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

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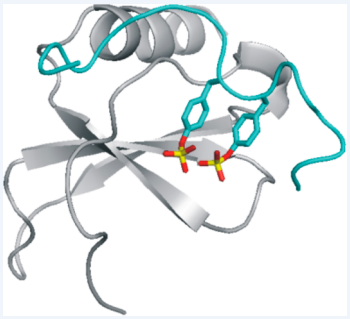
CONSPECTUS: Post-translational modification of proteins plays critical roles in regulating structure, stability, localization, and function. Sulfation of the phenolic side chain of tyrosine residues to form sulfotyrosine (sTyr) is a widespread modification of extracellular and integral membrane proteins, influencing the activities of these proteins in cellular adhesion, blood clotting, inflammatory responses, and pathogen infection. Tyrosine sulfation commonly occurs in sequences containing clusters of tyrosine 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 tyrosine 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 tyrosine residues. Using this methodology, we have carried out orthogonal deprotection and sulfation of up to three tyrosine residues within a given sequence, allowing access to all eight sulfoforms of a given target from a single solid-phase synthesis.

With homogeneously 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 antithrombotic 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 residues to form sulfotyrosine (sTyr). Due to localization of the tyrosylprotein sulfotransferase (TPST) enzymes that catalyze tyrosine sulfation in the trans-Golgi network (Scheme 1), tyrosine 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]

While it has been suggested that up to 1% of all proteins are sulfated on tyrosine, 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]

In light of the difficulties in analyzing and controlling tyrosine 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

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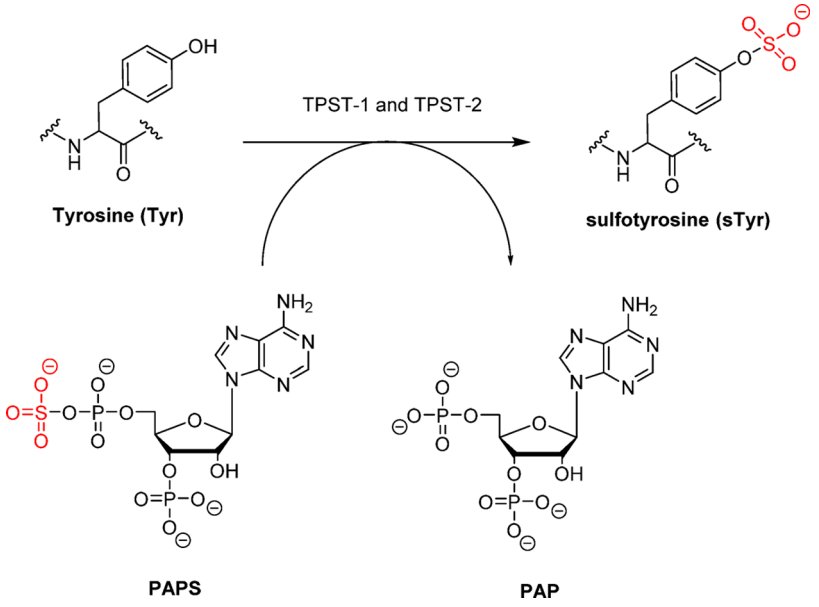
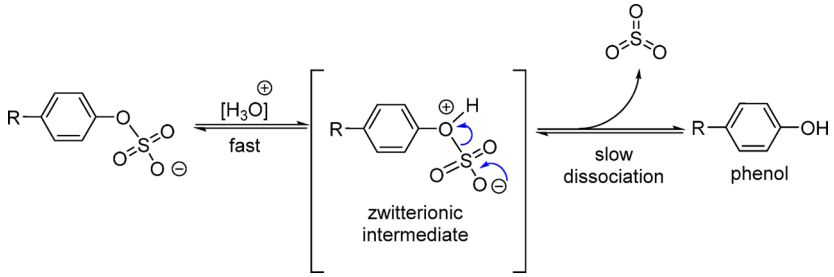
Scheme 1. Tyrosine Sulfation Facilitated by TPSTs in the trans-Golgi Network



Adapted from Moore.[3]

Scheme 2. Aryl Sulfate Hydrolysis in Acidic Aqueous Media



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 sulfotyrosine 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 tyrosine sulfation explored using synthetic sulfopeptides and sulfoproteins.

SYNTHESIS OF SULFOPEPTIDES USING SUITABLY PROTECTED SULFOTYROSINE "CASSETTES"

From a synthetic standpoint, the acid lability of the tyrosine 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. While 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 tyrosine-*O*-sulfate ester in the synthesis of sulfopeptides.

Early synthetic studies on sulfation of tyrosine residues within peptides relied on the use of nonselective 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 tyrosine residue was a major limitation of this strategy. The incorporation of preformed sulfotyrosine amino acid building blocks bearing counterions to stabilize the labile phenolic sulfate ester has also been employed in SPPS, for example, sodium,[9,12,13] barium,[14] and tetraalkylammonium salts.[15] While these amino acids have enabled the installation of sulfotyrosine 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 sulfotyrosine residues as robust diesters for use as amino acid "cassettes" in SPPS-based assembly protocols enabled the site-selective installation of sulfotyrosine 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.

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

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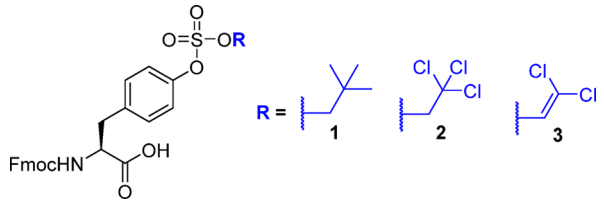


Figure 1. Fmoc-protected tyrosine cassettes containing nP (**1**), TCE (**2**), or DCV (**3**)-derived sulfate diesters.

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 sulfotyrosine 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, for example, 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.

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 tyrosine sulfation on function to be determined, as discussed later in this Account.

SITE SELECTIVE SOLID-PHASE SYNTHESIS OF HOMOGENEOUS SULFOPEPTIDES

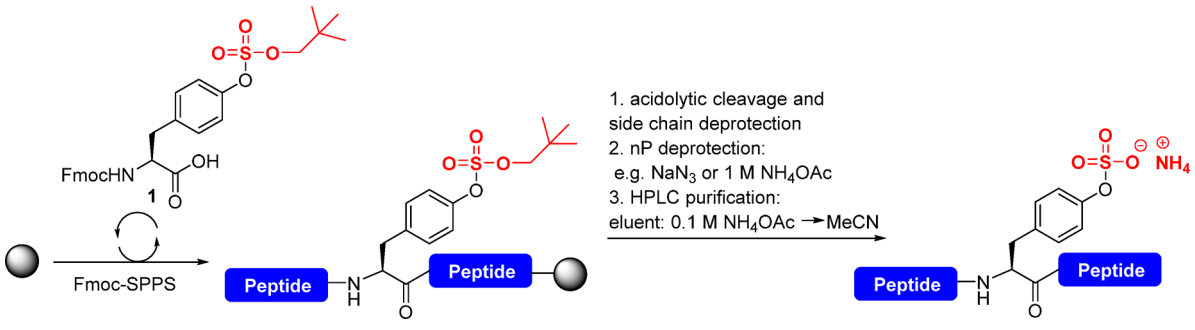
Many sulfopeptides and sulfoproteins possess multiple sulfotyrosine residues. Despite the significant advances made with the cassette strategy, there remains the need for an efficient method for the preparation of peptides bearing a variety of different sulfation patterns. Ideally, these sulfopeptides 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 tyrosine residues.[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 tyrosine phenol, *tert*-butyldimethylsilyl ether (TBS) and allyl 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 tyrosine residues. The resin was then separated into four parts for solid-phase synthetic manipulations. Deprotection of the allyl ether from Tyr26 was carried out using Pd(0). Subsequent acidolytic cleavage from the resin and concomitant side chain deprotection (including the TBS moiety from Tyr28) afforded nonsulfated CCR2(18−31), **18**. Synthesis of CCR2(18−31) with sulfation at Tyr26 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 sTyr28 form of CCR2(18−31) was achieved via TBAF-mediated removal of the silyl ether of **17** followed by sulfation with **19**. Deallylation of Tyr26 followed by side chain deprotection and cleavage from the resin then provided **21**. Finally, CCR2(18−31) sulfated at both Tyr26 and Tyr28 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 tyrosine 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

Scheme 3. Generalized Strategy for the Synthesis of Homogeneous Sulfopeptides Using the Neopentyl-Protected Sulfotyrosine Cassette 1



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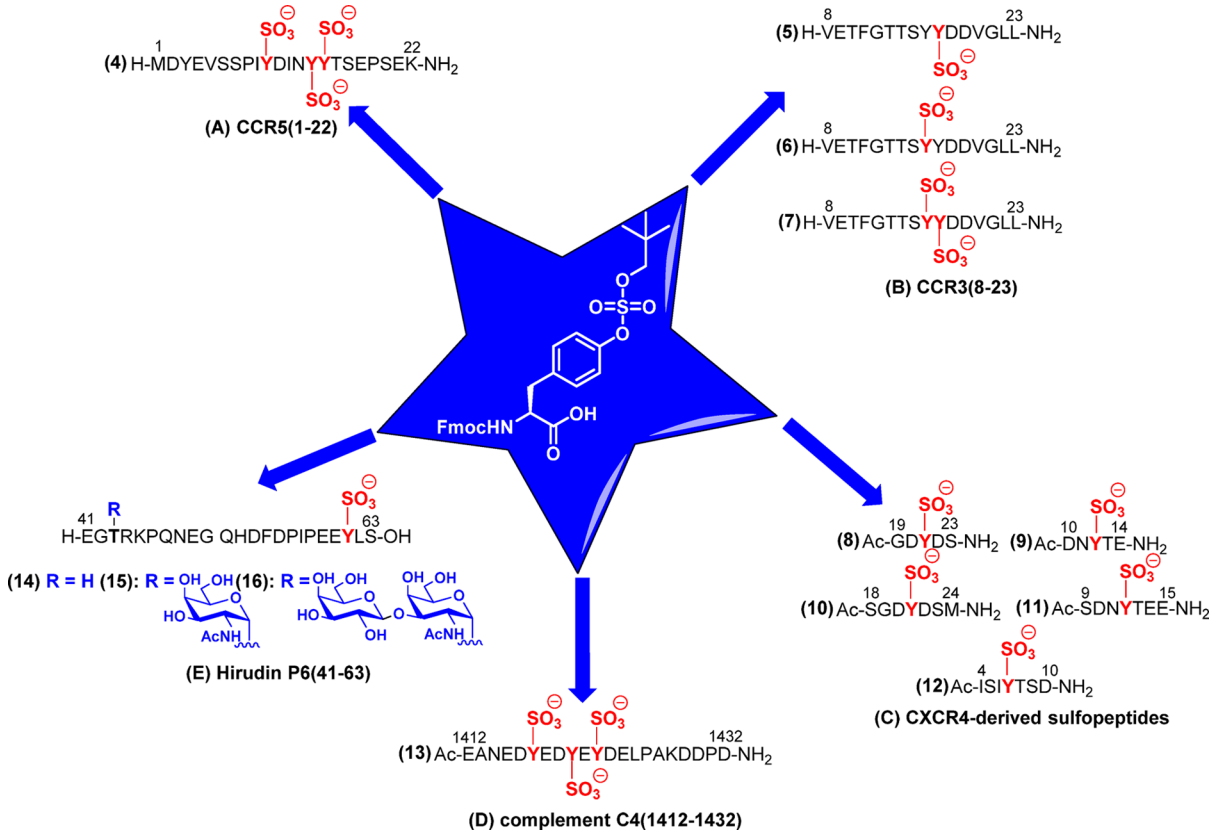
Scheme 4. Homogeneous Sulfopeptide Targets Synthesized Using the Cassette Strategy[a]



[a](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]

required the introduction of a third orthogonal protecting group for the side chain of tyrosine. For this purpose, we focused on the photolabile *o*-nitrobenzyl ether, which is completely orthogonal to the allyl 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, we assembled a resin-bound peptide library 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.

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.

EFFECTS OF TYROSINE SULFATION OF CHEMOKINE RECEPTORS ON CHEMOKINE BINDING AND ACTIVITY

Tyrosine 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 tyrosine residues. Commonly, these tyrosine residues undergo sulfation, which affects various aspects of receptor activity.

Identification of a Conserved Receptor-Sulfotyrosine 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 colored 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.

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

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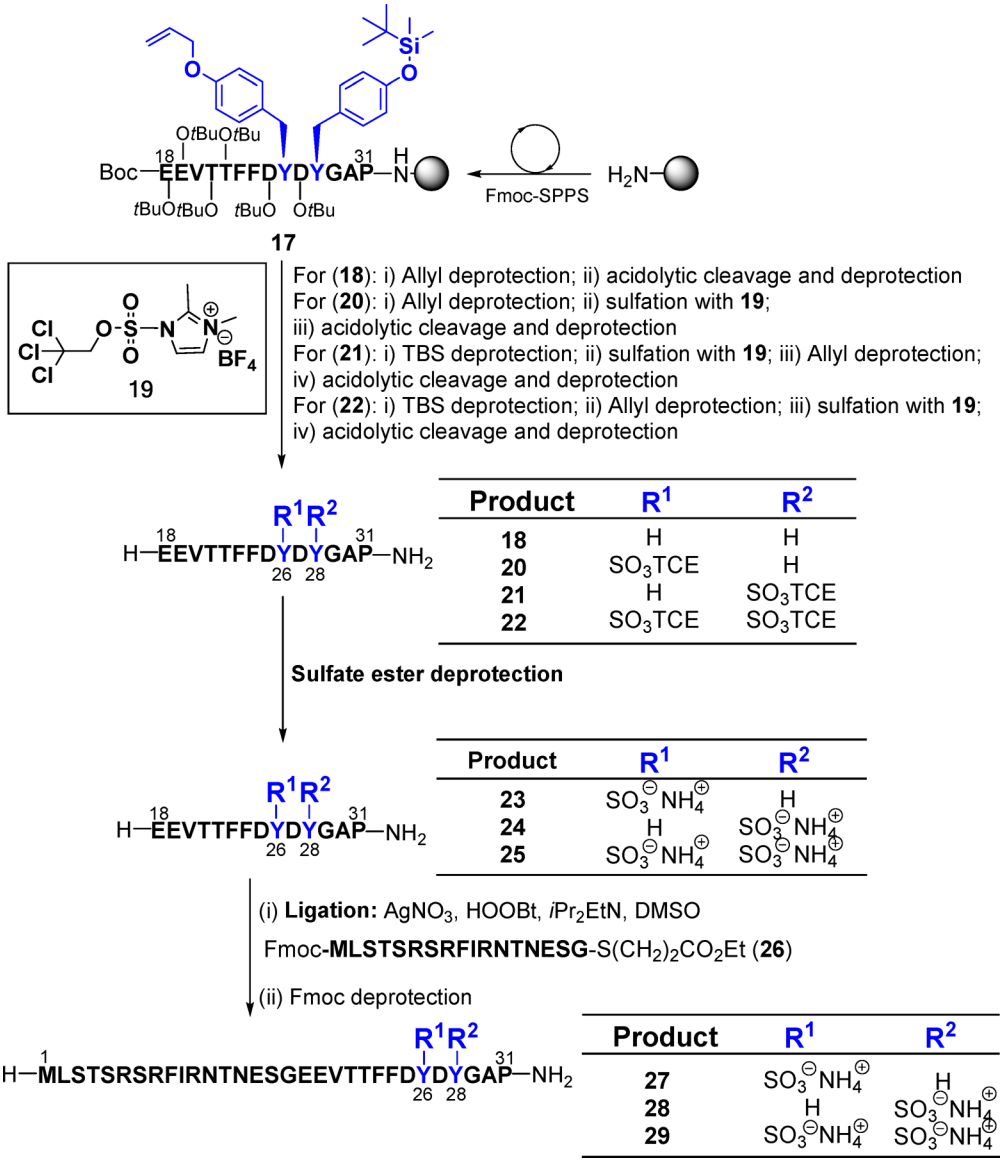
Scheme 5. Synthesis of Differentially Sulfated Peptides Derived from the N-Terminal Domain of the Chemokine Receptor CCR2 via a Divergent Solid-Phase Synthetic Strategy[21]



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	$SO_3^-NH_4^+$	H
24	H	$SO_3^-NH_4^+$
25	$SO_3^-NH_4^+$	$SO_3^-NH_4^+$

Product	R^1	R^2
27	$SO_3^-NH_4^+$	H
28	H	$SO_3^-NH_4^+$
29	$SO_3^-NH_4^+$	$SO_3^-NH_4^+$

receptor sulfotyrosine is the N-loop/β3 cleft, and the structures reveal specific salt bridges, hydrogen bonds, hydrophobic interactions, and cation–π interactions involved in sulfotyrosine recognition (Figure 3A,B). 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,B).[46] These results suggest that binding of chemokine to the sulfotyrosine residues in the N-terminal tail of the receptor may guide the subsequent interactions with transmembrane helices and thereby influence signaling.

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 polarization 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 the active

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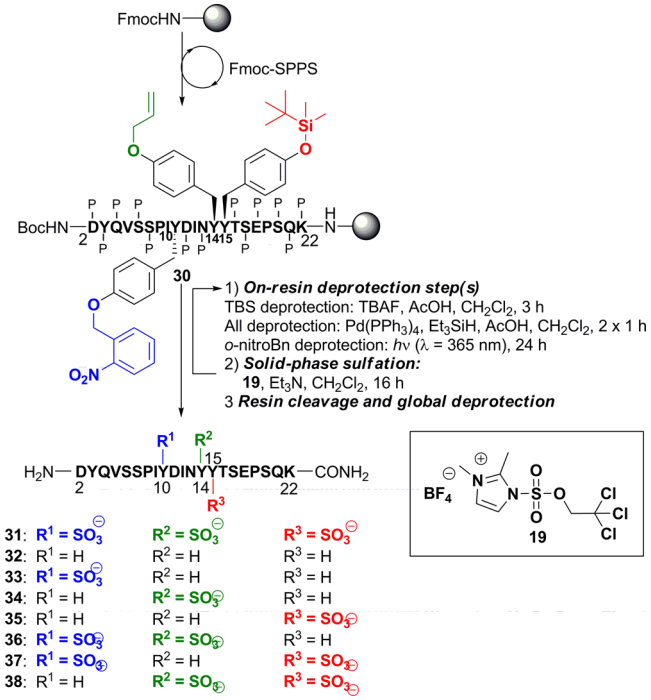
Scheme 6. Divergent solid-Phase Synthesis of Eight Possible Sulfoforms of CCR5(2−22) 31−38 with Variation in Sulfation Pattern at Tyr10, Tyr14 and Tyr15[35a]



[a]P = standard amino acid side chain protecting groups for Fmoc-SPPS.

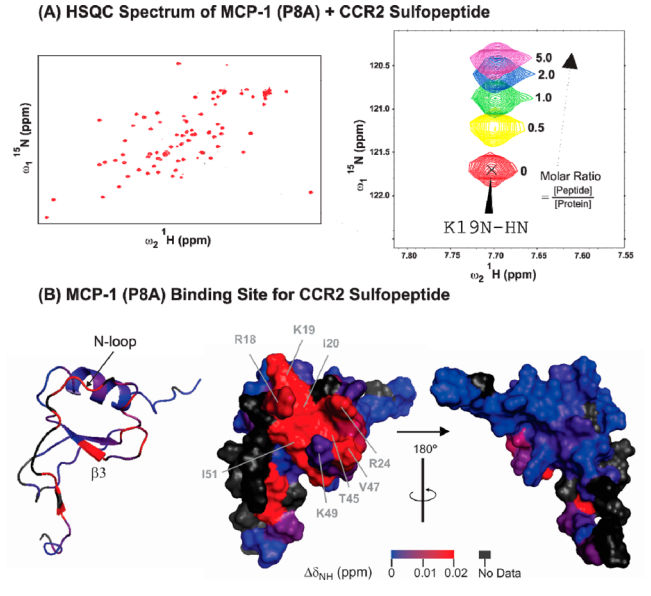


Figure 2. NMR identification of the receptor sulfopeptide binding site of a chemokine.[41] (A) HSQC spectrum of the chemokine MCP-1 (monomeric mutant P8A) including an expanded region illustrating the shift of one peak upon addition of a sulfopeptide from receptor CCR2. (B) Structure of monomeric MCP-1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

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.

Tyrosine 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 tyrosine 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 tyrosine 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 3−30-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 tyrosine 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 tyrosine 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.

Tyrosine 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. Tyrosine sulfation is one possible mechanism that could regulate the responses of receptors among possible ligands.[39] We have found that differently sulfated states of a receptor peptide can have distinct selectivity profiles among 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 3−10-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 among a range of potential chemokine ligands.

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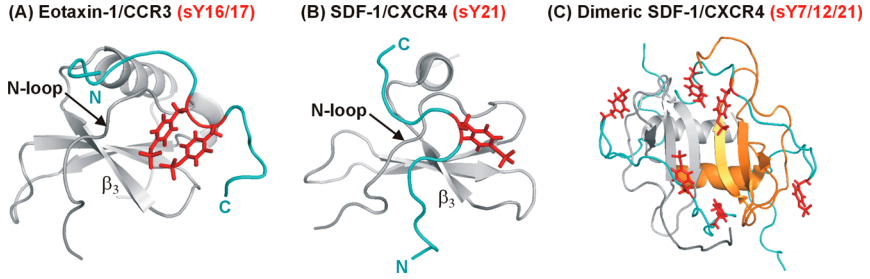

Figure 3. Structures of chemokine receptor sulfopeptides bound to cognate chemokines. (A) Doubly sulfated peptide 7 derived from receptor CCR3 bound to the CC chemokine eotaxin-1.[46] (B, C) Triply sulfated peptide from receptor CXCR4 bound to the CXC chemokine SDF-1,[44] showing (B) one protomer unit in the same orientation as that in panel A with the associated part of the peptide and (C) overall structure of the dimeric complex with the whole peptide. Chemokines are shown as gray or orange ribbons. Peptides are cyan with sulfotyrosine residues as red sticks.

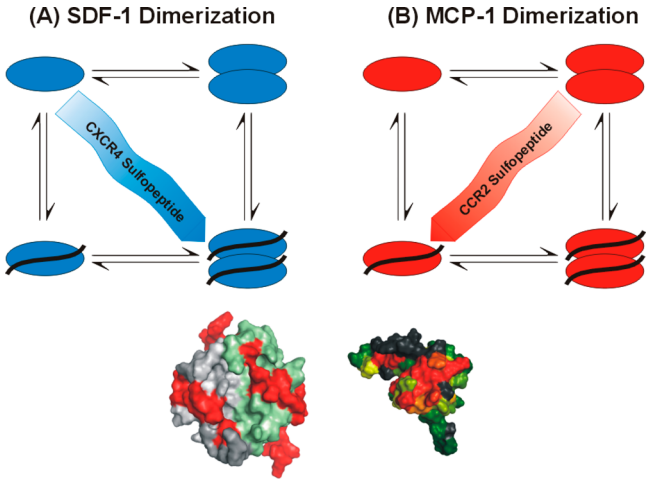

Figure 4. Cooperativity between chemokine dimerization and receptor sulfopeptide binding.[50] Schematic thermodynamic cycles showing that (A) a CXCR4 sulfopeptide enhances dimerization of the chemokine SDF-1 and (B) a CCR2 sulfopeptide favors dissociation of the MCP-1 dimer to the active monomeric state.

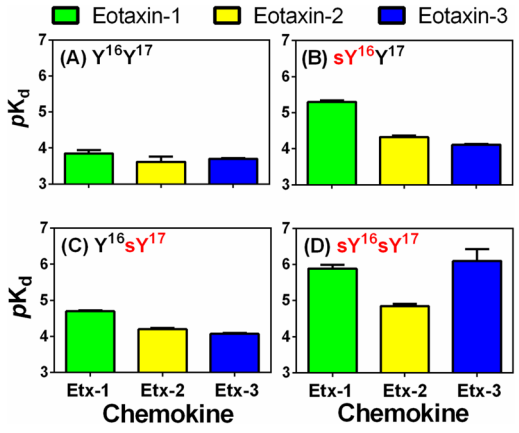

Figure 5. Tyrosine sulfation affects chemokine binding selectivity. Affinities, expressed as $pK_d = -\log(K_d)$, of (A) unsulfated, (B) Tyr16 sulfated, (C) Tyr17 sulfated, and (D) doubly sulfated forms of a CCR3 peptide for each of three cognate chemokines of CCR3: (green) eotaxin-1; (yellow) eotaxin-2; and (blue) eotaxin-3.

EFFECTS OF SITE-SELECTIVE SULFATION ON THE INTERACTIONS OF HIV gp120 WITH PEPTIDES FROM THE FUSION CO-RECEPTOR 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 co-receptors, the chemokine receptors CCR5 or CXCR4.[33,34] Farzan and co-workers 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 tyrosine residues (Tyr3, 10, 14, and 15) that are all potentially sulfated (Figure 6), although Tyr3 appears to be less important than the other

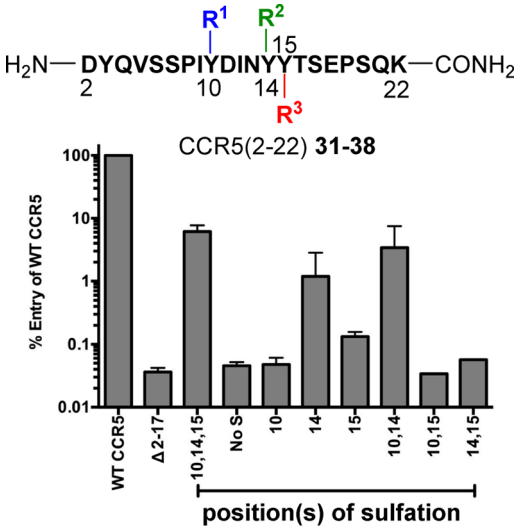

Figure 6. Reconstitution of HIV-1 entry into U87-CD4 cells expressing N-terminally truncated CCR5(Δ2−17) by treatment with differentially sulfated CCR5 peptides **31**−**38**.[35]

three tyrosine residues for facilitating HIV entry.[38] *In vitro*, Tyr14 and Tyr15 undergo TPST-catalyzed sulfation most rapidly, followed by Tyr10 and finally Tyr3.[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 Tyr10, Tyr14, and Tyr15 (**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 Tyr14 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 Tyr14 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

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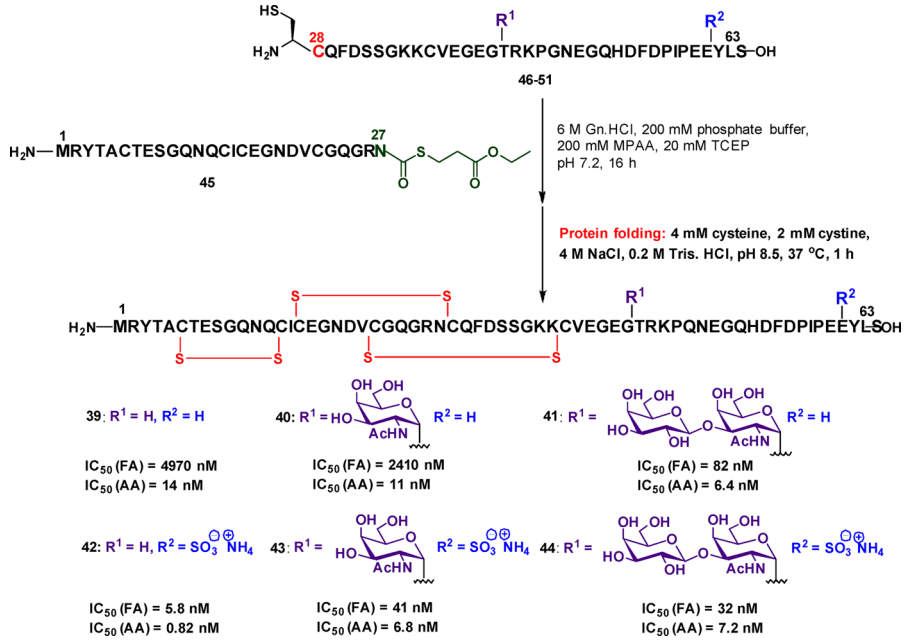
Scheme 7. Synthesis of Homogeneously Sulfated and Glycosylated Variants of Hirudin P6 Together with the Inhibitory Activities against the Fibrinogenolytic Activity (FA) and Amidolytic Activity (AA) of Human Thrombin[53]



interactions are critically dependent on sulfation of Tyr14 and may be enhanced by sulfation of the other tyrosine 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]

SYNTHESIS OF HOMOGENEOUS LEECH-DERIVED ANTI-THROMBOTIC SULFOPROTEINS VIA NATIVE CHEMICAL LIGATION

An emerging area within our laboratories involves the study of antithrombotic proteins from hematophagous 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 antithrombotic 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 anticoagulant agents. Like HIRV1, HP6 is known to possess a sulfotyrosine 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 Asn27 and Cys28 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 tyrosine 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 sulfotyrosine modification, the addition of a monosaccharide (in **40**) and disaccharide (in **41**) to Thr43 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.

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 sulfotyrosine modifications are discovered through proteomic endeavors it is likely that additional roles of this important post-translational modification will be discovered.

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Notes

The authors declare no competing financial interest.

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 received his B.Sc. (Hons) degree from the University of Canterbury, New Zealand, in 2002. In 2003, he was awarded a Gates Scholarship to undertake his Ph.D. at the University of Cambridge, where he graduated with a Ph.D. 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 focuses on utilizing 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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