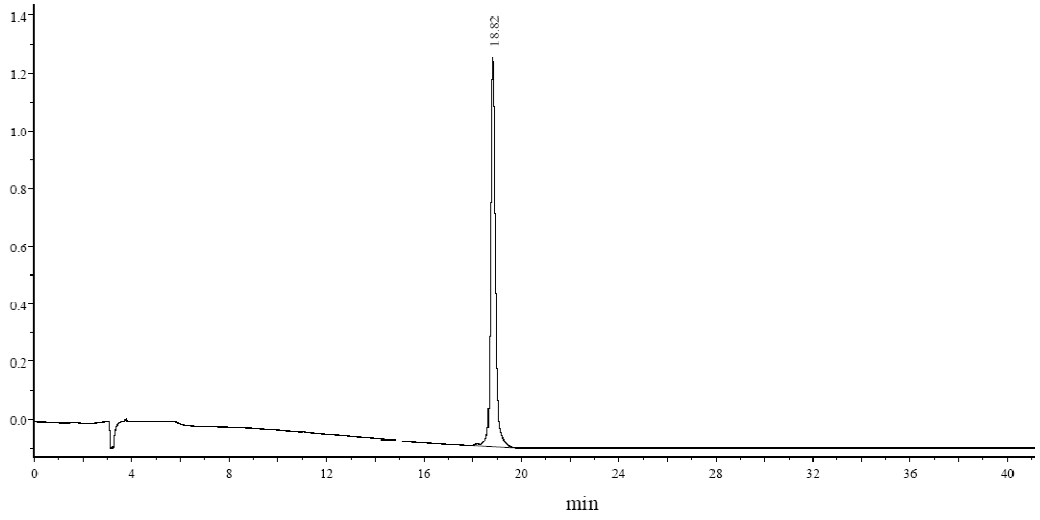
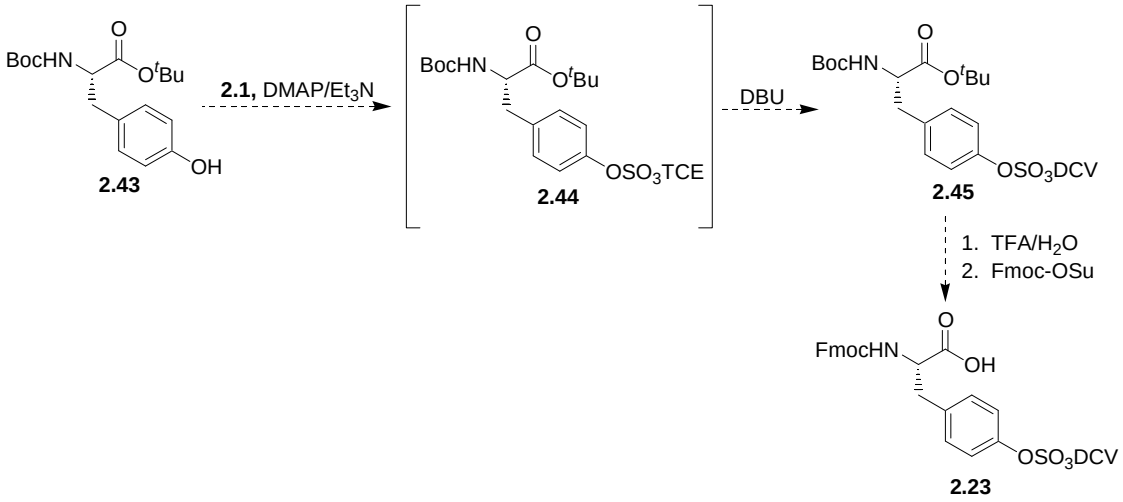


Figure 2.30. Analytical HPLC chromatogram of pure AcTTPDsYGHsYDDKDTLDLN-$TPVDKNH_2$ (**2.41**).

2.3.9 An improved synthesis of amino acid 2.23.

Before attempting to prepare any other sTyr peptides, we wished to devise a more economical approach to the synthesis of the key amino acid **2.23**. In SPPS, the amino acid building blocks are used in considerable excess and so it is important that economical methods are available for their preparation. Our current approach utilizes reagent **2.22** whose preparation involves a three step synthesis and requires expensive trimethyloxonium tetrafluoroborate (**2.20**). We wished to devise a method for preparing **2.23** without using reagent **2.22**. The key to the preparation of **2.22** in high yield was the use of DBU to effect elimination of HCl from **2.17** to give compound **2.18** (**Scheme 2.6**). We reasoned that if we incorporated our TCE sulfate using $Cl_3CCH_2OSO_2Cl$ (**2.1**) into amino acid **2.43** and then subjected the resulting amino acid **2.44** to DBU we should be able to obtain amino acid **2.45** (**Scheme 2.10**). Removal of the Boc and *tert*-butyl groups using TFA followed by reaction with Fmoc-OSu should give **2.23**.

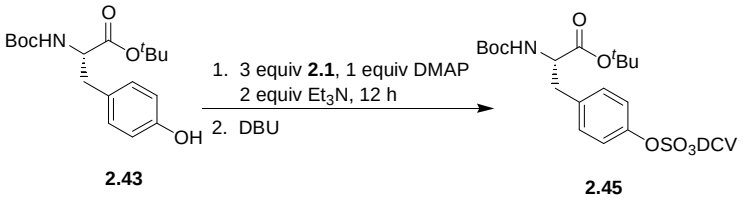


Scheme 2.10. Potential alternative route to amino acid **2.23**.

Compound **2.44** was prepared using our optimized conditions (**2.43**/DMAP/Et_3N) for the introduction of **2.1** into phenol derivatives[98] except ether was used as solvent instead of THF since we anticipated that the triethylammonium hydrochloride formed in the reaction would precipitate out better in ether and so be easier to remove. The reaction was allowed to proceed overnight (about 12 h, reaction complete as determined by TLC) and the mixture was filtered. Compound **2.44** was not purified. One equiv of DBU was added to the filtrate every hour until all of **2.44** was consumed as determined by TLC (this required 3 equiv of DBU). The hydrochloride salt of DBU that was produced during the reaction was formed as thick, glue-like oil. Nevertheless, the mixture was filtered and the filtrate was concentrated and the residue was purified by silica gel flash chromatography. We were pleased to find that this gave compound **2.45** albeit in a modest 62% yield (**Table 2.4**, entry 1). We then attempted the synthesis using THF as solvent hoping that the hydrochloride salt of DBU would form a precipitate and be easier to remove by filtration. This did indeed turn out to be

the case; however a slightly larger quantity of DBU (3.5 equiv) was required to consume all of **2.44**. We also performed an aqueous workup of the reaction using 2% aq. H_3PO_4 (to remove unreacted DBU) since Quagliato *et al.* have reported that the Boc group will withstand these mildly acidic conditions.[107] This gave **2.45** in a 81% yield. This reaction was repeated except 5 equiv of DBU was used to ensure complete conversion of **2.44** to **2.45**. An aq. workup was performed after both steps of the synthesis except 0.05 M phosphate buffer (pH 7.2) was used instead of 2% H_3PO_4 to ensure no loss of the Boc or *tert*-butyl groups. This gave **2.45** in an 88% yield.

Table 2.4. Synthesis of compound 2.45.



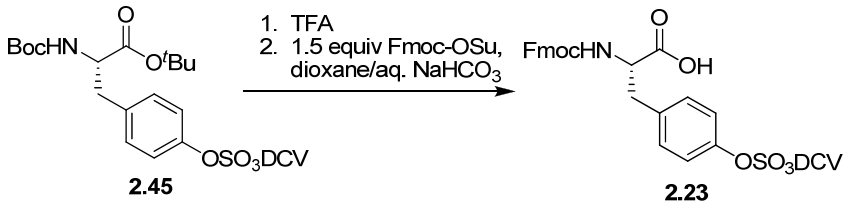
2.43 **2.45**

Entry	Solvent	Work up	DBU	% Yield
1	Et_2O	None	3 equiv[a]	62
2	THF	2% H_3PO_4	3.5 equiv[b]	81
3	THF	Phosphate buffer, pH 7.2	5 equiv[c]	88

[a]Added as one equiv every hour. Total reaction time of step 2 was 4 h. [b]Added as one equiv every hour with the last 0.5 equiv added after hour 3. Total reaction time of step 2 was 5 h. [c]Added as one equiv every hour. Total reaction time of step 2 was 6 h

An attempt to simultaneously remove the *tert*-butyl and Boc groups by treating **2.45** with 95% aqueous TFA for 1 hour followed by Fmoc installation using Fmoc-OSu according to a previously reported procedure[108] resulted in a moderate yield of **2.23** (57% yield).

However, Nowick *et al.*[109] and others[110] reported that simultaneous removal of Boc and *tert*-butyl groups can be accomplished in high yield by subjecting the amino acid to repetitive treatments with neat TFA. Thus, **2.45** was subjected to neat TFA for 2 h and then all of the TFA was removed by high vacuum rotary evaporation. This was repeated and then a third treatment was performed overnight. The TFA was removed again and the residue was triturated with $CHCl_3$ to give a white solid which upon treatment with FmocOSu gave **2.23** in a 85% yield (overall yield of 75%) (**Scheme 2.11**)



Scheme 2.11. Synthesis of **2.23** from **2.45**.

The enantiopurity of **2.23** synthesized from **2.45** was determined via the synthesis of dipeptides peptides Ac-Y(SO_3DCV)-$A_{(L)}$-NH_2 (**2.28LL**) and Ac-Y(SO_3DCV)-$A_{(DL)}$-NH_2 (**2.28LD/2.28LL**) followed by analysis by HPLC and ^{1}H-NMR as previously described in section 2.3.6. The HPLC chromatograms of these peptides are shown in Figures **2.31** and **2.32** and the ^{1}H-NMR spectra are shown in **Figures 2.33** and **2.34**. Comparing the ^{1}H-NMR spectra of **2.28LL (Figure 2.34**) to that of the **2.28LD/2.28LL** mixture (**Figure 2.33**) it appears that none of the undesired diastereomeric peptide (the DL isomer) is present in **Figure 2.34** which suggests that **2.33** was prepared in very high ee. On the other hand, the HPLC chromatogram of **2.28LL (Figure 2.32**) exhibits a large peak corresponding to the

desired LL dipeptide (at t_R = 29.2 min) and a very small peak (at t_R = 30.6 min) which, from the HPLC chromatogram of the **2.28LD/2.28LL** mixture (**Figure 2.31**), appears to be the undesired DL dipeptide. Nevertheless, integration of these peaks indicates that **2.23** was still obtain in very good ee (> 98 %).

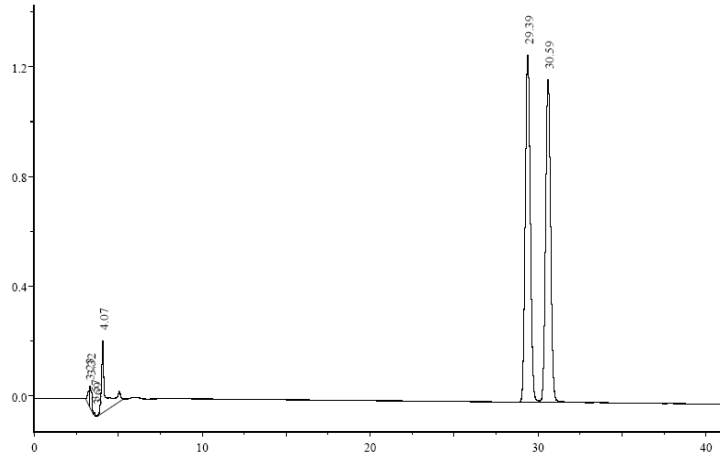


Figure 2.31. Analytical HPLC chromatogram of diastereomeric dipeptides Ac-Y(SO_3DCV)-$A_{(DL)}$-NH_2 (**2.28LD** and **2.28LL**). Amino acid **2.23**, prepared using the route outlined in **Scheme 2.10**, was used to prepare this mixture.

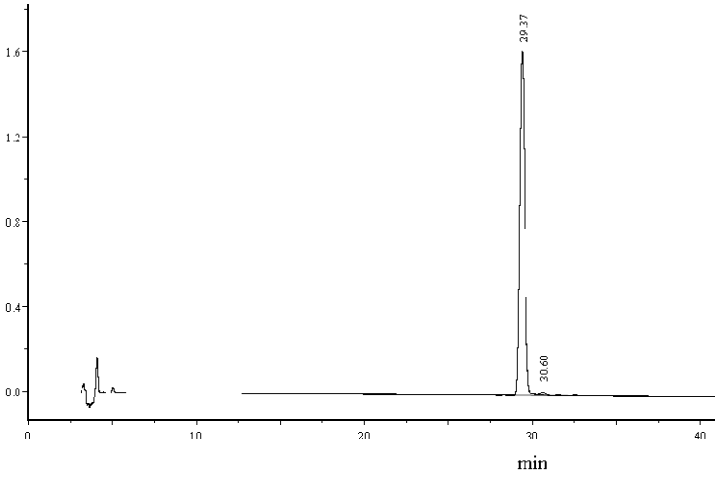


Figure 2.32. Analytical HPLC chromatogram of dipeptide Ac-Y(SO_3DCV)-$A_{(L)}$-NH_2 (**2.28LL**). Amino acid **2.23**, prepared using the route outlined in Scheme **2.10**, was used to prepare this peptide.

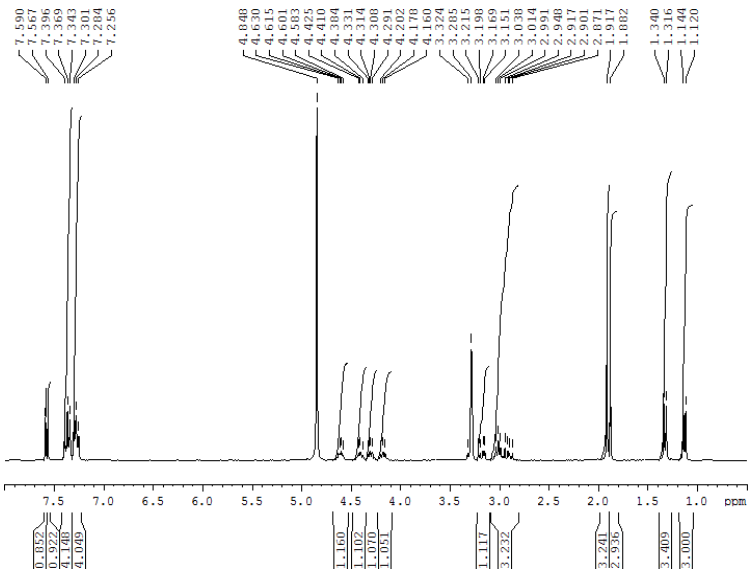


Figure 2.33. ^{1}H NMR (CD_3OD) spectrum of diastereomeric dipeptides Ac-Y(SO_3DCV)-$A_{(DL)}$-NH_2 (**2.28LD** and **2.28LL**). Amino acid **2.23**, obtained using the route outlined in Scheme **2.10**, was used to prepare this mixture.

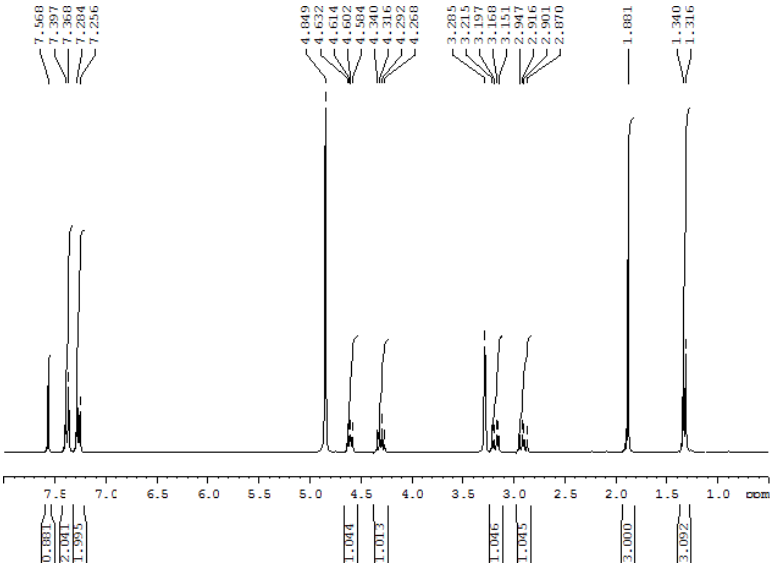


Figure 2.34. ^{1}H NMR spectrum (CD_3OD) of dipeptide Ac-Y(SO_3DCV)-$A_{(DL)}$-NH_2 (**2.28LL**). Amino acid **2.23**, obtained using the route outlined in Scheme **2.10**, was used to prepare this peptide.

2.3.10 Exploring alternative methods for DCV removal.

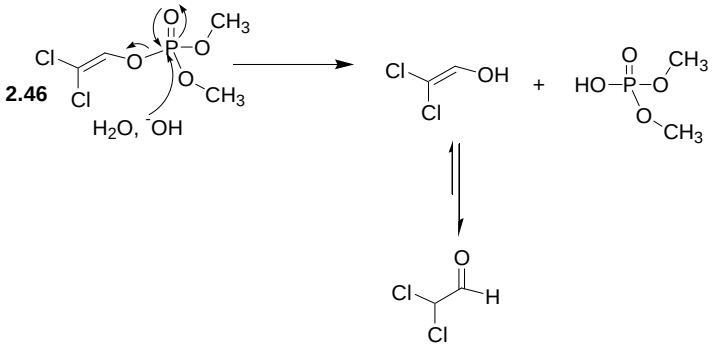
One of the concerns of our approach to sTyr peptides was that our sulfate deprotection conditions (hydrogenolysis) would result in partial reduction of the indole ring in tryptophan residues to give 2,3-dihydrotryptophan or even octahydrotryptophan. This phenomenon has been reported by several groups upon subjecting Trp-bearing peptides or Trp derivatives to H_2-Pd/C[111], formic acid-Pd/C,[112, 113] formic acid/Pd black,[114] or ammonium formate/$Pd(OAc)_2$.[113] Therefore we embarked on a study to find methods other than to Pd/C-ammonium formate-H_2 for removing the DCV group using **2.14** as a model substrate.

We initially examined conditions that have been used to remove trichloroethyl-based protecting groups. Zinc/triethylamine[115] or zinc/triethylamine in the presence of acetylacetone,[116] which have been used to remove the 2,2,2-trichloroethoxycarbonyl (Troc) group from amines and TCE moieties from TCE-protected phosphates, failed to effect deprotection (**Table 2.5**, entries 1 and 2). Zn(Cu)/DMF[117] (entry 3) successfully removed the DCV. However, the reaction turned deep brown upon completion with the formation of a heavy precipitate which we anticipated would be problematic when applied to peptides. Tributylphospine has been used remove the 1,1-dimethyl-2,2,2-trichloroethyl group from protected phosphates.[118] However, this was not successful with the DCV group. Ogilvie e*t al.* utilized tetra-*n*-butylammonium fluoride (TBAF) to remove the TCE and phenyl groups from protected phosphates and they suggested that reactions via the attack of fluoride ion on the phosphorus atom displacing the alcohols.[119] However, TBAF in presence of triethylamine or TBAF in presence of a nucleophilic catalyst and in less polar solvents failed to result in loss of the DCV group (entries 5-7). Replacing TBAF with tetra-*n*-

butylammonium iodide (TBAI), a stronger nucleophile, did not result in deprotection (entries 8, 9). Self-catalyzed transesterification with 2-dimethylaminoethanol at room temperature was adopted by Barton *et al.* to cleave a protected peptide linked to Merrifield resin linked via an ester bond.[120] The resulting ester with 2-dimethylaminoethanol was easily hydrolyzed using aqueous sodium carbonate or simply aqueous DMF. We thought that this procedure might also be effective to remove the DCV group from **2.14**. Unfortunately, 2-dimethylaminoethanol in DMF alone or in the presence of DBU failed to give any reaction (entries 10, 11).

Table 2.5. Attempts to deprotect DCV using **2.14** as model compound

Entry	Reaction Conditions	Outcome
1	20 equiv Zn, 6 equiv Et_3N, MeOH, rt for 24 h	No reaction
2	20 equiv Zn, 6 equiv Et_3N, 10 equiv acetyl-acetone,MeOH, rt for 24 h	No reaction
3	20 equiv Zn-Cu couple, 19 equiv Et_3N, 10 equiv acetyl-acetone/DMF, rt for 24 h	Loss of DCV group
4	3 equiv $(Bu)_3P$, 2 equiv Et_3N, DMF, rt for 24 h	No reaction
5	1 equiv TBAF, 2 equiv Et_3N, dioxane: H_2O 5:1, rt for 24 h	No reaction
6	1 equiv TBAF, 1 equiv DMAP, DMF, rt for 24 h	No reaction
7	1 equiv TBAF, 1 equiv DMAP, NMP, rt for 24 h	No reaction
8	5 equiv TBAI, DMF: H_2O 1:1, rt for 24 h	No reaction
9	5 equiv TBAI, 1 equiv DMAP, NMP:H_2O 1:1, rt for 24 h	No reaction
10	10 equiv $(CH_3)_2NCH_2CH_2OH$, DMF, rt for 24 h	No reaction
11	10 equiv $(CH_3)_2NCH_2CH_2OH$, 2 equiv DBU, DMF, rt for 24 h	No reaction



Scheme 2.12. Mechanism of degradation of Dichlorvos

Dichlorvos or 2,2-dichlorovinyl dimethyl phosphate (**2.46**, DDVP) is an organophosphorus compound widely used as a fumigant in public health, to control household pests and for protecting stored product from insects.[121] DDVP contains a DCV group bonded to phosphorus and interestingly a number of studies showing that this bond is very labile bond and DDVP is hydrolyzed at neutral pH through the attack of water at the phosphorus atom and the dichlorovinylate acts as leaving group (**Scheme 2.12**).[121-123] The rate of hydrolysis increases with increasing pH.[124-126] These results encouraged us to examine whether we could remove the DCV group from peptide **2.31** by hydrolysis. Surprisingly, no appreciable deprotection occurred in phosphate buffer at pH 5.5, 7.0 and 9.0 at 40 ^{0}C for 24 h (**Table 2.6**, entries 1-3). Changing the buffer to ammonium acetate (pH 7.0) together with an increase in the concentration did not cause any hydrolysis (entry 4). No loss of the DCV group in peptide **2.31** occurred even when it was exposed to 4 equiv NaOH in dioxane-water mixtures (0.1 N NaOH) at 40 ^{o}C for 24 h (entries 5 and 6).

Table 2.6. Attempts to remove the DCV from peptide DADEY(SO_3DCV)LNH_2 (**2.31**) by hydrolysis.

Entry	Reaction Conditions	Outcome
1	0.1 M Phosphate buffer, pH= 5.5, 10 mL for 10 mg **2.31** at 40 ^oC for 24 h	No reaction
2	0.1 M Phosphate buffer, pH= 7.0, 10 mL for 10 mg **2.31** at 40 ^oC for 24 h	No reaction
3	0.1 M Phosphate buffer, pH= 9.0, 10 mL for 10 mg **2.31** at 40 ^oC for 24 h	No reaction
4	1 M CH_3COONH_4, pH= 7.0, 10 mL for 10 mg **2.31** at 40 ^oC for 24 h	No reaction
5	4 equiv NaOH in H_2O:dioxane 1:4 (0.1M NaOH) at 40 ^oC for 24 h, 10 mg **2.31**	No reaction
6	4 equiv NaOH in H_2O:dioxane 4:1 (0.1 N NaOH) at 40 ^oC for 24 h, 10 mg **2.31**	No reaction

After performing these studies we became aware of a paper by Medzihradszky-Schweiger who reported that hydrogenolysis of carbobenzyloxy (CBz) protecting groups in Trp-bearing peptides using H_2 and 10% Pd/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.[127] This prompted us to examine whether the DCV group could be removed under basic hydrogenolysis conditions. Peptide **2.31** was suspended in water and 2 drops of concentrated ammonium hydroxide or 5 equiv of triethylamine (1 equiv per acidic group in the peptide plus one extra equiv) was added which resulted in solublization of the peptide. Hydrogenolysis of the mixture using H_2 (balloon pressure) as hydrogen source cleanly gave the desired peptide **2.30** in essentially quantitative yields (**Table 2.7**, entries 1 and 2) as indicated by HPLC. $Pd(OH)_2$ worked as well as Pd/C as catalyst (entry 3).

Table 2.7. Removal of the DCV from peptide DADEY(SO_3DCV)LNH_2 (**2.31**) by hydrogenolysis in the presence of base.

Entry	Reaction Conditions	% Yield **2.30**[a]
1	30 wt% of 10 % Pd/C, H_2, H_2O, 2 drops of ammonium hydroxide, rt, O/N	99
2	30 wt % of 10 % Pd/C, H_2,5 equiv Et_3N, H_2O, rt , O/N	99
3	50 wt % of $Pd(OH)_2$, H_2, 5 equiv Et_3N, H_2O, rt , O/N	99

[a]Crude yield based on HPLC monitoring of the reaction.

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 (**2.47**) and subjected it to 50 wt % of $Pd(OH)_2$, H_2 (balloon pressure), 2 equiv Et_3N in H_2O at rt for 16 h. **Figure 2.35** and **2.36** show the ^{1}H-NMR spectrum of peptide **2.47** before and after the reduction, respectively. No reduction of the indole ring has occurred confirming the results of Medzihradszky-Schweiger.

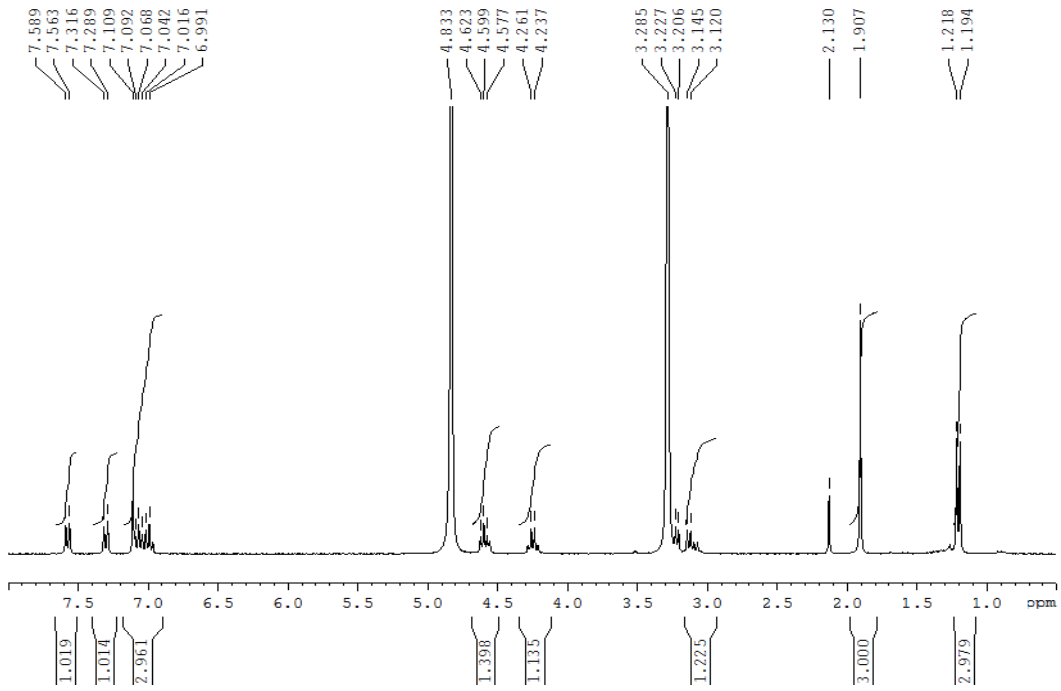


Figure 2.35. ^{1}H-NMR (CD_3OD) spectrum of peptide **2.47**.

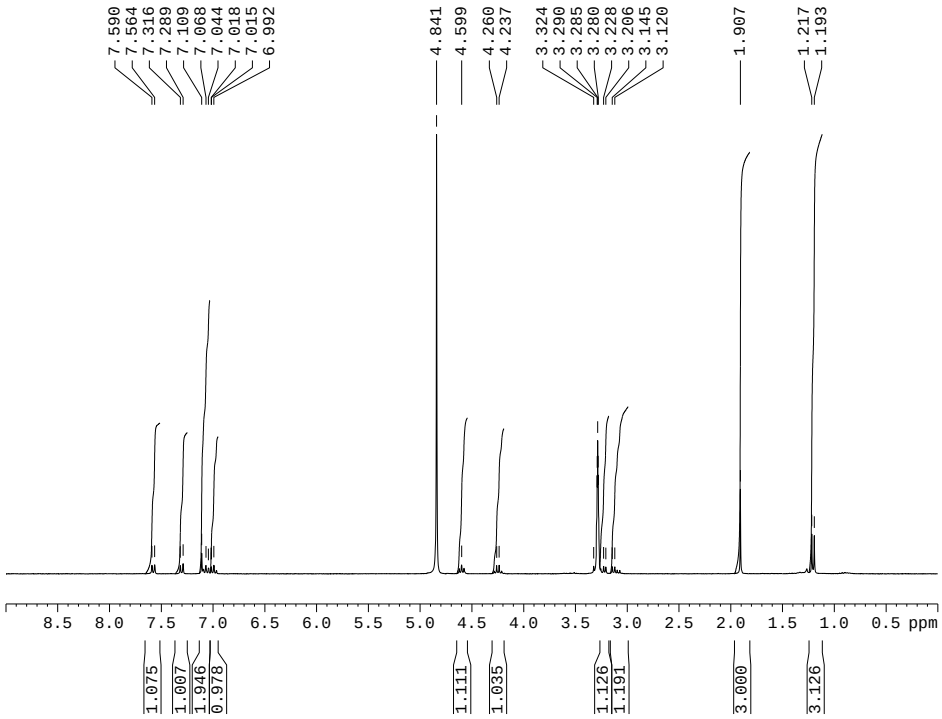


Figure 2.36. ^{1}H-NMR (CD_3OD) spectrum of peptide **2.47** after being subjected to 50 wt% of $Pd(OH)_2$, H_2, 2 equiv Et_3N for 16 h.

2.3.11 Exploring the stability of the DCV group to cleavage cocktails containing sulfur-based scavengers.

There were two other potential problems with our approach to sTyr peptides. Both involved peptides bearing sulfur-containing amino acids. It is well-known that sulfur-containing compounds can poison Pd catalysts rendering them ineffective. So we were initially concerned that we would not be able to use our methodology for peptides bearing methionine or cysteine. However, Medzihradszky-Schweiger reported that hydrogenolysis of carbobenzyloxy (CBz) protecting groups in sulfur-containing peptides using H_2 and 10% Pd/C (MeOH as solvent) proceeds readily when done in the presence of bases such as Et_3N. So after confirming that we could remove the DCV group under these conditions (discussed above) this was no longer a concern to us.[127] The other potential problem concerned the oxidation of Met residues when cleaving Met-bearing peptides from the polymer support. With Met-bearing peptides it is usually crucial that the cleavage cocktails contain sulfur-based reagents such as ethanedithiol (EDT) or thioanisole as these help suppress oxidation of the side chain of Met. Even for peptides that do not contain Met it is common to add these reagents to the cleavage cocktails since they trap the highly reactive carbocations (i.e. *tert*-butyl cations from *tert*-butyl protecting groups) produced during the cleavage process. Sulfur containing scavengers like EDT and thioanisole are among the most powerful and common scavengers. So far we have been adding triisopropylsilane (TIPS) to the cleavage cocktail to scavenge the cations. However, for Met-containing peptides EDT or thioanisole will also have to be present. As mentioned in Chapter 1 (section 1.3.3) the use of EDT or thioanisole is prohibited when unprotected tyrosine sulfate(s) is used to construct sTyr

peptides since an unprotected sulfate group is unstable to such reagents.[94] However, we did not know if the DCV group would be stable to cleavage cocktails containing EDT or thioanisole. To determine this, peptide **2.31** was subjected to variety of commonly used cleavage cocktails that contain EDT or thioanisole (**Table 2.8**) for 2 h and then examined by HPLC. No peptide resulting from loss of the DCV group was detected indicating that the DCV group is stable to these cleavage cocktails.

Table 2.8. Different cleavage cocktails used for stability studies with peptide **2.31**.

Reagent	Composition
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
TFA:TIS:Thioanisole:H_2O	92.5:2.5:2.5:2.5

2.3.12 Synthesis of $CXCR6_{1-20}$

Although the studies outlined in sections 2.3.10 and 2.3.11 suggested that we could use our methodology for the synthesis of sTyr peptides that also have Trp as well as residues with sulfur-containing side chains we decided to confirm this by constructing some challenging sTyr peptides bearing these residues. First, we turned our attention to the synthesis of a peptide that corresponds to residues 1-20 of the N-terminal region of CXCR6: $AcMAEHDsY_6HEDsY_{10}GFSSFNDSSQNH_2$ (**2.48**) (see chapter 1, section 1.2.2.3 for a discussion of CXCR6). This peptide bears a methionine residue at position 1. As mentioned in Chapter 1, the *N*-terminal region of CXCR6 contains two sTyr residues at positions 6 and

10. The significance of the presence of tyrosine sulfates in CXCR6 is not well understood. To our knowledge, no sulfated peptides corresponding to any part of the N-terminal region of CXCR6 have been synthesized. This turned out to be an extremely challenging peptide to prepare.

Our first attempt to synthesize CXCR6 employed our usual conditions discussed earlier using Rink amide resin (0.71 mmol/g), 2-MP for Fmoc removal, HBTU/HOBt as coupling reagents (one time for 1.5 h) 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 material obtained after cleavage showed many peaks (**Figure 2.37**). The mass spectrum of the mixture did indeed show that the desired peptide, $AcMAEHD_{(DCV)}YHED_{(DCV)}YGFSSFNDSSQNH_2$ (**2.49**), was present along with many other peptides.

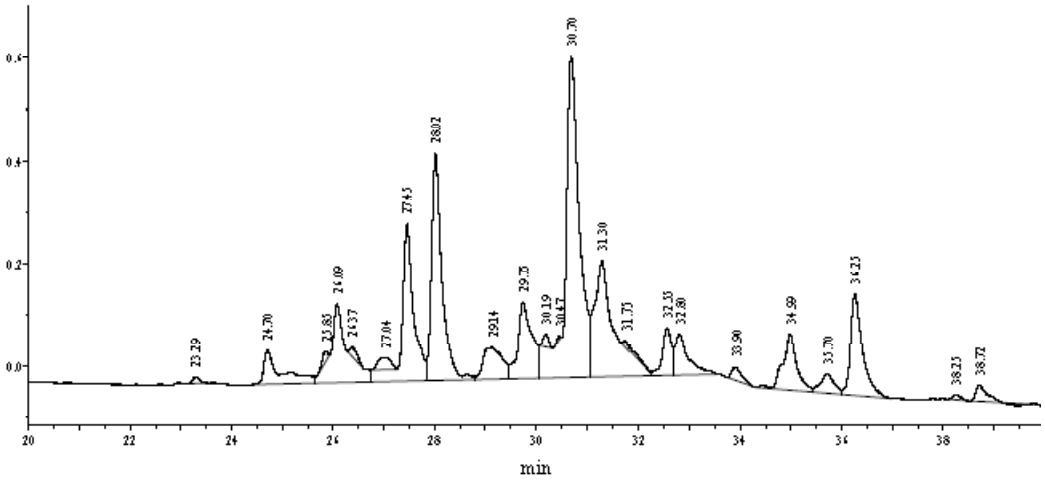


Figure 2.37. HPLC chromatogram of the material obtained after cleavage from the support from our first attempt to prepare peptide **2.49**. HBTU/HOBt used as coupling reagents (one time for 1.5 h) followed by a capping step.

We then attempted to prepare **2.49** using HCTU/HOCt as coupling agents and performing double couplings (2 x 45 min) followed by a capping step. Unfortunately, this did not improve the synthesis as indicated from the HPLC trace of the mixture after cleavage from the support (**Figure 2.38**). The mass spectrum of the mixture showed that our desired peptide, **2.49**, was again present. Two other significant peaks in the mass spectrum were identified as having resulted from two truncated peptides corresponding to the residues 9-20 and 10-20.

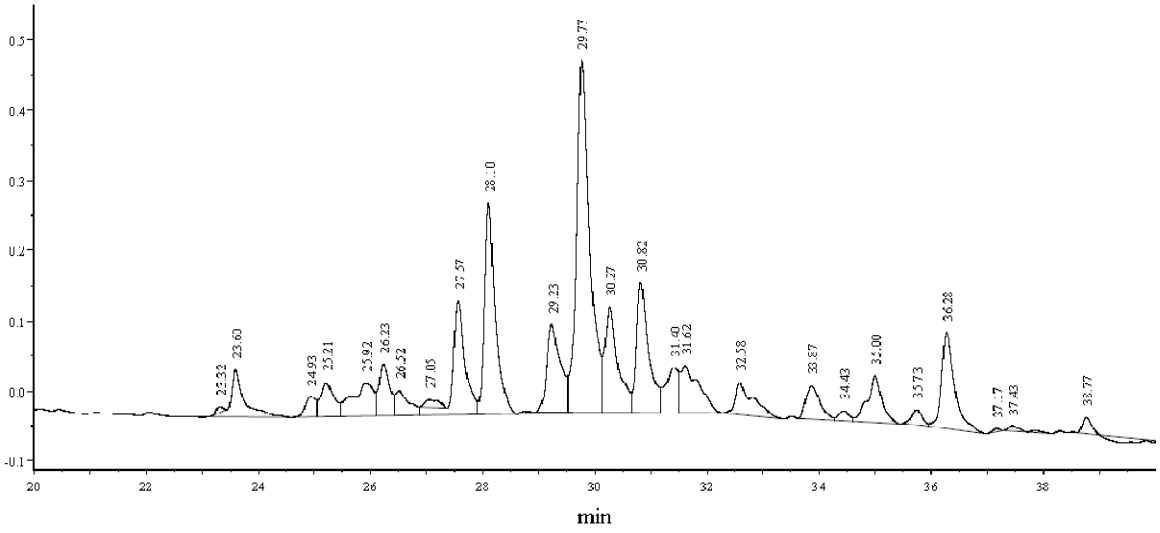


Figure 2.38. HPLC chromatogram of the material obtained after cleavage from the support from our second attempt to prepare peptide **2.49**. HCTU/HOCt as coupling agents and performing double couplings (2 x 45 min) followed by a capping step.

Since two truncated peptides corresponding to the residues 9-20 and 10-20 were obtained in our previous attempt, for our third attempt we incorporated Glu8 and Asp9 using HATU/HOAt as coupling agents while the reminder of the amino acids where incorporated using HCTU/HOCt, double coupling followed by capping. The crude HPLC trace showed

some improvement in that the largest major peak (t_R = 30.73 min) corresponded to peptide **2.49** (**Figure 2.39**). However, many other peptides were present indicating that incorporation of other amino acids besides residues 8 and 9 were challenging.

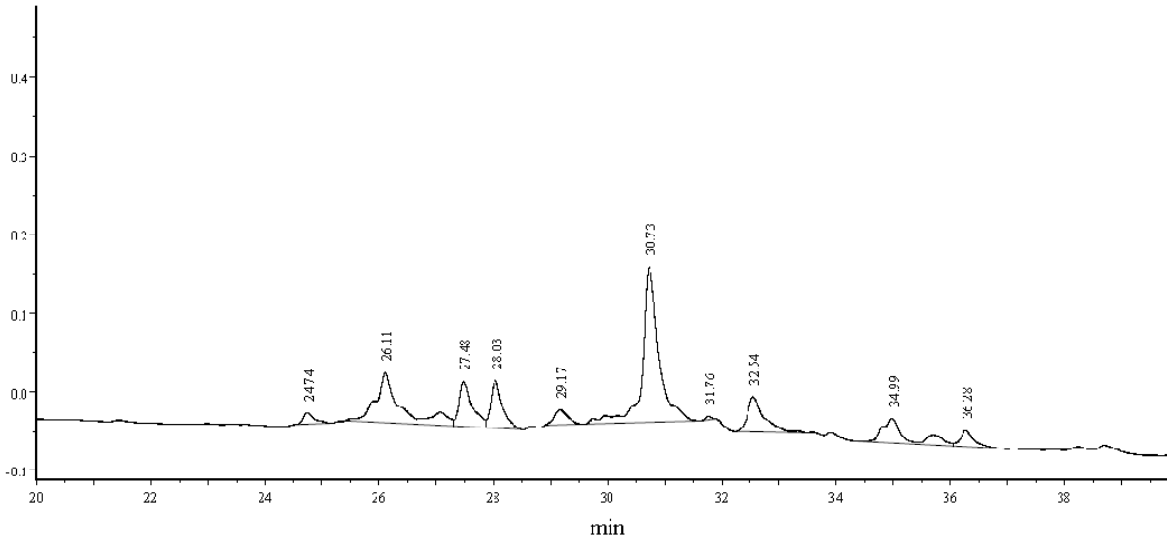


Figure 2.39. HPLC chromatogram of the material obtained after cleavage from the support from our third attempt to prepare peptide **2.49**. Glu8 and Asp9 were incorporated using using HATU/HOAt as coupling agents while the reminder of the amino acids where incorporated using HCTU/HOCt.

One of the major problems involved 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. Over the years a number of techniques have been developed to overcome the problem of aggregation such as the use of mixed solvent systems for resin swelling and coupling,[128-131] running the reactions at elevated temperature,[132] adding chaotropic salts to the solvent,[133-136]

and replacing the hydrogen of some of the amide moieties with a dimethoxybenzyl group. [137, 138] More recently, a unique approach involving tethering an amido group of the amide backbone with the side chain hydroxyl group of a serine or threonine residue via an aldehyde or ketone to form oxazolidines has been developed.[139-141] The formed oxazolidines have structural features analogous to proline and are thus called "pseudoproline". When pseudoproline is incorporated into a peptide chain it induces a kink in the peptide backbone which disrupts the intermolecular or intramolecular aggregations commonly experienced during peptide synthesis and this can result in an improvement in the yield of the peptide (**Figure 2.40**). 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.

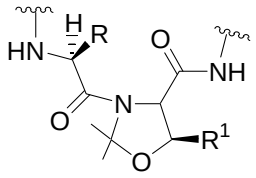
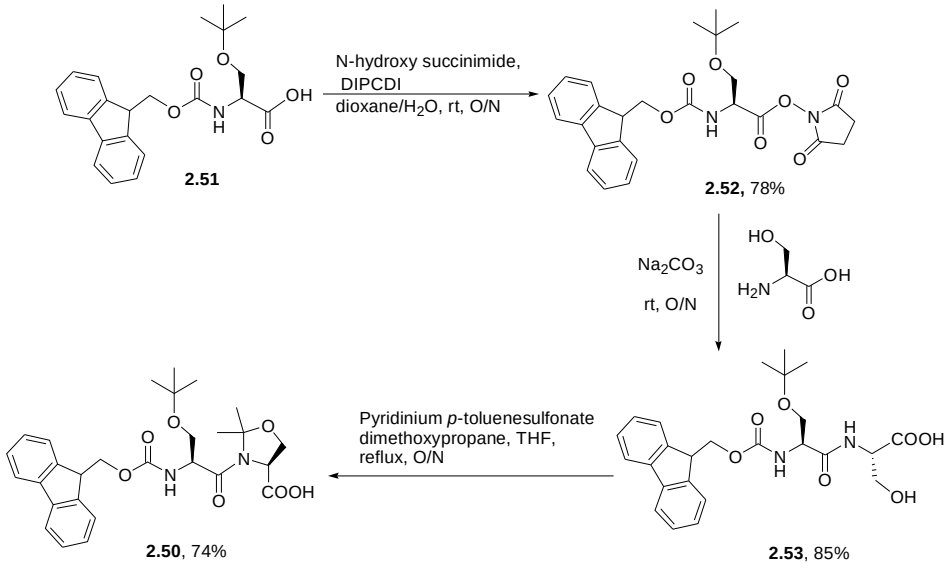


Figure 2.40. Incorporating pseudoproline ($\Psi^{Me,Me}$Pro shown) into a peptide induces a kink in the peptide backbone. R^1 = H (if derived from serine) or R^1 = CH_3 (if derived from threonine).

Consequently we decided to incorporate one of these pseudoproline residues during the synthesis of the **2.49**. There are four possible sites within **2.49** where pseudoproline can be incorporated: Ser13, Ser14, Ser18, and Ser19. Because the latter two are near the C-terminus it would be of minor beneficial effect to replace either of them with pseudoproline. So we decided to incorporate the pseudoproline at residues 13 or 14. Pseudoprolines are

incorporated into peptides as dipeptides: Fmoc-AA-ΨPro-OH. FmocSer(tBu)$\Psi^{Me,Me}$ProOH (**2.50**, **Scheme 2.13**) is a known compound and is even commercially available. Therefore we elected to replace Ser14 with $\Psi^{Me,Me}$Pro. Although **2.50** is commercially available it is very expensive so we decided to synthesize it according to **Scheme 2.13**.[139-141] The succinimide ester of FmocSer(O^{t}Bu)OH (**2.52**) was prepared through the reaction of the amino acid **2.51** with *N*-hydroxysuccinimide in the presence of diisopropylcarbodiimide (DIPCDI). Compound **2.52** coupled smoothly with unprotected serine to form dipeptide FmocSer(O^{t}Bu)SerOH (**2.53**) in a good yield. Reaction of **2.53** with 2,2-dimethoxypropane in the presence of pyridinium *p*-toluenesulfonate gave pseudoproline dipeptide **2.50** in 74% overall yield.



Scheme 2.13. Synthesis of FmocSer(tBu)$\Psi^{Me,Me}$ProOH (**2.50**).

Our fourth attempt to synthesize **2.49** employed conditions that were the same as our third attempt except dipeptide **2.50** was used. Interestingly, the use of pseudoproline **2.50** resulted in a truly dramatic improvement in the quality of crude **2.49** as indicated by the HPLC trace of the crude product which showed a single major peak corresponding to our target compound (**Figure 2.41**) as well as a few other minor peaks. We also repeated the synthesis using HATU/HOAt to incorporate pseudoproline **2.50** (2 x 20 min) while the rest of the amino acids were incorporated using HCTU/HOCt (2 x 20 min) and the results were almost identical to those shown in **Figure 2.41**.

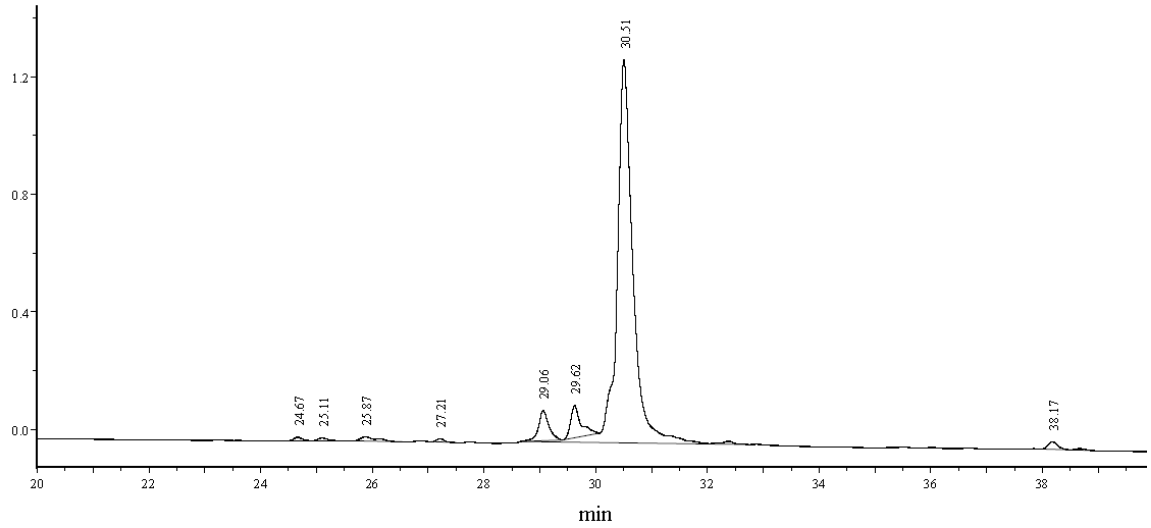


Figure 2.41. Analytical HPLC chromatogram of the material obtained after cleavage from the support from our fourth attempt to prepare peptide **2.49**. Dipeptide **2.50** was used. Double couplings (2 x 45 min) were performed and HATU/HOAT was used to incorporate Glu8, Asp9 and dipeptide **2.50** and HCTU/HOCt for the incorporation of all other residues. A capping step was also performed at the end of each coupling.

The removal of the DCV groups in **2.49** was achieved using 50% w/w $Pd(OH)_2$ under H_2 atmosphere (balloon pressure) in water/methanol (1:1) containing 11 equivalents of Et_3N and stirring for 24 h. The reaction mixture was centrifuged and the crude peptide was collected through decantation of the supernatant. The HPLC trace of the crude peptide composed of mainly of one major peak corresponding to our wanted compound and there is no sign of desulfation, attack of the 2-MP at sulfur atom of sulfate and/or oxidation of methionine. After HPLC purification (**Figure 2.42**), peptide **2.48** was obtained in a very respectable 41 % overall yield (> 97 % purity).

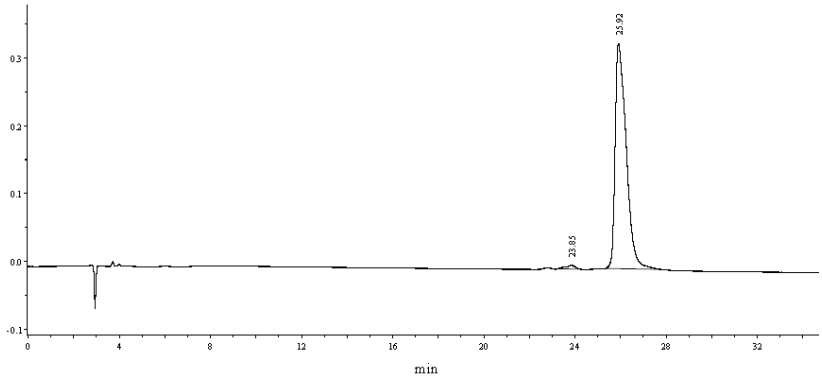


Figure 2.42. Analytical HPLC chromatogram of pure AcMAEHDsYHEDsYGFSSFNDSSQNH$_2$ (**2.48**).

2.3.13 Synthesis of DARC$_{8-42}$

For the synthesis of a Trp-containing peptide we decided to construct a peptide corresponding to residues 8-42 of the *N*-terminus region of the chemokine receptor DARC (see Chapter 1, section 1.2.2.2 for a discussion on DARC): AcAELSPSTENS$_{17}$S$_{18}$QLDFEDV**W**$_{26}$NS$_{28}$S$_{29}$**s**$\mathbf{Y}_{30}$GVNDSFPDGDs$\mathbf{Y}_{41}$DNH$_2$ (**2.54**).

Peptide **2.54** is 35-residue peptide containing sTyr residues at positions 30 and 41 as well as Trp at position 26. To our knowledge, no *sulfated* peptides corresponding to any part of the *N*-terminal region of DARC have been synthesized. If successful, we believe that this would be the largest multisulfated peptide ever made.

Our strategy for synthesis of DCV-protected precursor to peptide **2.54**, peptide AcAELSPSTENS$_{17}$S$_{18}$QLDFEDVWNS$_{28}$S$_{29(\mathbf{DCV})}$**Y**GVNDSFPDGD$_{(\mathbf{DCV})}$**Y**D-NH$_2$ (**2.55**), was to use HATU/HOAt to incorporate pseudoproline dipeptide **2.50** at positions 17 and 18 (Ser17-Ser18) and 28 and 29 (Ser28-Ser29) while incorporating all other amino acids using HCTU/HOCt. Double coupling (2 x 20 min) and capping after each coupling would also be employed. Again, Fmoc deprotection and cleavage from the support were done using our usual protocols (using 2-MP for Fmoc removal (3 x 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 2.43**). Interestingly, the ratio between the two peaks and appearance of new peaks occurred upon incubation of the peptide mixture in ammonium acetate buffer at pH = 9 for several hours. Upon HPLC separation and MS analysis of these two peaks we found that the peak at t_R= 23.7 min corresponded to peptide **2.55** while the peak at t_R= 26.3 differed from **2.55** by just 18 mass units suggesting loss of a water molecule.

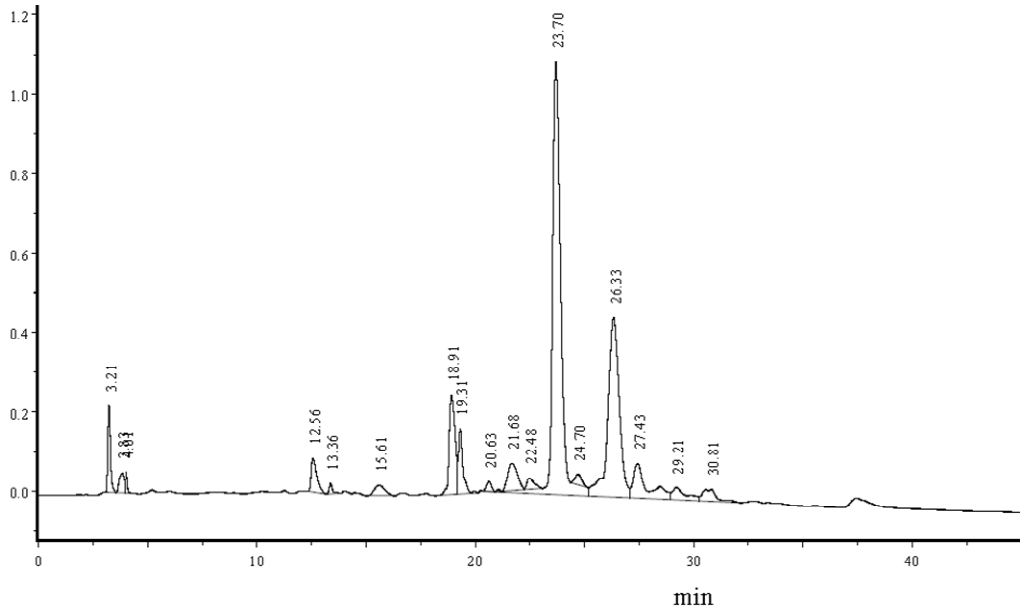
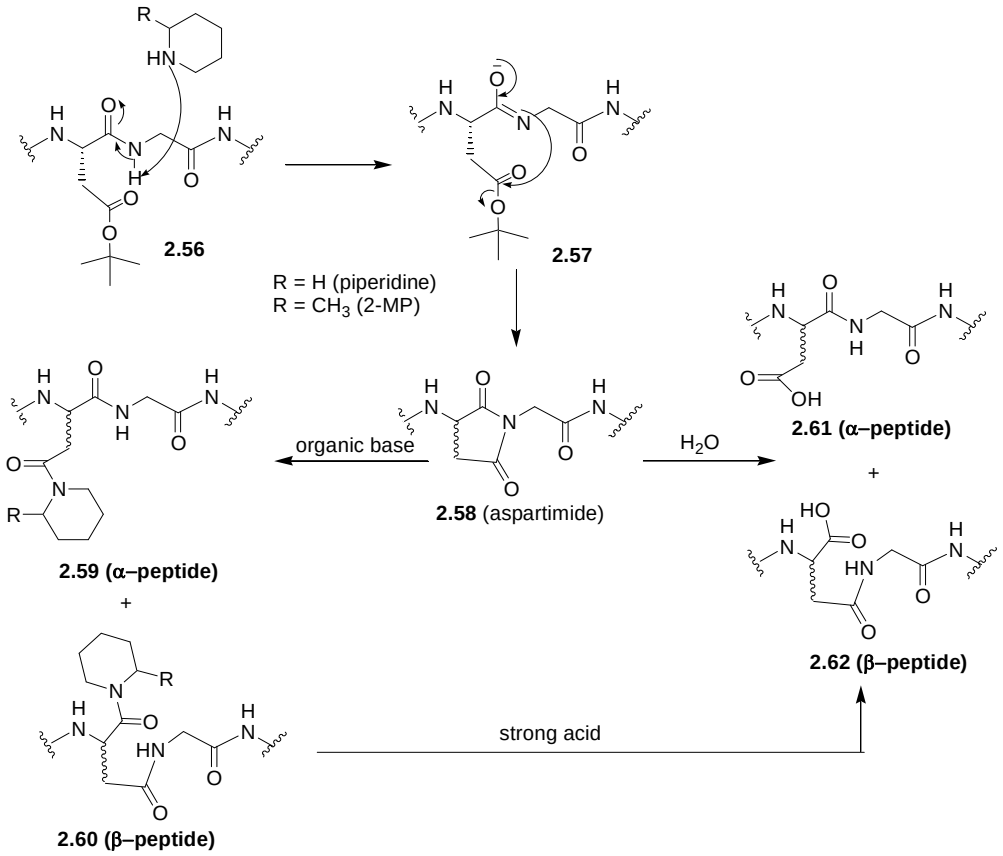


Figure 2.43. Analytical HPLC chromatogram of the material obtained after cleavage from the support from our first attempt to prepare peptide **2.55**. HATU/HOAt was used to incorporate pseudoproline dipeptide **2.50** at positions 17 and 18 (Ser17-Ser18) and 28 and 29 (Ser28-Ser29). For all other amino acids HCTU/HOCt was used as coupling agent. Double coupling (2 x 20 min) and capping after each coupling were also employed.

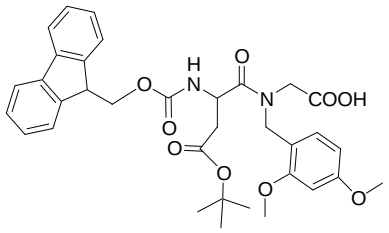
Upon close inspection of the target sequence we noticed that Asp38 was on the N-terminus side of Gly39 (Asp38-Gly39). Asp-Gly sequences within peptides are known to be very susceptible to aspartimide formation as outlined in **Scheme 2.14**.[142] The reaction involves attack of the nitrogen of the amide backbone on the carbonyl of the Asp residue and formation of the aspartimide (**2.58** in **Scheme 2.14**). This reaction occurs most readily during peptide synthesis when the Asp residue is protected (as illustrated in S**cheme 2.14**). The presence of a *tert*-butyl protecting group (as opposed to a benzyl ester protecting group) on the Asp side chain is known to help suppress but not completely prevent this reaction.[143] The reaction is base-catalyzed and so this can be a major problem during Fmoc-based SPPS. However, this reaction is also known to take place even when the Asp side chain is

unprotected such as during peptide cleavage from the support and it has even been reported that this can even occur during storage in a buffered solution.[142] The rate of the reaction depends very much on what amino acid is C-terminal to the Asp residue but occurs most readily if that amino acid is glycine.[142] When this reaction does occur during Fmoc-based SPPS peptide synthesis the resulting aspartimide can react with piperidine (if piperidine is used for Fmoc removal) to give tertiary amides (**2.59** and **2.60** in **Scheme 2.14**). Depending on what cleavage cocktail is used (and exposure time), the resulting tertiary amide can be hydrolyzed to the desired α-Asp-containing peptide and to the undesired β-Asp peptide (**2.61** and **2.62** in **Scheme 2.14**) but this does not occur very readily. The aspartimide can also be hydrolyzed to the α-Asp and β-Asp peptides. The aspartimide residue is also quite susceptible to epimerization.[143] Because peaks at t_R= 23.7 and 26.3 min (**Figure 2.43**) correspond to that of peptide **2.55** and **2.55** minus water (or something with a MW = 18), this suggests that imide formation occurred during treatment with the cleavage cocktail or 2-MP is simply too sterically hindered to attack the imide and the imide survived the peptide synthesis. We should also point out that Asn residues can also undergo succinimide formation in the presence of acid and this is also sequence dependent and occurs most readily with Asn-Gly sequences. However, if this occurred in our peptide then we would have seen in the mass spectrum a peptide that was *17* mass units less than **2.55**.



Scheme 2.14. Mechanism of aspartimide formation using an Asp-Gly peptide an example.

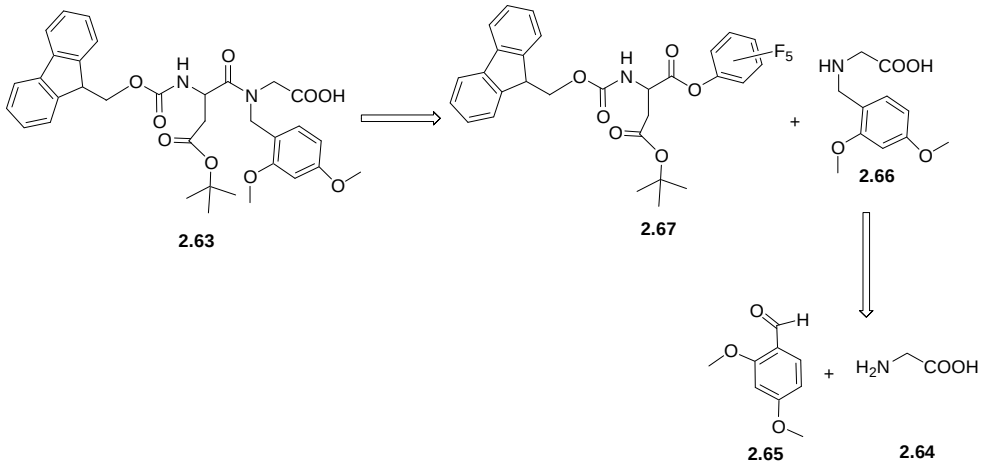
Although there is more than one way to avoid aspartimide formation including adding HOBt and dinitrophenol (DNP) to the piperidine/DMF solution and/or protecting the β-carboxy side chain with very bulky groups such as the 1-adamantyl (1-Ada) or 3-methylpent-3-yl (OMpe) groups, apparently the most effective way is *N*-backbone protection with the 2-hydroxy-4-methoxybenzyl (Hmb) group or, preferably, the 2,4-dimethoxybenzyl (Dmb) group.[142, 143] Consequently, we decided to attempt to overcome the proposed aspartimide formation problem by incorporating residues Asp38 and Gly39 as the protected dipeptide FmocAsp(O^{t}Bu)Gly(DMB)OH (**2.63**, **Figure 2.44**).



2.63

Figure 2.44. Structure of FmocAsp(O^{t}Bu)Gly(DMB)OH (**2.63**).

Dipeptide **2.63** is commercially available from Novabiochem. However, it is very expensive so we decided to prepare it ourselves. Surprisingly, there is only one report showing the synthesis of dipeptide **2.63** without much detail about the synthesis and characterization of the intermediates and final product.[144] The synthesis of **2.63** involved forming known amine **2.66** by a reductive amination reaction between glycine (**2.64)** and aldehyde **2.65**, and then reacting amine **2.66** with aspartate derivative **2.67** (**Scheme 2.15**).



2.63 **2.67** **2.66** **2.65** **2.64**

Scheme 2.15. Retrosynthesis of dipeptide **2.63**.

Compound **2.66** has been prepared by Sasaki *et al.* in a 38% yield by reacting glycine (**2.64**) with aldehyde **2.65** in 50% aqueous methanol in the presence of Pd/C and H_2.[145] We were able to repeat this reaction obtaining **2.66** in an identical yield. Several attempts to improve the yield using $NaBH_4$ as reductant (used in the preparation of the Hmb analogue of **2.66**[146]) or using ethyl or benzyl glycine esters as substrates and using $NaCNBH_3$ or $NaBH(OAc)_3$ as reductants[147, 148] either failed or gave the amine products in low yields. Coupling of **2.66** with pentafluorophenyl (Pfp) ester **2.67** in the presence of bis(trimethylsilyl)acetamide (*BSA*) (to introduce the trimethylsilyl group as a temporary carboxyl protecting group[144]) went smoothly to give the desired dipeptide **2.63** in a good yield (71%).

Our second attempt to prepare **2.55** was performed under the same conditions as our first attempt except dipeptide FmocAsp(OtBu)Gly(Dmb)OH (**2.63**) was substituted for Asp38 and Gly39. The HPLC trace of the crude product after cleavage from the resin indicated little or no aspartimide formation had occurred as indicated by the absence of a significant peak at t_R= 23.7 min (**Figure 2.45**). Removal of the DCV groups was achieved by subjecting crude **2.55** to 50% w/w of $Pd(OH)_2$ in the presence of Et_3N (17 equiv), and H_2 (balloon pressure) in water:methanol (1:1) for 24 h. After workup, the HPLC trace of the crude peptide showed mainly one major peak corresponding to our wanted compound and there was no sign of desulfation, attack of the 2-MP at the sulfur atom of the sulfate and/or tryptophan destruction (**Figure 2.46**). After HPLC purification, we obtained peptide **2.54** in 32% yield and in 98% purity (**Figure 2.47**).

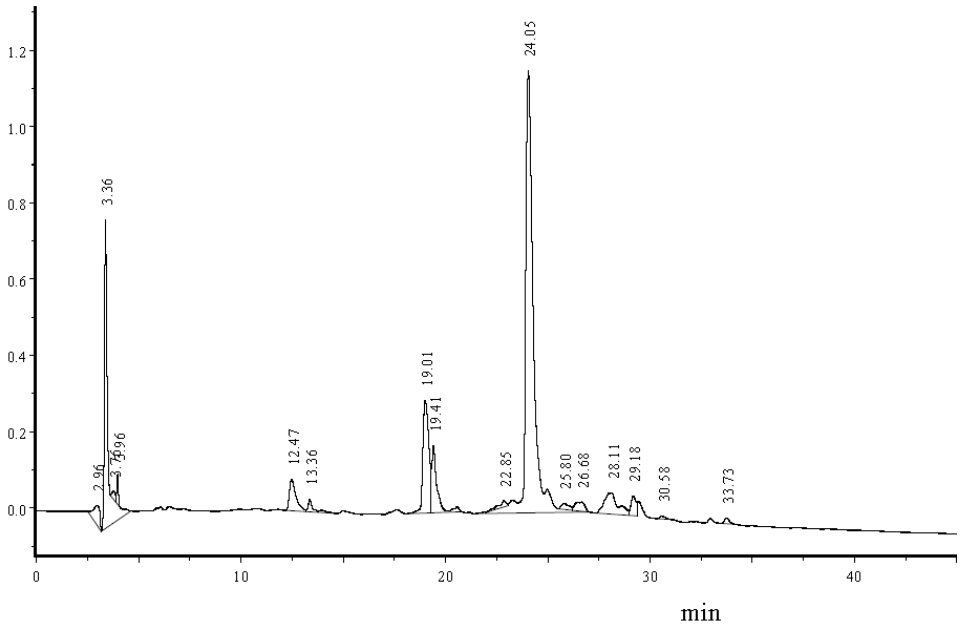


Figure 2.45. HPLC chromatogram of the material obtained after cleavage from the support from our second attempt to prepare peptide **2.55**. Conditions were identical to those used during our first attempt except FmocAsp(OtBu)Gly(Dmb)OH (**2.63**) was substituted for Asp38 and Gly39.

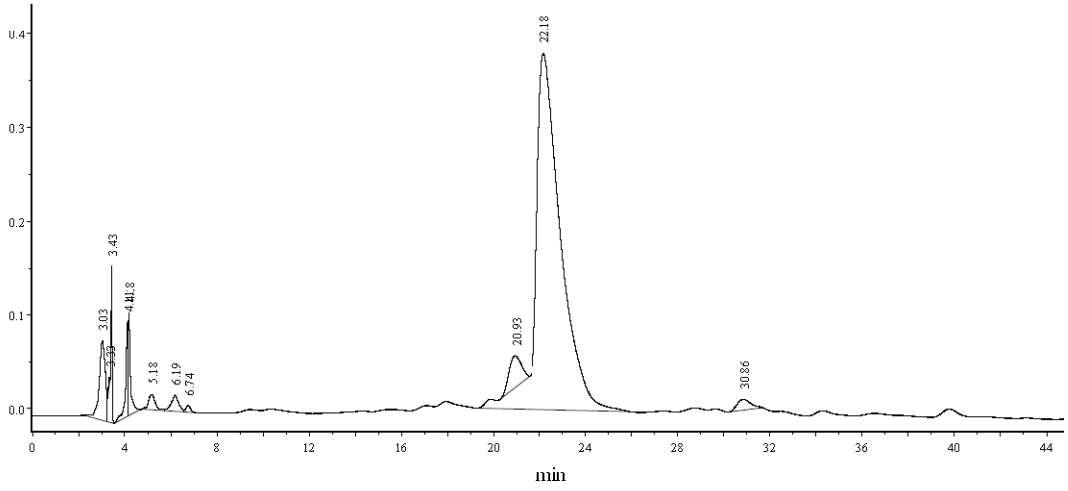


Figure 2.46. Analytical HPLC chromatogram of the crude peptide **2.54**.

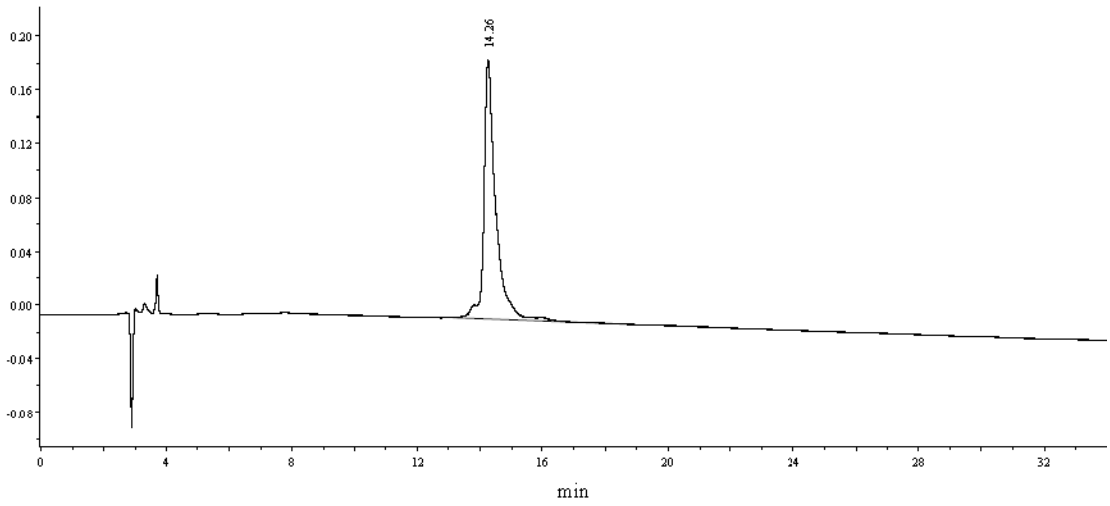


Figure 2.47. Analytical HPLC chromatogram of purified peptide **2.54**.

2.3.14 Shorter Fmoc deprotection times with 2-MP and using piperidine instead of 2-MP.

So far we have been removing the Fmoc group by subjecting the peptide to 3 x 10 min of 20% 2-MP in DMF, the total reaction time being 30 minutes. This reaction time was chosen 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.[101] When using piperidine to remove the Fmoc group a 2 x 10 minute (total time 20 min) treatment is standard. Hence we do a 30 minute treatment using 2-MP. However, we never examined whether a shorter reaction time would suffice. Some researchers use reaction times shorter than 20 minutes, such as in two steps of 3 min and 11 min each, to remove Fmoc groups during SPPS using piperidine.[108] To determine if shorter reaction times could be employed using 2-MP we prepared the 12-residue crude peptide AcISDRD$_{(DCV)}$YMGWMDF-NH_2 (**2.56**) which (when the DCV group is removed)

corresponds to residues 1-12 in CCK-12. Cholecystokinin (CCK), originally isolated from porcine intestine as a 33-residue peptide, has been shown to display various biological effects including stimulation of both pancreatic exocrine secretion and gallbladder contraction. We set up two parallel experiments. In both we used 2-MP for Fmoc removal except in one we used our standard 3 x 10 min deprotection time and the other we used a 3 min + 11 min deprotection time. After cleavage from the resin, the crude peptides were examined by analytical HPLC (**Figures 2.48 A** and **B)**. The chromatograms are identical indicating that shorter Fmoc deprotection times could be used with 2-MP.

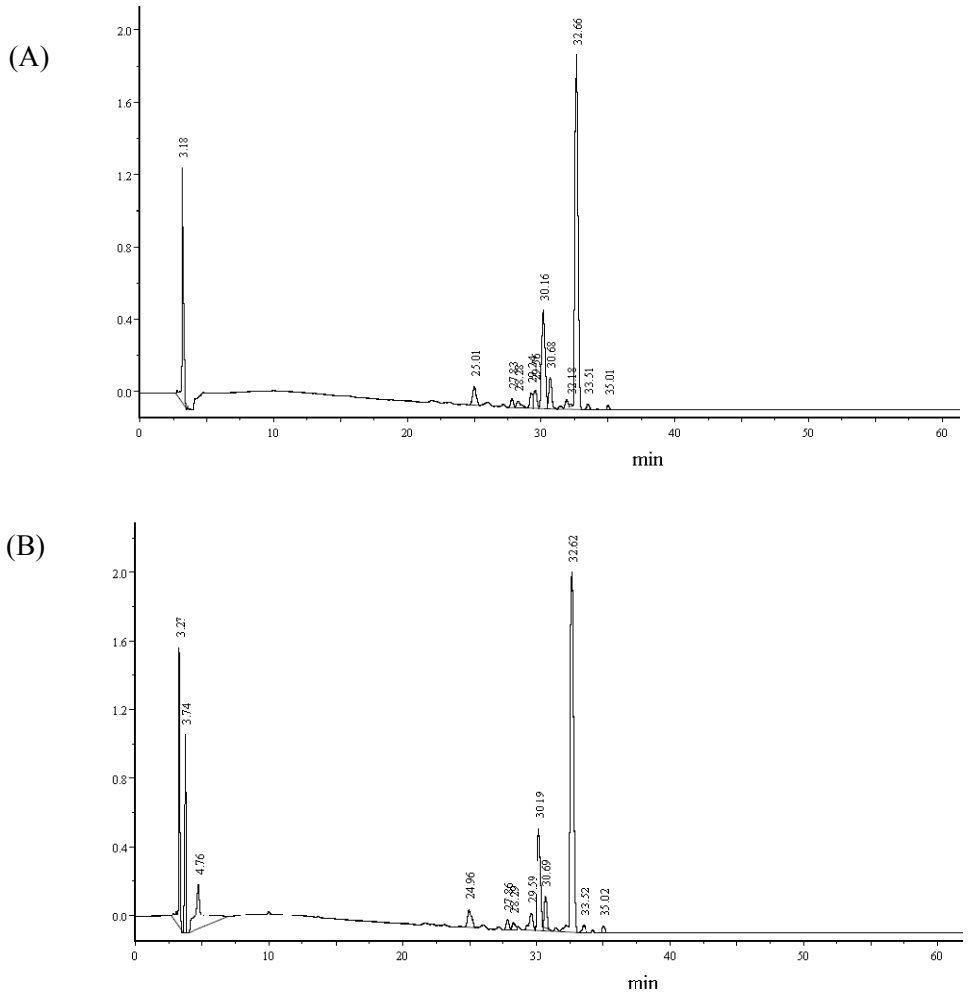


Figure 2.48. Analytical HPLC chromatogram of crude peptide **2.56.** A: Removing the Fmoc groups using 2-MP and a 3 x 10 min protocol. B: Removing the Fmoc groups using 2-MP and a 3 min + 11 min protocol.

Since piperidine reacts with DCV-protected sulfates by attack on the sulfur atom and displacement of the DCV group, we had assumed that piperidine could not be used with our protocol for preparing sTyr peptides (see section 2.3.2). However, Hari and Miller have shown that resin-supported (Wang) sulfonates exhibit superior stability to organic bases compared to their solution counterparts.[149] This report prompted us to examine whether piperidine could be used in place of 2-MP when using the DCV sulfate protecting group for

sTyr peptide synthesis. Thus, the CCK-12 peptide (**2.56**) was prepared using the same protocol as described above except piperidine was used for Fmoc removal using a 3 min + 11 min protocol. The HPLC trace of the crude peptide (**Figure 2.49**) is identical to those obtained using 2-MP (**Figures 2.48 A** and **B**). The mass spectrum of the crude peptide indicated that no product was formed resulting from attack of piperidine at sulfur. Although these results suggest that the resin-supported DCV sulfates are not as sensitive toward piperidine as we initially thought, it is important to point out that this study was done on only a 12-mer peptide and the DCV-protected sTyr residue was not incorporated until the seventh residue. Thus the DCV group was only exposed to a total of 84 min to piperidine. It is possible that during the synthesis of larger sTyr peptides where the DCV-protected tyrosine residue is incorporated at an early stage in the synthesis, some loss of the DCV group will occur. During our stability studies on DCV ester **2.14** (section 2.3.2) in 20% piperidine/DMF-d_7 we noted that **2.14** did not begin to exhibit significant decomposition until the 4 h mark. So if we use the shorter deprotection times (3 min + 11 min) as discussed above, it should be possible to use piperidine for Fmoc removal and incorporate 17 residues after the incorporation of the first DCV-protected sTyr residue before significant problems appear. If the resin is indeed capable of protecting the DCV esters to some extent then it may be possible to incorporate even more than 17 residues before significant decomposition of the DCV group appears. Further studies will be required to ascertain if this is indeed the case. In any case, as discussed in section 2.3.16 (see below), there is really no advantage to using piperidine over 2-MP during Fmoc-based SPPS.

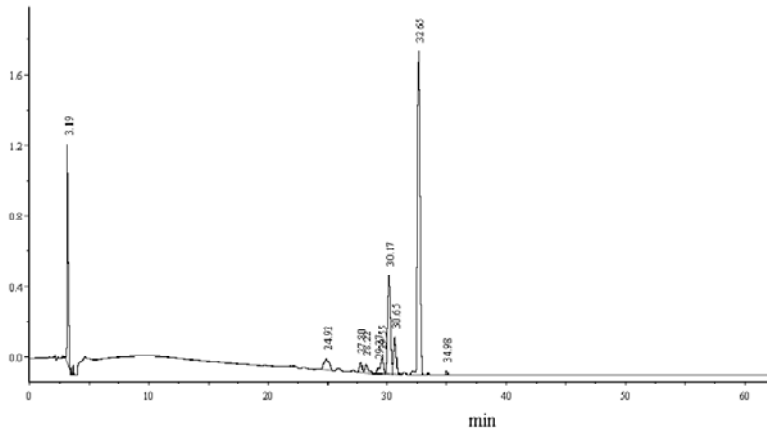
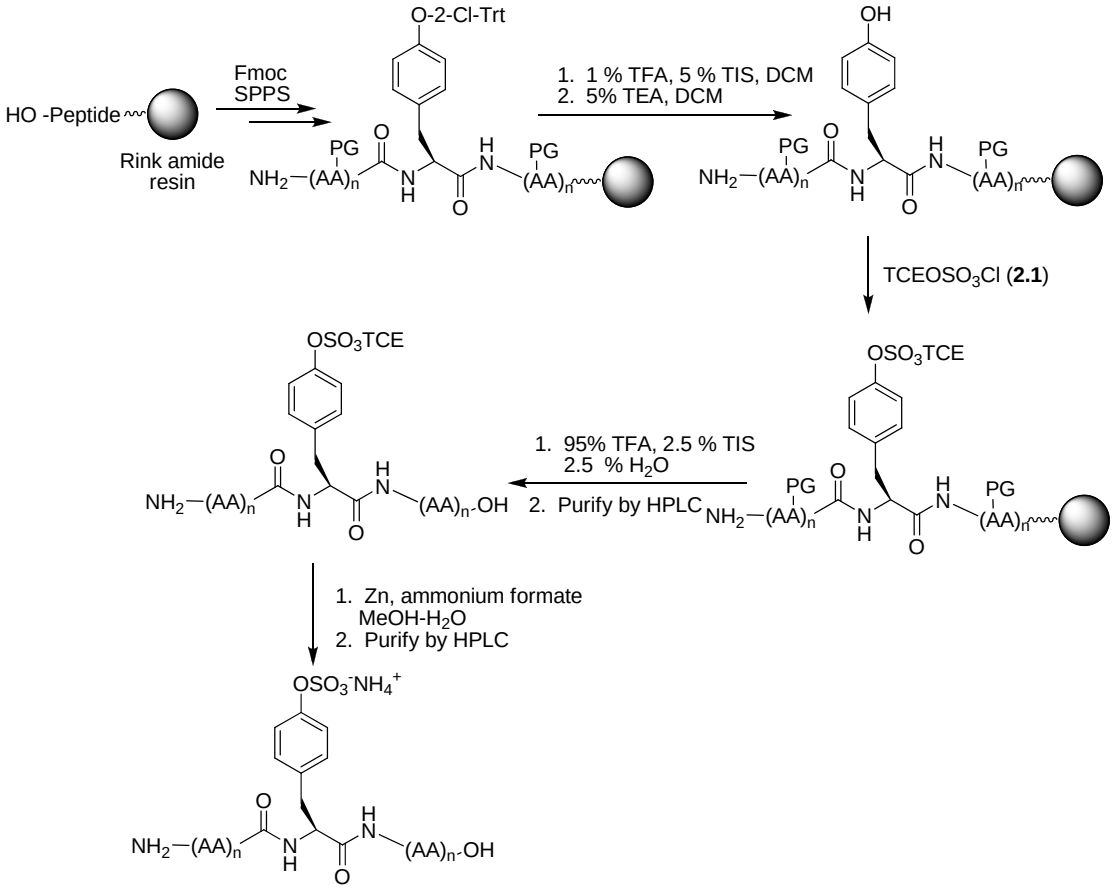


Figure 2.49. Analytical HPLC chromatogram of crude peptide **2.56**. The Fmoc groups were removed using piperidine (3 min + 11 min protocol).

2.3.15 Recent work by other groups on the synthesis of sTyr peptides.

While our work was in progress, two other groups reported new approaches to the Fmoc-based SPPS of sTyr peptides. One was reported by the Liskamp group at the University of Utrecht in the Netherlands.[150] Their approach is outlined in **Scheme 2.16**. In this approach the tyrosine residues that are to eventually be sulfated are introduced with their phenolic hydroxyl groups protected with the highly acid labile 2-chlorotrityl (2-Cl-Trt) group. After the peptide is constructed the 2-Cl-Trt group is selectively removed using 1 % TFA, 5% TIS in DCM which leaves all other side chain protecting groups intact and the peptide bound to the resin. The resulting peptide is then sulfated using our sulfating agent **2.1**. The peptide is then cleaved from the resin and the protecting group on the side chains of the other amino acids are removed using 95% TFA, 2.5% TIPS, 2.5% H_2O. The resulting peptide is purified by HPLC. The TCE group is then removed using Zn/ammonium formate and the final peptide purified by HPLC. Using this procedure they synthesized mono and

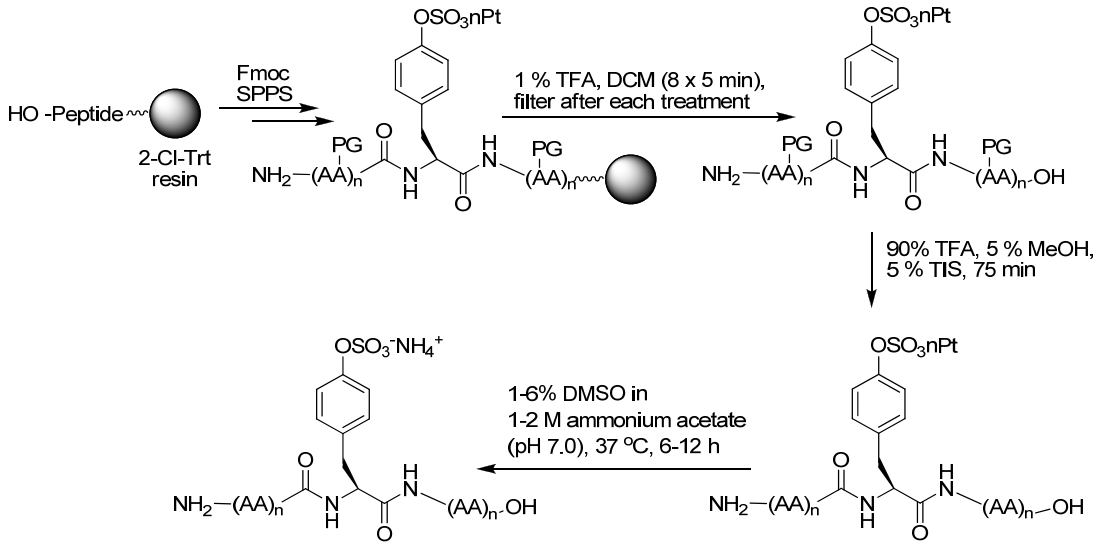
disulfated peptides corresponding to residues 7-28 in C5aR (peptide **2.41**). No yield was reported and no HPLC traces were given of the crude peptides after cleavage from the support. Although this procedure appears to work well in that the desired peptides were obtained in good purity, it requires two extra manipulations of the peptide (removal of the 2-Cl-Trt group, sulfation of the tyrosine residues) compared to our procedure. Moreover, the peptides had to be purified by HPLC twice: after introduction of the TCE-protected suflate group and after its deprotection.



Scheme 2.16. Liskamp's approach to the synthesis of sTyr peptides.

The second recently reported approach, developed by Simpson and Widlanski at Indiana University in the USA, is very similar to our approach in that the sulfate group is introduced at the beginning of the synthesis as a protected sulfodiester.[108] The neopentyl (nPt) group is used to protect the sulfate moiety. FmocTyr(OSO_3nPt)OH, which is used to introduce the protected sTyr residue, is prepared by subjecting BocTyrOtBu (**2.43**) to one equiv NaHMDS at -78 ^oC followed by the addition of $(CH_3)_3CCH_2OSO_2Cl$ then allowing the mixture to warm to rt followed by purification by flash chromatography. The resulting fully protected material, BocTyr(OSO_3nPt)OtBu, is then subjected to aq. TFA and then Fmoc-OSu to give FmocTyr(OSO_3nPt)OH in an overall yield of 66% (compared to 75% yield we obtained for our key building block (**2.23**) using the procedures outlined in **Scheme 2.10.**). Fmoc-based SPPS is used to incorporate the protected sTyr residue and piperidine is used for Fmoc removal (two treatments of 3 min + 11 min) (**Scheme 2.17**). After cleavage from the support, the nPt group is removed by dissolving the crude peptide in 0.5-3.0 mL of DMSO and then adding 50 mL of 1-2 M ammonium acetate (pH 7.0) and heating for 6-12 h at 37 ^oC. This results in loss of the nPt group by an assisted S_N1 reaction with methyl participation and rearrangement. The peptide is purified by loading the entire mixture (> 50 mL) directly into an HPLC equipped with a 2.0 cm x 25 cm reversed-phase column. Using this procedure and mainly manual SPPS they prepared mono and disulfated peptides corresponding to residues 8-23 of chemokine receptor CCR3, the CCK-12 sulfated peptide and peptide **2.35** in a very good yields and purity. For these syntheses, the highly acid labile 2-chlorotrityl resin (2-Cl-Trt, which gives carboxyl C-termini) or the Sieber amide resin (which gives carboxamide C-termini) were used and the peptides were removed from the support by treating the resin-

bound peptide 8 times with 1-2% TFA in DCM (filtered after each treatment). After the final filtration the combined filtrates were concentrated and the side chain protecting groups were removed by treating the peptides with 90% TFA, 5% MeOH. 5% TIPS (or 88% TFA, 4% MeOH. 54% TIPS, 4% thioanisole for Met-containing peptides). The approach was not tested using less acid labile resins such as the more commonly used (and cheaper) Rink amide resin that we used. The main advantages of this approach over ours are the very mild conditions that are used to remove the nPt group and, after removing the nPt group, being able to load the mixture directly into an HPLC for purification.



Scheme 2.17. Simpson and Widlanski's procedure for preparing sTyr peptides.

2.3.16 More about 2-MP versus piperidine for Fmoc removal

Both Liskamp's and Widlanski's procedures allow for the use of piperidine to remove the Fmoc group. Although this may appear to be an advantage over our approach, which requires the use of 2-MP for Fmoc removal (at least we suspect so for the synthesis of large

sTyr peptides), we do not think that this is an advantage. First of all, we have already shown that Fmoc deprotection times using 2-MP can be reduced from 30 minutes (3 x 10 min) to 14 minutes (3 min + 11 min). Moreover, as pointed out by Hachmann and Lebl[101] piperidine is a controlled substance. According to United States Code, Title 21, Chapter 13, Subchapter I, Part C-Registration of Manufacturers, Distributors, and Dispensers of Controlled Substances, the distribution of piperidine is carefully monitored. As pointed out by Hachmann and Lebl, piperidine is supposed to be stored in a locked cabinet with restricted access, its use has to be reported and, therefore (as Hachmann and Lebl pointed out), the use of piperidine can be a nuisance especially for peptide synthesis companies that deal with large quantities of piperidine. None of these restrictions or regulations apply to 2-MP. It is these restrictions that prompted Hachmann and Lebl to examine other bases such as 2-MP for Fmoc removal. 2-MP is about 25% cheaper than piperidine. For example, from Aldrich Chemical Co., 1.18 L (1 kg) of 2-MP (cat. # W424401, 98%, highest purity available from Aldrich) is $113.50 CDN ($ 96.00/L) while 1 L of piperidine (cat. # 104094, 99%, lowest purity and cheapest available from Aldrich) is $129.50 CDN. We obtained 2.96 L (3 kg) of 2-MP (98% pure) from Waterstone Technologies in the USA for $180.00 USD ($60.81/L) three years ago and we are still using this same batch (no further purification of it was required).

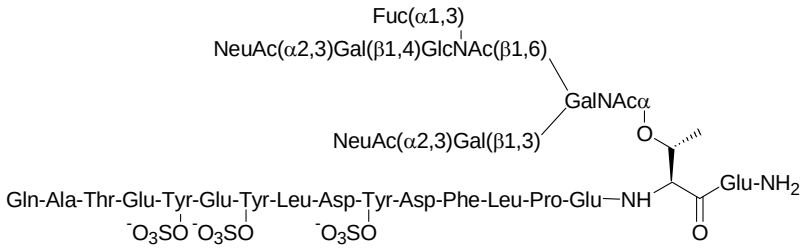
2.4 Summary, Conclusions and Future Studies

Several novel and important results were obtained from our studies. First of all, we developed a new protecting group for sulfates, namely, the dichlorovinyl (DCV) group. This was accomplished by conducting a careful analysis of the reaction of a TCE-protected sulfate ester with piperidine and 2-MP. By constructing a unique sulfuryl imidazolium reagent,

2.22, we were able to incorporate DCV-protected sulfate esters into compounds such as **2.25** to give DCV sulfate esters such as sTyr derivative **2.23**. We should point out that reagent **2.22** should allow us to incorporate DCV-protected sulfate esters into other biomolecules such as carbohydrates and steroids. Sulfated carbohydrates and steroids are important biomolecules involved in a variety of crucial biochemical processes. We also developed a more economical synthesis of amino acid **2.23** that did not require reagent **2.22**. We developed a new and general approach to Fmoc-based SPPS of sTyr peptides using amino acid **2.23** and 2-MP for Fmoc removal. We expect that with our approach, as well as with other approaches that were recently developed, sTyr peptides will now be readily accessible and their synthesis will no longer be the challenge that it has been for the last approximately 50 years. We are pleased to point out that Dr. Peter White, manager of product development at Novabiochem, the world's leading manufacturer and supplier of amino acids and peptide synthesis reagents, has contacted us and expressed a desire to commercialize amino acid **2.23**. We have also shown that 2-MP is an economical and practical replacement for piperidine during SPPS.

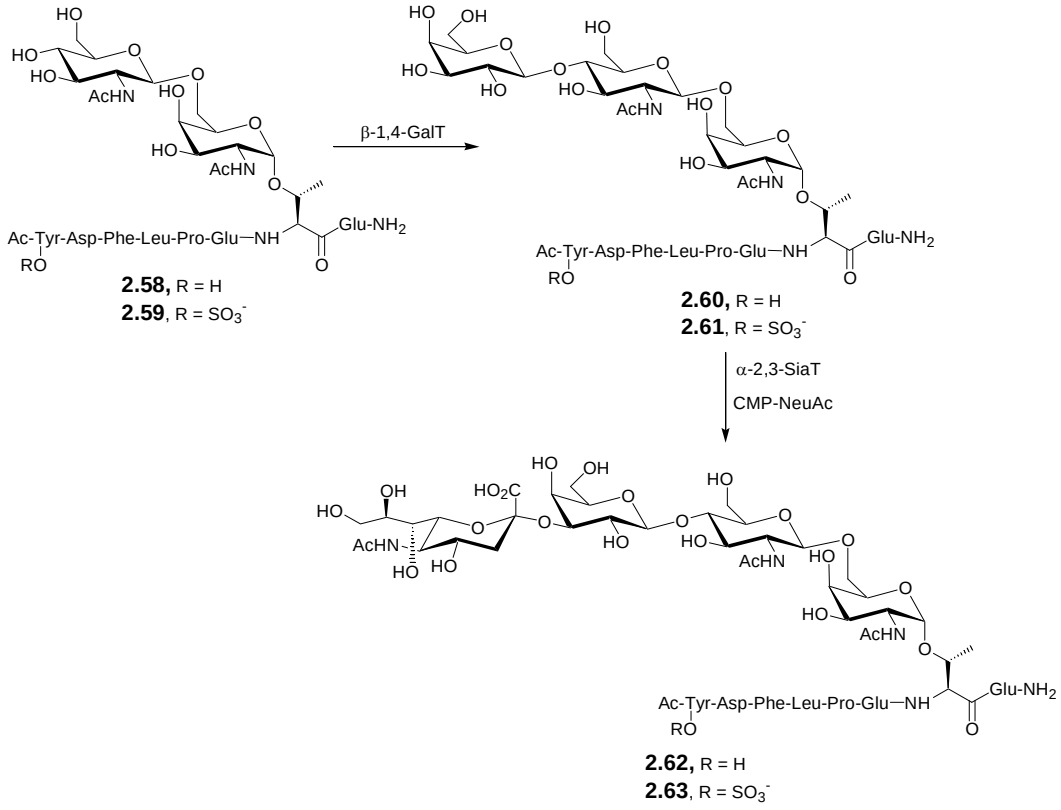
Future studies will examine whether the sTyr peptide synthesis strategy can be applied to the synthesis of peptide glycans. For example, PSGL-1 is actually a glycoprotein. Part of the *N*-terminal structure of PSGL-1 (**2.57**) is shown in **Figure 2.50**. Several groups have been tackling its synthesis. The most notable work in this area has been done by Chi-Huey Wong's group at Scripps West. Because of its complexity, the Wong group has focused on using enzymes for introducing the carbohydrate residues in the latter part of their syntheses. For example, during an attempted synthesis of glycopeptides **2.62** and **2.63** (a

partial synthesis of **2.57**) they were attempting to convert compounds **2.58** and **2.59** to compounds **2.60** and **2.61** using bovine β-1,4-galactosyltransferase (β-1,4-GalT) and then convert these compounds to **2.62** and **2.63** using rat α2,3-sialyltransferase (α-2,3SiaT) and CMP-NeuAc (**Scheme 2.18**).[151, 152] The β1,4-GalT reaction proceeded readily with nonsulfated substrate **2.58** but very sluggishly with sulfated substrate **2.59**. The α-2,3SiaT reaction also proceeded readily with nonsulfated substrate **2.60** but not at all with sulfated substrate **2.61**. A structural study indicated that there are no significant structural differences between substrates **2.58** and **2.59** and **2.60** and **2.61**. It was hypothesized that the difference in reactivity between the nonsulfated and sulfated substrates was caused by unfavorable electrostatic interactions of the anionic sulfate group on the peptide portion with the enzymes. We anticipate that such unfavorable interactions will be eliminated if the sulfate group is protected. Consequently, the Taylor group will be embarking on the synthesis of the DCV-protected analog of **2.58** and examine its ability to act as a substrate for β-1,4-GalT and α-2,3SiaT. Should these studies be successful, then removal of the DCV group would give the desired compound **2.63**. Successful results here would provide us with the impetus to prepare the fully trisulfated compound **2.64** (**Figure 2.51**).



2.57

Figure 2.50. The N-terminal structure of PSGL-1. (abbreviations: Fuc stands for fucose, GlcNAc stands for *N*-acetylglucosamine, Gal stands for galactose, NeuAc stands for *N*-acetylneuramic acid)



Scheme 2.18. The Wong's groups attempted synthesis of compound **2.63.**

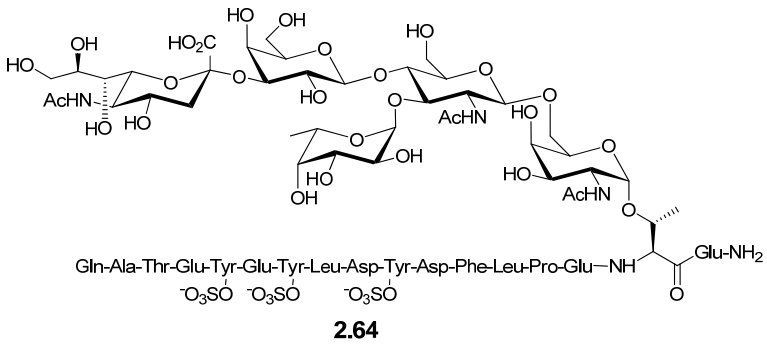


Figure 2.51. Structure of compound **2.64.**

2.5 Experimental

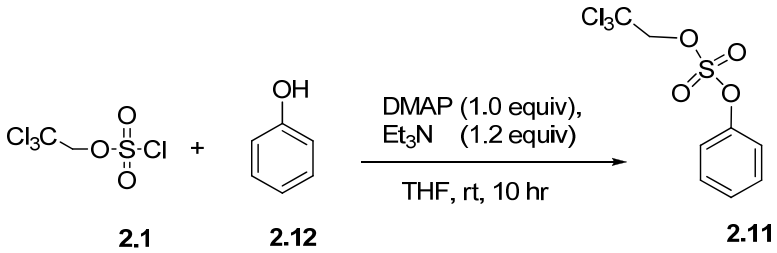
2.5.1 General Information:

All starting materials and reagents unless otherwise noted were obtained from Aldrich Chemical Company (Oakville, ON, Canada). 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). Amino acids were used for all peptide syntheses unless stated otherwise. All automated SPPS was performed using the Rink amide resin and were performed on a Quartet peptide synthesizer from Protein Technologies (USA) on a 25 μM scale. All reactions were carried out under argon with freshly distilled solvents. Tetrahydrofuran (THF) and Et_2O were distilled from sodium metal in the presence of benzophenone under argon. CH_2Cl_2 was distilled from calcium hydride under nitrogen. DMF was dried and distilled over calcium hydride under vacuum and stored over 4 angstrom sieves under argon. Flash chromatography was performed using silica gel 60Å (234-400 mesh) obtained from Silicycle (Laval, Quebec, Canada). Chemical shifts (δ) for ^1H NMR spectra run in $CDCl_3$ are reported in ppm relative to the internal standard

tetramethylsilane (TMS). Chemical shifts (δ) for ^{1}H NMR spectra run in DMSO-d_6 are reported in ppm relative to residual solvent protons (δ 2.49). Chemical shifts (δ) for ^{1}H NMR spectra run in CD_3OD are reported in ppm relative to residual solvent protons (δ 3.30). ^{1}H NMR spectra for hexa and octapeptides were run on a Brüker Avance 500 MHz instrument using D_2O as solvent and utilizing gradient water suppression method.[153] 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). ^{13}C NMR spectra run in DMSO-d_6, chemical shifts are reported in ppm relative to DMSO-d_6 (δ 39.5). ^{13}C NMR spectra run in CD_3OD, chemical shifts are reported in ppm relative to CD_3OD (δ 49.5). For the ^{19}F NMR spectra chemicals shifts are reported relative to an external fluoroform standard. Analytical and semipreparative RP-HPLC was achieved using Waters 600 controller equipped with a Waters 2487 detector. Analytical HPLC was performed with a Vydac 218TP54 C18 column (5 μm, 4.6 mm x 250 mm) and/or Higgins PROTO 200 C18 column (5 μm, 4.6 mm x 250 mm) using a 1.0 mL/min flow rate. Semi-preparative HPLC was conducted on Vydac 218TP1022 C18 column (10 μm, 22 mm x 250 mm) using an 8.0 mL/min flow rate. Electron impact (EI) 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 +ve ion work or 1:1 CH_3CN/H_2O + 0.5% ammonium hydroxide for –ve ion work.

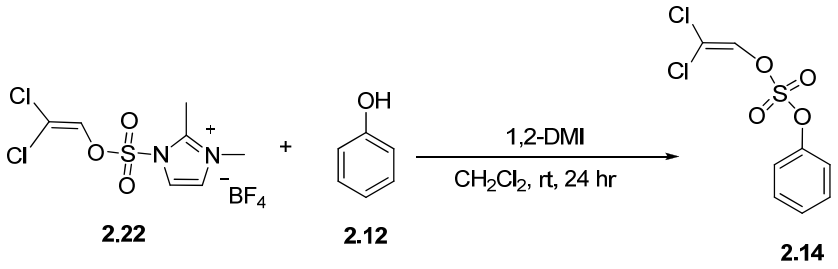
2.5.2 Synthesis of non-peptidyl compounds and dipeptides

2,2,2-Trichloroethyl phenyl sulfate (2.11).[98]



To a solution of phenol (**2.12**) (0.50 g, 5.31 mmol, 1 equiv), Et_3N (0.88 mL, 6.37 mmol, 1.2 equiv) and DMAP (0.60 g, 5.31 mmol, 1.00 equiv) in dry THF (20 mL) was added drop by drop over a period of 15 minutes a solution of 2,2,2-trichloroethoxy sulfurylchloride (**2.1**)[100] (1.50 g, 6.37 mmol, 1.20 equiv) in dry THF (10 mL). The solution was stirred for 10 h. EtOAc was added (100 mL) and the solution washed with H_2O (40 mL), 0.5 N HCl (2 x 40 mL), H_2O (40 mL) and sat. brine (40 mL). The organic layer was dried ($MgSO_4$), concentrated *in vacuo*, and the residue was subjected to flash chromatography (ethyl acetate:hexane 10:90) to yield pure compound **2.1** (1.4 g, 86%). NMR spectra were identical to that reported.[98] ^1H NMR (300 MHz, $CDCl_3$): δ 7.50-7.45 (m, 2H, H_{Ar}), 7.40-7.37 (m, 3H, H_{Ar}), 4.85 (s, 2H, H_{TCE}); ^{13}C NMR (75 MHz, $CDCl_3$): δ 150.1, 130.2, 127.9, 121.1, 92.4, 80.4.

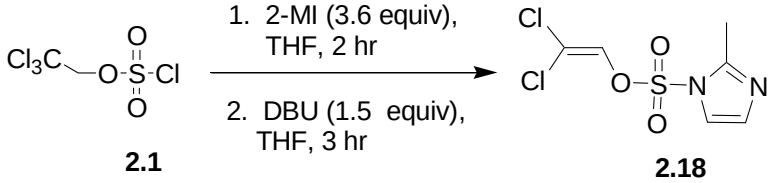
2,2-Dichlorovinyl phenyl sulfate (2.14).



2.22 **2.12** **2.14**

To a solution of phenol (**2.12**) (0.50 g, 5.31 mmol, 1.00 equiv) in CH_2Cl_2 (20 mL) at 0 ^oC was added 1,2-DMI (1.50 g, 15.9 mmol, 3.00 equiv) followed by compound **2.22** (5.70 g, 15.9 mmol, 3.00 equiv). The reaction was allowed to come to room temperature and stirred for 5 h. The reaction was then diluted with 20 mL of CH_2Cl_2, washed with sat. $NaHCO_3$ (2 x 10 mL), brine (2 x 10 mL) followed by H_2O (2 x 10 mL). The organic layer was dried ($MgSO_4$), concentrated and the residue subjected to flash chromatography (ethyl acetate:hexane, 1:9) which gave compound **2.14** as a colorless oil (1.32 g, 92% yield). ^1H NMR (300 MHz, $CDCl_3$): δ 7.46-7.41 (m, 2H, H_{Ar}), 7.38-7.35 (m, 1H, H_{Ar}), 7.33-7.29 (m, 2H, H_{Ar}), 7.15 (s, 1H, H_{DCV}); ^{13}C NMR (75 MHz, $CDCl_3$): δ 150.0, 133.6, 130.2, 128.2, 121.1, 117.3; HRMS (EI^+): calculated for $C_8H_6Cl_2O_4S$ $(M)^+$ 267.9364, found 267.9373.

1-(2,2-Dichlorovinyloxysulfuryl) 2-methylimidazole (2.18).

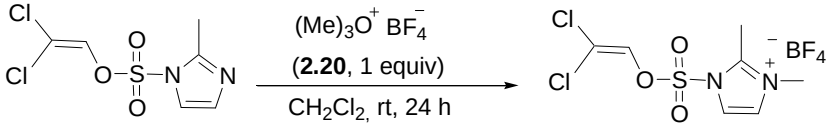


2.1 **2.18**

To a solution of 2-MI (4.00 g, 49.4 mmol, 3.60 equiv) in dry THF (28 mL) at 0 ^oC was added dropwise a solution of compound **2.1** (3.40 g, 13.7 mmol, 1.00 equiv) in dry THF (36 mL). The reaction mixture was allowed to stir for 1 h at 0 ^oC followed by 1 h at room

temperature. The reaction mixture was then filtered and the filtrate was cooled (ice bath). DBU (3.10 mL, 20.6 mmol, 1.50 equiv) was added dropwise and reaction mixture was allowed to stir for another 1 h at 0 ^{o}C followed by 2 h at room temperature. The reaction mixture was filtered after the addition of ether (130 mL) and the filtrate was washed with phosphate buffer (pH = 7.2 x 30 mL), brine (2 x 30 mL), dried ($MgSO_4$) and concentrated *in vauco*. Flash chromatography of the residue (ethyl acetate:hexane, 2:3) furnished compound **2.18** as a yellow oil (3.1 g, 88%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.21 (d, J = 1.2 Hz, 1H, H_{imi}), 6.92 (s, 1H, H_{DCV}), 6.90 (d, J = 1.2 Hz, 1H, H_{imi}), 2.59 (s, 3H, CH_{3imi}); ^{13}C NMR (75 MHz, $CDCl_3$): δ 145.8, 131.8, 127.8, 120.3, 119.5, 14.2; HRMS (EI^+): calculated for $C_6H_6Cl_2N_2O_3S$ $(M)^+$ 255.9476, found 255.9476.

1-(2,2-Dichlorovinyloxysulfuryl) 2,3-dimethylimidazolium tetrafluoroborate (2.22).



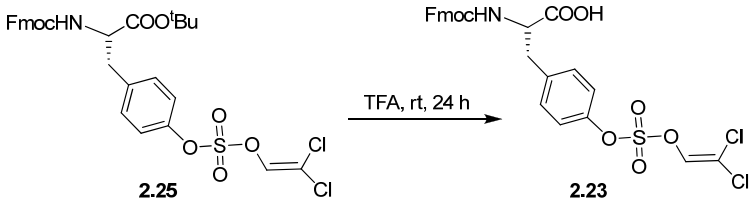
2.18 **2.22**

A solution of compound **2.18** (3.00 g, 11.7 mmol, 1.00 equiv) in CH_2Cl_2 (33 mL) was added dropwise to a suspension of trimethyloxonium tetrafluoroborate (**2.20**) (1.70 g, 11.7 mmol, 1.00 equiv) in CH_2Cl_2 (16 mL) at 0 ^{o}C. The reaction mixture is allowed to come slowly to room temperature and then stirred overnight. The solvent was evaporated *in vacuo* and the residue was triturated with hot THF (5 mL). The mixture was filtered and the filter cake was dried to give pure **2.22** (4.00 g, 95%). MP= 123-127 ^{o}C (decomp.). ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.27 (d, J = 2.4, 1H, H_{imi}), 7.98 (s, 2H, H_{imi} & H_{DCV}), 3.87 (s, 3H, $CH_{3\text{-}imi}$), 2.84 (s, 3H, $CH_{3\text{-}imi}$); ^{13}C NMR (75 MHz, DMSO-d_6): δ 149.6, 134.5, 124.5, 122.2,

121.4, 36.5, 12.2; ^{19}F NMR (282 MHz, DMSO-d_6): δ -148.04; HRMS (ESI^+): calculated for $C_7H_9Cl_2N_2O_3S$ $(M\text{-}BF_4)^+$ 270.9701, found 270.9711.

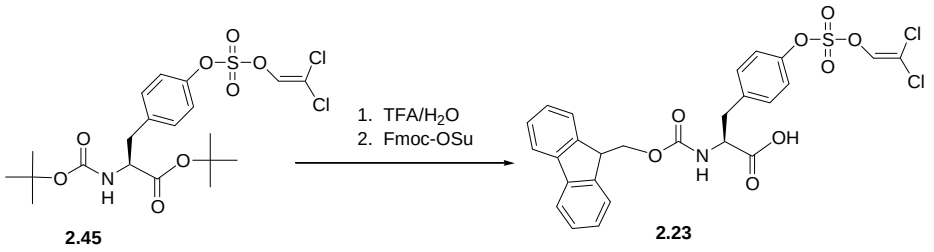
N^{α}-[(Fluoren-9-yl)methoxylcarbonyl]-L-tyrosine dichlorovinyl sulfate (2.23).

Method A: From **2.25**



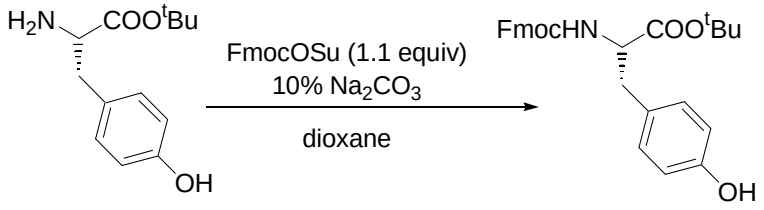
Compound **2.25** (1.30 g, 2.04 mmol, 1.00 equiv) was dissolved in trifluoroacetic acid (TFA) (5.7 mL) and the resulting solution was stirred at room temperature for 16 h. The TFA was removed under reduced pressure and the residue was subjected to flash chromatography (CH_2Cl_2:MeOH, 95:5) which gave **2.23** as a white foam (1.09 g, 92% yield). ^{1}H NMR (300 MHz, DMSO-d_6): δ 12.90 (br-s, 1H, $COOH_{Tyr}$), 8.07 (s, 1H, H_{DCV}), 7.88 (d, J = 7.5 Hz, 2H, H_{Fmoc}), 7.76 (d, J = 8.4 Hz, 1H, NH_{Tyr}), 7.66-7.63 (m, 2H, H_{Fmoc}), 7.45-7.27 (m, 8H, $H_{Fmoc,}$ and H_{Tyr}), 4.23-4.17 (m, 4H, $CH_{2\text{-}Fmoc,}$ CH_{Fmoc} and CH_{Tyr}), 3.17-2.88 (m, 2H, $CH_{2\text{-}Tyr}$); ^{13}C NMR (75 MHz, $CDCl_3$): δ 173.5, 156.4, 148.63, 144.2, 141.2, 139.1, 135.4, 131.6, 128.1, 127.5, 125.7, 125.6, 121.4, 120.5, 118.2, 66.1, 55.6, 47.1, 36.1; HRMS (ESI^+): calculated for $C_{26}H_{22}Cl_2NO_8S$ $(M+H)^+$ 578.0443, found 578.0445.

Method B: From **2.45**



Compound **2.45** (6.00 g, 11.7 mmol, 1.00 equiv) 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 aq. solution of sodium carbonate (42.0 mL, 3.72 g, 35.1 mmol, 3.0 equiv) and the mixture was cooled using an ice bath. A solution of Fmoc-OSu (5.92 g, 17.6 mmol, 1.50 equiv) in dioxane (42 mL) was added and the reaction was allowed to warm to rt then stirred overnight. The reaction mixture was acidified using 1 M HCl (to pH = 2), extracted with EtOAc (3 x 100 mL), dried ($MgSO_4$), filtered and concentrated by rotary evaporation. The residue was subjected to flash chromatography (100% CH_2Cl_2 to 5% MeOH in CH_2Cl_2) which gave pure **2.23** as off-white foam 5.75 g (85%). ^{1}H NMR and ^{13}C NMR data were identical to **2.23** prepared by method A.

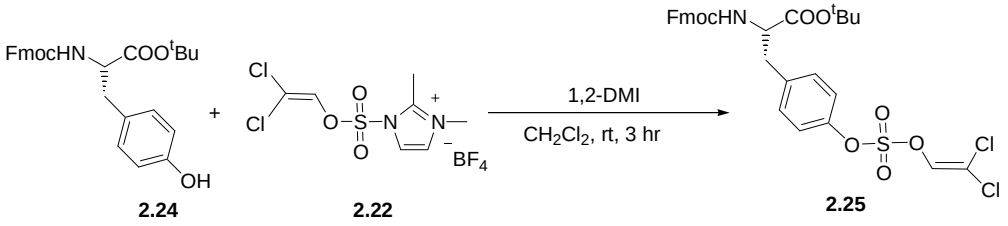
N^{α}-[(Fluoren-9-yl)methoxylcarbonyl]-L-tyrosine *tert*-butyl ester (FmocTyrO^{t}Bu, 2.24).[103]



2.24

This was prepared according to the procedure of Wang *et al*.[103] Fmoc-OSu (1.56 g, 4.63 mmol, 1.10 equiv) was added to a solution of L-tyrosine *tert*-butyl ester (1.00 g, 4.21 mmol, 1.00 equiv) in 14 mL of dioxane and 12 mL of 10% Na_2CO_3 at 0 ^{o}C (ice bath). The mixture was stirred for 4 h at 0 ^{o}C then 12 h at room temperature. The mixture was extracted with ethyl acetate (2 x 50 mL), and the combinded organics were washed with brine (2 x 20 mL) and H_2O (2 x 20 mL). The organic layer was dried (Na_2SO_4), concentrated, and the residue purified by flash chromatography (ether: hexane, 1:1) to give pure compound FmocTyrO^{t}Bu (**2.24**) (1.74 g, 90% yield). NMR spectra were identical to that reported.[103] ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.78 (d, J = 7.2 Hz, 2H, H_{Fmoc}), 7.59 (d, J = 7.2 Hz, 2H, H_{Fmoc}), 7.42 (dd, J = 7.5 and 7.2 Hz, 2H, H_{Fmoc}), 7.32 (dd, J = 7.2 and 7.5 Hz, 2H, H_{Fmoc}), 7.02 (d, J = 8.1 Hz, 2H, H_{Tyr}), 6.75 (d, J = 8.1 Hz, 2H, H_{Tyr}), 5.40 (s, 1H, OH_{Tyr}), 5.33 (d, J = 8.1 Hz, 1H, NH_{Tyr}), 4.54-4.36 (m, 3H, $CH_{2\text{-}Fmoc,}$and CH_{Tyr}), 4.22 (dd, J_1 =J_2 = 6.9 Hz, 1H, CH_{Fmoc}), 3.04 (m, 2H, $CH_{2\text{-}Tyr}$), 1.45 (s, 9H, $H_{\textit{tert}\text{-but}}$); ^{13}C NMR (75 MHz, $CDCl_3$): δ 171.0, 155.9, 155.1, 143.7, 141.3, 130.6, 127.7, 127.6, 127.1, 125.1, 120.0, 115.4, 82.6, 67.1, 55.4, 47.2, 37.6, 28.0.

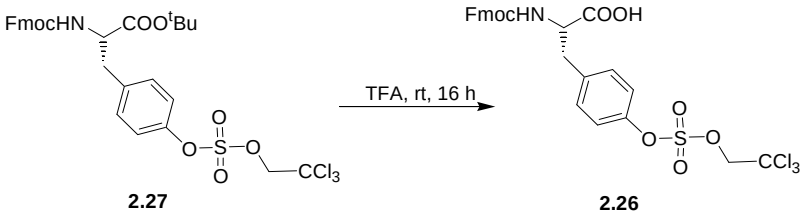
***tert*-Butyl N$^{\alpha}$-[(Fluoren-9-yl)methoxylcarbonyl]-L-tyrosine dichlorovinyl sulfate (2.25).**



2.24 **2.22** **2.25**

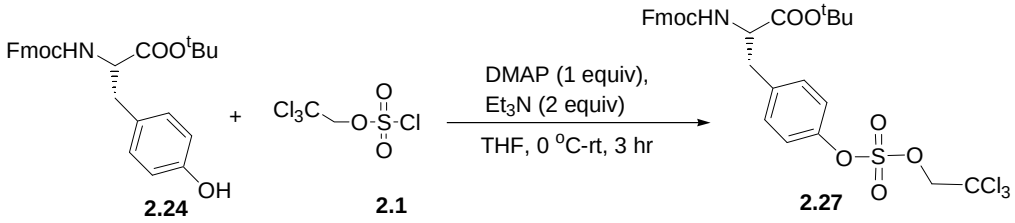
To a solution of FmocTyrO^{t}Bu (**2.24**) (1.00 g, 2.17 mmol, 1.00 equiv) in CH_2Cl_2 (8.7 mL) at 0 ^oC was added 1,2-DMI (0.62 g, 6.52 mmol, 3.00 equiv) followed by portionwise addition of compound **2.22** (2.33 g, 6.52 mmol, 3.00 equiv). The reaction mixture was allowed to come to room temperature and then allowed to stir for 3 h. The reaction mixture was diluted with another 8 mL of CH_2Cl_2, washed with brine (3 x 5 mL) and H_2O (3 x 5 mL) then dried (Na_2SO_4) and concentrated. The residue was purified using flash chromatography (ethyl acetate:hexane, 20:80) to give pure **2.25** (1.29 g, 94% yield). ^1H NMR (300 MHz, $CDCl_3$): δ 7.80 (d, J = 7.5 Hz, 2H, H_{Fmoc}), 7.61 (d, J = 7.2 Hz, 2H, H_{Fmoc}), 7.44 (dd, J_1 = J_2 = 7.2 Hz, 2H, H_{Fmoc}), 7.35 (dd, J = 7.2 and 7.5 Hz, 2H, H_{Fmoc}), 7.28-7.25 (m, 4H, H_{Tyr}), 7.18 (s, 1H, H_{DCV}), 5.39 (d, J = 7.8 Hz, 1H, NH_{Tyr}), 4.58-4.37 (m, 3H, $CH_{2\text{-}Fmoc,}$and CH_{Tyr}), 4.24 (dd, J_1 = J_2 = 6.6 Hz, 1H, CH_{Fmoc}), 3.14-4.13 (m, 2H, $CH_{2\text{-}Tyr}$), 1.44 (s, 9H, $H_{\textit{tert}\text{-but}}$); ^{13}C NMR (75 MHz, $CDCl_3$): δ 170.1, 155.4, 148.9, 143.8, 143.6, 141.3, 136.6, 133.5, 131.2, 127.7, 127.0, 125.0, 124.9, 120.9, 112.0, 119.9, 117.4, 82.7, 66.8, 54.9, 47.1, 37.8, 27.9; HRMS (ESI$^+$): calculated for $C_{30}H_{30}Cl_2NO_8S$ $(M+H)^+$ 634.1069, found 634.1061.

N$^{\alpha}$-[(Fluoren-9-yl)methoxylcarbonyl]-L-tyrosine trichloroethyl sulfate (2.26).



Compound **2.27** (0.50 g, 0.74 mmol, 1.0 equiv) was dissolved in TFA (3.3 mL) and the resulting solution was stirred at room temperature for 16 h. The TFA was removed under reduced pressure and the residue was subjected to flash chromatography (CH_2Cl_2: MeOH, 95:5) which gave pure compound **2.26** (0.41 g, 89% yield)**.** ^{1}H NMR (300 MHz, DMSO-d_6): δ 12.77 (br-s, 1H, $COOH_{Tyr}$), 7.84 (d, J = 7.2 Hz, 2H, H_{Fmoc}), 7.72 (d, J = 8.1 Hz, 1H, NH_{Tyr}), 7.63-7.59 (m, 2H, H_{Fmoc}), 7.38-7.23 (m, 8H, $H_{Fmoc,}$ and H_{Tyr}), 5.30 (s, 2H, H_{TCE}), 4.18-4.12 (m, 4H, $CH_{2\text{-}Fmoc,}$ CH_{Fmoc} and CH_{Tyr}), 3.13-2.84 (m, 2H, $CH_{2\text{-}Tyr}$); ^{13}C NMR (75 MHz, $CDCl_3$): δ 173.5, 156.4, 148.6, 144.2, 144.21, 141.17, 138.7, 131.4, 128.1, 127.5, 125.7, 125.6, 121.6, 120.5, 93.4, 80.5, 66.1, 55.7, 47.0, 36.2; HRMS (ESI$^+$): calculated for $C_{26}H_{23}Cl_3NO_8S$ $(M+H)^+$ 614.0210, found 614.0217.

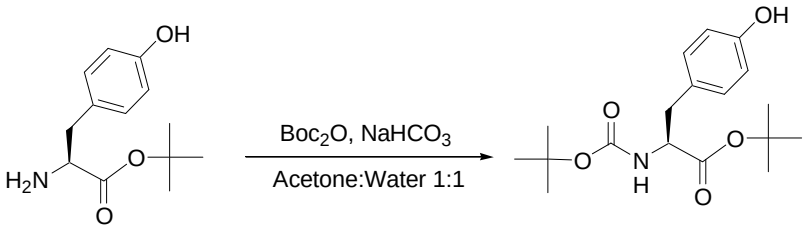
***tert*-Butyl N$^{\alpha}$-[(Fluoren-9-yl)methoxylcarbonyl]-L-tyrosine trichloroethyl sulfate (2.27).**



To a solution of FmocTyrO^{t}Bu (**2.24**) (0.50 g, 1.08 mmol, 1.00 equiv) and 2,2,2-trichloroethoxy sulfurylchloride **2.1** (0.80 g, 3.26 mmol, 3.00 equiv) in dry THF (2 mL) at 0 ^{o}C (ice bath) was added dropwise a solution of DMAP (0.13 g, 1.08 mmol, 1.00 equiv) and

Et_3N (0.30 mL, 2.17 mmol, 2.00 equiv) in dry THF (2.3 mL). The ice bath was removed and the reaction was stirred for 3 h. The mixture was filtered and the filtrate was concentrated *in vacuo*. Flash chromatography of the residue (ethyl acetate:hexane, 1:3) gave pure **2.27** (0.72 g, 98% yield). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.80 (d, J = 7.2 Hz, 2H, H_{Fmoc}), 7.61 (d, J = 7.5 Hz, 2H, H_{Fmoc}), 7.43 (dd, J = 7.2 and 7.5 Hz, 2H, H_{Fmoc}), 7.37-7.22 (m, 6H, $H_{Fmoc,}$ H_{Tyr}), 5.38 (d, J = 7.5 Hz, 1H, NH_{Tyr}), 4.83 (s, 2H, H_{TCE}), 4.57-4.39 (m, 3H, $CH_{2\text{-}Fmoc,}$and CH_{Tyr}), 4.23 (dd, J_1 = 6.6 & J_2 = 6.9 Hz, 1H, CH_{Fmoc}), 3.13-3.12 (m, 2H, $CH_{2\text{-}Tyr}$), 1.43 (s, 9H, $H_{\textit{tert-but}}$); ^{13}C NMR (75 MHz, $CDCl_3$): δ 170.3, 155.6, 149.1, 143.9, 143.8, 141.37, 141.35, 136.5, 131.2, 127.8, 127.1, 125.15, 125.07, 121.0, 120.09, 120.06, 92.5, 82.7, 80.4, 66.9, 55.2, 47.2, 37.8, 28.0; HRMS (ESI$^+$): calculated for $C_{30}H_{31}Cl_3NO_8S$ $(M+H)^+$ 670.0836, found 670.0822.

N$^{\alpha}$-[tert-butoxylcarbonyl]-L-tyrosine *tert*-butyl ester (2.43).

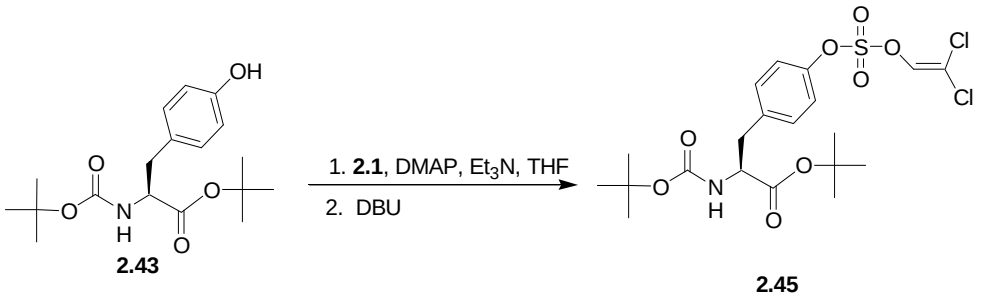


2.43

This was prepared according to the procedure of Stefan.[154] A solution of sodium bicarbonate (1.70 g, 20.2 mmol, 1.10 equiv) in water (49 mL) was added to a suspension of L-tyrosine *tert*-butyl ester (4.35 g, 18.3 mmol, 1.00 equiv) in acetone (39 mL). A solution of di-*tert*-butyldicarbonate (4.40 g, 20.2 mmol, 1.10 equiv) in acetone (10 mL) was added dropwise and the resulting solution was stirred at rt overnight. The acetone was removed by rotary evaporation and the residue was dissolved in ethyl acetate (100 mL). This solution was washed with 0.1 N HCl (25 mL) then dried (Na_2SO_4), filtered and concentrated by rotary

evaporation to yield 6.10 g (99% yield) of **2.43** as a yellowish-white solid, MP =114-115 ^{o}C (reported MP = 115.8-116.7 ^{o}C). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.10 (d, J = 8.3 Hz, 2H, H_{Tyr}), 6.71 (d, J = 8.4 Hz, 2H, H_{Tyr}), 4.95 (d, J = 6.9 Hz, 1H, NH_{Tyr}), 4.77 (s, 1H, OH), 4.38-4.36 (m, 1H, CH_{Tyr}), 2.96-2.94 (m, 2H, $CH_{2\text{-}Tyr}$), 1.40 (s, 9H, $H_{tert\text{-}but}$), 1.39 (s, 9H, $H_{tert\text{-}but}$). ^{13}C NMR (75 MHz, $CDCl_3$): δ 171.4, 155.5, 155.4, 130.5, 127.4, 115.4, 82.3, 80.1, 55.1, 37.6, 28.3, 27.9.

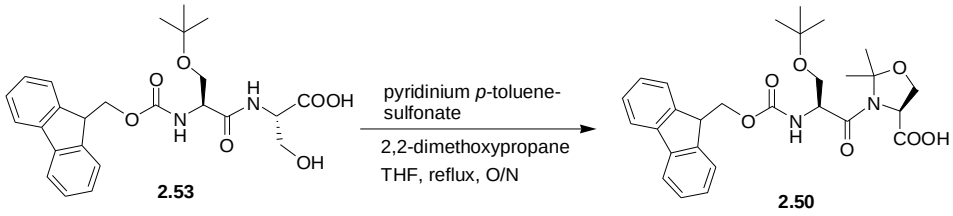
***tert*-Butyl N$^{\alpha}$-[*tert*-butoxylcarbonyl]-L-tyrosine dichlorovinyl sulfate (2.45).**



To a solution of **2.43** (6.00 g, 17.7 mmol, 1.00 equiv) in dry THF (24 mL) at 0 ^{o}C was added reagent **2.1** (15.7 g, 53.3 mmol, 3.00 equiv) followed by a solution of DMAP (2.17 g, 17.7 mmol, 1.00 equiv) and Et_3N (4.9 mL, 35.5 mmol, 2.00 equiv) 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 x 100 mL) and brine (2 x 100 mL) then dried ($MgSO_4$), filtered concentrated by rotary evaporation. The residue was dissolved in THF (72 mL) and 1 equiv of DBU (2.5 mL, 17 mmol) was added at 1 hour intervals over 4 h for a total of 5 equiv DBU. After 6 h the reaction mixture was filtered and the filtrate was diluted with EtOAc (2 x 100 mL). This solution was washed with phosphate buffer (pH = 7.2, 2 x 100 mL), and brine (2 x 100 mL) then dried ($MgSO_4$), filtered and concentrated by rotary evaporation. The

residue was purified by flash chromatography using ethyl acetate:*n*-hexane (15:85) to yield 8.01 g of **2.45** 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, $CH_{2\text{-}Tyr}$), 1.39 (s, 9H, $H_{tert\text{-}but}$), 1.36 (s, 9H, $H_{tert\text{-}but}$); ^{13}C NMR (75 MHz, $CDCl_3$): δ 170.5, 154.9, 148.8, 137.1, 133.6, 131.2, 120.8, 117.2, 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.

FmocSer(tBu)Ser(Ψ^{Me,Me}Pro)OH (2.50)[155]

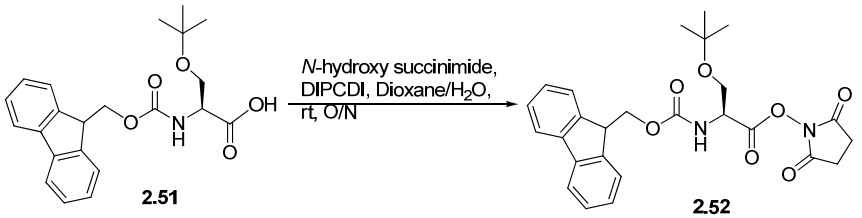


2.53 **2.50**

To a solution of dipeptide **2.53** (4.00 g, 8.50 mmol, 1.00 equiv) in THF (170 mL) was added pyridinium *p*-toluenesulfonate (0.43 g, 1.70 mmol, 0.20 equiv) followed by 2,2-dimethoxypropane (5.2 mL, 42.5 mmol, 5.00 equiv). The resulting solution was refluxed under Ar overnight during which the condensate was passed over 4 Å molecular sieves before returning to the reaction vessel. After cooling, triethylamine (0.36 mL, 2.6 mmol, 0.31 equiv) was added and the solution was stirred for 5 min afterward the solution was concentrated to dryness by rotary evaporation. The residue was taken up in ethyl acetate (250 mL), washed with water (3 x 120 mL), dried ($MgSO_4$), filtered and concentrated to dryness by rotary evaporation. Flash chromatography of the residue (1% MeOH/CH_2Cl_2 to 10% methanol/CH_2Cl_2) gave a crude foamy product which was dissolved in ether (5.5 mL) and isopropanol (2.7 mL). Pentane (27 mL) was added and the mixture stirred for 15 min.

More pentane (24 mL) was added over 1 h and resulting mixture was allowed to stir at room temperature for overnight. After an additional 2 h of stirring at 0 ^{o}C, the resulting solid was filtered and dried which gave 3.21 g of **2.50** as an off-white solid (74% yield). MP = 156-158 ^{o}C. ^{1}H NMR (300 MHz, CD_3OD): δ 7.76 (d, J = 7.4 Hz, 2H, H_{Fmoc}), 7.63-7.59 (m, 2H, H_{Fmoc}), 7.35 (dd, J_1=J_2 7.3 Hz, 2H, H_{Fmoc}), 7.28 (dd, J = 7.2 and 7.1 Hz, 2H, H_{Fmoc}), 4.99 (s, 1H, NH), 4.44-4.18 (m, 6H, $CH_{2\text{-}Fmoc}$, CH_{Fmoc}, $CH_{2\text{-}Asp}$ and $CH_{Peptide}$), 3.50-3.40 (m, 2H, $CH_{2\text{-}Asp}$), 1.63 (s, 3H, CH_3), 1.51 (s, 3H, CH_3), 1.15 (s, 9H, $H_{tert\text{-}but}$); ^{13}C NMR (75 MHz, DMSO-*d6*): δ 168.2, 155.6, 144.3, 144.1, 141.1, 128.1, 127.5, 126.0, 125.9, 120.5, 95.8, 73.4, 67.0, 66.4, 63.7, 53.3, 47.0, 27.4, 25.7, 23.1. HRMS (ESI^+): calculated for $C_{28}H_{35}N_2O_7$ $(M+H)^+$ 511.2444, found 511.2432. The analytical HPLC chromatogram (linear gradient of 30:70 CH_3CN: (0.1%TFA) water to 80:20 CH_3CN: (0.1%TFA) water over 30 min showed a single peak at t_R= 27.5 min.

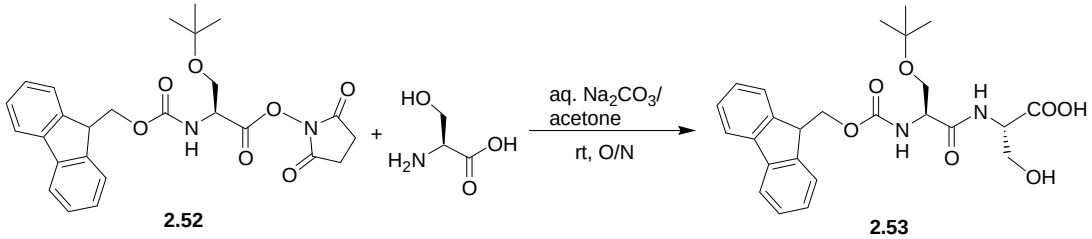
N-α-Fmoc-O-*tert*-butyl-L-serine N-hydroxysuccinimide ester (2.52)[155]



To a solution of FmocSer(OtBu)OH (**2.51**, 2.87 g, 7.50 mmol, 1.00 equiv) in dioxane (20 mL) was added a solution of *N*-hydroxysuccinimide (1.58 g, 13.6 mmol, 1.82 equiv) in dioxane:water (5.5 mL, 9:1) followed by DIPCDI (1.5 mL, 9.75 mmol, 1.3 equiv). The reaction mixture was stirred overnight at rt then concentrated by rotary evaporation. The residue was then dissolved in ethyl acetate and washed with 0.001 M HCl (3 x 25 mL, pH = 3), dried ($MgSO_4$), filtered and concentrated *in vacuo* leaving an off-white solid. The residue

was recrystallized using ethanol/*n*-hexane to give 3.31 g of pure **2.52** as a white solid (92% yield). MP = 81-83 ^{o}C. ^{1}H NMR (300 MHz, CD_3OD): δ 7.79 (d, J = 7.4 Hz, 2H, H_{Fmoc}), 7.66-7.65 (m, 2H, H_{Fmoc}), 7.38 (dd, $J_1 = J_2$ = 7.4 Hz, 2H, H_{Fmoc}), 7.30 (dd, J = 7.4 and 7.3 Hz, 2H, H_{Fmoc}), 7.79-7.76 (m, 1H, H_{Asp}), 4.39-4.22 (m, 3H, $CH_{2\text{-}Fmoc}$, and CH_{Fmoc}), 3.88-3.72 (m, 2H, $CH_{2\text{-}Asp}$), 2.82 (s, 4H, H_{Succ}), 1.21 (s, 9H, $H_{tert\text{-}but}$); ^{13}C NMR (75 MHz, CD_3OD): δ 167.7, 166.7, 156.8, 143.8, 143.7, 141.1, 127.4, 126.8, 124.8, 119.5, 73.6, 66.9, 61.3, 53.5, 46.8, 26.1, 25.1.

FmocSer(OtBu)SerOH (2.53)[155]

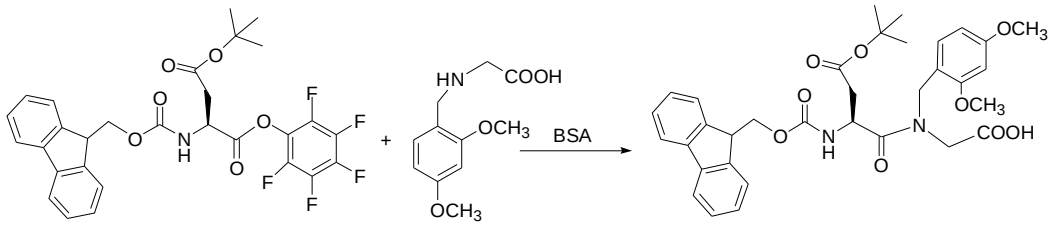


2.52 **2.53**

L-Serine (1.86 g, 17.7 mmol, 2.00 equiv) was dissolved in aq. sodium carbonate (65 mL, 3.76 g, 35.5 mmol, 4.00 equiv) and added dropwise to a solution of **2.52** (4.27 g, 8.88 mmol, 1.00 equiv) in acetone (50 mL). After stirring overnight at rt the reaction mixture was acidified to pH = 2-3 using conc. HCl, extracted with ethyl acetate (3 x 100 mL) and the combined organics were dried ($MgSO_4$), filtered and concentrated by RV. The residue was subjected to FC (1% CH_3OH in CH_2Cl_2 to 10% CH_3OH in CH_2Cl_2) which gave 3.52 g of pure **2.53** (85% yield). MP = 88-91 ^{o}C. ^{1}H NMR (300 MHz, CD_3OD): δ 7.77 (d, J = 7.4 Hz, 2H, H_{Fmoc}), 7.63-7.59 (m, 2H, H_{Fmoc}), 7.36 (dd, J = 7.2 and 7.4 Hz, 2H, H_{Fmoc}), 7.28 (dd, J = 7.3 and 7.4 Hz, 2H, H_{Fmoc}), 4.45-4.19 (m, 5H, $CH_{2\text{-}Fmoc}$, CH_{Fmoc} and 2 $CH_{Peptide}$), 3.93-3.78 (m, 2H, $CH_{2\text{-}Asp}$), 3.62-3.59 (m, 2H, $CH_{2\text{-}Asp}$), 1.18 (s, 9H, $H_{tert\text{-}but}$); ^{13}C NMR (75 MHz,

CD_3OD): δ 171.2, 157.0, 143.9, 143.7, 141.1, 127.4, 126.8, 124.8, 119.5, 73.5, 66.7, 61.7, 61.6, 55.1, 54.7, 46.9, 26.2. The analytical HPLC chromatogram (linear gradient of 30:70 CH_3CN: (0.1%TFA) water to 80:20 CH_3CN: (0.1%TFA) water over 30 min) showed a single peak at t_R= 15.5 min.

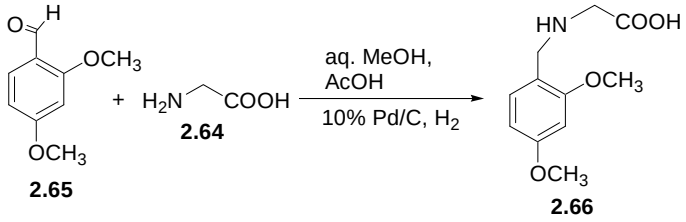
Fmoc-Asp(OtBu)Gly(DMB)OH (2.63)[144]



To a suspension of **2.66** (0.26 g, 1.04 mmol, 1.20 equiv) in CH_2Cl_2 (2 mL) was added bis(trimethylsilyl)acetamide (BSA) (0.64 mL, 2.6 mmol, 3.0 equiv) and the mixture was stirred for 20 min until the suspension became a clear solution. Compound **2.67** (0.50 g, 0.87 mmol, 1.0 equiv) was added and the mixture stirred overnight at rt. The mixture was diluted with DCM (2 mL) and a 10% of citric acid solution (1.6 mL) was added and the mixture stirred for 5 min. The layers were separated and the organic layer was dried ($MgSO_4$), filtered and concentrated by RV. The residue was subjected to FC (1% MeOH:$CHCl_3$ to 4% MeOH:$CHCl_3$) which gave a crude foamy product. This material was the dissolved in ether (4 mL) and precipitated using pentane which gave pure 0.38 g **2.63** as a white solid (71% yield). MP = 87-90 ^oC. ^1H NMR (300 MHz, CD_3OD): δ 7.76 (d, J = 7.3 Hz, 2H, H_{Fmoc}), 7.62 (d, J = 7.3 Hz, 2H, H_{Fmoc}), 7.35-7.26 (m, 4H, H_{Fmoc}), 7.09 (dd, J = 8.4 and 8.3 Hz, 1H, $H_{2,4\text{-dimethoxybenzyl}}$), 6.49-6.35 (m, 2H, $H_{2,4\text{-dimethoxybenzyl}}$), 5.29-5.24 (m, 1H, H_{oxaz}), 4.67-4.50 (m, 2H, $CH_{peptide}$), 4.38-3.97 (m, 5H, $CH_{peptide}$), 3.76 (d, J = 11 Hz, 3H, OCH_3), 3.70 (d, J = 8 Hz,

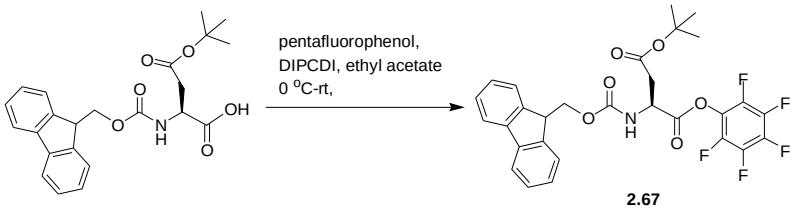
3H, OCH_3), 2.46-2.75 (m, 2H, $CH_{2\text{-Asp)}}$, 1.39 (d, J = 7.5 Hz, 9H, $H_{tert\text{-}butyl}$); ^{13}C NMR (75 MHz, $CDCl_3$): δ 171.9, 171.8, 171.1, 170.8, 170.0, 169.9, 161.1, 160.8, 158.8, 158.7, 156.5, 156.3, 143.8, 143.7, 141.97, 141.97, 141.94, 130.6, 129.9, 127.3, 126.7, 124.9, 124.8, 119.5, 116.2, 115.6, 104.2, 104.0, 98.6, 97.7, 80.8. 54.43. 54.30, 48.13, 47.00, 44.54, 38.05, 37.75, 26.84. HRMS (ESI^+): calculated for $C_{34}H_{38}N_2O_9Na$ $(M+Na)^+$ 641.2465, found 641.2475. The analytical HPLC chromatogram (linear gradient of 40:60 CH_3CN: (0.1%TFA) water to 80:20 CH_3CN: (0.1%TFA) water over 30 min showed a single peak at t_R= 25.4 min.

_N_-(4-dimethoxybenzyl)glycine (2.66)[145]



A 50% aq. methanol solution (~ 200 mL) was added to gylcine (**2.64**, 1.50 g, 20 mmol, 1.0 equiv) and 2,4-dimethoxybenzaldehyde (**2.65**, 3.35 g, 20 mmol, 1.0 equiv) until all of the solid material dissolved. Acetic acid (1 mL) was added and the mixture was subjected to hydrogenation using 10% Pd/C (350 mg) and hydrogen gas (balloon pressure) for 6 h. The catalyst was removed by filtration and the solution concentrated by RV. The residue was dissolved in *n*-butanol and washed with water. The organic phase was concentrated and the residue was triturated with ether to give 1.71 g of **2.66** as a white solid (38% yield). MP= 188-191 ^oC.^1H NMR (300 MHz, CD_3OD): δ 7.24 (d, J = 8.4 Hz, 1H, H_{Ar}), 6.60 (d, J = 2.1 Hz, 1H, H_{Ar}), 6.53 (dd, J = 8.4 and 2.1 Hz, 1H, H_{Ar}), 4.13 (s, 2H, CH_2), 3.88 (s, 3H, OCH_3), 3.79 (s, 3H, OCH_3), 3.39 (s, 2H, CH_2) ; ^{13}C NMR (75 MHz, CD_3OD): 169.5, 162.6, 159.2, 132.3, 111.3, 104.7, 98.0, 54.7, 54.5, 47.9, 46.1.

N-α-Fmoc-O-*tert*-butyl-L-aspartic acid pentafluorophenyl ester (2.67)



To an ice-cooled solution of FmocAsp(OtBu)OH (1.00 g, 2.43 mmol, 1.00 equiv) and pentafluorophenol (0.45 g, 2.43 mmol, 1.00 equiv) in ethyl acetate was added DIPCDI (0.30, 2.43 mmol, 1.00 equiv) and the mixture was stirred at 0 °C for 1 h and followed by an additional 1 h at rt. The mixture was filtered and the solvent was removed by RV leaving a solid residue. Recrystallization of the residue using n-hexane gave 1.26 g of pure **2.67** (90% yield). MP = 95-96 °C (reported MP= 97-100 °C). ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.74 (d, *J* = 7.4 Hz, 2H, H_{Fmoc}), 7.58 (d, *J* = 6.93 Hz, 2H, H_{Fmoc}), 7.38 (dd, *J* = 7.2 and 7.4 Hz, 2H, H_{Fmoc}), 7.29 (dd, *J* = 7.3 and 7.4 Hz, 2H, H_{Fmoc}), 5.96 (d, *J* = 8.9 Hz, 1H, NH), 4.99-4.96 (m, 1H, H_{Asp}), 4.50-4.21 (m, 3H, $CH_{2\text{-}Fmoc,}$and CH_{Fmoc}), 3.17-2.85 (m, 2H, $CH_{2\text{-}Asp}$), 1.47 (s, 9H, $H_{tert\text{-}but}$); ^{13}C NMR (75 MHz, $CDCl_3$): δ 169.7, 167.4, 155.8, 143.7, 143.5, 141.3, 127.7, 127.1, 125.1, 120.0, 82.7, 67.5, 50.2, 47.0, 37.6, 28.0. ^{19}F NMR (282 MHz, DMSO-d_6): δ -152, -157, -162.

2.5.3 Determination of the enantiopurity of compounds 2.23 and 2.26.

The enantiopurity of compounds **2.23** and **2.26** was determined by constructing the following dipeptides and analyzing them by HPLC and ^{1}H-NMR: $AcY(SO_3DCV)A_{(DL)}NH_2$ (**2.28LD**/**2.28LL**), $AcY(SO_3DCV)A_{(L)}NH_2$ (**2.28LL**), $AcY(SO_3TCE)A_{(DL)}NH_2$ (**2.29DL**/**2.29LL**) and $AcY(SO_3TCE)A_{(L)}NH_2$ (**2.29LL**). Manual Fmoc-SPPS was

performed using a plastic syringe equipped with a porous filter and the Rink amide resin (50 mg, loading of 0.71 mmol/g, preswollen in DMF for 1 h before use). Fmoc-(L)-Ala was used for the synthesis of **2.28LL** and **2.29LL** and racemic Fmoc-(D,L)-Ala was used for the synthesis of the diastereomeric mixtures **2.28LD/2.28LL** and **2.29LD/2.2LL.** Amino acids were coupled using the following reagents, molar equivalents and reaction times: Fmoc-AA-OH (4.0 equiv), HBTU (4.0 equiv), HOBt (4.0 equiv) and DIPEA (4.0 equiv) in less than 1 ml of DMF (stirred 10 min at room temperature before being added to the resin) and then shaken with resin (2 x 90 min for first amino acids and 1 x 90 min for second amino acids). Fmoc removal was achieved using 20% 2-MP/DMF (1-2 mL, 3 x 10 min). After the removal of the terminal Fmoc group the resin was washed with DMF (3 mL, 5 x 3 min) and then treated with 2 mL of 2:1 pyridine: acetic anhydride solution for 3 h then washed with DMF (3 mL, 5 x 3 min), methanol (3 mL, 5 x 3 min) and CH_2Cl_2 (3 mL, 5 x 3 min) then dried. The dipeptides were cleaved from the resin by shaking with a cleavage cocktail consisting of TFA:triisopropylsilane (TIPS) (98:2, 2 mL) for 2.5 h after which the mixture was filtered. The resin was treated with another 2 mL of the cocktail for 30 min, filtered, and then the resin was washed with successive portions of the cleavage cocktail (3 x 0.5 mL). The combined filtrates were concentrated under reduced pressure and the residue subjected to flash chromatography (CH_2Cl_2:MeOH, 9:1) which gave the desired dipeptides. The resulting dipeptides were analyzed using analytical C-18 RP-HPLC eluting with CH_3CN/H_2O (0.1% TFA) employing a linear gradient of 20% to 40% CH_3CN over 1 h with the detector set to 220 nm.

AcY(SO$_3$DCV)A$_{(DL)}$NH$_2$ (2.28LD/2.28LL). ^{1}H NMR (300 MHz, CD_3OD): δ 7.58 (s, 1H, H_{DCV}), 7.56 (s, 1H, H_{DCV}), 7.36-7.25 (m, 8H, H_{Tyr}), 4.61 (dd, J_1 = 5.4 and J_2 = 9.0 Hz, 1H, $CH_{peptide}$), 4.41 (dd, J_1 = 7.5 and J_2 = 7.8 Hz, 1H, $CH_{peptide}$), 4.30 (dd, J = 6.9 and 14.1 Hz, 1H, $CH_{peptide}$), 4.17 (dd, J = 7.2 and 14.4 Hz, 1H, $CH_{peptide}$), 3.18 (dd, J = 5.4 and 14.1 Hz, 1H, $CH_{2\text{-}Tyr}$), 3.08-2.87 (m, 3H, $CH_{2\text{-}Tyr}$), 1.91 (s, 3H, H_{Ac}), 1.88 (s, 3H, H_{Ac}), 1.32 (d, J = 7.2 Hz, 3H, H_{Ala}), 1.13 (d, J = 7.5 Hz, 3H, H_{Ala}). HRMS (ESI$^+$): calculated for $C_{16}H_{20}Cl_2N_3O_7S$ $(M+H)^+$ 468.0399, found 468.0409. Analytical HPLC showed two peaks with t_R= 33.2, 35.0 min. See **Figures 2.2** and **2.3** for the ^{1}H-NMR spectrum and HPLC chromatogram.

AcY(SO$_3$DCV)A$_{(L)}$NH$_2$ (2.28LL). ^{1}H NMR (300 MHz, CD_3OD): δ 7.56 (s, 1H, H_{DCV}), 7.38 (d, J = 8.7 Hz, 2H, H_{Tyr}), 7.26 (d, J = 8.7 Hz, 2H, H_{Tyr}), 4.60 (dd, J = 5.4 and 9.0 Hz, 1H, $CH_{peptide}$), 4.30 (dd, J = 7.2 and 14.4 Hz, 1H, $CH_{peptide}$), 3.18 (dd, J = 5.4 and 13.8 Hz, 1H, $CH_{2\text{-}Tyr}$), 2.90 (dd, J = 9.0 and 13.8 Hz, 1H, $CH_{2\text{-}Tyr}$), 1.88 (s, 3H, H_{Ac}), 1.32 (d, J = 7.2 Hz, 3H, H_{Ala}); HRMS (ESI$^+$): calculated for $C_{16}H_{20}Cl_2N_3O_7S$ $(M+H)^+$ 468.0399, found 468.0409. Analytical HPLC showed one peak t_R= 33.6 min. See **Figures 2.4** and **2.5** for the ^{1}H-NMR spectrum and HPLC chromatogram.

AcY(SO$_3$TCE)A(DL)NH$_2$ (2.29DL/2.29LL). ^{1}H NMR (300 MHz, CD_3OD): δ 7.38-7.28 (m, 8H, H_{Tyr}), 5.06 (s, 2H, H_{TCE}), 5.04 (s, 2H, H_{TCE}), 4.59 (dd, J = 5.4 and 7.7 Hz, 1H, $CH_{peptide}$), 4.40 (dd, J_1 = J_2 = 7.8 Hz, 1H, $CH_{peptide}$), 4.30 (dd, J = 7.2 and 14.4 Hz, 1H, $CH_{peptide}$), 4.17 (dd, J = 7.2 and 14.4 Hz, 1H, $CH_{peptide}$), 3.17 (dd, J = 5.7 and 14.1 Hz, 1H, $CH_{2\text{-}Tyr}$), 3.03-2.87 (m, 3H, $CH_{2\text{-}Tyr}$), 1.91 (s, 3H, H_{Ac}), 1.88 (s, 3H, H_{Ac}), 1.32 (d, J = 7.2 Hz, 3H, H_{Ala}), 1.13 (d, J = 7.5 Hz, 3H, H_{Ala}); HRMS (ESI$^+$): calculated for $C_{16}H_{20}Cl_2N_3O_7S$ $(M+H)^+$ 468.0399, found 468.0409 and $C_{16}H_{21}Cl_3N_3O_7S$ $(M+H)^+$ 504.0166, found 504.0172.

Analytical HPLC showed four peaks t_R= 34.9, 36.8, 38.9, 40.4 min. See **Figures 2.6** and **2.7** for the ^{1}H-NMR spectrum and HPLC chromatogram.

AcY(SO$_3$TCE)A$_{(L)}$NH$_2$ (2.29LL). ^{1}H NMR (300 MHz, CD_3OD): δ 7.36 (d, *J* = 8.7 Hz, 2H, H_{Tyr}), 7.31 (d, *J* = 8.7 Hz, 2H, H_{Tyr}), 5.04 (s, 2H, H_{TCE}), 4.60 (dd, *J* = 5.7 and 9.0 Hz, 1H, $CH_{peptide}$), 4.32-4.26 (m, 1H, $CH_{peptide}$), 3.17 (dd, *J* = 5.7 and 14.1 Hz, 1H, $CH_{2\text{-}Tyr}$), 2.97-2.83 (m, 1H, $CH_{2\text{-}Tyr}$), 1.88 (s, 3H, H_{Ac}), 1.32 (d, *J* = 7.2 Hz, 3H, H_{Ala}), HRMS (ESI$^+$): calculated for $C_{16}H_{20}Cl_2N_3O_7S$ $(M+H)^+$ 468.0399, found 468.0409 and $C_{16}H_{21}Cl_3N_3O_7S$ $(M+H)^+$ 504.0166, found 504.0172. Analytical HPLC showed four peaks t_R= 34.9, 38.8 min. See **Figures 2.8** and **2.9** for the ^{1}H-NMR spectrum and HPLC chromatogram.

2.5.4 Synthesis of crude hexapeptides 2.16, 2.31 and 2.32.

Manual SPPS was performed using the same resin and procedures described for the synthesis of dipeptides **2.28** and **2.29**. Coupling of the first amino acid was carried out with the pentafluorophenyl ester of FmocLeu (4.0 equiv), HOBt (4.0 equiv) and DIPEA (4.0 equiv) in DMF (stirred 10 min at room temperature before being added to the resin) and then shaken with resin (2 x 90 min). All subsequent couplings were performed using HBTU (4.0 equiv), HOBt (4.0 equiv), DIPEA (4.0 equiv) and Fmoc-AA-OH (4.0 equiv). *tert*-Butyl protection was used for the side chains of Asp, Glu and Tyr. After the final Fmoc deprotection the resin was washed with DMF (3 mL, 5 x 3 min), methanol (3 mL, 5 x 3 min), ethanol (3 mL, 5 x 3 min) and diethyl ether (3 mL, 5 x 3 min) then left exposed to air to dry.

Cleavage of the peptides from the resin was achieved by shaking with a cleavage cocktail consisting of TFA:TIPS (98:2, 2 mL) for 2.5 h after which the mixture was filtered. The resin was treated with another 2 mL of the cocktail for 30 min, filtered, and then the

resin was washed with successive portions of the cleavage cocktail (3 x 0.5 mL). The combined filtrates were concentrated to half volume under reduced pressure. The material was transferred to a 50 mL centrifuge tube and *t*-butyl methyl ether was added which resulted in the precipitation of the peptides. The mixture was cooled in a dry ice-acetone bath for 30 min then centrifuged (5000 rpm, -4 ^{o}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 resulting crude peptides were analyzed by analytical RP-HPLC (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). The analytical HPLC of crude DADEY(SO_3DCV)LNH_2 (**2.31**) obtained using amino acid **2.23** showed mainly a single peak at t_R= 26.0 min (see **Figure 2.10**). This was used without further purification for the synthesis of peptide **2.30**. The analytical HPLC of the mixture of crude DADEY(SO_3DCV)LNH_2 (**2.31**) and DADEY(SO_3TCE)LNH_2 (**2.32**) obtained using amino acid **2.26** showed mainly two major peaks at t_R= 27.9 and 28.7 min in an *apparent* 1:1 ratio (see **Figure 2.11**). This was used without further purification for the synthesis of peptide **2.30**.

DADEYLNH$_2$ (2.16).

Lyophilization after the post cleavage manipulation afforded almost pure peptide in a 98% yield. ^{1}H NMR of the aromatic region (500 MHz, D_2O): δ 6.80 (d, J = 8.5 Hz, 2H, H_{Tyr}), 6.48 (d, J = 8.5 Hz, 2H, H_{Tyr}) (see **Figure 2.14**); HRMS (ESI$^-$): calculated for $C_{31}H_{44}Cl_2N_7O_{13}$ $(M-H)^-$ 722.2997, found 722.2993. Analytical HPLC showed a single peak

at t_R= 14.5 min (linear gradient of 5 to 95 CH_3CN:H_2O (0.1% TFA) over 60 min) (see **Figure 2.1, B**).

2.5.5 General procedure for the removal of the DCV- or TCE-protecting groups in peptides 2.31, 2.32, 2.34, 2.36, 2.39 and 2.42.

Ammonium formate (9 equiv for peptides **2.31**, **2.32** and **2.36**, 21 equiv for peptide **2.34**) was added to a solution of peptide in HPLC grade methanol (approximately 1 mL per 10 mg peptide) followed by 30 wt% of 10% Pd/C. The reaction was fitted with a balloon filled with H_2, stirred at rt and the reaction was monitored by HPLC for the disappearance of starting material (CH_3CN/H_2O (0.1% TFA) as eluent, linear gradient from 5 to 95 CH_3CN in 60 min). For peptide **2.31** (when amino acid **2.23** was used) the reaction was complete after 1 h. For the mixture of peptides **2.31** and **2.32** (when amino acid **2.26** was used) the reaction was complete after 3 h. For peptides **2.34** and **2.36**, after 2 h another 20 wt% of 10% Pd/C was added. For peptide **2.34** the reaction was complete after an additional 3 h while for peptide **2.36** it was complete after an additional 1 h. For peptides **2.39** and **2.42** ammonium formate (30 equiv and 19 equiv respectively) was added to a solution of the peptide in methanol (approximately 2 mL per 10 mg peptide) followed by 50 wt% of 10% Pd/C and the reaction was allowed to stir under H_2 atmosphere for 15 h at room temperature.

After the reactions were complete, the mixture was transferred to an Eppendorf tube and centrifuged in a microcentrifuge to pellet the Pd/C. The supernatant was removed and concentrated under vacuum at room temperature and the crude material purified by semi-preparative RP-HPLC.

2.5.6 Synthesis of DADEsYLNH$_2$ (2.30).

Deprotection of the DCV group from peptide **2.31** (when amino acid **2.23** was used) or the DCV/TCE from **2.31**/**2.32** mixture (when amino acid **2.26** was used) was performed using the general procedure described in section 2.5.5. The analytical HPLC chromatogram of crude deprotected peptide **2.30** exhibited essentially a single major peak (t_R= 14.14 min; linear gradient from 5% to 95% CH_3CN in H_2O (0.1% TFA) over 60 min, λ = 220 nm) (see **Figure 2.12**). To further confirm that no desulfation occurred during DCV deprotection a sample of the crude peptide **2.30** was spiked with peptide **2.16** (DADEYLNH$_2$) and then analyzed by analytical HPLC. The chromatogram exhibited *two* peaks corresponding to peptides **2.16** and **2.30** at t_R= 15.2 min and 14.1 min respectively (linear gradient from 5% to 95% CH_3CN in H_2O (0.1% TFA) over 60 min, λ = 220 nm) (see **Figure 2.13**). Purification of peptide **2.30** by semi-preparative RP-HPLC (CH_3CN/20 mM ammonium acetate, pH = 6.8, linear gradient from 5% to 20% CH_3CN over 40 min, t_R= 13.5 min, λ = 220 nm) afforded peptide **2.30** as a flocculent white powder. When using amino acid **2.23** the yield was 71%. Analytical HPLC of this material using a linear gradient of 1:99 CH_3CN:20 mM ammonium acetate to 80:20 CH_3CN:20 mM ammonium acetate over 40 min showed single peak at t_R = 11.0 min. ^{1}H NMR of the aromatic region (see **Figure 2.15**) (500 MHz, D_2O): δ 6.95 (d, *J* = 8.5 Hz, 2H, $H_{s\text{-}Tyr}$), 6.89 (d, *J* = 8.0 Hz, 2H, $H_{s\text{-}Tyr}$); HRMS (ESI$^-$): calculated for $C_{31}H_{44}N_7O_{16}S$ (M-$^+$NH$_4$)$^-$ 802.2565, found 802.2561. When using amino acid **2.26** the yield was 45%. However the analytical HPLC chromatogram indicated that this material was contaminated with a small amount of impurity which could not be removed. The ^{1}H NMR of

the aromatic region and HRMS of this material were identical to that of peptide **2.30** that was obtained using amino acid **2.23**.

2.5.7 Synthesis of AcsYEsYLDsYDFNH$_2$ (2.33, PSGL-1$_{5\text{-}12,\ trisulfated}$), AcYEsYLDYDFNH$_2$ (2.35, PSGL-1$_{5\text{-}12,\ monosulfated\ at\ Tyr7}$) and AcYEYLDYDFNH$_2$ (2.37, PSGL-1$_{5\text{-}12,\ nonsulfated}$).

Automated SPPS was used. For the first amino acid the following protocol was used:

Step	Operation	Volume (mL)	Duration (min)	Reps
1	DMF wash	1	10	3
2	20% 2-MP/DMF	1	10	3
3	DMF wash	1	0.5	6
4	Coupling[a]	1	90	1
5	Coupling[a]	1	90	1
6	DMF wash	1	0.5	3

[a]A solution of Fmoc-AA-OPfp (4 equiv), HOBt (4 equiv) and DIPEA (4 equiv) in DMF (stirred 10 min at room temperature) was added manually to the resin.

For subsequent amino acids the following protocol was used:

Step	Operation	Volume (mL)	Duration (min)	Reps
1	DMF wash	1	0.5	6
2	20% 2-MP/DMF	1	10	3
3	DMF wash	1	0.5	6
4	Coupling [a]	1	90	1
5	DMF wash	1	0.5	3

[a]Addition of Fmoc-AA-OH (5 equiv) in DMF followed by addition of HOBt (5 equiv)/HBTU (5 equiv)/DIPEA (5 equiv) in DMF.

For the last amino acid the following protocol was used:

Step	Operation	Volume (mL)	Duration (min)	Reps
1	DMF wash	1	0.5	6
2	20% 2-MP/DMF	1	10	3
3	DMF wash	1	0.5	6
4	Coupling[a]	1	90	1
5	DMF wash	1	0.5	6
6	20% 2-MP/DMF	1	10	3
7	DMF	1	0.5	6
8	Acetylation[b]	1	180	1
9	DMF wash	1	10	3
10	MeOH wash	1	10	3
11	DCM wash	1	10	3
12	Dry	1	60	1

[a]Addition of Fmoc-AA-OH (5 equiv) in DMF followed by addition of HOBt (5 equiv)/HBTU (5 equiv)/DIPEA (5 equiv) in DMF. [b]A 1 mL 2:1 mixture of pyridine:acetic anhydride was added manually to the resin.

Peptides were cleaved form the support using the procedure outlined in section 2.5.4. This gave crude peptides AcY(SO_3DCV)EY(SO_3DCV)LDY(SO_3DCV)DFNH_2 (**2.34**), and AcYEY(SO_3DCV)LDYDFNH_2 (**2.36**) and AcYEYLDYDFNH_2 (**2.37**). Peptides **2.34** and **2.36** 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). The analytical HPLC chromatogram of crude **2.34** showed mainly one major peak in the HPLC chromatogram (t_R= 44.3 min) (see **Figure 2.16**). This was used without further purification for the synthesis of peptide **2.33**. The analytical HPLC chromatogram of crude **2.36** showed

mainly one major peak in the HPLC chromatogram, (t_R= 31.6 min) (see **Figure 2.18**). This was used without further purification for the synthesis of peptide **2.35**.

Peptide **2.33** was prepared from peptide **2.34** using the general deprotection procedure described in section 2.5.5. Preparative HPLC purification (CH_3CN/ 20 mM ammonium acetate as eluent, linear gradient from 1% to 30% CH_3CN over 30 min, t_R= 21.4 min, λ = 220 nm) afforded pure **2.33** as a flocculent white powder (16.9 mg, 46%). The analytical HPLC chromatogram (linear gradient of 1:99, CH_3CN:20 mM ammonium acetate to 80:20 CH_3CN:20 mM ammonium acetate over 40 min) showed a single peak at t_R= 13.7 min (see **Figure 2.17**). ^{1}H NMR of the aromatic region (see **Figure 2.21**) (500 MHz, D_2O): δ 7.03-6.89 (m, 9H, $4H_{s\text{-}Tyr}$ and $5H_{Ph}$), 6.85-6.79 (m, 6H, $4H_{s\text{-}Tyr}$), 6.72 (d, J = 8.0 Hz, 2H, $H_{s\text{-}Tyr}$); HRMS (ESI$^-$): calculated for $C_{57}H_{66}N_9O_{27}S_3$ $(M\text{-}3^+NH_4)^{3-}$ 468.1076, found 468.1070.

Peptide **2.35** was prepared from peptide **2.36** using the general deprotection procedure described in section 2.5.5. Preparative HPLC purification of the crude peptide (CH_3CN/20 mM ammonium acetate as eluent, linear gradient from 1% to 50% CH_3CN over 30 min, t_R= 19.5 min, λ = 220 nm) afforded pure **2.35** as a flocculent white powder (19.9 mg, 63%). The analytical HPLC chromatogram (linear gradient of 1:99 CH_3CN:20 mM ammonium acetate to 80:20 CH_3CN:20 mM ammonium acetate over 40 min) showed a single peak at t_R= 15.9 min (see **Figure 2.19**). ^{1}H NMR of the aromatic region (see **Figure 2.22**) (500 MHz, D_2O): δ 7.00-6.87 (m, 9H, $4H_{s\text{-}Tyr}$ and $5H_{Ph}$), 6.68 (d, J = 8.0 Hz, 2H, H_{Tyr}), 6.56 (d, J = 7.5 Hz, 2H, H_{Tyr}), 6.41 (dd, J = 8.0 and 9 Hz, 4H, H_{Tyr}); HRMS (ESI$^-$): calculated for $C_{57}H_{67}N_9O_{21}S$ $(M\text{-}H\text{-}^+NH_4)^{2-}$ 622.7086, found 622.7083.

Preparative HPLC purification of crude **2.37** (CH_3CN/20 mM ammonium acetate as eluent, linear gradient from 1 % to 50 % CH_3CN over 30 min, t_R= 21.3 min, λ = 220 nm) afforded **2.37** as a flocculent white powder (20.0 mg, 68%). The analytical HPLC chromatogram (linear gradient of 1:99 CH_3CN:20 mM ammonium acetate to 80:20 CH_3CN:20 mM ammonium acetate over 40 min) showed a single peak at t_R= 16.5 min (see **Figure 2.20**). ^{1}H NMR of the aromatic region (see **Figure 2.23**) (500 MHz, D_2O): δ 7.01-6.90 (m, 5H, H_{Ph}), 6.75 (d, *J* = 8.0 Hz, 2H, H_{Tyr}), 6.70 (d, *J* = 8.0 Hz, 2H, H_{Tyr}), 6.52 (d, *J* = 8.0 Hz, 2H, H_{Tyr}), 6.44 (dd, $J_1 = J_2$ = 8.0 Hz, 4H, H_{Tyr}), 6.39 (d, *J* = 8.0 Hz, 2H, H_{Tyr}); HRMS (ESI$^-$): calculated for $C_{57}H_{68}N_6O_{18}$ $(M-H)^-$ 1166.4682, found 1166.4684.

2.5.8 Synthesis of Ac-DADSENSSFsY_{23}sY_{24}sY_{25}DsY_{27}LDEVAFNH_2 (2.38, $D6_{14-33,\ tetrasulfated}$) and Ac-TTPDsYGHsYDDKDTLDLNTPVDKNH_2 (2.41, $C5aR_{7-28}$).

Automated SPPS was used. For the first amino acid the following protocol was used:

Step	Operation	Volume (mL)	Duration (min)	Reps
1	DMF wash	1	10	3
2	20% 2-MP/DMF	1	10	3
3	DMF wash	1	0.5	6
4	Coupling[a]	1	90	1
5	Coupling[a]	1	90	1
6	DMF wash	1	0.5	6
7	Capping[b]	2	10	1
8	DMF wash	1	0.5	6

[a]A solution of FmocPheOPfp for **2.38** or FmocLys(Boc)OPfp for **2.41** (4.0 equiv), HOCt (4.0 equiv) and DIPEA (4.0 equiv) in DMF (stirred 1-2 min at room temperature) was added manually to the resin. [b]2:1:3 solution of pyridine:acetic anhydride:DMF.

For subsequent amino acids the following protocol was employed:

Step	Operation	Volume (mL)	Duration (min)	Reps
1	DMF wash	1	0.5	3
2	20% 2-MP/DMF	1	10	3
3	DMF wash	1	0.5	6
4	Coupling[a]	1	45	2
5	Coupling[a]	1	45	2
6	DMF wash	1	0.5	6
7	Capping[b]	2	10	1
8	DMF wash	1	0.5	6

[a]For peptide **2.38** and **2.41**: Addition of Fmoc-AA-OH (5.0 equiv) in DMF followed by addition of HOCt (5 equiv)/HCTU (5 equiv)/DIPEA (5 equiv) in DMF [b]2:1:3 solution of pyridine:acetic anhydride :DMF

For peptide **2.38**, residues 23-25 were incorporated using the following protocol:

Step	Operation	Volume (mL)	Duration (min)	Reps
1	DMF wash	1	0.5	3
2	20% 2-MP/DMF	1	10	3
3	DMF wash	1	0.5	6
4	Coupling[a]	1	45	1
5	Coupling[a]	1	45	1
6	DMF wash	1	0.5	6
7	Capping[b]	2	10	1
8	DMF wash	1	0.5	6

[a]A solution of Fmoc-AA-OH (5.0 equiv), HATU (5.0 equiv) and DIPEA (5.0 equiv), in 1 mL of DMF (stirred 1-2 min at room temperature) was manually added to the resin. [b]2:1:3 solution of pyridine:acetic anhydride:DMF.

For the last amino acid the following protocol was used:

Step	Operation	Volume (mL)	Duration (Min)	Reps
1	DMF wash	1	0.5	6
2	20% 2-MP/DMF	1	10	3
3	DMF wash	1	0.5	6
4	Coupling[a]	1	45	2
5	Coupling[a]	1	45	2
6	DMF wash	1	0.5	6
7	20% 2-MP/DMF	1	10	3
8	DMF wash	1	0.5	6
9	Capping	2	60	1
10	DMF wash	1	10	3
11	DCM wash	1	10	3
12	Dry	1	60	1

[a]For peptide **2.38** and **2.41**: Addition of Fmoc-AA-OH (5.0 equiv) in DMF followed by addition of HOCt (5 equiv)/HCTU (5 equiv)/DIPEA (5 equiv) in DMF [b]2:1:3 solution of pyridine:acetic anhydride :DMF

The peptides were cleaved from the resin using the procedure described in section 2.5.4**.** The resulting crude peptides $AcDADSENSSF_{(DCV)}Y_{23(DCV)}Y_{24(DCV)}Y_{25}D_{(DCV)}Y_{27}LDEVAFNH_2$ (**2.39**), and $AcTTPD_{(DCV)}YGH_{(DCV)}YDDKDTLDLNTPVDKNH_2$ (**2.42**), were analyzed by analytical 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). The analytical HPLC chromatogram of crude **2.39** showed mainly one major peak in the HPLC chromatogram (t_R = 44.9 min) (see **Figure 2.27**). This was used without further purification for the synthesis of peptide **2.38**. The analytical HPLC chromatogram of crude **2.42** showed mainly one major peak in the HPLC

chromatogram, (t_R = 28.9 min) (see **Figure 2.29**). This was used without further purification for the synthesis of peptide **2.41**.

Peptide **2.38** was prepared from peptide **2.39** (20 mg) using the general deprotection procedure described in section 2.5.5. Preparative HPLC purification (CH_3CN/ 100 mM ammonium acetate (pH = 9) as eluent, linear gradient from 10% to 30% CH_3CN over 60 min, t_R= 23.0 min, λ = 220 nm) afforded pure **2.38** as a flocculent white powder after lyophilization (9.7 mg, 39% from resin loading). The analytical HPLC chromatogram (linear gradient of 10:90 CH_3CN:100 mM ammonium acetate to 40:60 CH_3CN:100 mM ammonium acetate over 40 min) showed a single peak at t_R= 14.2 min (see **Figure 2.28**). LRMS (ESI^-): calculated for $C_{112}H_{143}N_{22}O_{53}S_4$ $(M-H)^{-1}$ 2771.8059, found 2771.7405.

Peptide **2.41** was prepared from peptide **2.42** (20 mg) using the general deprotection procedure described in section 2.5.5. Preparative RP-HPLC purification (CH_3CN/ 100 mM ammonium acetate (pH = 9) as eluent, linear gradient from 5% to 20% CH_3CN over 40 min, t_R= 27.0 min, λ = 220 nm) afforded pure **2.41** as a flocculent white powder after lyophilization (14.0 mg, 58% from resin loading). The analytical HPLC chromatogram (linear gradient of 5:95 CH_3CN:100 mM ammonium acetate to 30:70 CH_3CN:100 mM ammonium acetate over 40 min) showed a single peak at t_R= 18.8 min (see **Figure 2.30**). LRMS (ESI^-): calculated for $C_{111}H_{165}N_{28}O_{48}S_2$ $(M-H)^{-1}$ 2722.0778, found 2722.0144.

2.5.9 Synthesis of AcMAEHDsY$_6$HEDsY$_{10}$GFSSFNDSSQNH$_2$ (2.48, CXCR6$_{1-20}$)

Automated SPPS was used. The following general protocol was used for all amino acid unless mentioned otherwise.

Step	Operation	Volume (mL)	Duration (Min)	Reps
1	DMF[a]	1	0.5	3
2	20% 2-MP	1	10	3
3	DMF wash	1	0.5	6
4	Coupling[b]	1	20	1
5	Coupling[b]	1	20	1
6	DMF wash	1	0.5	6
7	Capping[c]	2	10	1
8	DMF wash	1	0.5	6

[a] For the first AA duration is extended 10 minX 3 times. [b]Fmoc-AA-OH (5 equiv) in DMF followed by addition of a solution of HOCt (5 equiv), HCTU (5 equiv), DIPEA (5 equiv) in DMF. [c]2:1:3 solution of pyridine:acetic anhydride :DMF

Residues Glu8, Asp9 and pseudoproline **2.50** (position 13-14) were incorporated into the growing peptide chain using the following protocol:

Step	Operation	Volume (mL)	Duration (Min)	Rep
1	DMF wash	1	0.5	3
2	20% 2-MP	1	10	3
3	DMF wash	1	0.5	6
4	Coupling[a]	1	45	2
4	Coupling[a]	1	45	2
6	DMF wash	1	0.5	6
7	Capping[b]	2	10	1
9	DMF wash	1	0.5	6

[a]A solution of Fmoc-AA-OH (4 equiv) or pseudoproline dipeptide **2.50** HATU (4 equiv) and DIPEA (4 equiv) in DMF was added manually to the resin. [b]2:1:3 solution of pyridine:acetic anhydride :DMF

The last amino acid was attached to the growing peptide and acetylated using the same procedure described for insertion of the last amino acid in **2.39** (section 2.5.8). Cleavage of the peptide from the resin was achieved by mixing the resin for 2.5 h with the cleavage cocktail (TFA:TIS:H_2O:EDT 92.5:2.5:2.5:2.5), followed by 5 min washing time with the same cocktail. After cleavage from the support the crude peptide, $AcMAEHD_{(DCV)}YHED_{(DCV)}YGFSSFNDSSQNH_2$ (**2.49**), was isolated using the same procedure as that described in section 2.5.4. Crude peptide, **2.49**, was analyzed by analytical 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. The analytical HPLC chromatogram of crude **2.49** showed mainly one major peak in the HPLC chromatogram (t_R = 30.5 min) (see **Figure 2.41**). Peptide **2.49** was used without further purification for the synthesis of peptide **2.48**. Peptide **2.48** was prepared from peptide **2.49** by dissolving **2.49** (10 mg) in H_2O (1 mL) containing of Et_3N (5.56 µL, 11 equiv) 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 rt under hydrogen gas (balloon pressure) for 24 h. 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 preparative HPLC (CH_3CN/20 mM ammonium acetate as eluent, linear gradient of 10 to 15 % CH_3CN over 30 min) which gave 3.8 mg of pure **2.48** as a flocculent white powder after lyophilization (41% yield). The analytical HPLC chromatogram (linear gradient of 10:90 CH_3CN:20 mM ammonium acetate to 15:85 CH_3CN:20 mM ammonium acetate over 30 min) showed a single peak at t_R= 25.9 min (see

Figure 2.42). LRMS (ESI⁻): calculated for $C_{103}H_{134}N_{27}O_{45}S_3$ $(M-H)^{-1}$ 2564.8195, found 2564.5745.

2.5.10 Synthesis of AcAELSPSTENSSQLDFEDVWNSSsY$_{30}$GVNDSFPDGDsY$_{41}$DNH$_2$ (2.54, DARC$_{8-42}$).

Automated SPPS was used. The general protocol used for peptide **2.48** (section 2.5.9) was used for peptide **2.54** except for the last amino acid and residues Asp38, Glu39, Ser17, Ser18, Ser28 and Ser29. The last amino acid was attached to the growing peptide and acetylated using the same procedure described for the insertion of the last amino acid in **2.38** and **2.41** (section 2.5.8.). Asp38 and Glu39 were incorporated as dipeptide **2.63** and residues Ser28 and Ser29 and Ser17-and Ser18 were incorporated as pseudoproline dipeptide **2.50** using the same protocol described in section 2.5.9 for the incorporation of **2.50** in peptide **2.48**. The peptide was cleaved from the support using the same procedure described for peptide **2.48** (section 2.5.9). The resulting crude peptide AcAELSPSTENSSQLDFEDVWNS$_{28}$S$_{29(DCV)}$YGVNDSFPDGD$_{(DCV)}$YD-NH$_2$ (**2.55**), was 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. The analytical HPLC chromatogram of crude **2.49** showed mainly one major peak in the HPLC chromatogram (t_R = 24.05 min) (see **Figure 2.45**).

Peptide **2.54** was prepared from peptide **2.55** using the same procedure described for peptide **2.49** (section 2.5.9) except 17 equiv of triethylamine was used. Peptide **2.54** was purified using preparative HPLC (CH_3CN/20 mm ammonium acetate as eluent, linear

gradient from 15% to 20% CH_3CN over 60 min, t_R= 15.8 min) which gave 2.9 mg of pure **2.54** (32% yield). The analytical HPLC chromatogram (linear gradient of 15:85 CH_3CN:20 mM ammonium acetate to 25:75 CH_3CN:20 mM ammonium acetate over 30 min) showed a single peak at t_R= 14.26 min (see **Figure 2.47**). LRMS (ESI^-): calculated for $C_{169}H_{234}N_{41}O_{74}S_2$ $(M-H)^{-1}$ 4085.5255, found 4085.2175.

2.5.11 Crude AcISDRD$_{(DCV)}$YMGWMDFNH$_2$ (2.56)

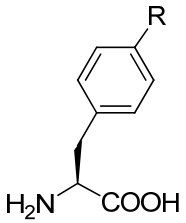
Automated SPPS was used employing the general protocol described above for peptide **2.48** (section 2.5.9) but using Fmoc deprotection times of either 3 x 10 min or 3 min + 11 min and using either 2-MP or piperidine. Peptide cleavage and post cleavage manipulations were performed as described for peptide **2.48** (section 2.5.9). The crude peptides (**2.56**) were analyzed by analytical HPLC using 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 2.48** and **2.49**)

Chapter 3

Synthesis of a Peptide Corresponding to PSGL-1_{43-50} Containing the Non-Hydrolyzable Sulfotyrosine Mimic 4-(Sulfonomethyl)phenylalanine using a Sulfonate protecting Group Strategy

3.1 Introduction

The development of a facile method for the synthesis of sTyr-containing peptides is important since these peptides can be used for determining the requirement for sTyr residues within sTyr-bearing proteins. However, such peptides have limitations as far as drug *development* is concerned due to the hydrolytic lability of the sulfate group. For drug development, a stable substitute for the sulfate group is preferred. One of the most widely used non-hydrolyzable sTyr mimics is 4-(sulfonomethyl)phenylalanine (**3.2, Smp**). It retains almost the same dimensions and charge as sTyr but it is much more stable than sTyr.

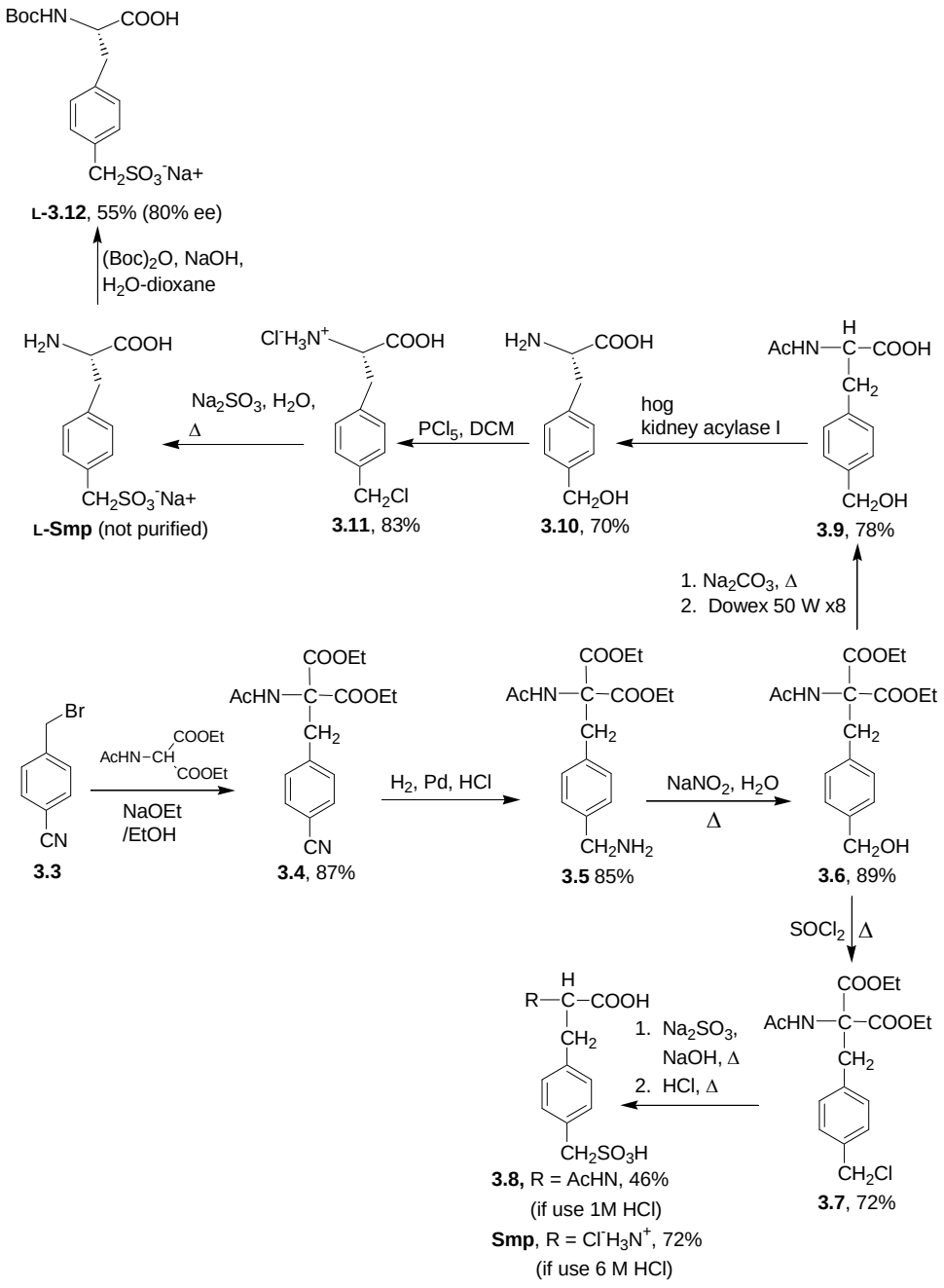


3.1 R = OSO_3H (sulfotyrosine)
3.2 R = CH_2SO_3H (4-(sulfonomethyl)phenylalanine, Smp)

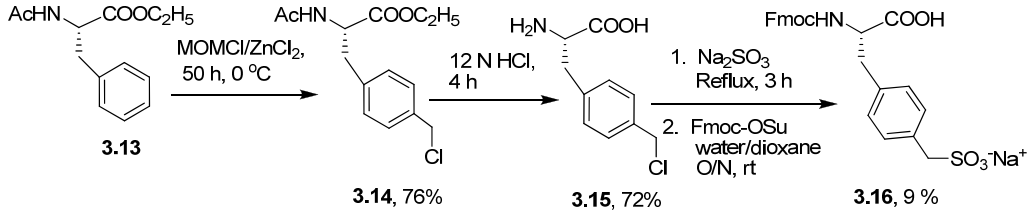
Figure 3.1. Sulfotyrosine (sTyr) and 4-(sulfonomethyl)phenylalanine (Smp).

Marseigne and Roques, 1988, were the first to propose Smp as a stable replacement for sTyr.[156] They developed a racemic synthesis of AcSmp (**3.8** in **Scheme 3.1**) and using Boc solution phase peptide synthesis constructed Ac(D,L)Smp-Nle-Gly-Trp-Nle-Asp-Phe-

NH_2 a heptapeptide that corresponds to residues 27-33 in CCK. In this peptide, sTyr27 in CCK has been replaced with a Smp group and the Met residues at positions 28 and 31 have been replaced with Norleucine (Nle). The D,L mixture of peptide isomers were separated by chromatography. The L-isomer displayed an affinity for pancreatic binding sites (K_i = 1.7 nM) almost equal to that of its sTyr analog.[157] Moreover, it was a full agonist in the stimulation of pancreatic amylase secretion as well as in the induction of guinea pig ileum contractions (EC_{50} = 3.2 nM).[157] In 1991, Roques and coworkers reported a synthesis of Smp in which racemic precursor **3.9** (S**cheme 3.1**) was subjected to a kinetic resolution using hog kidney acylase (**Scheme 3.1**) to give **3.10** predominantly as the L-isomer. Compound **3.10** was then converted into L-BocSmp (**3.12**, 80% ee). This amino acid was then incorporated into Ac-Asp-Smp-Nle-Gly-Trp-Nle-Asp-Phe-NH_2 an octapeptide that corresponds to residues 26-33 in CCK using SPPS and Boc chemistry.[158] This compound proved to be a full agonist in the stimulation of pancreatic amylase secretion.



Scheme 3.1. Synthesis of Smp building blocks by Marseigne and Roques.

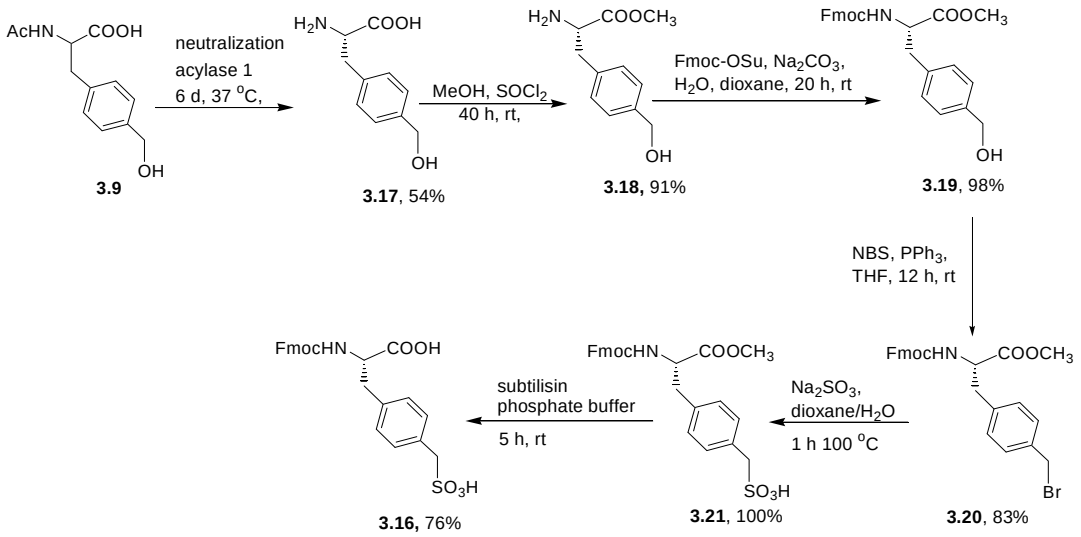


Scheme 3.2. Synthesis of building block **3.19** according to Rivier and coworkers' procedure.[159]

In 1993, Rivier and coworkers reported an enantioselective synthesis of L-FmocSmp (**3.16**) (**Scheme 3.2**). This synthesis appears to be very straightforward starting from readily available Ac-L-PheOEt (**3.13**) though the yield of the last two steps was low (9% yield of pure product obtained over these two steps). These workers then used **3.16** as a building block to incorporate Smp into a variety of CCK analogs using SPPS. For example they prepared a peptide corresponding to residues 1-33 in CCK. In this peptide Tyr27 was replaced with Smp, Met residues 7, 28 and 31 were replaced with Nle and Trp30 was replaced with 2-naphthylalanine (Nal). The crude peptide preparation was extremely complex. Nevertheless, they were able to obtain pure peptide after multiple HPLC columns.[159] This peptide was 30 times less potent than CCK-8 in the stimulation of pancreatic amylase secretion.[159] They suggested that this reduction in activity compared to CCK-8 was due to the large number of unnatural amino acids present in their modified peptide.

In 2007, Herzner and Kunz wished to construct a peptide corresponding to part of the N-terminus of PSGL-1 (see chapter 1, section 1.2.1 for a discussion of PSGL-1) except that they wished to replace the sTyr residues with Smp.[160] They reported that they were unable to

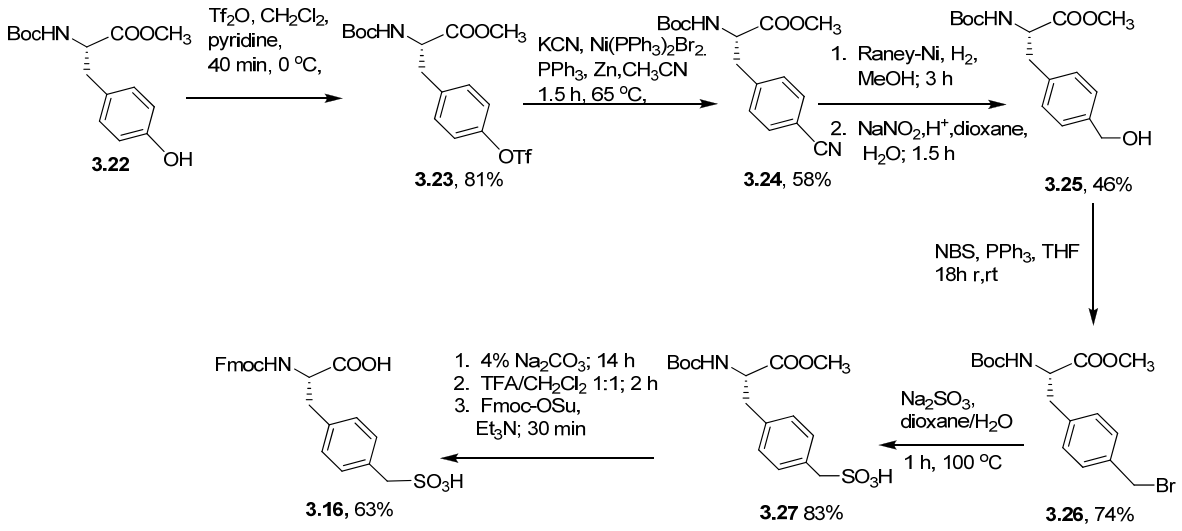
repeat Rivier and coworkers' synthesis of amino acid **3.16**. So they embarked on a study to develop a more reliable synthesis of this key building block. Their first trial started with hydroxymethyl derivative **3.9** which was prepared following Marseigne and Roques chemistry in five steps starting from *p*-methyl-benzonitrile (**Scheme 3.1**).[156] Resolution of the D,L-mixture was done using an acylase enzyme followed by carboxyl protection and Fmoc installation. Reaction of hydroxymethyl derivative **3.25** with *N*-bromosuccinimide and triphenylphosphine provided the corresponding alkylbromide **3.26** in a good yield. Installation of the sulfonate group followed by enzymatic hydrolysis of the ester afforded the desired building block (**Scheme 3.3**). Because this route involves many steps and the low yield of the resolution step, the authors decided to explore other routes.



Scheme 3.3. Synthesis of building block **3.16** according to Herzner and Kunz (route 1).

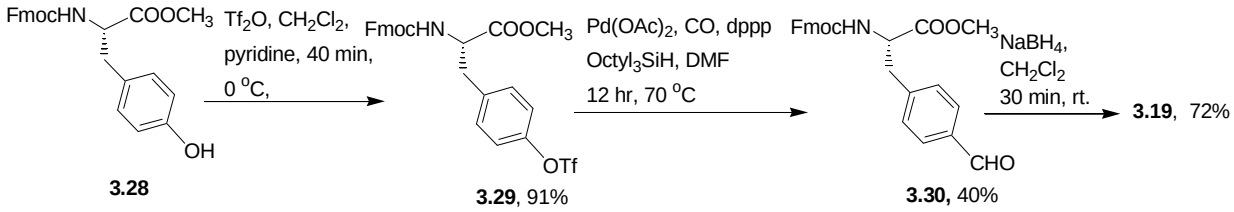
Their next route started with the synthesis of the triflate derivative **3.23** which in turn was reacted with potassium cyanide in a nickel-catalyzed reaction to give the corresponding

cyano derivative **3.24** in a 58% yield (**Scheme 3.4**). Reduction of the cyano group using Raney-Nickel followed by diazotization and hydrolysis gave the corresponding hydroxymethyl derivative **3.25** in low yield. The rest of manipulations were done in analogy to **Scheme 3.3** expect that there are extra two steps involving Boc removal and Fmoc installation. Again the synthesis is relatively long and proceeds in low overall yield.



Scheme 3.4. Synthesis of building block **3.19** according to Herzner and Kunz (route 2).

In attempt to improve the yield of compound **3.19** (in **Scheme 3.3**) Herzner and Kunz performed a palladium-catalyzed reductive carbonylation of Fmoc-protected tyrosine derivative **3.29** to give formyl derivative **3.30** (**Scheme 3.5**). Unfortunately, the reaction proceeded in low yield. The reduction of the formyl group using sodium borohydride occurred smoothly to furnish the hydroxymethyl derivative **3.19** which was subsequently manipulated in the same way as described in **Scheme 3.3**.



Scheme 3.5. Synthesis of building block 3.19 according to Herzner and Kunz (route 3).

Having the building block **3.16** in hand, they decided to explore the utility of it in the SPPS of a protected octapeptide corresponding to residues 43-50 in PSGL-1 (**3.31**, **Figure 3.2**). The coupling of the first four amino acids proceeded smoothly. However, coupling of the fifth amino acid (the second Smp) turned out to be difficult and even after performing double couplings for 16 h the yield did not exceed 50%. Furthermore, the coupling of the seventh amino acid was difficult and the yield did not exceed 34% even after double couplings for 16 h. The researchers did not comment if the difficulty in the synthesis was due to the use of **3.16** or if it was due to the peptide sequence itself. The overall yield was only 5.5% after a difficult HPLC purification.

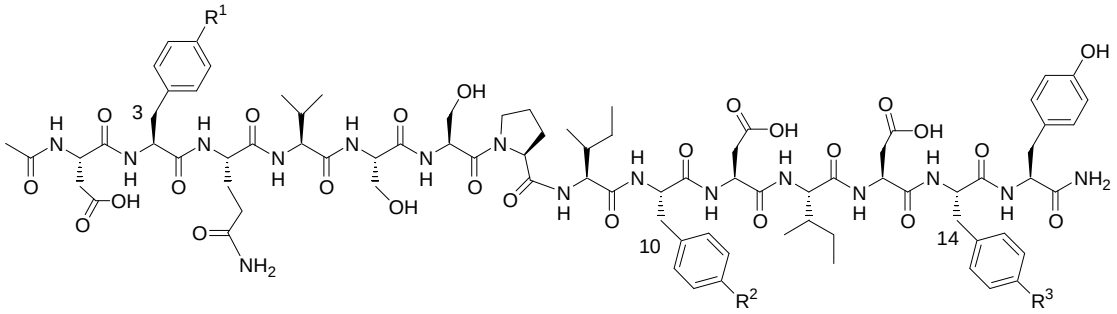
$$Ac\text{-}Ala_{43}\text{-}Thr(Bn)\text{-}Glu(OBn)\text{-}Phe(CH_2SO_3H)\text{-}Glu(OBn)\text{-}Phe(CH_2SO_3H)\text{-}Leu\text{-}Asp(OBn)_{50}\text{-}OH$$

Figure 3.2. Partially protected peptide corresponding to residues Ala^{43}-Asp^{50} of PSGL-1 (**3.31**).

Very recently (2008), Bewley *et al.* examined Smp as a replacement for the sTyr residues in CCR5. It has been shown that the HIV-1 co-receptor, CCR5, has two sTyr residues at positions 10 and 14, whose presence is required for binding HIV-1 gp-120 and

mediating viral entry.[161] It has been suggested that peptides or other compounds that can interact with these sTyr residues may be used as anti-HIV drugs since they might be able to compete with HIV-1 for binding to CCR5. When they tried to develop an assay to screen for sTyr-based inhibitors of CCR5 Nt-gp120 interactions they found that the sTyr residues lacked sufficient chemical stability which made their assay results questionable (they also mentioned that the large scale synthesis of sTyr peptides in the quantities and quality required for their assays was difficult using the methods that had been published at that time). In order to find an alternative substitute for the sulfate groups, they prepared a number of peptides (**Figure 3.3**) where the sTyr residues in peptide **3.32** were replaced with phosphotyrosine (pTyr) at one of the two positions (**3.33** and **3.34**, **Figure 3.3**) or both of them (**3.35**), or the two sulfate groups were replaced with Smp (**3.36**) (no mention was made as to how the Smp-bearing peptide was constructed) and tested them for their ability to bind to the CD4-gp120 complex using saturation transfer difference NMR and surface plasmon resonance.[162] The phosphate groups generally diminished the binding affinity as illustrated by the complete loss of binding affinity of **3.35**. Based on the results of the binding affinities of **3.33** and **3.34** it was concluded that position 10 is more accommodating to the phosphate than position 14. Such an observation can be reasoned by the fact that the binding pocket for sTyr14 contains only one positive charge and hence cannot accommodate the double charged phosphate group very well. Most importantly, they found that sulfonate analogue **3.36** had only a slightly diminished affinity compared to **3.32**. The slight reduction of the binding occurs because a hydrogen bond that is formed between the ester oxygen (S-O-C oxygen) in the sTyr residue and one of the residues in binding site is completely lost in the sulfonate

analogue. On the top of that, there is a steric clash between the methylene hydrogens of the sulfonate residue and the active site. Overall, the authors decided that Smp is the best substitute for sTyr.

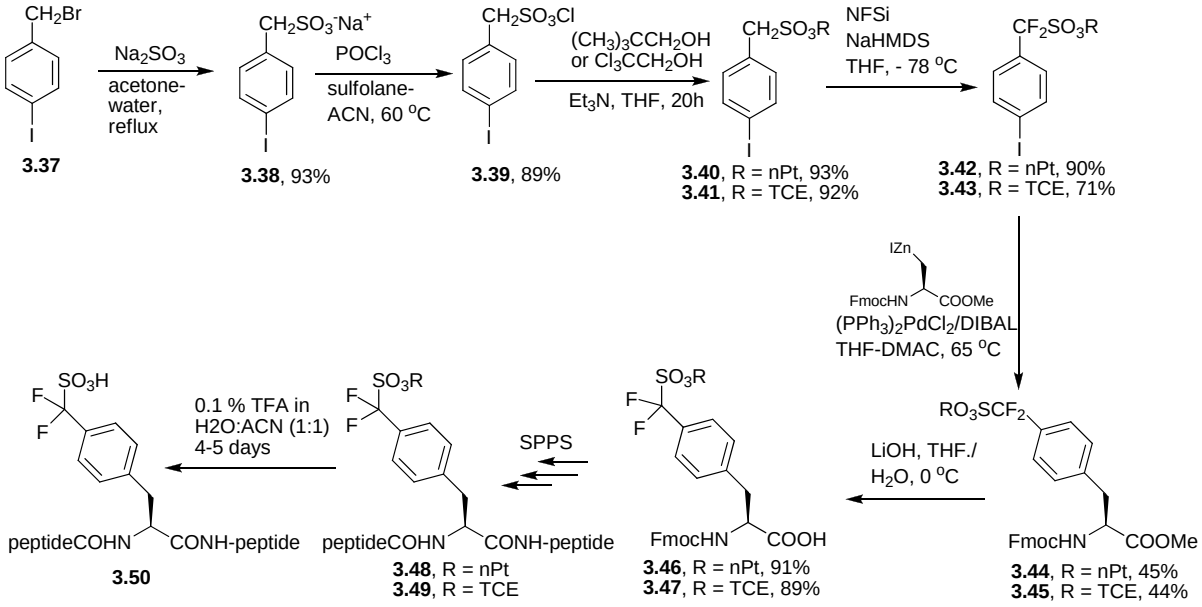


3.32 R^1= OH, R^2=R^3=OSO_3^-
3.33 R^1= OH, R^2=OSO_3^-,R^3=OPO_3^{2-}
3.34 R^1= OH, R^2=OPO_3^{2-},R^3=OSO_3^-
3.35 R^1= OH, R^2=R^3=OPO_3^{2-}
3.36 R^1= OH, R^2=R^3=$CH_2SO_3^-$

Figure 3.3. CCR5 Nt peptide and analogue

It is clear for the above discussion that Smp is a useful non-hydrolyzable sTyr mimic. However, it is also clear from the above discussion that there are issues concerning its synthesis and its incorporation into peptides. Several years ago, our group reported the enantioselective synthesis of compounds **3.46** and **3.47** using the approach outlined in **Scheme 3.6**.[163] These building blocks were used to incorporate L-[sulfono(difluoromethyl)]phenylalanine (F_2Smp) into peptides.[163] The syntheses were done manually using HATU/HOAt as coupling agent and 20% piperidine in DMF for Fmoc deprotection. The HPLC traces of the crude TCE-protected peptides of type **3.49** exhibited numerous peaks compared to the relatively clean neopentyl protected peptide (**3.48**). The

removal of the neopentyl group was achieved using 1:1 CH_3CN:0.1% TFA (H_2O) for 4-5 days at rt which gave the F_2Smp-bearing peptides (**3.50**) in good yield.



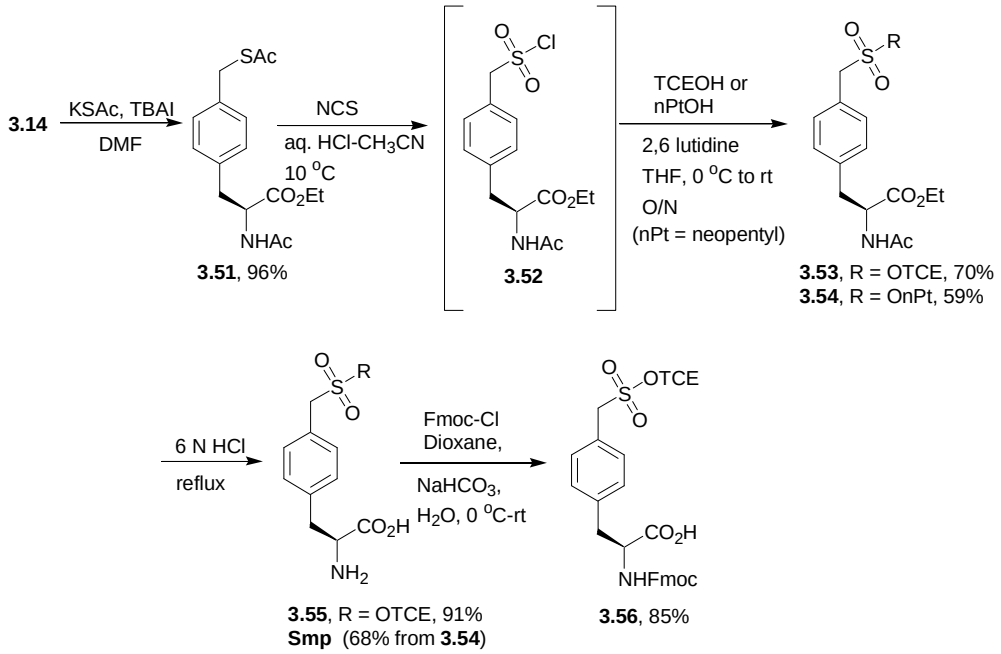
Scheme 3.6. Synthesis of sulfotyrosine mimics **3.51** and **3.52**.

The ease with which F_2Smp was incorporated into peptides has prompted us to examine whether a similar approach, where the sulfonate moiety is incorporated as a protected ester, could be used for the synthesis of peptides bearing Smp. We wished to construct FmocSmp with the sulfonate group protected and then use this as a building block to prepare Smp-bearing peptides. Dr. Bryan Hill, a post-doc in the Taylor lab attempted to prepare FmocSmp derivatives with the sulfonate moiety protected with either a neopentyl or a TCE group (**Scheme 3.7**). The TCE group has never been examined as a protecting group for sulfonate esters; however, as discussed in chapter 2, this moiety is an effective protecting

group for sulfate esters and is removed under very mild reducing conditions. It was anticipated that it might be suitable as a protecting group for the sulfonate group in Smp during SPPS if we used 2-methylpiperidine (2-MP) for Fmoc removal as we did for the synthesis of sTyr peptides (see Chapter 2). The key step in Dr. Hill's approach to these compounds was the selective deprotection of the carboxylic acid and amino functionalities in **3.53** and **3.54** without loss of the sulfonate protecting group (**Scheme 3.7**). We were pleased to find that this could be achieved with the TCE-protected compound **3.53** by refluxing **3.53** in 6 N HCl for 12 h which gave amino acid **3.55** in a 91% yield. This reaction demonstrates the remarkable stability of TCE-protected sulfonates to acid. However, subjecting the neopentyl ester **3.54** to the same conditions resulted in hydrolysis of the neopentyl ester to give Smp in 68% yield. He was unable to find conditions that would allow us to selectively remove the ethyl and acetyl groups without removing the neopentyl group. TCE-protected FmocSmp (**3.56**) was obtained in an 85% yield from **3.55** by reacting **3.55** with Fmoc-Cl under standard Schotten-Baumann conditions.

3.2 Objectives

The objective of the work carried out in this chapter is to determine if Smp-bearing peptides can be prepared in good yield and purity using compound **3.56** as the building block.



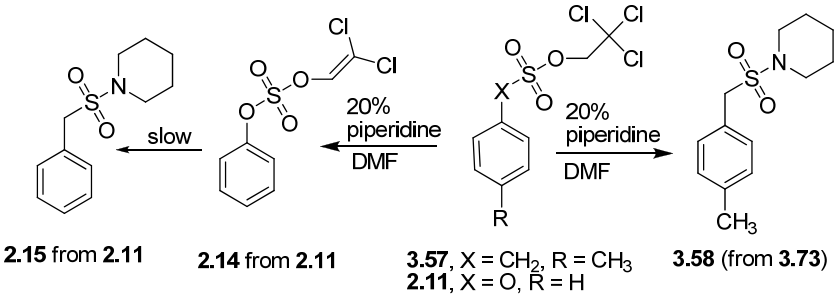
Scheme 3.7. Synthesis of amino acid derivative **3.56.**

3.3 Result and Discussion

3.3.1 Stability of TCE-protected phenylsulfonates

Before attempting to prepare peptides using compound **3.56** we determined conditions for removing the TCE group and we examined the stability of TCE sulfonate esters by subjecting model sulfonate **3.57** (**Scheme 3.8**) to a variety of conditions. The TCE group in **3.57** was readily removed using mild reducing conditions such as Zn/ammonium formate in MeOH, Zn in HOAc and hydrogenolysis (H_2 or ammonium formate with 10% Pd/C). Not surprisingly, compound **3.57** is stable to 100% TFA with no detectable reaction occurring even after several days and to TFA containing scavenging reagents such as triisopropylsilane (TIPS), anisole and thioanisole. It is also stable to mild reducing agents such as $NaBH_4$. It is stable to sterically hindered organic bases such as triethylamine but slowly decomposes in the presence of an excess of stronger or less sterically hindered

organic bases. For example, ^{1}H NMR studies in 20% piperidine/DMF-d_7 revealed that ester **3.57** undergoes nucleophilic attack by piperidine on the sulfur atom to give compound **3.58** (**Scheme 3.8**) which was isolated and also characterized by mass spectrometry and ^{1}H NMR. Compound **3.57** was completely consumed within 24 hours. This is in contrast to the previously mentioned aryl sulfate **2.11** which undergoes a relatively rapid elimination of HCl to give dichlorovinyl (DCV) ester **2.14** followed by a slower substitution reaction to give **2.15** and other decomposition products.



Scheme 3.8. Decompostion of sulfate **2.11** and sulfonate **3.57** in presence of 20%piperidine.

We have also shown (see chapter 2) that although 20% 2-methylpiperidine (2-MP) in DMF also converts sulfate ester **2.11** into DCV sulfate ester **2.14**, no further reaction between 2-MP and ester **2.14** occurs even after several days. Furthermore, 2-MP was used in the preparation of number of difficult peptides and worked as well as piperidine during SPPS (see chapter 2). This encouraged us to examine the stability of compound **3.57** in the presence of 20% 2-MP. ^{1}H NMR studies of ester **3.57** in 20% 2-MP/DMF-d_7 revealed that although attack of 2-MP on the sulfur atom still occurs, the reaction is considerably slower compared to when piperidine is used and even after 8 hours only 8% of **3.57** had reacted.

These results together with fact that sulfonates exhibit superior on-resin stability compared to in solution as illustrated by Hari and Miller[149] suggested to us that amino acid **3.56** could be used in SPPS if 2-MP was used as the base for Fmoc removal.

3.3.2 Determination of the enantiopurity of compounds 3.56

Although Dr. Bryan Hill had prepared amino acid derivative **3.56** he had not determined its enantiopurity. The enantiopurity of **3.56** were determined by constructing dipeptides Ac-Phe(*p*-CH_2SO_3TCE)-$A_{(L)}$-NH_2 (**3.59LL**) and Ac-Phe(*p*-CH_2SO_3TCE)-$A_{(DL)}$-NH_2 (**3.59LL/3.59DL**) and comparing their HPLC chromatograms and ^{1}H-NMR spectra. These dipeptides were prepared using the same protocol described for the preparation of hexapeptide **2.16**.

As expected, the diastereomeric dipeptide mixture Ac-Phe(*p*-SO_3TCE)-$A_{(DL)}$-NH_2 showed two peaks in the HPLC trace and two sets of peaks in the ^{1}H-NMR spectrum corresponding to peptides **3.59LL** and **3.59LD** (**Figure 3.4** and **3.5**). Peptide Ac-Phe(*p*-SO_3TCE)-$A_{(L)}$-NH_2 on the other hand showed only one peak in the HPLC trace and one set of peaks in the ^{1}H-NMR spectrum corresponding to peptide **3.59LL** (**Figures 3.6** and **3.7**) indicating that amino acid **3.56** was obtained in > 98% ee.

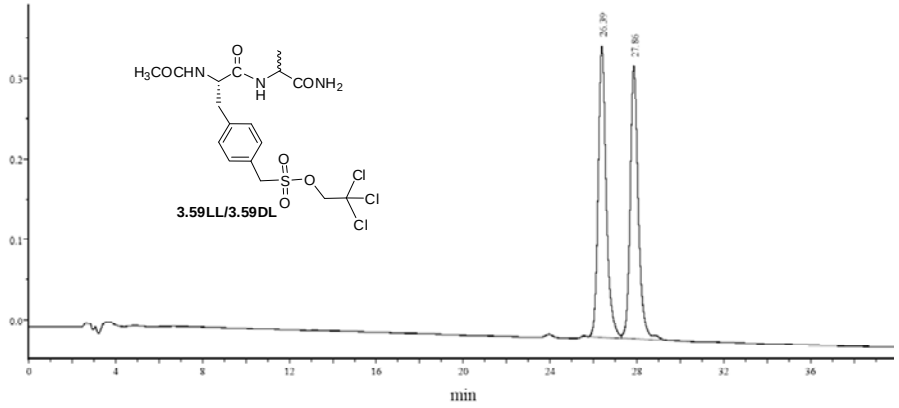


Figure 3.4 Analytical HPLC chromatogram of diastereomeric dipeptides **3.59LD** and **3.59LL**

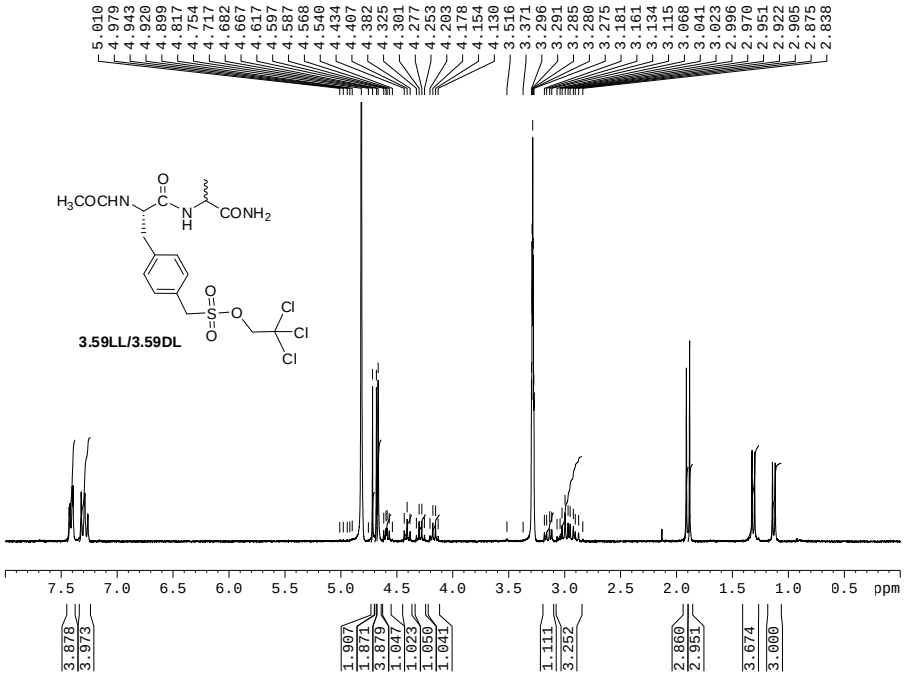


Figure 3.5. 300 MHz ^{1}H NMR (CD_3OD) spectrum of diastereomeric dipeptides **3.59LD** and **3.59LL.**

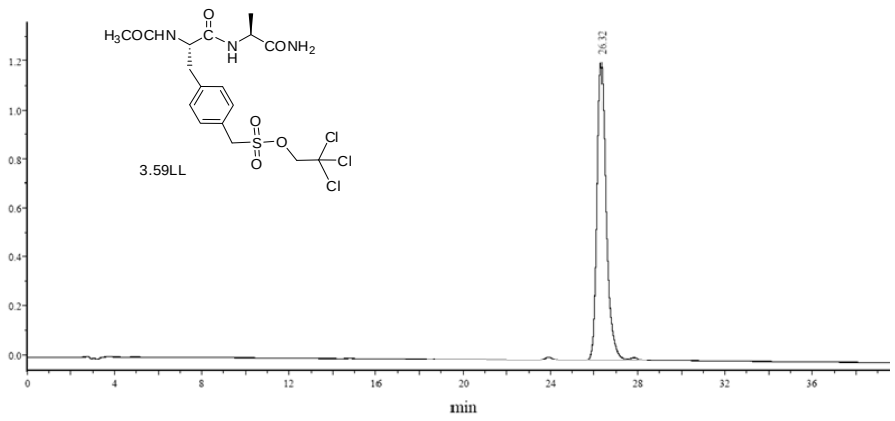


Figure 3.6. Analytical HPLC chromatogram of dipeptide **3.59LL.**

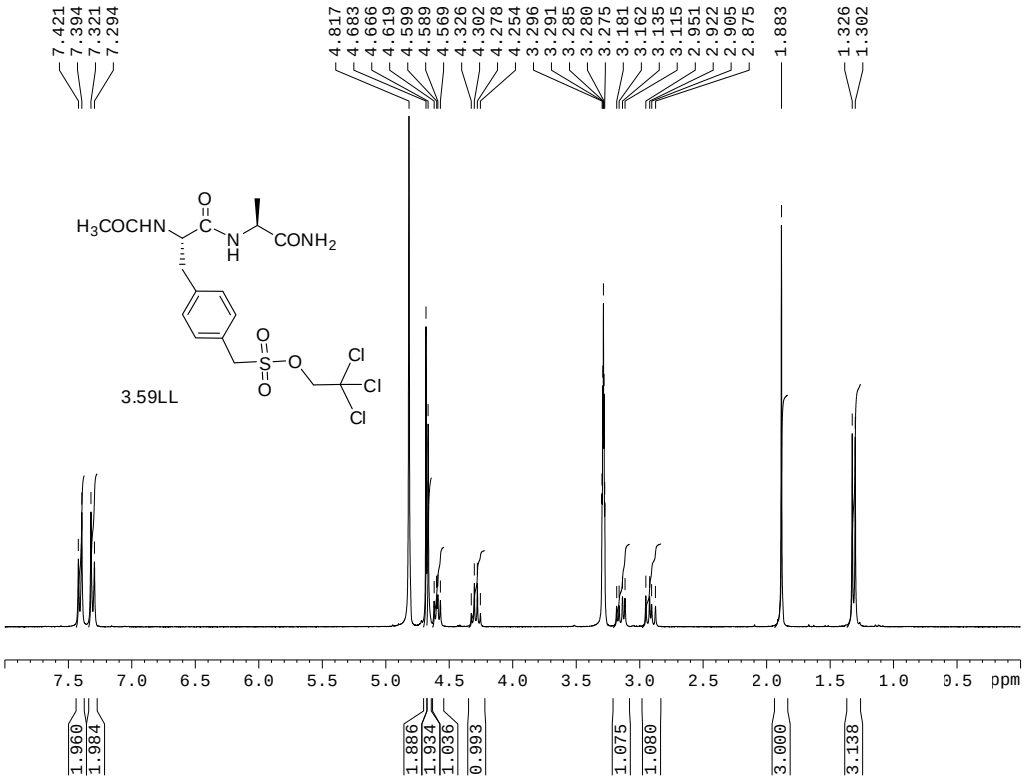


Figure 3.7. 300 MHz ^{1}H NMR (CD_3OD) spectrum of dipeptide **3.59LL.**

3.3.3 Synthesis of a model sulfonated peptide corresponds to residues 43-50 of PSGL-1 (3.60)

In order to demonstrate the utility of our TCE-protected FmocSmp in peptide synthesis we prepared an octapeptide, Ac-Ala-Thr-Glu-Phe(*p*-$CH_2SO_3^-$)-Glu-Phe(*p*-$CH_2SO_3^-$)-Leu-Asp-NH_2 (**3.60**), which corresponds to residues 43-50 of P-selectin glycoprotein ligand-1 (PSGL-1) in which the sTyr residues at positions 46 and 48 are replaced with Smp. This was chosen as a model peptide since Herzner and Kunz (see section 3.1.1) reported difficulties in preparing benzyl-protected version of **3.60** (**3.31** in **Figure 3.2**) using sulfonate-unprotected amino acid **3.16**.[160] Automated SPPS was performed using the Rink amide resin and HBTU/HOBt as coupling reagents. The removal of the Fmoc group was removed using 3 x 10 min of 20% 2-MP/DMF. After the completion of the peptide chain, the completed peptide was cleaved from the resin using 98% TFA/2% TIPS then precipitated in ether. The analytical HPLC chromatogram of the crude peptide consisted of one major peak plus a few minor peaks (**Figure 3.8**). Furthermore, the $^-$ESI mass spectrum of the crude peptide indicated that the major product in the crude mixture was desired TCE-protected peptide Ac-Ala-Thr-Glu-Phe(*p*-CH_2SO_3TCE)-Glu-Phe(*p*-CH_2SO_3TCE)-Leu-Asp-NH_2 (**3.61**) and no peaks corresponding to peptides that had undergone substitution of the TCE group with 2-MP were detected. After subjecting crude peptide **3.61** to H_2 (balloon), 30 wt. % of 10% Pd/C, and 15 equiv. ammonium formate for 6 h, HPLC analysis of the crude reaction mixture revealed one major peak plus a variety of minor peaks (**Figure 3.9**). Purification by semi-preparative RP-HPLC gave pure peptide **3.60** in a 60% yield in 95% purity (**Figure 3.10**)

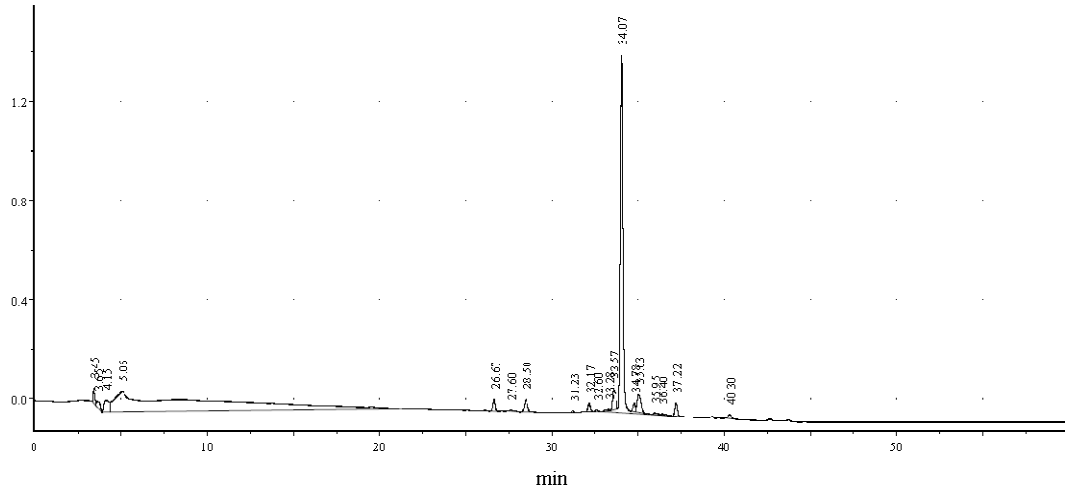


Figure 3.8. Analytical HPLC chromatogram of the crude peptide Ac-Ala-Thr-Glu-Phe(*p*-CH_2SO_3TCE)-Glu-Phe(*p*-CH_2SO_3TCE)-Leu-Asp-NH_2 (**3.61,** t_R= 34.0 min).

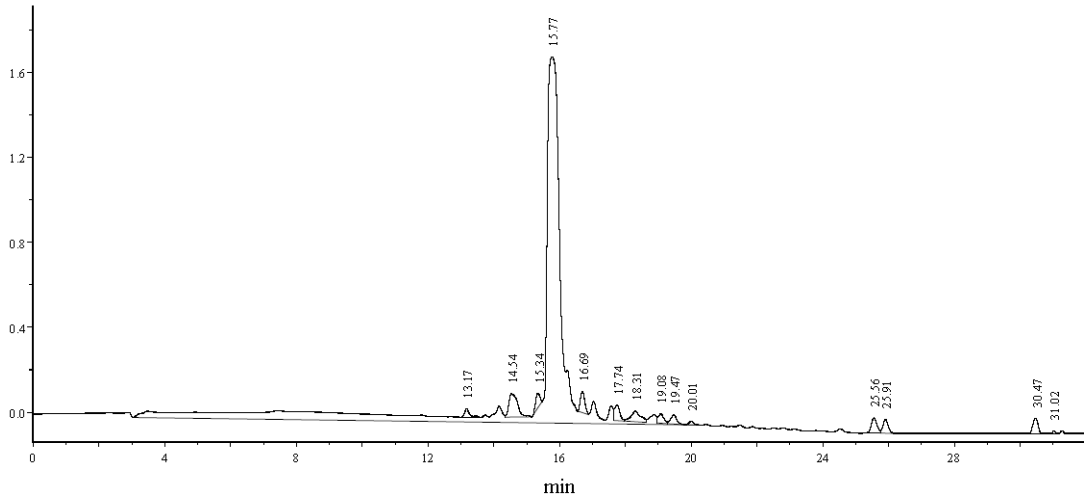


Figure 3.9. Analytical HPLC chromatogram of the crude peptide Ac-Ala-Thr-Glu-Phe(*p*-$CH_2SO_3^-$)-Glu-Phe(*p*-$CH_2SO_3^-$)-Leu-Asp-NH_2 (**3.60,** t_R = 15.7 min) prepared from TCE protected amino acid **3.61.**

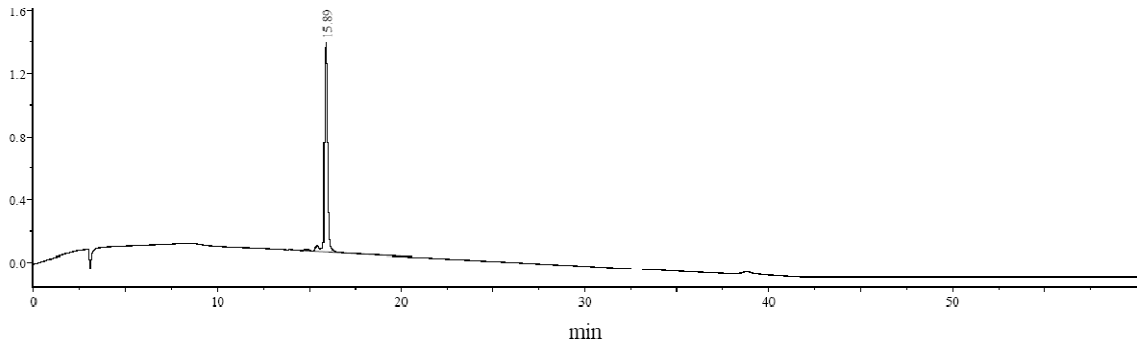
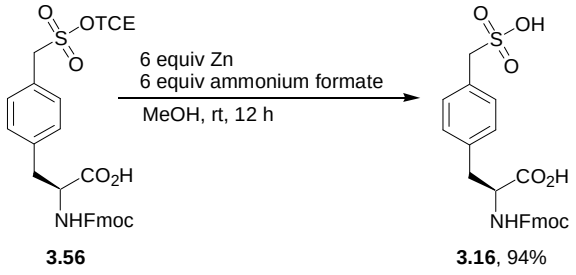


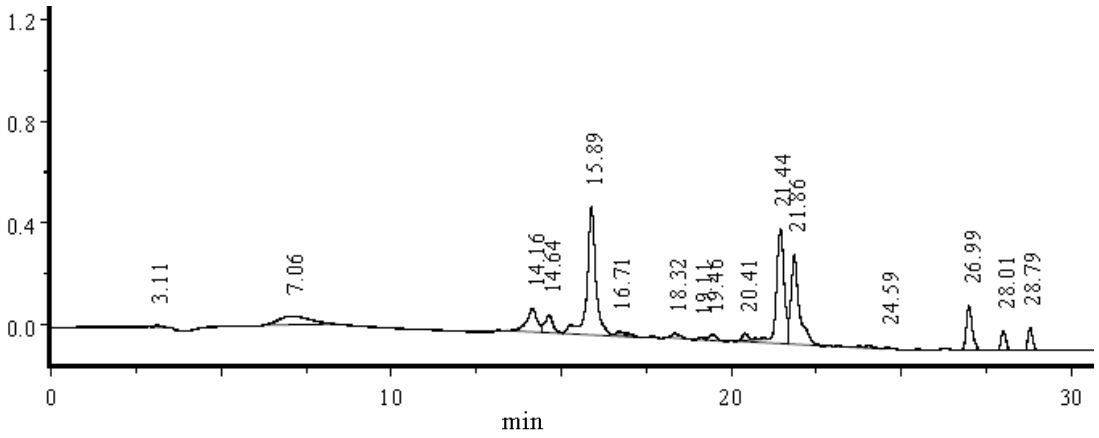
Figure 3.10. Analytical HPLC chromatogram of purified peptide Ac-Ala-Thr-Glu-Phe(*p*-$CH_2SO_3^-$)-Glu-Phe(*p*-$CH_2SO_3^-$)-Leu-Asp-NH_2 (**3.60,** t_R = 15.88 min) prepared using TCE protected amino acid **3.56.**

To compare our methodology to the previously reported methodology in which Smp is incorporated unprotected we also prepared peptide **3.60** using the identical procedure except sulfonate-unprotected amino acid **3.6** was used as a building block. We found that amino acid **3.16** could be prepared in 94% yield from compound **3.56** by subjecting **3.56** to Zn/ammonium formate in MeOH (**Scheme 3.9**). HPLC analysis of the crude peptide showed many peaks (**Figure 3.10**) and after purification using semi-preparative RP-HPLC pure peptide **3.60** was obtained in only a 19% yield.



3.56 **3.16**, 94%

Scheme 3.9. Synthesis of **3.16** from **3.56.**



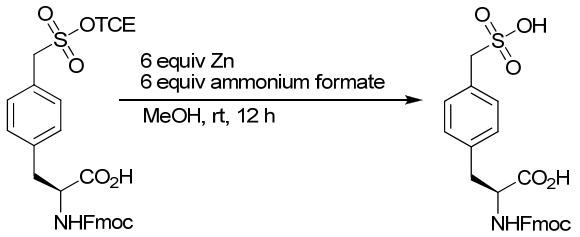
Scheme 3.10. Analytical HPLC chromatogram of the crude peptideAc-Ala-Thr-Glu-Phe(*p*-$CH_2SO_3^-$)-Glu-Phe(*p*-$CH_2SO_3^-$)-Leu-Asp-NH_2 (**3.60,** t_R=15.76 min) prepared from unprotected amino acid **3.16.**

3.4 Summary

We have shown that TCE-protected sulfonates are stable to a variety of conditions such as strong acid, mild bases and reducing agents but are not stable to an excess of organic bases such as piperidine. We demonstrated that amino acid **3.56** is an effective building block for the solid phase synthesis of Smp-bearing peptides when using 2-MP as base and that this approach provided the targeted peptide in higher yield compared to when sulfonate-unprotected amino acid **3.16** was used. We expect that the TCE group will be useful as a protecting group for sulfonates in general and will be especially effective in situations where stability to strongly acidic conditions is required. Future studies will involve the preparation of other Smp-bearing peptides such as those corresponding the N-terminus of the DARC protein (see chapter 1, section 1.2.2.2) and then examine them for their ability to prevent DBL binding to Duffy positive red blood cells.

3.5 Experimental

Synthesis of FmocPhe(*p*-CH_2SO_3H)OH (3.16).



3.56 **3.16**

To a solution of amino acid **3.56** (1.0 g, 1.63 mmol) in HPLC grade methanol (3 mL) was added ammonium formate (0.61 g, 9.78 mmol) followed by Zn dust (0.64 g, 9.78 mmol). After stirring overnight, the reaction mixture was filtered through Celite® and concentrated by rotary evaporation. Compound **3.16** was purified by flash chromatography using CH_2Cl_2/MeOH/AcOH/H_2O (50:8:1:1), and the solvent system was shifted to (CH_2Cl_2/MeOH/ AcOH /H_2O 7:3:0.3:0.6) when compound **3.16** started to elute from the column. This gave pure **3.16** as a white solid (0.73 g, 94%). Mp = 182-185 ^oC; ^1H NMR (300 MHz, CD_3OD): δ 7.76 (2H, d, J = 7.5 Hz); 7.59 (2H, d, J = 7.2 Hz), 7.38-7.25 (6H, m), 7.15 (2H, d, J = 7.8 Hz), 4.35-4.27 (2H, m), 4.24-4.18 (1H, m), 4.15-4.11 (1H, m), 3.97 (2H, s), 3.14 (1H, dd, J_1 = 9.3 Hz & J_2 = 4.8 Hz), 2.94 (1H, dd, J_1 = 8.4 Hz & J_2 = 5.4 Hz); ^{13}C NMR (75 MHz, DMSO-d_6): δ 174.1, 156.1, 144.3, 144.2, 141.1, 136.6, 133.6, 130.3, 128.8, 128.0, 127.5, 125.8, 125.7, 120.5, 65.98, 57.6, 56.5, 47.0, 36.9; LR$^-$ESIMS *m/z* (relative intensity): 480 ([M-H]$^-$, 100), 481 (M, 32); HR$^-$ESIMS calculated for $C_{25}H_{22}NO_7S$ $(M-H)^-$ 480.1117, found 480.1104.

Determination of the enantiopurity of amino acid 3.56.

The enantiopurity of the building block **3.56** was determined by synthesizing dipeptides Ac-Phe(*p*-SO_3TCE)-$A_{(L)}$-NH_2 (**3.59LL**) and Ac-Phe(*p*-SO_3TCE)-$A_{(DL)}$-NH_2 (**3.59LL/3.59DL**) and analyzing them by HPLC and ^{1}H NMR. The synthesis of the dipeptides was done in the same way as described for hexapeptide **2.16** and dipeptides **2.28** and **2.29** using Rink amide as solid support, HBTU/HOBt as coupling agent and 2-MP for Fmoc removal (chapter 2, section 2.5.4). After coupling of the last amino acid and Fmoc removal, an acetylation step was conducted using acetic anhydride/pyridine/DMF mixture. The cleavage from the resin and post cleavage manipulations were performed in the same way as described before for **2.28** and **2.29**. The crudes products after removal of the cleavage cocktail were purified by flash chromatography to yield the pure dipeptide products. The resulting dipeptides were analyzed using analytical C-18 RP-HPLC eluting with CH_3CN/20 mM ammonium acetate (pH = 6.8) employing a linear gradient of 20% to 35% CH_3CN over 40 min with the detector set to 220 nm (see **Figures 3.4** and **3.6**).

Ac-Phe(*p*-CH_2SO_3TCE)-$A_{(L)}$-NH_2 (3.59LL)

^{1}H NMR (300 MHz, CD_3OD): δ 7.42-7.39 (4H, m), 7.32-7.26 (4H, m), 4.71 (2H, s), 4.68 (2H, s), 4.66 (4H, s), 4.59 (1H, dd, J_1 = 5.9 Hz & J_2 = 2.8 Hz), 4.40 (1H, t, J = 7.7 Hz), 4.28 (1H, dd, J_1 = 7.3 Hz & J_2 = 7.1 Hz), 4.16 (1H, dd, J_1 = 7.2 Hz & J_2 = 7.0 Hz), 3.14 (1H, dd, J_1 = 8.0 Hz & J_2 = 5.8 Hz), 3.0-2.8 (3H, m), 1.91 (3H, s), 1.88 (3H, s), 1.31 (3H, d, J = 7.1 Hz), 1.12 (3H, d, J = 7.3 Hz). HRMS (ESI^+): calculated for $C_{17}H_{23}Cl_3N_3O_6S$ $(M+H)^+$ 502.0373, found 502.0385. The ^{1}H NMR spectrum is shown in **Figure 3.7**.

Ac-Phe(*p*-CH_2SO_3TCE)-$A_{(DL)}$-NH_2 (3.59LL/3.59DL)

^{1}H NMR (300 MHz, CD_3OD): δ 7.40 (2H, d, J = 8.1 Hz), 7.30 (2H, d, J = 8.1 Hz), 4.68 (2H, s), 4.66 (2H, s), 4.59 (1H, dd, J_1 = 5.9 Hz & J_2 = 2.9 Hz), 4.28 (1H, dd, J_1 = J_2 = 7.1 Hz), 3.14 (1H, dd, J_1 = 8.0 Hz & J_2 = 5.8 Hz), 2.95-2.87 (1H, m), 1.88 (3H, s), 1.31 (3H, d, J = 7.1 Hz),; HRMS (ESI^+): calculated for $C_{17}H_{23}Cl_3N_3O_6S$ $(M+H)^+$ 502.0373, found 502.0385. The ^{1}H NMR spectrum is shown in **Figure 3.5**.

Synthesis of crude peptides AcATEPF(*p*-CH_2SO_3TCE)EF(*p*-CH_2SO_3TCE)LDNH_2 (3.61), and AcATEPF(*p*-CH_2SO_3H)EF(*p*-CH_2SO_3H)LDNH_2 (3.60 using amino acid 3.16 as building block).

The synthesis of these peptides was done automatically using a Quartet automated peptide synthesizer at 25 μM scale employing the same protocols as shown in the synthesis of peptides **2.34** and **2.37** (chapter 2, section 2.5.7) where the first amino acid is coupled as pentafluorophenyl activated ester using 4 equivalents excess. Coupling cycle for the remaining amino acids composed of a DMF wash, deprotection of Fmoc using 2-MP (3 x 10 min), coupling of the amino acid using HBTU/HOBt/DIPEA and finally a DMF wash. The coupling of the last amino acid involved an extra step of acetylation after removal of the Fmoc group. Cleavage from the resin using TFA:TIPS 95:5 followed by precipitation of the peptide using *tert*-butyl methyl ether was also performed as described before ((chapter 2, section 2.5.4). The peptide pellet that remained after centrifugation was suspended in water and lyophilized to give crude peptides **3.60** and **3.61** from amino acids **3.16** and **3.56,** respectively.

Synthesis of peptide 3.60 from peptide 3.61.

To a solution of crude peptide **3.61** (30 mg) in HPLC grade methanol (3 mL) was added ammonium formate (19.4 mg, 15 equiv), followed by 10% Pd/C (9 mg, 30% wt.). The reaction was fitted with a balloon filled with H_2, stirred at rt and the reaction was monitored by HPLC for the disappearance of peptide **3.61** (CH_3CN/H_2O 0.1% TFA) as eluent, linear gradient from 5 to 95 CH_3CN in 60 min). After 6 h, the reaction mixture was transferred into an Eppendorf tube and centrifuged to pellet the Pd/C. The supernatant was removed and the pellet was washed two more times with 3 mL of methanol. The combined supernatants were concentrated under reduced pressure. Peptide **3.60** was purified using semipreparative RP-HPLC with a UV detector set at 220 nm. A linear gradient of 1% CH_3CN/99% 100 mM ammonium acetate (pH = 9) to 10% CH_3CN/90 % 100 mM ammonium acetate over 60 min was used as eluent (t_R = 39.3 min). Fractions containing peptide **3.60** were pooled, concentrated by high vacuum rotary evaporation and the residue was dissolved in water and repeatedly lyophilized until a constant weight was obtained. Peptide **3.60** was obtained as flocculent white powder (16.7 mg, 60% yield based on resin loading). Peptide **3.60** was 95% pure as determined by analytical RP-HPLC, t_R= 15.8 min, 1% to 40% CH_3CN in 0.1 M ammonium acetate (pH = 9) over 60 min, λ = 220 nm (**Figure 3.10**). HRMS ($^-$ESI): calculated for $C_{49}H_{68}N_6O_{22}S_2$ $(M-H)^-$ 1198.3920, found 1198.3917.

Chapter 4

Sulfotyrosine containing peptides as inhibitors of PTP1B.

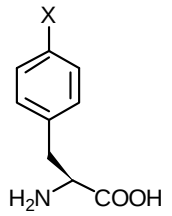
4.1 Introduction

The signalling process mediated by protein phosphorylation is controlled by the opposing but complementary actions of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs).[164] While PTKs catalyze the transfer of the phosphate moiety from ATP to the phenolic group of the tyrosine residue(s), PTPs catalyze the hydrolysis of the formed phosphate bond to yield the reverse of the aforementioned reaction.[164] It is well accepted now that kinases are involved in controlling the amplitude of a signalling response, whereas phosphatases control its rate and duration.[165,166] On the other hand, the imbalance in between PTKs and PTPs is linked to many disease states including cancer, diabetes, obesity and inflammation and autoimmunity.[167-169]

Among the 107 members of the PTP family[170], protein tyrosine phosphatase 1B (PTP1B) has garnered the most attention as far as inhibitor design is concerned.[171, 172] PTP1B is known for its negative regulatory role in both insulin and leptin signalling. PTP1B dephosphorylates activated insulin receptor (IR), insulin receptor substrates (IRS) and the downstream result is the inhibition of glucose uptake.[173-175] This is supported by the fact that overexpression of PTP1B in cell culture resulted in decreased insulin-stimulated IR phosphorylation.[176-178] Diabetic mice showed a decrease in fat, plasma and blood glucose level upon treatment with PTP1B antisense oligonucleotides.[179] Most significantly, PTP1B double-knockout mice showed an enhanced sensitivity to insulin, have improved glycemic

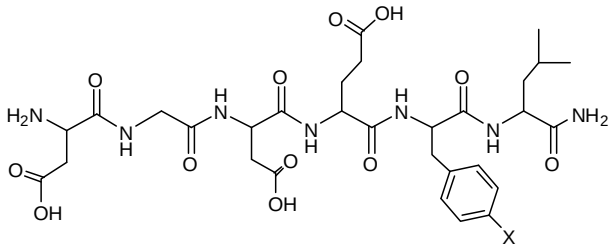
control and are more resistance to weight gain when fed with high-diet compared to wild-type. Such effects most likely occurred because the increased energy expenditure resulted from enhanced leptin sensitivity.[180, 181] Altogether these data suggesting that inhibitors to PTP1B could be developed into drugs for the treatment of metabolic diseases such as type-2 diabetes and obesity.

Free phosphotyrosine (pTyr) is a relatively poor PTP1B substrate (**4.1**, K_m = 5 mM) in comparison to peptides bearing pTyr such as DADEpYL, a hexapeptide corresponding to a region in the epidermal growth factor receptor (K_m = 3.6 μM).[182] Peptides that do not bear a phosphate group do not bind to PTP1B. These studies underscore the importance of both the phosphate group and residues in addition to the pTyr residue to substrate binding. Phosphate esters are not considered to be suitable as drug candidates due to their hydrolytic liability and poor cell permeability. Hence, the search for a suitable pTyr mimic for drug design has been a major part of PTP1B research.[183] The phosphate group and numerous phosphate mimics have been incorporated into peptide and non-peptidyl platforms and tested as inhibitors for PTP1B.[183] Numerous pTyr mimics have been evaluated in the context of the DADEXL hexapeptide (X = pTyr mimic).[183]



4.1, X = $OP(O)_2OH$ (pTyr or pY)
4.2, X = $CH_2P(O)_2OH$ (Pmp)
4.3, X = $CF_2P(O)_2OH$ (F_2Pmp)
4.4, X = $OS(O)_2OH$ (sTyr or sY)
4.5, X = $CH_2S(O)_2OH$ (Smp)
4.6, X = $CF_2S(O)_2OH$ (F_2Smp)

The most straightforward approach to preparing a stable pTyr mimic is to replace the bridging oxygen by a methylene group to give phosphonomethyl phenylalanine (**4.2**, Pmp). Insertion of amino acid **4.2** into the DADEXL hexapeptide template yielded peptide (**4.8**) resulted in a 25-fold lower affinity for PTP1B (IC_{50}= 200 μM) compared to its parent peptide bearing **4.7**. The loss of affinity was suggested to be due to the increase in pK_a of the phosphonate group and a net reduction in formal charge of the phosphonate. In addition, the decrease in the affinity of the phosphonate may be reasoned by the loss of hydrogen bonding interaction between the phosphate ester bridging oxygen atom and nearby residues.[184]

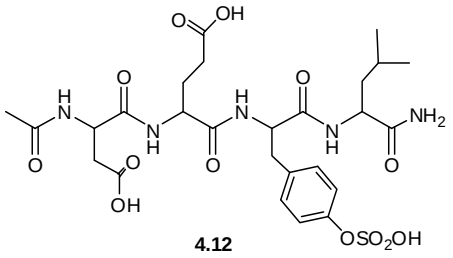


4.7, X = $OP(O)_2OH$ **4.10,** X = $CH_2S(O)_2OH$
4.8, X = $CH_2P(O)_2OH$ **4.11,** X = $CF_2S(O)_2OH$
4.9, X = $CF_2P(O)_2OH$

In attempt to enhance the affinity of the phosphonate towards PTP1B, two α-fluorines were incorporated into Pmp to give the corresponding difluorphosphonomethylphenylalanine (**4.3**, F_2Pmp) bearing a difluoromethylenephosphonyl (DFMP) group. Upon insertion of F_2Pmp into the hexapeptide platform (**4.9**) it showed an IC_{50} = 100 nM. F_2Pmp is one of the most effective phosphate mimics ever obtained, showing an approximate 1000-fold enhancement in affinity relative to the analogous peptides bearing Pmp (peptide **4.8**).[184] This difference in affinity may be due to the possibility that the enzyme prefers to bind the dianionic form of the phosphonate inhibitor as opposed to the monoanionic form. At the pH

under which the inhibitor studies were performed (pH 6.5-7), the fluorinated phosphonate **4.9** exists almost entirely in the dianionic form while the phosphonate group in the non-fluorinated inhibitor **4.8** would exist mainly in the monoanionic form. However, kinetic studies performed with these inhibitors over a broad pH range spanning the phosphonate and phosphate pK_a's revealed that the monoanionic and dianionic forms bind with equal affinity suggesting that the large difference in affinity between **4.8** and **4.9** is not due to the lower pK_a of the fluorinated phosphonate moiety. X-ray crystallographic studies of a small molecule inhibitor bearing a DFMP group bound to PTP1B suggest that the fluorines contribute to binding mainly by H-bonding with specific residues in the active site.[185]

Inhibitors bearing the DFMP group are not highly cell permeable which limits their use in drug development. Consequently, numerous less ionic phosphate mimics have been investigated.[183] Among these mimics is sTyr (**4.4**) which was examined by Desmarais and coworkers as a substitute for pTyr.[186] Surprisingly, the tetrapeptide (**4.12**) containing sTyr acted as good competitive inhibitor with an IC_{50} value in the low μM range which is in the range of the K_m's of the best phosphorylated substrates.[186]



4.12

The Taylor group developed sulfonomethylphenylalanine (**4.5**, Smp) and difluorosulfonomethylphenylalanine (**4.6**, F_2Smp) as hydrolytically stable pTyr mimics and incorporated them into the hexapeptide platform to give peptides **4.10** and **4.11** respectively and tested them against PTP1B.[187,188] Surprisingly, substitution of the methylene group in **4.10** with a difluoromethylene group (**4.11**) did not have the significant effect on binding as found with the phosphonates (IC_{50}'s = 24 μM for **4.11** versus 44 μM for **4.10**). It has been hypothesized that with the sulfonates the larger sulfur atom (compared to phosphorus) somehow restricts movement of the mimic in the active site which prevents the fluorine atoms from attaining beneficial interactions within the active site. The potency of peptides **4.10** and **4.11** have never been compared to the corresponding sTyr peptide DADE**sY**LNH_2 (**2.30**), since **2.30** has never been prepared until very recently (see chapter 2).

There are two objectives to the work in this chapter. One is to determine how sTyr compares to Smp and F_2Smp as a pTyr mimic in the context of PTP1B inhibition. This will be done by determining the IC_{50} of peptide **2.30** whose synthesis was described in chapter 2. Another objective of this work is to determine if the K_m's or some other parameter (k_{cat}, or k_{cat}/K_m) of phosphorylated peptide substrates can be used as a guide for developing peptide-based PTP1B inhibitors. If a peptide substrate exhibits a low K_m then will its sTyr analog exhibit a good affinity or should other kinetic parameters of the substrate (such as k_{cat} and k_{cat}/K_m) also be scrutinized? In an attempt to answer this question we will construct a series of sTyr peptides that are based on some recently reported pTyr-bearing peptidyl PTP1B substrates. The IC_{50}'s will be determined for these sTyr peptides and compared to the reported K_m's and other parameters of the corresponding pTyr substrates.

4.2 Results and discussion

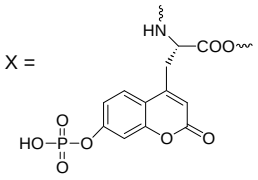
To determine if the K_m's of pTyr substrates can be used as a guideline for preparing peptide-based inhibitors bearing pTyr mimics we chose a series of known pTyr peptide substrates that differed in their sequence, number of residues, K_m's and k_{cat} values. We wish to prepare the sTyr analogs of these peptides and determine their IC_{50}'s and then compare the IC_{50}'s to the K_m's ((and k_{cat}/K_m's) of the pTyr peptidyl substrates. Very recently, the groups of Barrios[189] and Pei[190] have reported several novel peptide substrates for PTP1B. Among them are peptides **4.13** and **4.14** (**Table 4.1**).[189] These peptides contain a phosphorylated coumarin-bearing amino acid in place of pTyr. We specifically chose these two peptides for our studies since they have very similar K_m's but very different k_{cat}'s and consequently different k_{cat}/K_m values. The similar K_m values would *suggest* that they bind to the enzyme with equal affinity but the much higher k_{cat} value of peptide **4.13** suggests that the phosphate group in **4.13** is capable of obtaining an orientation in the active site that is more conducive to catalysis than that of peptide **4.14**. Peptide **4.15** (**Table 4.1**) was recently reported by Pei and coworkers.[190] It has a sequence that is very different from that of peptides **4.13** and **4.14** and also contains pTyr and not a phosphorylated coumarin residue. Its K_m is very similar to that of peptides **4.13** and **4.14** and has a k_{cat} that is similar to peptide **4.14**. Peptide **4.16** (**Table 4.1**) corresponds to residues 1159-1171 of the insulin receptor kinase.[183] Its sequence and number of residues is quite different from the other peptides mentioned above. It has two pTyr residues one which binds in the catalytic site and the other which binds in a second, non-catalytic pTyr binding site. It has a K_m (7.9 μM) (k_{cat} was not reported) that is much lower than peptides **4.13-4.15**. It's EC_{50} has been determined, using a substrate trapping

mutant of PTP1B, to be 210 nM. The monophosphorylated forms of peptide **4.16** exhibit K_m's that are more than 10-fold higher than that of peptide **4.16**.

Table 4.1. Kinetic parameters of compounds **4.13-4.16**.

Entry	Sequence	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M (M^{-1} s^{-1})
1	Ac-FnGA-**X**-QLEENH$_2$ (**4.13**)[a]	160	270	6.0 x 10^5
2	Ac-VFDQ-**X**-HESPNH$_2$ (**4.14**)[a]	11.5	280	4.1 x 10^4
3	EHTGH**pY**AA (**4.15**)[b]	14	290	4.7 x 10^4
4	ETD**pY$_p$Y**RKGGKGLL (**4.16**)[c]	NR[d]	7.9	NR[d]

[a]See ref. 187 [b]See ref. 188 [c]See ref. 183 [d]NR = not reported



The synthesis of peptide **2.30** was reported in chapter 2 (section 2.3.7). The sTyr analogues of peptide **4.13-4.16** (peptides **4.17-4.20**) were prepared using the optimized procedure described for the synthesis of residue 1-20 of N-terminal of CXCR6 (Chapter 2) applying HCTU/HOCt/DIPEA (5 equiv) for amino acid coupling, 2-MP (3 x 10 min) for Fmoc deprotection and TFA:TIPS:H_2O:EDT (92.5:2.5:2.5:2.5) for side chains protecting groups deprotection and cleavage from resin. Removal of the DCV groups was achieved using 50% w/w $Pd(OH)_2$ under H_2 atmosphere (balloon pressure) in water/methanol (1:1) containing Et_3N (one equiv of Et_3N per acidic amino acid and two equiv per DCV group). IC_{50} determinations were carried out under conditions similar to those reported by MerckFrosst applying 6,8-difluoro-4-methylumbelliferyl phosphate (DiFMUP) as substrate at K_m concentration (5μM).[191,192] The IC_{50} curves for peptide **2.30** and **4.17-4.20** are shown in **Figures 4.1-4.5**. The results are summarized in **Table 4.2**.

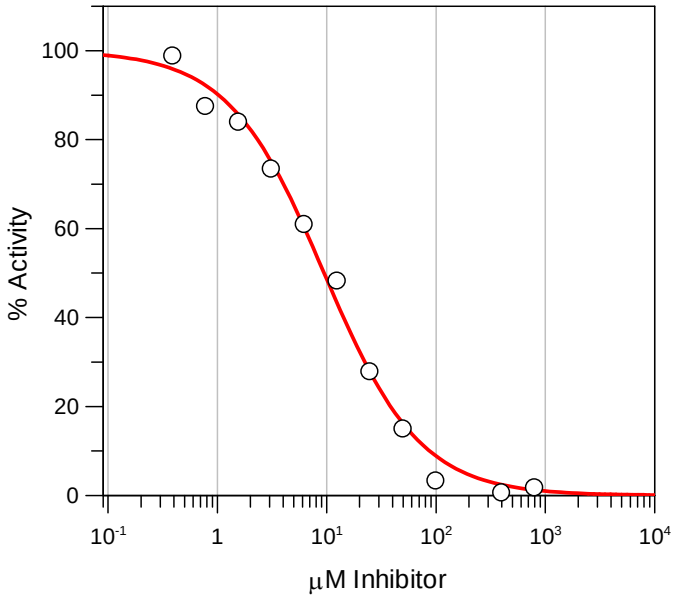


Figure 4.1. IC_{50} plot for peptide **2.30**. Inhibitor concentrations range from 0.39-800 µM. $IC_{50} = 9.4 \pm 0.6$ µM.

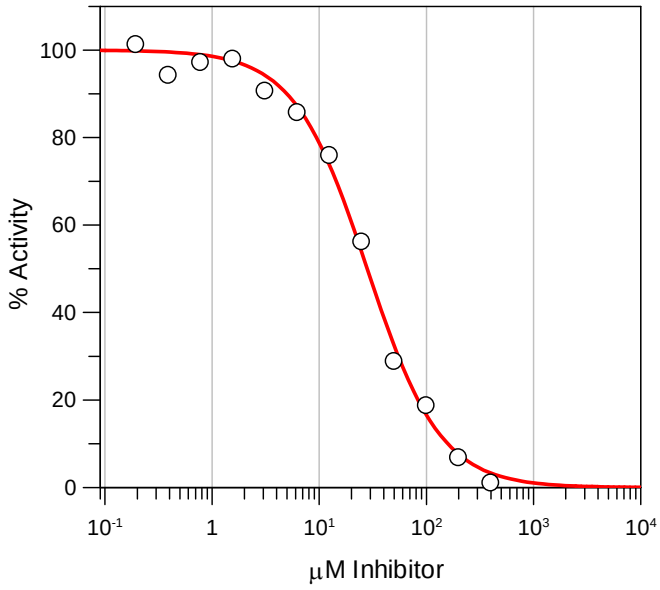


Figure 4.2. IC_{50} plot for peptide **4.17**. Inhibitor concentrations range from 0.19-400 µM. $IC_{50} = 28.0 \pm 1.4$ µM.

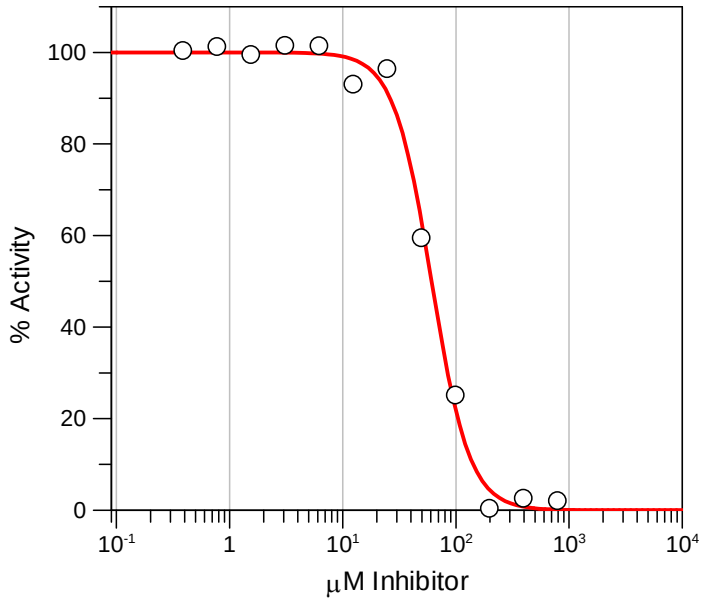


Figure 4.3. IC_{50} plot for peptide **4.18**. Inhibitor concentrations range from 0.39-800 μM. IC_{50} = 61.0 ± 2.4 μM.

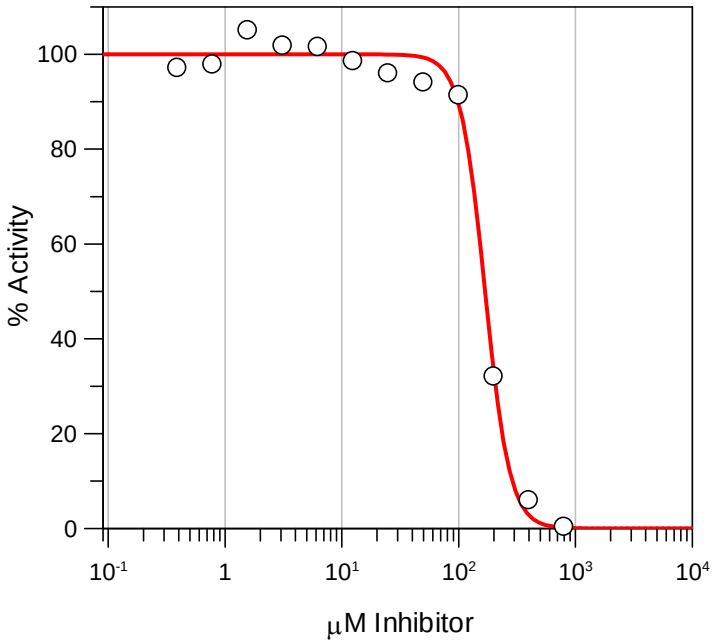


Figure 4.4. IC_{50} plot for peptide **4.19**. Inhibitor concentrations range from 0.39-800 μM. IC_{50} = 168 ± 5.4 μM.

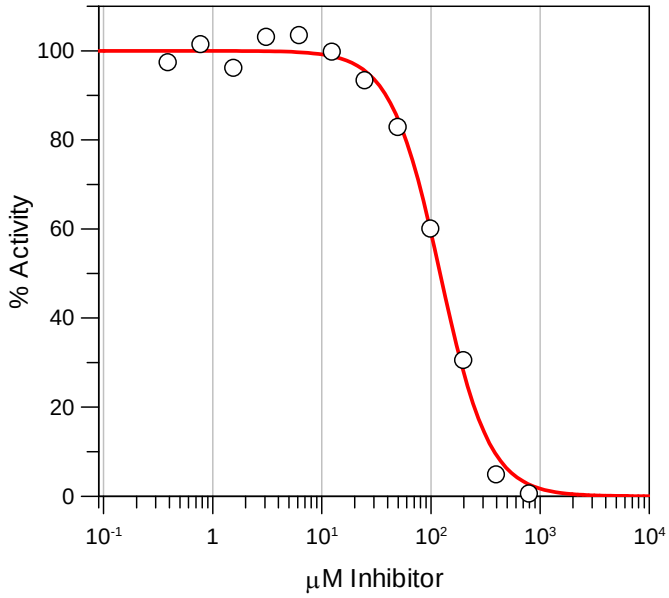


Figure 4.5. IC_{50} plot for peptide **4.20**. Inhibitor concentrations range from 0.39-800 μM. IC_{50} = 121.2 ± 5 μM.

Among the peptides examined, hexapeptide **2.30** (DADEsYLNH$_2$) was the most potent inhibitor of PTP1B with an IC_{50} = 9.4 μM. This IC_{50} is similar to the K_m values that have been reported for the analogous pTyr peptide **4.7** (5-20 μM).[182] Although **2.30** is not as potent as its diflouromethylenephosphonate analogue (about 100 times less potent than peptide **4.9**), it is slightly more potent than Smp (**4.10**) and F_2Smp (**4.11**). This confirms the results from our previous studies that the sulfur-containing inhibitors appear to be relatively insensitive to substitution at the position bridging the SO_3 group and the phenyl ring.[188]

Table 4.2. IC_{50}`s of peptides **2.30** and **4.17-4.20**.

Entry	Sequence	IC_{50} (μM)
1	DADE**sY**LNH_2 (**2.30**)	9.4
2	Ac-FnGA-**sY**-QLEENH_2 (**4.17**)	28
3	Ac-VFDQ-**sY**-HESPNH_2 (**4.18**)	61
4	EHTGH**sY**AANH_2 (**4.19**)	168
5	ETD**sY$_s$Y**RKGGKGLLNH_2 (**4.20**)	121

The IC_{50} of peptide **4.19** is only 1.7 times less than that of its analogous pTyr peptide **4.15**. However, it should be pointed out that the IC_{50} curve for this peptide was unusually steep (**Figure 4.4**) suggesting that inhibition could in part be due to inhibitor aggregation.[193] The IC_{50}`s for peptides **4.17** and **4.18** are 5-10-fold less than the $K_m$`s reported for the analogous phosphoryl coumarin-bearing peptides **4.13** and **4.14**. This suggests that the coumarin ring has a detrimental effect on substrate binding which makes it difficult to compare the effect of the sulfur versus phosphorus substitution (IC_{50} values of the sTyr peptides to the K_m values of the coumarin-bearing peptides). The k_{cat} value for peptide **4.13** is 14-fold greater than that of peptide **4.14**. Thus, at the very least, it appears that k_{cat} values of these coumarin-bearing peptide substrates should not be used as a guideline for preparing the analogous peptide inhibitors bearing a pTyr mimic. Also, once again it should pointed out that the IC_{50} curve for peptide **4.18** is unusually steep (**Figure 4.3**) suggesting that inhibition could in part be due to inhibitor aggregation.[193]

Surprisingly bis-sulfated peptide **4.20** exhibited a much higher IC_{50} compared to the K_m of its pTyr analog **4.16**. Since our studies with peptide **2.30** and those of Desmarais *et al.* [186] suggest that the sulfate group in sTyr peptides bind in the active site almost as well as the phosphate group in pTyr peptides (based on comparing IC_{50}'s to K_m's) then the difference

between the IC_{50} of peptide **4.20** and the K_m of peptide **4.16** could very likely be due to the presence of the extra sTyr residue in **4.20** As mentioned above, in peptide **4.16** one pTyr residue (pTyr1162) binds in the catalytic site and the other (pTyr 1163) binds in a second, non-catalytic pTyr binding site. The monophosphorylated forms of peptide **4.16** exhibit K_m's that are more than 10-fold higher (> 80 μM) than that of peptide **4.16**. The IC_{50} found for peptide **4.20** (121 μM) is closer to that of the K_m's found for the monophosphorylated versions of peptide **4.16** suggesting that the binding synergism found with the two pTyr residues in peptide **4.16** does not occur with the corresponding sulfated peptide. It is possible that the sTyr residue in peptide **4.20** that corresponds to pTyr residue 1163 in peptide **4.16** is not accommodated in the second, non-catalytic phosphate binding site very well. Determination of the IC_{50} values of the monosulfated versions of peptide **4.20** will help shed some light on this matter.

4.3 Experimental

4.3.1 General.

See section 2.5.1 for general information concerning syntheses. All reagents and buffers for for enzyme assays were obtained form Sigma unless stated otherwise. DiFMUP was obtained from Molecular Probes. PTP1B was obtained from (PROSPEC (Protein-Specialists), Rehovot, Israel). Enzyme assays were performed at 25 ^{o}C on a Molecular Devices Gemini microplate reader.

4.3.2 Synthesis of sulfotyrosine bearing peptides.

General procedure for the synthesis of peptides **4.17-4.20**

Automated SPPS was used as described in chapter 2. The general protocol used for peptide **2.48** (section 2.5.9) was used for all peptides. The last amino acids in peptides **4.17** and **4.18** were incorporated applying the procedure described for insertion of the last amino acid in **2.39** (section 2.5.8). The peptide was cleaved from the support using the same procedure described for peptide **2.48** (section 2.5.9). The resulting crude peptides 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. The analytical HPLC chromatogram of crude DCV protected peptides showed mainly one major peak. The removal of the DCV groups from DCV protected intermediates was achieved applying the same procedure described for peptide **2.49** (section 2.5.9) except one equiv of Et_3N per acidic amino acid and two equiv per DCV group were applied.

Ac-FnGAsYQLEENH$_2$ (**4.17**), Peptide **4.17** was purified using preparative HPLC (CH_3CN/20 mm ammonium acetate as eluent, linear gradient from 15% to 25% CH_3CN over 30 min, t_R= 20.0 min) which gave 26.4 mg of pure **4.17** (82% yield). The analytical HPLC chromatogram (linear gradient of 15:85 CH_3CN:20 mM ammonium acetate to 25:75 CH_3CN:20 mM ammonium acetate over 30 min) showed a single peak at t_R= 21.2 min. HRMS (ESI^-): calculated for $C_{52}H_{74}N_{11}O_{19}S$ $(M-H)^{-1}$ 1188.4883, found 1188.4883.

Ac-VFDQsYHESPNH$_2$ (**4.18**), Peptide **4.18** was purified using preparative HPLC (CH_3CN/20 mm ammonium acetate as eluent, linear gradient from 10% to 25% CH_3CN over 30 min, t_R= 16.5 min) which gave 27.2 mg of pure **4.18** (86% yield). The analytical HPLC chromatogram (linear gradient of 10:90 CH_3CN:20 mM ammonium acetate to 30:70

CH_3CN:20 mM ammonium acetate over 30 min) showed a single peak at t_R= 14.7 min. HRMS (ESI^-): calculated for $C_{50}H_{65}N_{12}O_{19}S$ $(M-H)^{-1}$ 1169.4210, found 1169.4209.

EHTGHsYAANH$_2$ (**4.19**), Peptide **4.19** was purified using preparative HPLC (CH_3CN/20 mm ammonium acetate as eluent, linear gradient from 5% to 15% CH_3CN over 30 min, t_R= 18.4 min) which gave 19.7 mg of pure **4.19** (82% yield). The analytical HPLC chromatogram (linear gradient of 5:95 CH_3CN:20 mM ammonium acetate to 15:85 CH_3CN:20 mM ammonium acetate over 30 min) showed a single peak at t_R= 13.5 min. HRMS (ESI^-): calculated for $C_{38}H_{53}N_{13}O_{15}S$ $(M-H)^{-1}$ 963.3505, found 963.3505.

ETDsY$_{1152}$sY$_{1153}$RKGGKGLLNH$_2$ (**4.20**). Peptide **4.20** was purified using semi-preparative HPLC (CH_3CN/20 mm ammonium acetate as eluent, linear gradient from 15% to 25% CH_3CN over 30 min, t_R= 15.5 min) which gave 30.5 mg of pure **4.20** (73% yield). The analytical HPLC chromatogram (linear gradient of 15:85 CH_3CN:20 mM ammonium acetate to 25:75 CH_3CN:20 mM ammonium acetate over 30 min) showed a single peak at t_R= 11.3 min. LRMS (ESI^-): calculated for $C_{67}H_{105}N_{19}O_{26}S$ $(M-2H)^{2-}$ 827.84655, found 827.7619.

4.3.3 IC_{50} and K_i determinations of peptides 2.30 and 4.17-4.20.

Stock solutions of the inhibitors were prepared in 50 mM Bis–Tris HCl, pH 6.3, 5 mM DTT, and 2 mM EDTA. 10 μL of each inhibitor stock solution was added to the wells of a 96-well microtiter plate containing 90 μL of 5.5 μM diFMUP in 50 mM Bis–Tris HCl, pH 6.3, 5 mM DTT, and 2 mM EDTA. The reactions were initiated at 25°C with 10 μL of a 33 nM solution of PTP1B in a buffer containing 50 mM Bis–Tris HCl, pH 6.3, 20% glycerol, 5 mM DTT, 2 mM EDTA, and 0.1% Triton X-100. The production of fluorescent product

diFMU was monitored for 10 min using a spectrofluorimeter platereader with excitation and emission at 360 nm and 460 nm, respectively. The initial rates of enzyme activity in relative fluorescence units per second (RFU/s) were used to determine the IC_{50}. The ratio of the initial rate in the presence of inhibitor (V_i) to that in the absence of inhibitor (V_o) was calculated and plotted as a semi-log curve in Grafit, from which the IC_{50} value was calculated based on the following equation: $V_i = V_o/[1 + ([I]/IC_{50})^S] + B$, where V_i is the initial rate of reaction at an inhibitor concentration of [I]; V_o is the velocity in the absence of inhibitor; B is background and *s* is the slope factor equal to V_o - B. The IC_{50} curves for compounds **2.30** and **4.17-4.20** were shown in **Figures 4.1-4.5**.

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