Homogeneous Sulfopeptides and Sulfoproteins: Synthetic Approaches and Applications to Characterize the Roles of Tyrosine Sulfation on Biochemical Function

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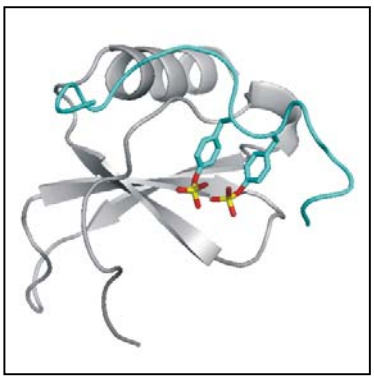


Post-translational modification of proteins plays critical roles in regulating structure, stability, localization and function. Sulfation of the phenolic side chain of tyrosine (Tyr) residues, to form sulfotyrosine (sTyr), is a widespread modification of extracellular and integral membrane proteins, influencing the activities of these proteins in cellular adhesion, blood clotting, inflammatory responses and pathogen infection. Tyr sulfation commonly occurs in sequences containing clusters of Tyr residues and is incomplete at each site, resulting in heterogeneous mixtures of

sulfoforms. Purification of individual sulfoforms is typically impractical. Therefore, the most promising approach to elucidate the influence of sulfation at each site is to prepare homogeneously sulfated proteins (or peptides) synthetically. This Account describes our recent progress in both development of such synthetic approaches and application of the resulting sulfopeptides and sulfoproteins to characterize the functional consequences of Tyr sulfation.

Initial synthetic studies used a cassette-based solid-phase peptide synthesis (SPPS) approach in which the side chain sulfate ester was protected to enable it to withstand Fmoc-based SPPS conditions. Subsequently, to address the need for efficient access to multiple sulfoforms of the same peptide, we developed a divergent solid-phase synthetic approach utilizing orthogonally side chain protected Tyr residues. Using this methodology we have carried out orthogonal deprotection and sulfation of up to three Tyr residues within a given sequence, allowing access to all eight sulfoforms of a given target from a single solid-phase synthesis.

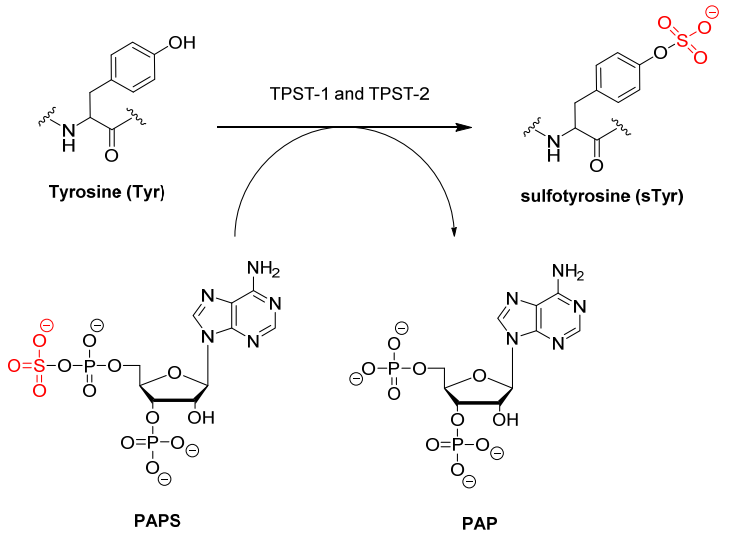
With homogenously sulfated peptides in hand, we have been able to probe the influence of tyrosine sulfation on biochemical function. Several of these studies focused on sulfated fragments of chemokine receptors, key mediators of leukocyte trafficking and inflammation. For the receptor CCR3, we showed that tyrosine sulfation enhances affinity and selectivity for binding to chemokine ligands and we determined the structural basis of these affinity enhancements by NMR spectroscopy. Using a library of CCR5 sulfopeptides, we demonstrated the critical importance of sulfation at one specific site for supporting HIV-1 infection.

Demonstrating the feasibility of producing homogeneously tyrosine-sulfated proteins, in addition to smaller peptides, we have used SPPS and native chemical ligation methods to synthesize the leech-derived anti-thrombotic protein hirudin P6, containing both tyrosine sulfation and

glycosylation. Sulfation greatly enhanced inhibitory activity against thrombin, whereas addition of glycans to the sulfated protein decreased inhibition, indicating functional interplay between different post-translational modifications. In addition, the success of the ligation approach suggests that larger sulfoproteins could potentially be obtained by ligation of synthetic sulfopeptides to expressed proteins, using intein-based technology.

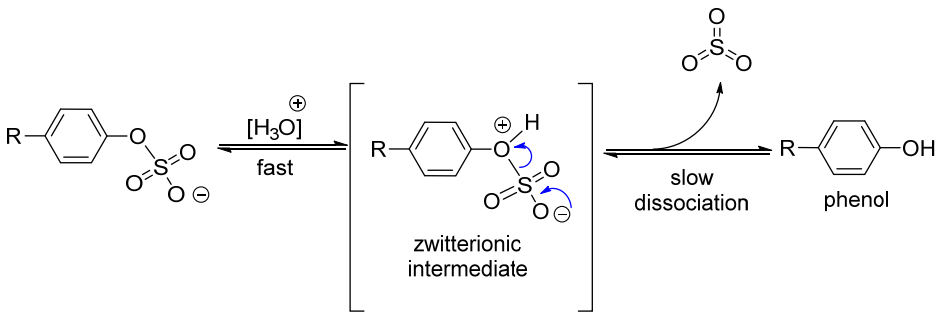
Introduction:

The covalent modification of amino acids during or after translation of a protein plays important structural and functional roles, with potential effects on folding, conformation, distribution, stability and activity.[1] One common post-translational modification (PTM) is sulfation of the hydroxyl moiety within the phenolic side chain of tyrosine (Tyr) residues to form sulfotyrosine (sTyr). Due to localization of the tyrosylprotein sulfotransferase (TPST) enzymes that catalyze Tyr sulfation in the *trans*-Golgi network (Scheme 1), Tyr *O*-sulfation occurs exclusively on extracellular and integral membrane proteins, such as those involved in hormone activity, blood clotting, inflammation and pathogen infection. In the few cases studied in detail, sulfation has generally been found to influence biological function by enhancing the affinities of modified proteins for binding partners. The biological effects of tyrosine sulfation have been reviewed in detail elsewhere.[2]



Scheme 1: Tyr sulfation facilitated by TPSTs in the *trans*-golgi network. Adapted from Moore.[3]

While it has been suggested that up to 1% of all proteins are sulfated on Tyr, this modification is rarely observed in the course of routine protein analysis, due to the inherent acid lability of the phenolic sulfate ester moiety through the loss of sulfur trioxide (Scheme 2).[4]



Scheme 2. Aryl sulfate hydrolysis in acidic aqueous media.

In light of the difficulties in analyzing and controlling Tyr sulfation of native or expressed proteins, there has been a strong motivation to develop methods to synthesize peptide model systems (fragments of proteins) in which the sulfation state and homogeneity can be carefully

controlled and which are amenable to structural and biophysical analysis. Consequently, we and others have focused on developing methods to make sTyr compatible with standard solid-phase peptide synthesis (SPPS) protocols, thereby enabling the robust synthesis of sulfopeptides with homogeneous sulfation patterns. In this account, we outline these synthetic approaches and summarize our studies on the functional consequences of Tyr sulfation explored using synthetic sulfopeptides and sulfoproteins.

1) Synthesis of sulfopeptides using suitably protected sTyr "cassettes"

From a synthetic standpoint, the acid lability of the Tyr sulfate ester moiety excludes the possibility of using an unprotected free sulfate ester in Boc-strategy SPPS due to repeated exposure to trifluoroacetic acid (TFA) during peptide construction and cleavage with HF. Whilst Fmoc-strategy SPPS employs alkaline conditions during peptide construction, prolonged treatment with TFA for cleavage of the peptide from the resin and removal of side-chain protecting groups also precludes the use of this synthetic strategy in combination with a free Tyr-*O*-sulfate ester in the synthesis of sulfopeptides.

Early synthetic studies on sulfation of Tyr residues within peptides relied on the use of non-selective sulfating reagents such as sulfur trioxide-pyridine[5,6] and sulfur trioxide-*N*,*N*-dimethylformamide[7-11] complexes for global sulfation of peptides. The inherent lack of selectivity of these reagents for the side chain of the Tyr residue was a major limitation of this strategy. The incorporation of pre-formed sTyr amino acid building blocks bearing counterions to stabilize the labile phenolic sulfate ester has also been employed in SPPS e.g. sodium,[9,12,13] barium[14] and tetraalkylammonium salts.[15] Whilst these amino acids have enabled the installation

of sTyr residues, the acidic cleavage conditions used to release the target peptide from the solid support leads to substantial loss of the acid labile sulfate monoester.[16,17]

To circumvent these issues, the use of orthogonal protection of sTyr residues as robust diesters for use as amino acid "cassettes" in SPPS-based assembly protocols enabled the site-selective installation of sTyr residues into target peptides (Figure 1).[18] A number of acid-stable aryl sulfate protecting groups, including neopentyl (nP),[18,19] 2,2,2-trifluoroethyl (TFE),[20] 2,2,2-trichloroethyl (TCE)[21-23] and 2,2-dichlorovinyl (DCV) sulfate esters[24,25] have been employed.

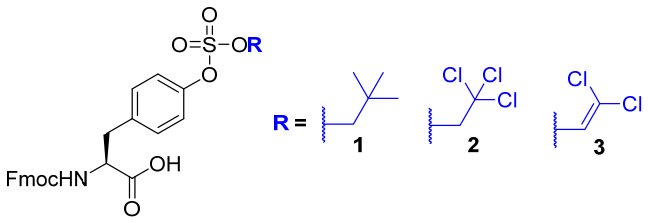
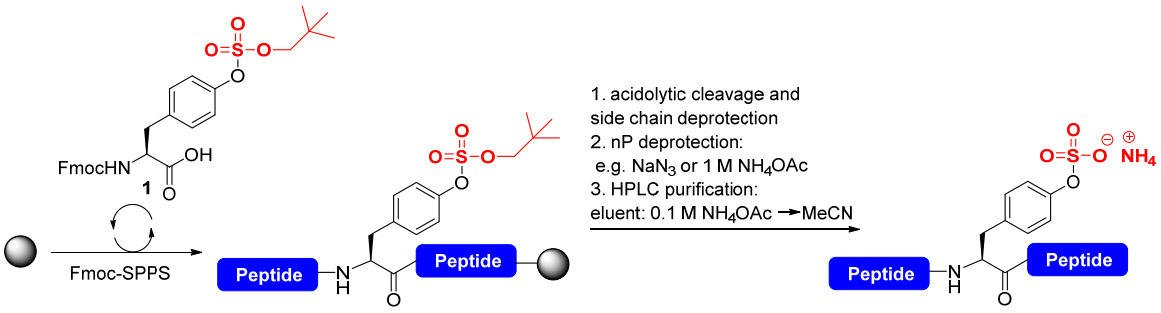


Figure 1. Fmoc-protected Tyr cassettes containing nP (**1**), TCE (**2**) or DCV (**3**)-derived sulfate diesters.

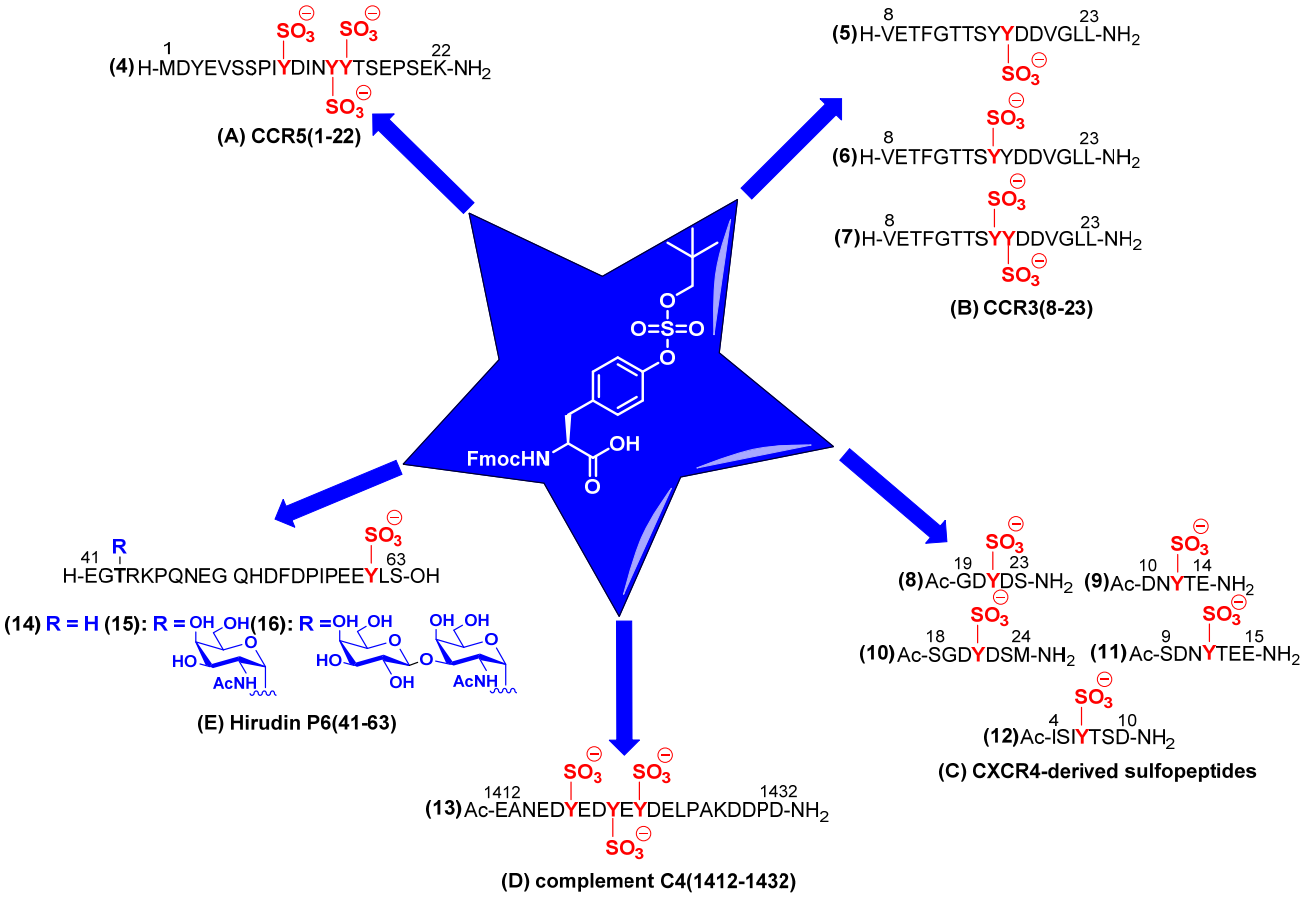
Our laboratories took inspiration from the work of Widlanski and co-workers who showed that the neopentyl(nP)-protected cassette Fmoc-Tyr(OSO_3nP)-OH (**1**)[18,19] could be installed into peptides under Fmoc-strategy SPPS conditions. Cassettes **2** and **3** have also been used in the assembly of peptides, however, the DCV- and TCE-sulfate esters are inherently unstable to the repeated piperidine treatments.[24] Synthesis of site specifically sulfated peptides *via* this cassette approach involves elongation of the peptide, including installation of sTyr cassette **1**, on a suitable solid support under standard Fmoc-SPPS conditions (Scheme 3). Following the assembly of the desired sulfopeptide, cleavage from the resin with concomitant side chain deprotection of acid labile protecting groups provides nP-protected sulfopeptides. Removal of

the nP protecting groups can be achieved using small nucleophilic reagents e.g. sodium azide[18] or ammonium acetate.[19] Following deprotection, the sulfopeptide target can be purified to homogeneity via HPLC, performed using gradients of ammonium acetate and acetonitrile to avoid acid-promoted hydrolysis of the sulfate esters.



Scheme 3. Generalized strategy for the synthesis of homogeneous sulfopeptides using the nP-protected sTyr cassette **1**.

We have extensively employed the cassette strategy described above for the assembly of homogeneously sulfated peptide fragments of a range of proteins as depicted in Scheme 4.[26-30] Access to each of these has enabled the effect of Tyr sulfation on function to be determined, as discussed later in this account.

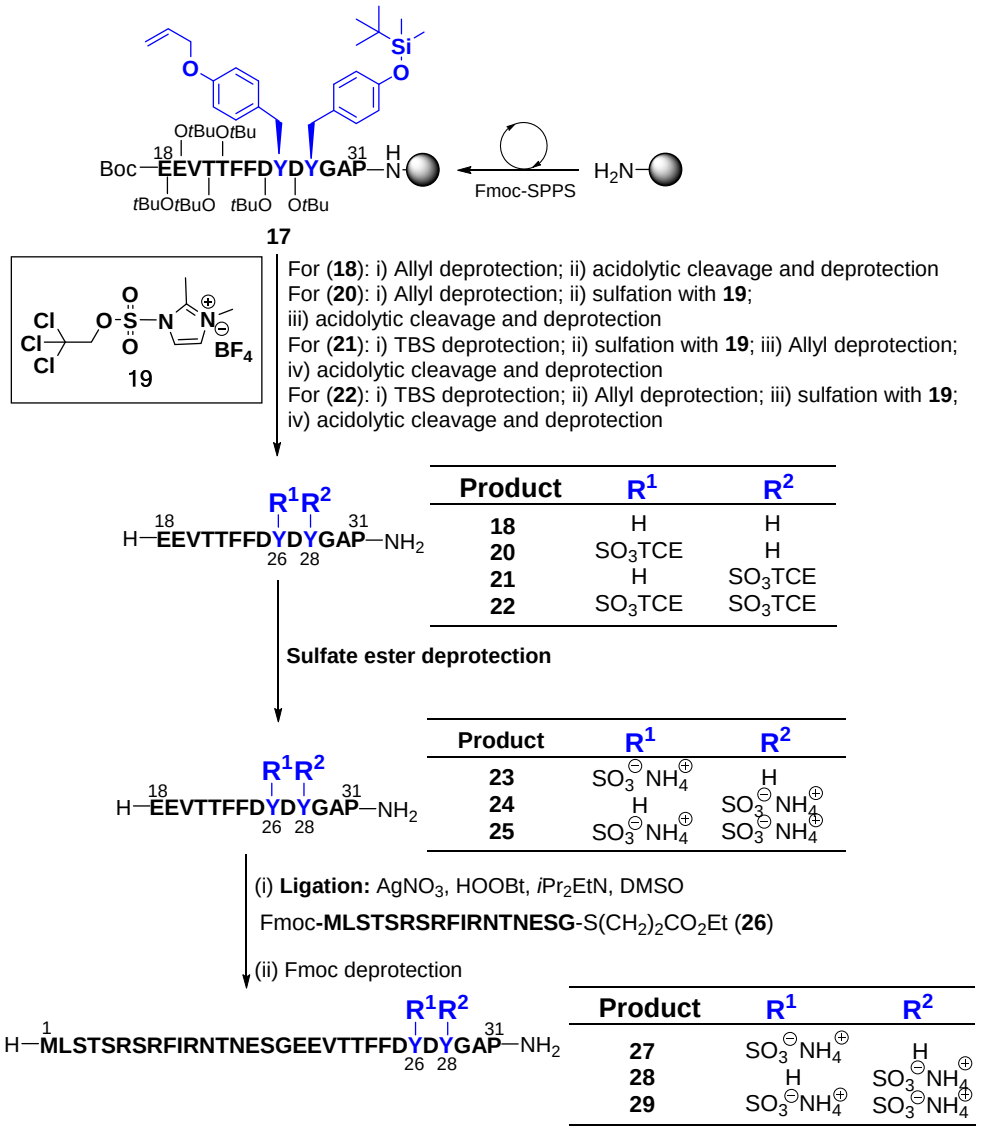


Scheme 4. Homogeneous sulfopeptide targets synthesized using the cassette strategy: (A) CCR5(1-22) (**4**)[30]; (B) differentially sulfated CCR3(8-23) peptides (**5-7**)[26]; (C) small CXCR4-derived sulfopeptides (**8-12**);[27] (D) triply-sulfated complement C4(1412-1432) (**13**);[28] (E) sulfated and glycosylated hirudin P6 (41-63) (**14-16**).[29]

2) Site Selective Solid-Phase Synthesis of Homogeneous Sulfopeptides

Many sulfopeptides and sulfoproteins possess multiple sTyr residues. Despite the significant advances made with the cassette strategy, there remains the need for an efficient method for the preparation of peptides bearing a variety of different sulfation patterns. Ideally, these sulfopeptides should be accessible from a single synthesis which cannot be achieved using the

cassette-based approach.[21,31] Therefore, we have developed a general and efficient method to gain rapid access to peptide libraries with well-defined sulfation patterns *via* a divergent SPPS approach using orthogonally side-chain protected Tyr residues.[21]



Product	R^1	R^2
18	H	H
20	SO_3TCE	H
21	H	SO_3TCE
22	SO_3TCE	SO_3TCE

Product	R^1	R^2
23	$SO_3^{\ominus}NH_4^{\oplus}$	H
24	H	$SO_3^{\ominus}NH_4^{\oplus}$
25	$SO_3^{\ominus}NH_4^{\oplus}$	$SO_3^{\ominus}NH_4^{\oplus}$

Product	R^1	R^2
27	$SO_3^{\ominus}NH_4^{\oplus}$	H
28	H	$SO_3^{\ominus}NH_4^{\oplus}$
29	$SO_3^{\ominus}NH_4^{\oplus}$	$SO_3^{\ominus}NH_4^{\oplus}$

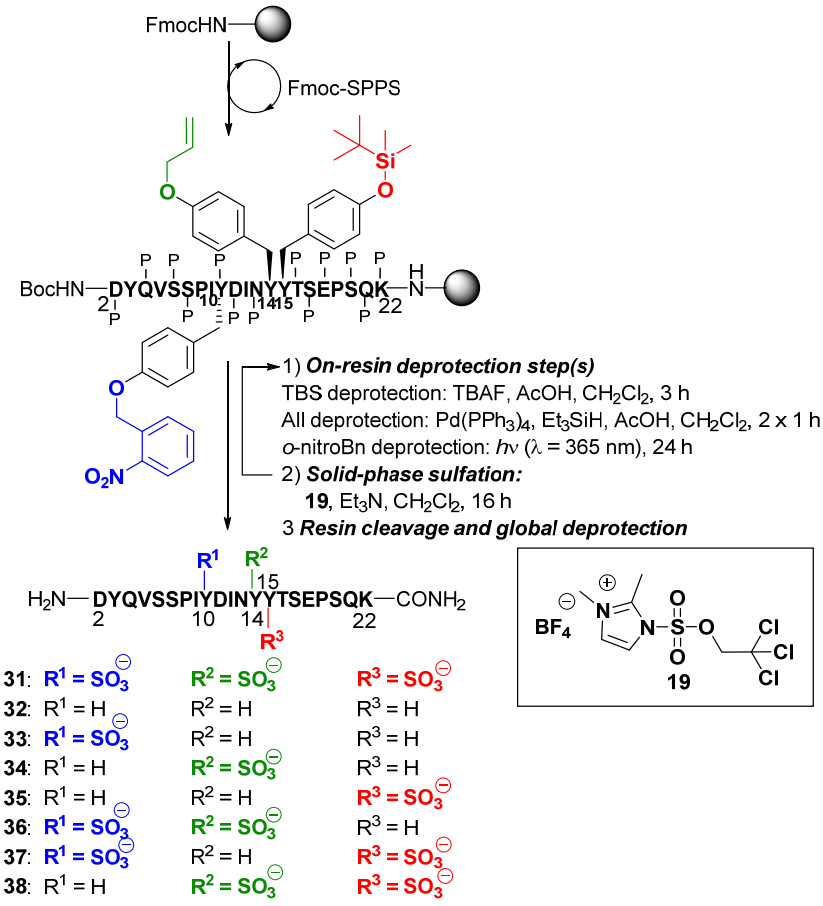
Scheme 5. Synthesis of differentially sulfated peptides derived from the N-terminal domain of the chemokine receptor CCR2 *via* a divergent solid-phase synthetic strategy.[21]

Initially, for the solid-phase assembly of a peptide bearing two sulfation sites, a 14 amino acid fragment of the N-terminal extracellular domain of the chemokine receptor CCR2 was selected (Scheme 5). We utilized two protecting groups for the Tyr phenol; *tert*-butyldimethylsilyl ether (TBS) and allyl (All) ether, compatible with Fmoc-SPPS and stable to the conditions used for sulfation, yet orthogonal to each other.[32] Resin-bound peptide **17**, corresponding to CCR2(18-31), was first synthesized on Rink amide resin *via* Fmoc-strategy SPPS, including incorporation of the orthogonally protected Tyr residues. The resin was then separated into four parts. Deprotection of the All ether from Tyr-26 was carried out using Pd(0). Subsequent acidolytic cleavage from the resin and concomitant side chain deprotection (including the TBS moiety from Tyr-28) afforded non-sulfated CCR2(18-31) **18**. Synthesis of CCR2(18-31) with sulfation at Tyr-26 began with removal of the allyl ether from **17**, followed by sulfation with Taylor's TCE-imidazolium-based sulfating reagent **19**.[22] Acidolytic side chain deprotection with concomitant TBS removal and cleavage from the resin afforded TCE-protected sulfate diester **20**. Synthesis of the sTyr-28 form of CCR2(18-31) was achieved *via* TBAF-mediated removal of the silyl ether of **17** followed by sulfation with **19**. Deallylation of Tyr-26 followed by side chain deprotection and cleavage from the resin then provided **21**. Finally, CCR2(18-31) sulfated at both Tyr-26 and Tyr-28 was accessed by sequential removal of the silyl and allyl ethers from **17** followed by global sulfation with **19** and acidic cleavage to provide **22**. From here, deprotection of the TCE-sulfate esters of **20-22** was smoothly effected by catalytic hydrogenolysis[25] and provided the desired sulfoforms of CCR2(18-31) **23-25** in excellent yields following HPLC purification.

Importantly, we have shown that sulfopeptide libraries generated from the solid-phase sulfation approach are amenable to further elaboration through the ligation-based extension of **23-25** *via* Ag(I)-promoted ligation chemistry with peptide thioester **26** corresponding to CCR2(1-17).[21]

Each of the differentially sulfated peptides underwent smooth ligation with **26** and following *in situ* Fmoc-deprotection, the library of differentially sulfated peptides **27-29**, corresponding to the entire N-terminal domain of CCR2, was obtained in good yields without any loss of the labile sulfate ester(s).

The success of the divergent solid-phase sulfation strategy for the rapid assembly of small sulfopeptide libraries led to expansion of the methodology to access all possible sulfated variants of peptides bearing three potential Tyr sulfation sites. Initially, our target sulfopeptides were those corresponding to the N-terminal extracellular domain of the chemokine receptor CCR5, in which three sulfotyrosine residues are critical for function as a co-receptor for HIV entry into human cells (*vide infra*).[33,34] The synthesis of the CCR5 sulfopeptide required the introduction of a third orthogonal protecting group for the side chain of Tyr. For this purpose we focused on the photolabile *o*-nitrobenzyl ether that is completely orthogonal to the All and TBS ethers.[35] Orthogonally protected resin-bound CCR5(2-22) **30** was synthesized using Fmoc-SPPS (Scheme 6). Using a combination of on-resin deprotection and sulfation reactions with **19**, similar to those described above, a resin-bound peptide library was assembled bearing all possible sulfation states at the three relevant tyrosine residues. Following acidolytic cleavage and deprotection of the TCE sulfate ester(s), the desired homogeneous sulfopeptide library **31-38** was accessed in good yields.



Scheme 6. Divergent solid-phase synthesis of eight possible sulfoforms of CCR5(2-22) **31-38** with variation in sulfation pattern at Tyr10, Tyr14 and Tyr15.[35] (P = standard amino acid side chain protecting groups for Fmoc-SPPS)

Gaining access to a number of homogeneously modified sulfopeptides and sulfoproteins using the methods described above has enabled us to study the effects of sulfation on the structure, binding and activity of sulfopeptides. A summary of the functional studies carried out with the synthetic sulfopeptides in our laboratories to date is presented below.

3) Effects of Tyr Sulfation of Chemokine Receptors on Chemokine Binding and Activity

Tyr Sulfation of Chemokine Receptors. Chemokine receptors are a family of G protein-coupled receptors expressed in leukocyte membranes.[36] Chemokine activation gives rise to migration of the leukocytes to the locations of chemokine expression, a critical aspect of normal immune surveillance as well as inflammatory responses. In addition, chemokine receptors play important roles in cancer metastasis[37] and in infection of cells with human immunodeficiency virus-1 (HIV-1) and the malarial parasite *Plasmodium vivax*.[38,39] The flexible N-terminal tail of chemokine receptors is the primary site for chemokine binding.[40] In most receptors the N-terminal tail contains two or more Tyr residues. Commonly, these Tyr residues undergo sulfation, which affects various aspects of receptor activity.

Identification of a Conserved Receptor-sTyr Binding Site on Chemokines. The availability of specifically-sulfated peptides derived from the N-terminal extracellular domain of chemokine receptors has enabled us and others to identify the binding sites of receptor sulfopeptides on chemokines using NMR spectroscopy.[19,26,41-46] Figure 2B shows the structure of the chemokine MCP-1 coloured to highlight the NMR spectral changes observed upon binding to doubly sulfated peptide **25** from the chemokine receptor CCR2.[41] In this chemokine-sulfopeptide complex (and in all the others that have been studied to date) the primary binding site is a shallow cleft defined by the "N-loop" and the third β-strand (β3) of the chemokine. The sequences of these two structural elements vary substantially between chemokines giving rise to differences in sulfopeptide binding affinity and selectivity.

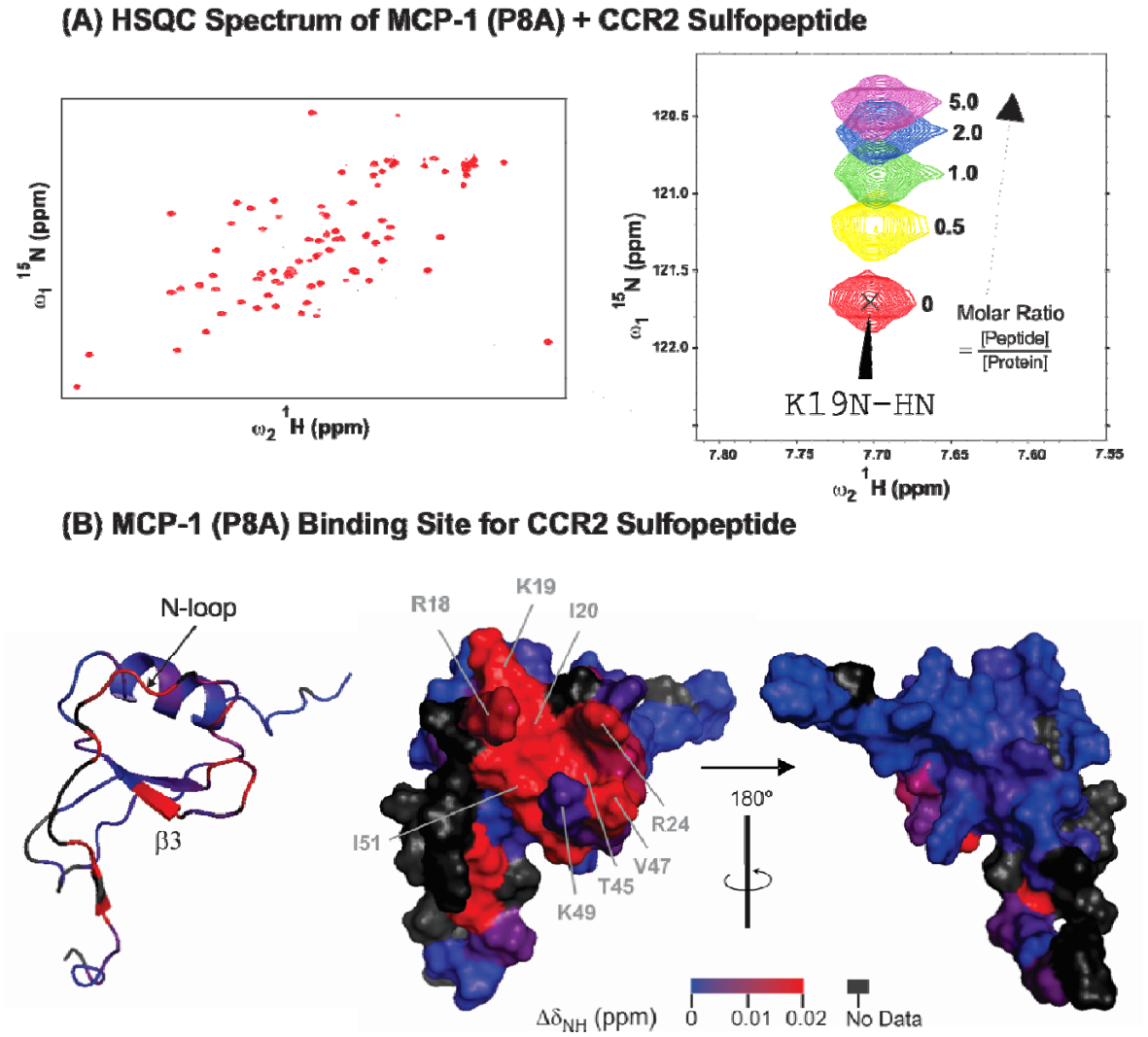


Figure 2. NMR identification of the receptor sulfopeptide binding site of a chemokine.[41] (A) HSQC spectrum of the chemokine MCP-1 (monomeric mutant P8A) including an expanded region illustrating the shift of one peak upon addition of a sulfopeptide from receptor CCR2. (B) Structure of monomeric MCP-1 coloured by NMR chemical shift changes upon sulfopeptide binding; red tones and residue labels highlight the N-loop/β3-strand binding site.

We and the group of Brian Volkman have each reported structures of chemokines (eotaxin-1/CCL11 and SDF-1/CXCL12) bound to sulfopeptides derived from the N-terminal tails of the

corresponding receptors [CCR3 and CXCR4, respectively].[44,46] In both cases, the primary binding site for receptor sTyr is the N-loop/β3 cleft and the structures reveal specific salt bridges, hydrogen bonds, hydrophobic interactions and cation-π interactions involved in sTyr recognition (Figure 3A and 3B). However, these structures also highlight some potentially important differences between the chemokine:receptor pairs. First, in the SDF-1:CXCR4 sulfopeptide structures, the chemokine is dimeric and binds to two peptide molecules that bridge the dimer interface (Figure 3C), whereas the eotaxin-1:CCR3 sulfopeptide structure is a 1:1 complex (Figure 3A). Dimerization of CXC chemokines such as SDF-1 is consistent with receptor binding and activation, whereas receptor activation by CC chemokines, such as eotaxin-1, requires a monomeric chemokine, as observed in our structural study. Second, the two structures reveal a dramatic (~150°) difference in the orientation of the receptor sulfopeptide relative to the chemokine, leading to the conclusion that different chemokines adopt different bound orientations on their cognate receptors (Figure 3A and 3B).[46] These results suggest that binding of chemokine to the sTyr residues in the N-terminal tail of the receptor may guide the subsequent interactions with transmembrane helices and thereby influence signaling.

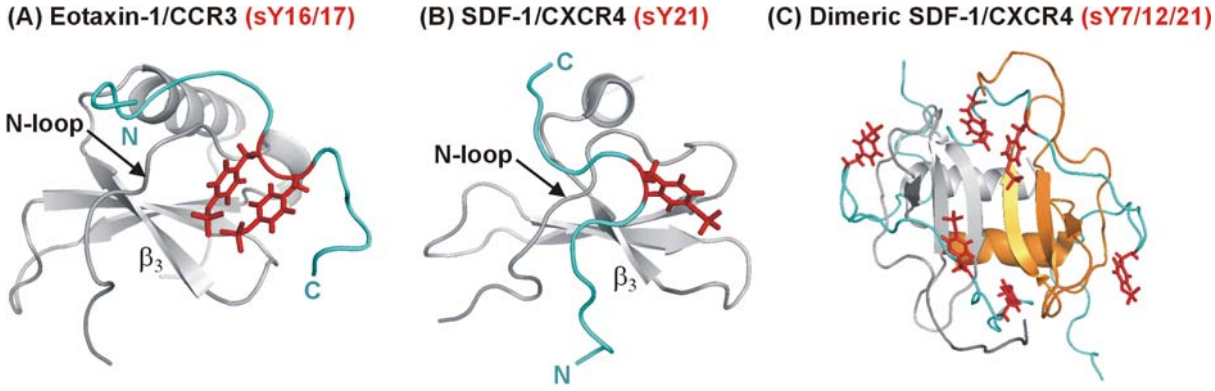


Figure 3. Structures of chemokine receptor sulfopeptides bound to cognate chemokines. (A) A doubly-sulfated peptide **7** derived from receptor CCR3 bound to the CC chemokine eotaxin-1.[46]

(B) and (C) A triply-sulfated peptide from receptor CXCR4 bound to the CXC chemokine SDF-1,[44] showing: (B) one protomer unit in the same orientation as (A) with the associated part of the peptide; and (C) the overall structure of the dimeric complex with the whole peptide. Chemokines are shown as grey or orange ribbons. Peptides are cyan with sTyr residues as red sticks.

Sulfated Receptor Peptides Can Regulate the Functional State of a Chemokine. Most chemokines form dimeric structures at high concentrations or when bound to glycosaminoglycans on endothelial surfaces, which facilitates chemokine localization and leukocyte chemotaxis.[47] NMR and fluorescence polarisation measurements have shown that a sulfopeptide from CXCR4 stabilizes the dimeric form of the chemokine SDF-1 relative to the monomeric form (Figure 4A).[27] Since the functionally important N-terminal region remains exposed in the dimeric form of CXC chemokines (such as SDF-1), it is feasible that these chemokines bind and activate their (sulfated) receptors as dimers.[48] In contrast, members of the CC chemokine family (such as MCP-1) form dimer structures in which the N-terminal region is buried such that CC chemokine dimers are inactive.[49] Consistent with this, we have observed that CCR2 sulfopeptides **23-25** increase the population of active monomeric state of MCP-1 at the expense of the inactive dimeric state (Figure 4B).[41,50] Thus, in the case of this CC chemokine, sulfopeptide binding and dimerization are negatively cooperative.

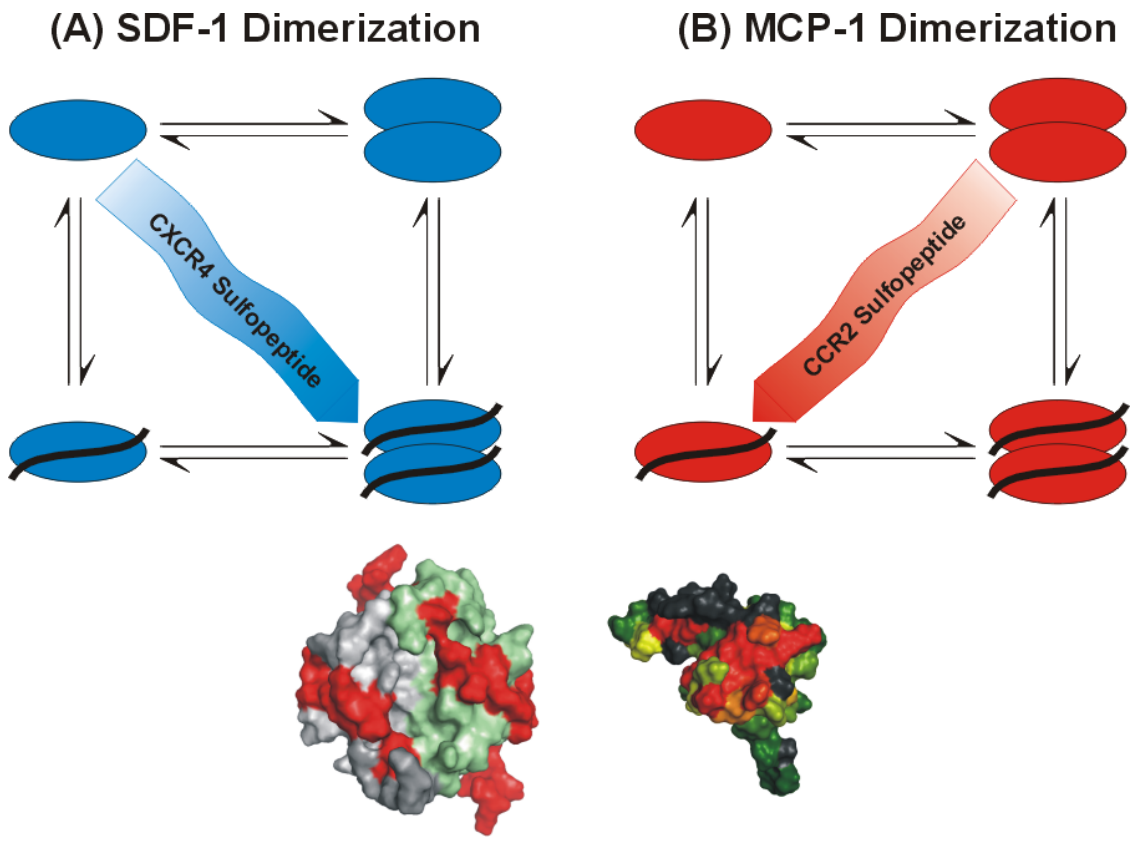


Figure 4. Cooperativity between chemokine dimerization and receptor sulfopeptide binding.[50] Schematic thermodynamic cycles showing that: (A) a CXCR4 sulfopeptide enhances dimerization of the chemokine SDF-1; (B) a CCR2 sulfopeptide favours dissociation of the MCP-1 dimer to the active monomeric state.

Tyr Sulfation Increases the Chemokine-Binding Affinity of Receptor Peptides. Purified, synthetic sulfopeptides serve as convenient models for the flexible, N-terminal tails of chemokine receptors and have been used to determine the contributions of specific Tyr sulfate moieties to chemokine binding affinity. Most of these studies have utilized NMR-based titrations, as in Figure 2A.[41] However, recently we have described the synthesis of a fluorescent sulfopeptide derived from a chemokine receptor and the application of this peptide in both direct and competitive fluorescence anisotropy assays.[51] These assays have substantial advantages over

NMR-based methods including higher precision, lower material requirements, and convenience of automation for high-throughput screening.

Binding studies using these biophysical methods have shown that the affinities of receptor-derived peptides for cognate chemokines is dramatically enhanced by sulfation of Tyr residues. For example, the fluorescence anisotropy assay showed that the monomeric chemokine MCP-1(P8A) binds to the non-sulfated form of a CCR2 peptide (**18**) with an equilibrium dissociation constant (K_d) of 8.6 ± 0.8 μM, to the two singly-sulfated forms of this peptide with affinities of 2.3 ± 0.4 μM (for **23**) and 5.4 ± 0.9 μM (for **24**), and to the doubly sulfated form **25** with an affinity of 0.31 ± 0.06 μM.[51] Similar sulfation-dependent affinity enhancements (on the order of 3- to 30-fold for each sulfate group) have been observed by NMR for the chemokine binding interactions of peptides derived from receptors CCR2, CCR3, CCR5 and CXCR4.[19,26,41-43] In most chemokine receptors there are two or more Tyr residues in the N-terminal regions, raising the possibility that the receptors could be differentially sulfated depending on the cell-type, tissue or genetic and environmental factors. We have observed that sulfation of different Tyr residues in a CCR3-derived peptide differentially affects the affinity of this peptide for cognate chemokines of CCR3.[19] Thus, differential sulfation of this receptor is a potential mechanism for regulating the chemokine responses of cells expressing this receptor.

Tyr Sulfation Can Modulate the Chemokine-Selectivity of Receptor Peptides. Chemokines and their receptors form a complex network in which most receptors can respond to several cognate chemokines and most chemokines can activate more than one receptor. Tyr sulfation is one possible mechanism that could regulate the responses of receptors amongst possible ligands.[39] We have found that differently sulfated states of a receptor peptide can have distinct

selectivity profiles amongst cognate chemokines (Figure 5).[26] For example, a non-sulfated CCR3-derived peptide has similar affinity for the three chemokines eotaxin-1, -2 and -3. However, addition of a single sulfate group gives two peptides (**5** and **6**) that bind 3- to 10-fold more tightly to eotaxin-1 than to the other two chemokines. Moreover, the corresponding doubly sulfated peptide (**7**) displays a different selectivity profile from any of the other peptides, binding 10-fold more tightly to eotaxin-1 and -3 than to eotaxin-2. These data provide proof-of-principle that differential sulfation of chemokine receptors could regulate their responses amongst a range of potential chemokine ligands.

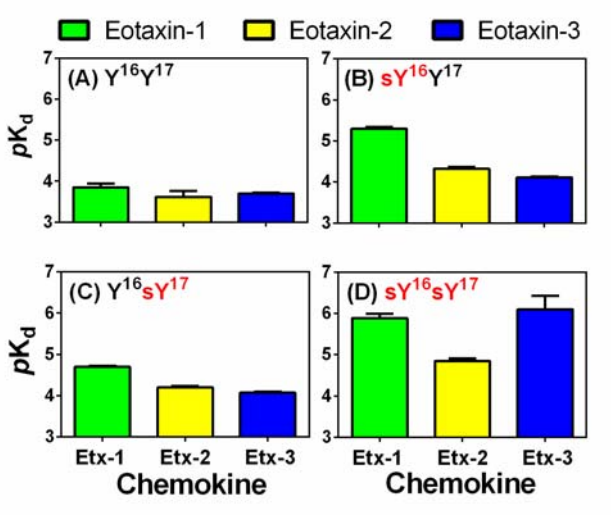


Figure 5. Tyr sulfation affects chemokine binding selectivity. Affinities, expressed as pK_d = -log(K_d), of (A) unsulfated, (B) Tyr-16 sulfated, (C) Tyr-17 sulfated and (D) doubly sulfated forms of a CCR3 peptide for each of three cognate chemokines of CCR3: (green) eotaxin-1; (yellow) eotaxin-2; and (blue) eotaxin-3.

4) Effects of Site-Selective Sulfation on the Interactions of HIV gp120 with Peptides from the Fusion Coreceptor CCR5

Infection of leukocytes by human immunodeficiency virus-1 (HIV-1) requires interaction of the viral envelope glycoprotein gp120 with host cell receptor CD4 and one of two fusion coreceptors – the chemokine receptors CCR5 or CXCR4.[33,34] Farzan and coworkers have elegantly shown that sulfation of CCR5 is required for cell entry by relevant strains of HIV-1.[38] The N-terminal tail of CCR5 contains four Tyr residues (Tyr-3, -10, -14 and -15) that are all potentially sulfated (Figure 6), although Tyr-3 appears to be less important than the other three Tyr residues for facilitating HIV entry.[38] *In vitro*, Tyr-14 and Tyr-15 undergo TPST-catalyzed sulfation most rapidly, followed by Tyr-10 and finally Tyr-3.[52]

Using the divergent solid-phase synthesis approach described above, we prepared a small library of peptides spanning CCR5 residues 2-22 and containing all possible combinations of sulfation at Tyr-10, Tyr-14 and Tyr-15 (**31**-**38**, Figure 6). Access to these peptides has allowed us to test the influence of sulfation at each site on gp120 binding and HIV fusion with target cells.[35] In a competitive binding assay, only peptides sulfated at Tyr-14 effectively displaced the anti-gp120 monoclonal antibody 17b from CD4-bound gp120 (in which the CCR5 binding site is exposed). Similarly, only peptides sulfated at Tyr-14 complemented an N-terminally-truncated form of CCR5 in supporting HIV-1 entry into target cells (Figure 6). In both assays, the most potent peptide was the triply-sulfated peptide, followed by the peptide sulfated at both positions 10 and 14. Thus, the results indicate that gp120 interactions are critically dependent on sulfation of Tyr-14 and may be enhanced by sulfation of the other Tyr residues. Interestingly, we have also shown that HIV strains that are resistant to the CCR5 antagonist maraviroc (MVC) have an increased reliance on sulfated CCR5 N-terminus for cell entry, and that synthetic triply sulfated CCR5 peptide (**4**) can inhibit HIV entry in the presence of MVC.[30]

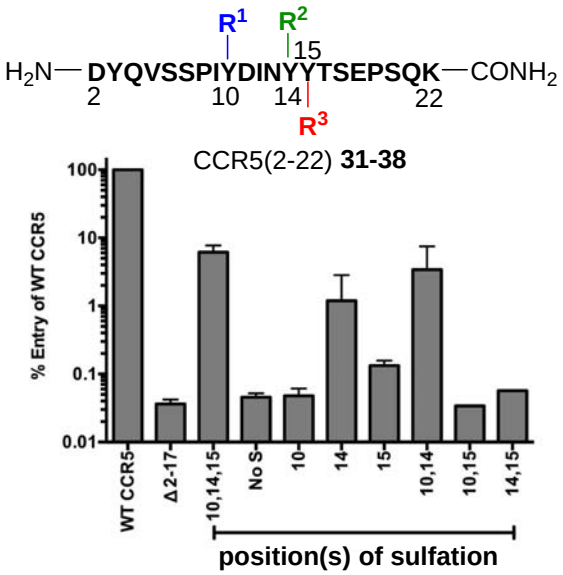
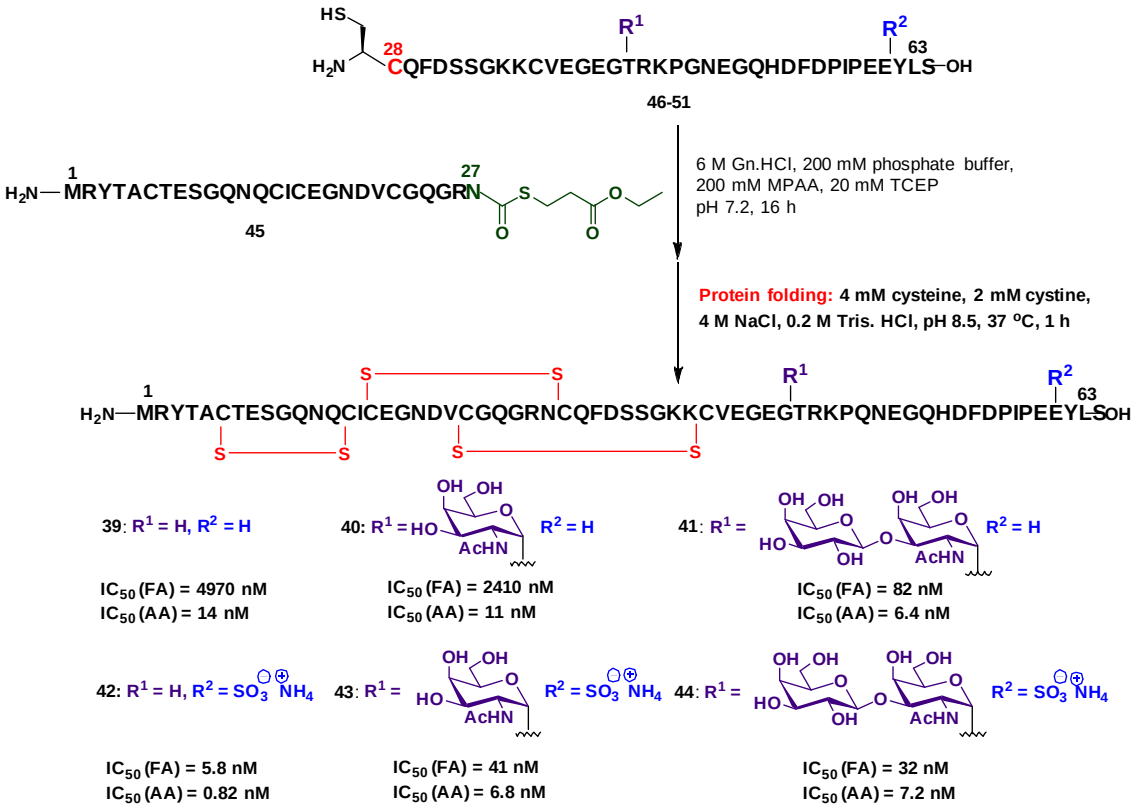


Figure 6. Reconstitution of HIV-1 entry into U87-CD4 cells expressing N-terminally truncated CCR5(Δ2-17) by treatment with differentially sulfated CCR5 peptides **31-38**.[35]

5) Synthesis of Homogeneous Leech-Derived Anti-Thrombotic Sulfoproteins *via* Native Chemical Ligation

An emerging area within our laboratories involves the study of anti-thrombotic proteins from hemaphatagous organisms[53,54] that hold potential as therapeutic leads for the treatment of thrombotic disorders.[55,56] An example is hirudin P6 (HP6), a 63 amino acid protein produced by the buffalo leech *Hirudinaria manillensis*.[57] HP6 consists of an N-terminal domain containing three disulfide bonds and an acidic C-terminal domain, reminiscent of the archetypal anti-thrombotic protein hirudin (HIRV1)[58,59] from the medicinal leech *H. medicinalis.* HIRV1 exhibits potent inhibition against human thrombin with several unmodified variants entering the clinic as anti-coagulant agents. Like HIRV1, HP6 is known to possess a sTyr residue on the C-terminus (at Tyr 61) but is also glycosylated at Thr43 with a mucin-type *O*-glycan. We set out to

interrogate the effect of both sulfation and glycosylation modifications on thrombin inhibitory activity through the chemical synthesis of a library of six modified HP6 proteins **39-44**.[53] We proposed the assembly of the library of modified HP6 proteins using our solid-phase sulfation method in concert with native chemical ligation. We disconnected the protein between Asn-27 and Cys-28 such that the protein could be assembled through common HP6(1-27) thioester **45** and HP6(28-63) peptides **46-51** possessing variation in sulfation state and glycan structure (Scheme 7). All six peptide targets (**46-51**) were synthesized from a single resin precursor bearing a TBS-protected Tyr residue using our divergent solid-phase sulfation strategy. The ligation-based assembly involved the reaction of thioester **45** with **46-51** and provided full length HP6 (glycosulfo)proteins (Scheme 7). These proteins were then successfully folded using a redox buffer to afford the natively folded HP6 proteins **39-44** bearing different homogeneous modifications. The sulfate and glycan modifications were shown to have a dramatic effect on both the fibrinogenolytic and amidolytic activity of human thrombin. Specifically, in the absence of a sTyr modification, the addition of a monosaccharide (in **40**) and disaccharide (in **41**) to Thr-43 led to an increase in inhibition against both activities of thrombin. However, addition of sulfate alone, without the presence of glycans, (in **42**) resulted in the most potent inhibitor of the fibrinogenolytic (FA; IC_{50} = 5.8 nM) and amidolytic activities (AA; IC_{50} = 820 pM) of thrombin. The addition of glycans to this sulfated protein (in **43** and **44**) led to a dramatic drop in the inhibitory activity of the protein. This interesting interplay between different post-translational modifications suggests that similar activity (and possible structural) modulation is possible in other proteins, an area of future investigation in our laboratories.



Scheme 7. Synthesis of homogeneously sulfated and glycosylated variants of hirudin P6 together with the inhibitory activities against the fibrinogenolytic activity (FA) and amidolytic activity (AA) of human thrombin.[51]

Outlook

The development of robust methods for the synthesis of homogeneously sulfated peptides and proteins has accelerated efforts to understand the importance of tyrosine sulfation for structure and function. As more peptides and proteins bearing sTyr modifications are discovered through proteomic endeavours it is likely that additional roles of this important post-translational modification will be discovered.

Biographies

Martin J. Stone received his B.Sc. and M.Sc.(hons) degrees from the University of Auckland, New Zealand (1986 and 1987, respectively) and his Ph.D. from Cambridge University, England (1991). After completing a postdoctoral fellowship at the Scripps Research Institute, La Jolla, CA, he joined the faculty of the Chemistry Department at Indiana University in 1995. In 2007 he moved to the Department of Biochemistry and Molecular Biology at Monash University, Melbourne, Australia, where he is currently Associate Professor and Director of the Monash Biomedical Science (MBio) Graduate Program. His research focuses on the structure-activity relationships of chemokines and chemokine receptors, including their regulation by post-translational modifications and the mechanisms and biological consequences of biased agonism.

Richard J. Payne BSc (Hons) degree from the University of Canterbury, New Zealand in 2002. In 2003 he was awarded a Gates Scholarship to undertake his PhD at the University of Cambridge, where he graduated with a PhD in 2006. After 18 months as a Lindemann Fellow at The Scripps Research Institute (La Jolla) he began his independent career (in January 2008) at The University of Sydney where he is currently Professor of Organic Chemistry and Chemical Biology and an Australian Research Council Future Fellow. Professor Payne's research focusses on utilising the power of synthetic organic chemistry to interrogate biological systems and address problems of medical significance. He has made significant breakthroughs in the development of new methodologies for the assembly of peptides and proteins bearing complex post-translational modifications, including vaccine and therapeutic candidates. As a result of his research endeavours he has been the recipient of several prestigious national awards including

the Royal Australian Chemical Institute Biota Medal in Medicinal Chemistry (2008), the Rennie Memorial Medal (2012), the Athel Beckwith Lectureship (2013), the Tregear Award for Peptide Science (2013) and the Le Févre Memorial Prize from the Australian Academy of Science (2014).

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