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Site-Selective Solid-Phase Synthesis of a CCR5 Sulfopeptide Library to Interrogate HIV Binding and Entry

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Abstract

Tyrosine (Tyr) sulfation is a common post-translational modification that is implicated in a variety of important biological processes, including the fusion and entry of human immunodeficiency virus type-1 (HIV-1). A number of sulfated Tyr (sTyr) residues on the N-terminus of the CCR5 chemokine receptor are involved in a crucial binding interaction with the gp120 HIV-1 envelope glycoprotein. Despite the established importance of these sTyr residues, the exact structural and functional role of this post-translational modification in HIV-1 infection is not fully understood. Detailed biological studies are hindered in part by the difficulty in accessing homogeneous sulfopeptides and sulfoproteins through biological expression and established synthetic techniques. Herein we describe an efficient approach to the synthesis of sulfopeptides bearing discrete sulfation patterns through the divergent, site-selective incorporation of sTyr residues on solid support. By employing three orthogonally protected Tyr building blocks and a solid-phase sulfation protocol, we demonstrate the synthesis of a library of target N-terminal CCR5(2-22) sulfoforms bearing discrete and differential sulfation at Tyr-10, Tyr-14 and Tyr-15, from a single resin-bound intermediate. We demonstrate the importance of distinct sites of Tyr sulfation in binding gp120 through a competitive binding assay between the synthetic CCR5 sulfopeptides and an anti-gp120 monoclonal antibody. These studies revealed a critical role of sulfation at Tyr-14 for binding and a possible additional role for sulfation at Tyr-10. N-terminal CCR5 variants bearing a sTyr residue at position 14 were also found to complement viral entry into cells expressing an N-terminally truncated CCR5 receptor. The results of these studies provide important insight into the intricate role of site-specific Tyr sulfation in CCR5 coreceptor function.

Introduction

Tyrosine (Tyr) sulfation is one of the most common post-translational modifications affecting secreted and transmembrane proteins, with estimates that more than 1% of all human proteins contain sulfated Tyr (sTyr) residues.[1-5] The sulfation process is mediated by tyrosylprotein sulfotransferase-1 and -2 (TPST-1 and TPST-2, respectively), enzymes located in the *trans*-Golgi network that catalyze the transfer of sulfate from 3´-phosphoadenosine 5´-phosphosulfate (PAPS) to the phenol moiety of a peptidyltyrosine residue.[6-8] sTyr residues

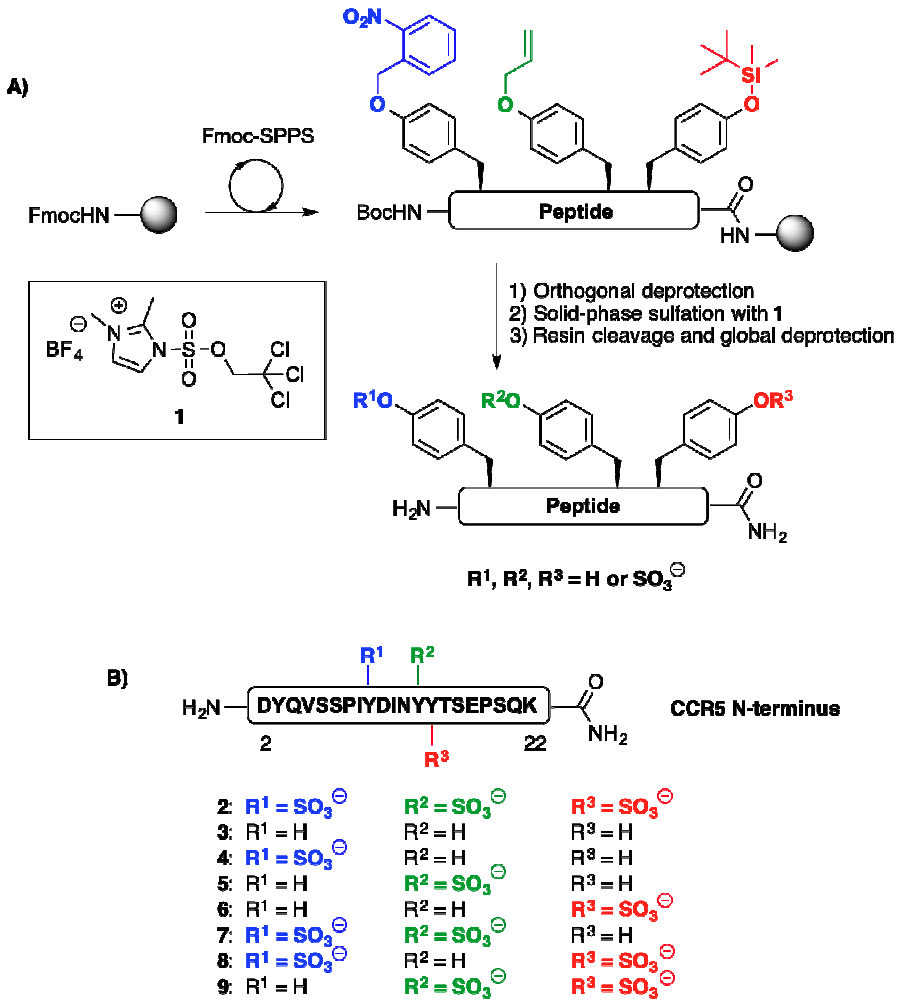
are implicated in a number of important physiological processes, including blood coagulation,[9-12] cell-cell interactions,[13] chemokine signalling through chemokine receptors[14-16] and viral entry into host cells.[2, 17-20] Indeed, Tyr sulfation is known to have a particularly significant role in mediating the fusion and entry of human immunodeficiency virus type 1 (HIV-1) into target cells. Multiple sulfation sites on the N-terminal fragment of the chemokine coreceptor CCR5 are thought to be involved in a high affinity binding interaction with the HIV-1 envelope glycoprotein gp120-cellular CD4 receptor complex that is essential for viral entry.[17] The crucial role of sTyr in CCR5-mediated HIV-1 infection is of particular interest for the broader development of CCR5 mimics and coreceptor antagonists as novel antivirals.[21-23]

Despite the biological importance of sulfation, the precise structural and functional role of sTyr residues in CCR5 and in the majority of other proteins known to be sulfated is not fully understood.[3] Detailed studies of the role of Tyr sulfation on biological activity are complicated by difficulties in accessing large quantities of high-purity, homogeneous sulfoproteins. This is owing to the fact that the Tyr sulfation process is not templated, but rather is controlled by the relative activities of TPSTs in the cell. Indeed, under the influence of the sulfotransferase enzymes, a heterogeneous mixture of different protein sulfoforms results, varying widely in the number and location of sTyr residues. The highly acid labile phenolic sulfate ester linkage further complicates the extraction and manipulation of sulfopeptides as well as the ability to access target compounds by standard chemical methods. As such, there is a strong demand for the development of new synthetic routes for the preparation of peptides and proteins bearing discrete Tyr sulfation patterns.

Early chemical methods for accessing sulfopeptides involved global sulfation protocols whereby unprotected Tyr residues were reacted with sulfur trioxide-DMF[24-28] or sulfur trioxide-pyridine complexes.[29, 30] The incorporation of pre-formed sTyr amino acid building blocks as the sodium[26, 31, 32] or tetraalkylammonium salts[33] in solid-phase peptide synthesis (SPPS) subsequently enabled the site-selective installation of sTyr residues. However, cleavage of the target peptide from the solid support and concomitant global side-chain deprotection under the standard acidic conditions employed in SPPS leads to substantial loss of the acid labile sulfate monoester.[34, 35] The orthogonal protection of sTyr residues as robust diesters for use in Fmoc-SPPS has therefore been explored as an alternative approach for cassette strategy incorporation of Tyr sulfation.[36] A number of acid stable aryl sulfate protecting groups, including neopentyl,[36, 37] 2,2,2-trifluoroethyl (TFE),[38] 2,2,2-trichloroethyl (TCE)[39-41] and 2,2-dichlorovinyl (DCV) sulfate esters,[42, 43] have been

employed in the solid-phase synthesis of target sulfopeptides. Liskamp and coworkers have further extended the use of sulfate diesters to encompass the global sulfation of free Tyr residues on resin-bound peptides *via* treatment with TCE chlorosulfate.[44] We have recently explored the use of sulfuryl imidazolium salts,[38, 45] such as Taylor's TCE sulfating reagent **1** (Scheme 1A)[45] and the corresponding TFE derivative,[38] for the total synthesis of the sulfoprotein hirudin P6[11, 12] as well as for the solid-phase sulfation of two Tyr residues within the N-terminal domain of the chemokine receptor CCR2.[39] This study enabled subsequent biochemical studies of the role of Tyr sulfation in chemokine recognition.[46-48]

Despite these advances, there are as yet no divergent synthetic approaches to sulfopeptide libraries of targets bearing more than two sulfation sites. Driven by our interest in the role of the multiply-sulfated N-terminal region of CCR5 (containing four possible Tyr sulfation sites, three of which are proposed to be important to CCR5 coreceptor function)[17] in binding gp120 and mediating HIV-1 entry, we were interested in developing a divergent, solid-phase sulfation strategy that would allow access to sulfopeptide libraries containing variable sulfation at three distinct sites. To this end, we envisaged the use of three orthogonal side-chain Tyr protecting groups, *ortho*-nitrobenzyl (*o*-Nb), allyl (All) and *tert*-butyl-dimethylsilyl (TBS) groups, which, after installation at the required position in the target peptide sequence, can be selectively and differentially deprotected in the presence of one another and all other side-chain protecting groups commonly employed in Fmoc-SPPS (Scheme 1A). We proposed that orthogonal deprotection(s), followed by site-selective solid-phase sulfation of the selectively unmasked Tyr residues with TCE sulfating reagent **1** would enable rapid access to all eight variants of a target sulfopeptide bearing three sulfation sites (Scheme 1A). Importantly, this methodology would enable access to a library of target N-terminal CCR5(2-22) sulfoforms (compounds **2**-**9**, Scheme 1B), bearing discrete and differential sulfation at Tyr-10, Tyr-14 and Tyr-15, from a single resin-bound peptide and without any intermediary purification steps. We describe herein the development of this novel methodology and demonstrate the high-yielding syntheses of eight differentially sulfated CCR5 targets to facilitate a systematic study of the role of sulfation in binding to the HIV-1 envelope glycoprotein gp120. We have also demonstrated that the sulfation pattern on the N-terminus of CCR5 heavily influences whether fragments of the N-terminal CCR5 domain can complement viral entry of an N-terminally truncated CCR5 receptor on T cells.



Scheme 1. A) Divergent solid-phase sulfation strategy employing three orthogonal protecting groups. B) Target N-terminal CCR5(2-22) sulfoforms **2**-**9**.

Results and discussion

Synthesis of target CCR5 sulfopeptides

In order to implement the proposed divergent solid-phase sulfation strategy, we first synthesized three pre-formed, orthogonally protected Tyr building blocks (**10**, **11**, and **12**), bearing side-chain *o*-nitrobenzyl, allyl and TBS-protection of the phenolic Tyr side chain, respectively (Figure 1, see Supporting Information for synthetic details). These building blocks could be accessed in gram quantities and in high enantiopurities from commercially available, protected Tyr precursors. With the requisite Tyr derivatives in hand, we next embarked upon the solid-phase synthesis of the target CCR5 sulfopeptides, comprised of residues 2-22 of the N-terminal domain, with a single substitution of Cys-20 to Ser to prevent

inter-peptide disulfide formation, together with the incorporation of three possible sulfation sites at Tyr-10, Tyr-14 and Tyr-15. Synthesis was performed using Rink amide resin and began with the loading of Fmoc-Lys(Boc)-OH. Elongation to residue Thr-16 (just prior to the first potential Tyr sulfation site) was accomplished using standard Fmoc-SPPS (see Scheme 2 and Supporting Information). Building block **12** was next incorporated using a slight excess of amino acid (1.5 eq.), in the presence of HATU (1.5 eq.) and *N,N*-diisopropylethylamine (DIPEA) (3.0 eq.) in *N,N*-dimethylformamide (DMF). Compound **11** bearing side-chain allylic protection was subsequently incorporated at position 14, using identical coupling conditions. Extension to Asp-11 using standard Fmoc-SPPS was followed by the coupling of *ortho*-nitrobenzyl Tyr derivative **10** using optimized conditions of (Benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate PyBOP (2.0 eq.), hydroxybenzotriazole (HOBt) (2.0 eq.) and *N*-methylmorpholine (NMM) (4.0 eq.) in DMF (see Supporting Information for details). The peptide was further elongated to CCR5(2-22) **13** and the resin split into eight equal portions to facilitate divergent solid-phase manipulations at Tyr-10, Tyr-14 and Tyr-15.

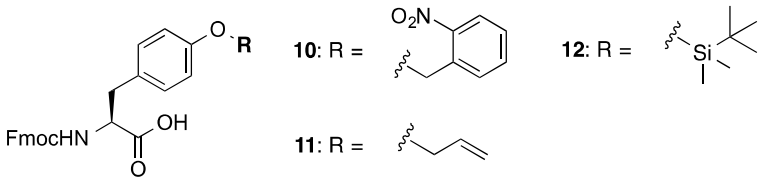
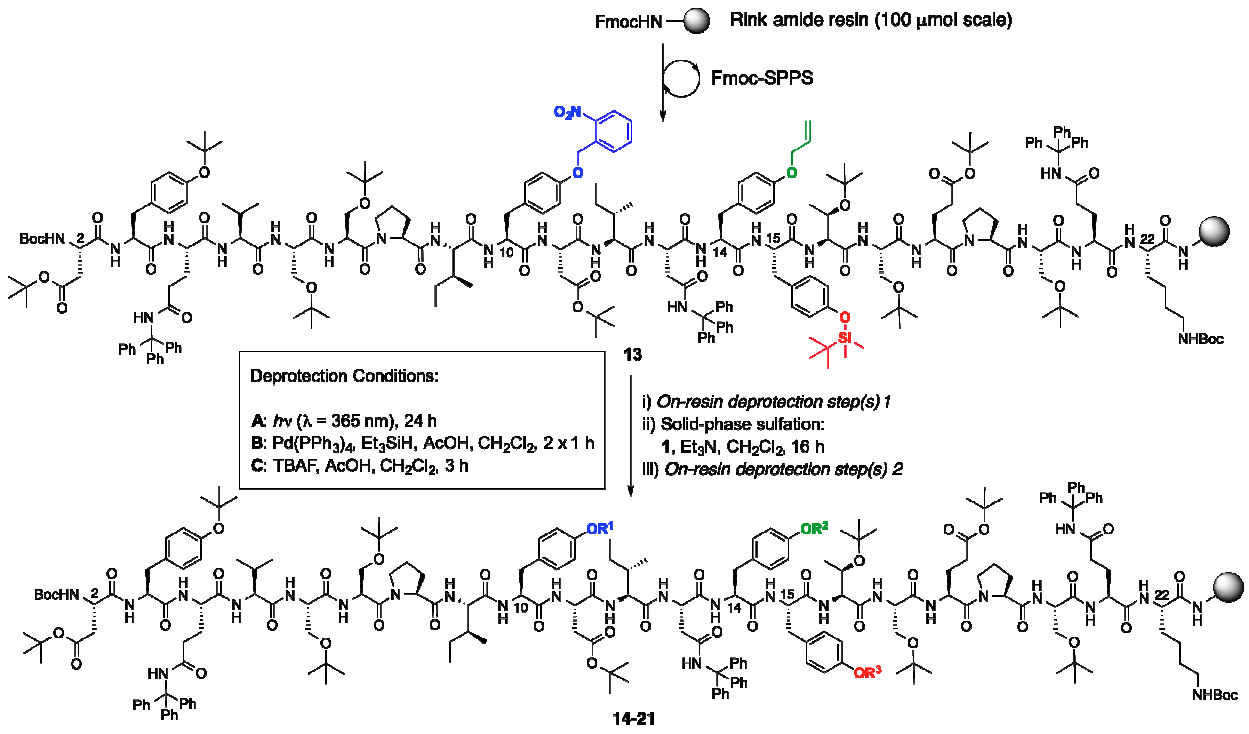


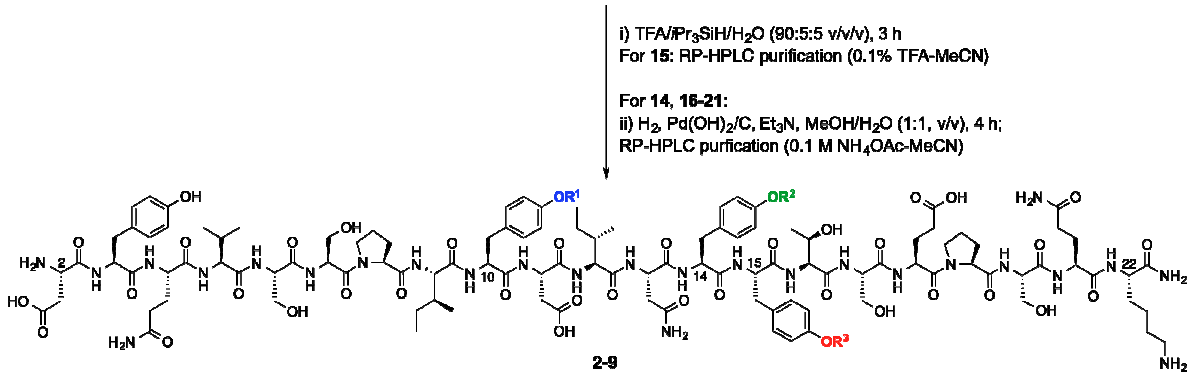
Figure 1. Fmoc-protected Tyr building blocks **10**, **11** and **12**.

From the fully protected resin-bound peptide **13**, we first embarked upon the synthesis of target CCR5(2-22) peptide **2**, a triply sulfated variant bearing sTyr residues at positions 10, 14 and 15. As construction of the desired sulfation pattern of peptide **2** would first require the removal of all three Tyr side-chain protecting groups, we hypothesized that this synthesis would enable us to affirm the orthogonality of the three Tyr protecting groups selected (Scheme 2). To probe our protecting group strategy, resin bound CCR5 peptide **13** was first irradiated with UV light at $\lambda = 365$ nm in DMF for 24 h (deprotection condition A). Gratifyingly, these conditions facilitated quantitative and selective removal of the side-chain *o*-nitrobenzyl group at Tyr-10, leaving the remaining phenolic protecting groups intact (as judged by HPLC-MS analysis of the cleavage of a small number of resin beads). Subsequent treatment with $Pd(PPh_3)_4$, triethylsilane (TES) and acetic acid in CH_2Cl_2 (deprotection condition B) effected clean removal of the allyl ether at Tyr-14. A final treatment with tetrabutylammonium fluoride (TBAF) buffered with acetic acid[39] (deprotection condition C)

resulted in the loss of the TBS protecting group at Tyr-15, affording a resin-bound peptide bearing three unprotected Tyr residues. Having demonstrated the iterative and selective deprotection of each Tyr protecting group, we next subjected the immobilized peptide to solid-phase sulfation using TCE imidazolium sulfating reagent **1** (8 eq. per free phenol) in the presence of triethylamine, to afford peptide **14** bearing TCE-protected sulfate diesters at positions 10, 14, and 15. Acidic cleavage of the peptide from the resin was followed by removal of the TCE protecting group *via* catalytic hydrogenolysis using $Pd(OH)_2$ on carbon.[43] HPLC purification in the presence of 0.1 M NH_4OAc (to avoid loss of the acid labile sulfate monoester) afforded the triply sulfated CCR5 variant **2** in 28% yield based on the original resin loading (over 47 linear steps).



Resin-bound Peptide	*On-resin Deprotection Step(s) 1*	*On-resin Deprotection Step(s) 2*	R^1 (Tyr-10)	R^2 (Tyr-14)	R^3 (Tyr-15)
14	A, B, C	-	SO_3TCE	SO_3TCE	SO_3TCE
15	A, B	-	H	H	TBS
16	A	B	SO_3TCE	H	TBS
17	B	A	H	SO_3TCE	TBS
18	C	A, B	H	H	SO_3TCE
19	A, B	-	SO_3TCE	SO_3TCE	TBS
20	A, C	B	SO_3TCE	H	SO_3TCE
21	B, C	A	H	SO_3TCE	SO_3TCE



(Sulfo)peptide	R^1 (Tyr-10)	R^2 (Tyr-14)	R^3 (Tyr-15)	Isolated Yield
2	$SO_3^-NH_4^+$	$SO_3^-NH_4^+$	$SO_3^-NH_4^+$	28%
3	H	H	H	60%
4	$SO_3^-NH_4^+$	H	H	42%
5	H	$SO_3^-NH_4^+$	H	37%
6	H	H	$SO_3^-NH_4^+$	31%
7	$SO_3^-NH_4^+$	$SO_3^-NH_4^+$	H	29%
8	$SO_3^-NH_4^+$	H	$SO_3^-NH_4^+$	20%
9	H	$SO_3^-NH_4^+$	$SO_3^-NH_4^+$	34%

Scheme 2. Divergent solid-phase synthesis of CCR5(2-22) (sulfo)peptides **2-9**.

With conditions in hand for both the selective, on-resin deprotection of each orthogonal Tyr side-chain protecting group as well as the subsequent installation of the TCE sulfate diester on the solid-phase, we proceeded to synthesize the remaining CCR5(2-22) sulfoforms of our proposed library using a combinatorial deprotection-solid-phase sulfation strategy. In each case the initial deprotection(s) (conditions A, B and/or C, on-resin deprotection step(s) 1, Scheme 2) were followed by solid-phase sulfation with imidazolium sulfating reagent **1** to install the appropriately positioned TCE-protected sulfate diesters onto the unmasked phenolic Tyr side-chains. A second round of deprotection reactions (on-resin deprotection step(s) 2, Scheme 2) then facilitated the removal of the remaining Tyr side-chain protecting groups at positions 10 and/or 14. It should be noted that in cases where Tyr-15 remained unsulfated (e.g. resin-bound peptides **15**, **16**, **17** and **19**), the side-chain TBS ether was left intact, as loss of this protecting group could be facilitated concomitantly with acidic cleavage from the resin and global side-chain deprotection.[39] Following solid-phase manipulations, the resin-bound peptides **15-21** were treated with an acidic cocktail of TFA/*i*Pr_3SiH/H_2O (90:5:5 v/v/v) to effect cleavage from the resin and removal of all remaining side-chain protecting groups. At this point, unsulfated variant **3** was obtained directly following HPLC purification in 60% yield based on the original resin loading. The crude, differentially TCE-sulfated peptides derived from resin-bound variants **16-21** were then treated with $Pd(OH)_2$ in the presence of H_2 to remove the TCE sulfate diester protecting group(s). Purification by HPLC afforded the six remaining sulfoforms **4-9** in good overall yields (20-42%, an average of 97-98% per step). Importantly, this synthetic approach represents the first divergent synthesis of all 8 distinct sulfoforms of a given sulfopeptide bearing three sites of sulfation, in this case the CCR5 N-terminus (bearing sulfation at Tyr-10, -14 and -15), from a single-resin bound peptide and serves as a proof of concept for the rapid synthesis of compound libraries of multiply sulfated peptide and protein targets.

Binding interaction of N-terminal CCR5 sulfoforms with gp120

With the eight target sulfoforms of the N-terminal region of CCR5 in hand (compounds **2-9**), we were next interested in investigating the effect that discrete CCR5 sulfation patterns had on mediating binding with the HIV-1 Env glycoprotein gp120 and facilitating viral entry. Although previous studies have examined the role of specific sTyr residues in CCR5 binding,[17, 19, 20] we were interested in carrying out the first systematic

screen of multiple sulfation patterns, including a detailed analysis of the role of sulfation at Tyr-15. To evaluate the ability of our synthetic CCR5 peptides to bind gp120, we first carried out a competitive binding assay between peptides **2**-**9** and the anti-gp120 monoclonal antibody 17b[49] (Figure 2A). In this ELISA-based assay, recombinant gp120 was captured on plates coated with recombinant human CD4 lacking its transmembrane domain (rsCD4), which exposes the binding site on gp120 for CCR5. The protocol for producing rsCD4 is provided in detail in the Methods section. Conveniently, the epitope on gp120 for 17b overlaps with the CCR5 binding site, such that the displacement of antibody 17b from gp120 upon treatment with the sulfopeptide is indicative of the ability of the sulfopeptide to bind to gp120. The results in Figure 2A show that unsulfated peptide **3** could not displace the binding of 17b to gp120, confirming that sulfation is essential for gp120 binding to CCR5. Only peptides sulfated at Tyr-10, -14, and -15 (peptide **2**), Tyr-10 and -14 (peptide **7**), Tyr -14 and 15 (peptide **9**), and Tyr-14 (peptide **5**) could inhibit 17b binding to gp120, albeit to variable levels, whereas peptides sulfated at Tyr -10 and -15 (peptide **8**), and -15 alone (peptide **6**), could not. However, the most potent inhibition of antibody binding was exhibited by triply sulfated peptide **2**. Together, these results suggest that Tyr-14 is the most critical sulfation site for gp120 binding to CCR5, but that sulfation of Tyr-10 and Tyr-15 may further enhance gp120 binding affinity.

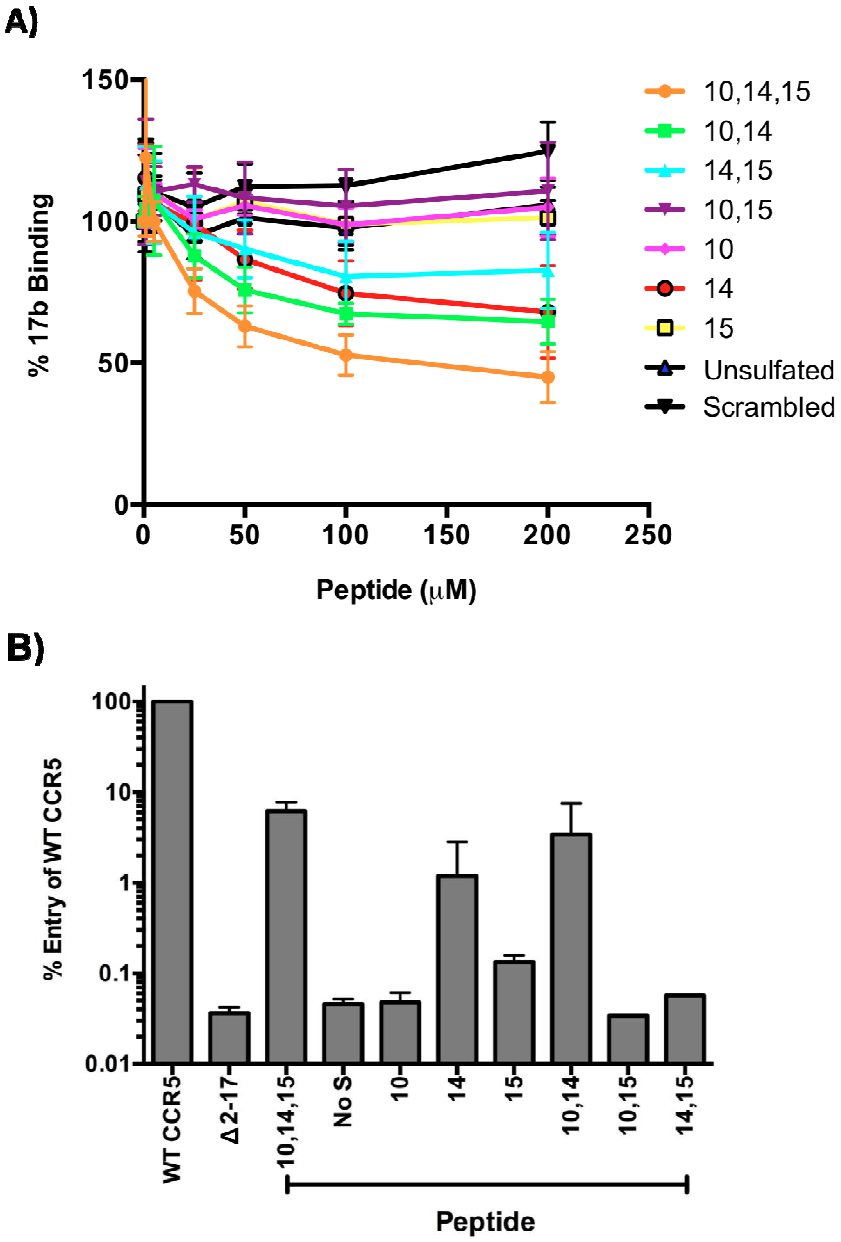


Figure 2. A) CCR5 sulfoforms inhibit binding of 17b to gp120. gp120 (recombinant) from the HIV-1 Bal strain was incubated on plates coated with recombinant soluble CD4 prior to addition of CCR5 sulfoforms. 17b was then added to the wells followed by sheep anti-human horseradish peroxidase (HRP) and absorbance$_{450}$ values were read. Binding curves were constructed where % binding = 100 – [($_{Abs}$17b + peptide – $_{Abs}$17b + no peptide) x 100]. Data points represent the mean and standard error of the mean from 5 independent experiments. **B) CCR5 sulfoforms can reconstitute HIV-1 entry in cells expressing N-terminally truncated CCR5.** U87-CD4 cells expressing an N-terminally truncated CCR5 (Δ2-17) were incubated with CCR5 peptide sulfoforms (100 μM concentration) for 30 min prior to infection with luciferase reporter viruses pseudotyped with HIV-1 YU-2 Env. Entry is expressed as a percentage of entry obtained in U87-CD4 cells expressing an equivalent amount of wild type CCR5. The data is the mean and standard deviation of triplicate infections from two independent experiments.

To determine whether the pattern of sulfated Tyr residues in CCR5 that are implicated in gp120 binding are also functionally important for HIV-1 entry into cells, we next undertook a series of CCR5 N-terminus complementation assays using luciferase reporter HIV-1 pseudotyped with gp120 from the YU2 HIV-1 strain (Figure 2B). These assays are based on those previously described by Farzan and coworkers.[20] Briefly, cells expressing CD4 together with N-terminally truncated (and thus non-functional) CCR5 (Δ2-17) were inoculated with virus in the presence or absence of each sulfopeptide. Any reconstitution of HIV-1 entry into the cells by addition of a synthetic sulfopeptide to the media would signify a "functional" sulfopeptide that is capable of mediating HIV-1 entry into cells after gp120 binding. When compared to cells expressing wild type (WT) CCR5, only HIV-1 that was treated with CCR5 variants sulfated at Tyr-10, -14, and -15 (peptide **2**), Tyr-10 and -14 (peptide **7**) and Tyr-14 (peptide **5**) could restore HIV-1 entry, with the triply sulfated peptide **2** and peptide **7** sulfated at Tyr-10 and -14 showing the greatest levels of restoration (Figure 2B). Not unexpectedly, the overall levels of restored virus entry were markedly lower than those mediated by WT CCR5. Together, the results of these functional studies largely mirror those from the gp120 binding studies (Figure 2A), and suggest a critical role for sTyr14 in CCR5 in mediating HIV-1 entry, and a possible additional role for sTyr10 for gp120 binding and entry. The observed importance of Tyr sulfation at positions 10 and 14 is corroborated by the original complementation assays performed by Farzan and coworkers,[20] which demonstrated that a CCR5 N-terminal peptide bearing sTyr residues at positions 10 and 14 could functionally reconstitute a CCR5 variant lacking the critical N-terminal region. Dragic and coworkers have additionally demonstrated that CCR5 N-terminal peptides bearing sTyr residues at positions 10 and 14 (but not the singly sulfated variants) were capable of binding gp120-CD4 complexes.[19] The observed binding motifs may be a direct result of the structural conformation of CCR5 when in complex with gp120 and CD4, which has recently been interrogated using NMR techniques.[50, 51] These studies show that residues 9-15 of the CCR5 N-terminus adopt a helical conformation, which positions Tyr-10 and Tyr-14 on the same face of the helix, enabling efficient interaction of these two residues with gp120. Saturation transfer difference (STD) NMR studies have also shown that sTyr-14 is positioned closer to gp120 than sTyr-10, and is thought to make a number of important hydrogen bonding interactions through its appended sulfate group.[50, 51] Tyr-15, on the other hand, is closely packed with neighboring hydrophobic residues and is pointed toward the host cell membrane,[50] so the enhanced binding and inhibition by the triply-sulfated peptide **2** relative

to doubly-sulfated peptide **7** bearing sTyr residues at positions 10 and 14 may occur *via* an indirect structural effect.

Conclusion

In summary, we have developed the first solid-phase sulfation strategy to allow for the divergent and site-selective incorporation of sTyr at three possible sulfation sites in a target peptide. By incorporating three orthogonally-protected Tyr residues into a single resin-bound peptide, we were able to access a library of eight sulfoforms of an N-terminal fragment of the HIV-1 coreceptor CCR5 (bearing differential sulfation at Tyr-10, -14 and -15). This was achieved through iterative solid-phase deprotection(s), followed by solid-phase sulfation. This technique enabled the rapid and high-yielding construction of CCR5(2-22) variants **2**-**9**. These distinct sulfoforms were subsequently utilized in the first comprehensive interrogation of the ability of CCR5(2-22) variants bearing discrete Tyr sulfate modifications at positions 10, 14 and 15, to bind gp120 and mediate HIV-1 entry into host cells. A number of sulfated CCR5 variants, particularly those bearing a sTyr residue at position 14, were shown to bind gp120 and enhance viral entry into cells expressing a truncated CCR5 coreceptor that lacks the crucial N-terminus. The results of these studies provide important insight into the functional role of site-specific Tyr sulfation and should prove useful in the development of CCR5 mimetics and antagonists as novel anti-viral therapies. In addition, the generality of the solid-phase synthetic methodology should facilitate the construction of other sulfopeptide and sulfoprotein libraries to interrogate the importance of Tyr sulfate modifications in a number of diverse systems, including other chemokine receptors, decoy receptors and complement proteins,[52] in the future.

Methods

Synthesis of N-terminal CCR5 peptides

General Fmoc-SPPS:

***Resin loading*:** Rink amide resin (100-200 mesh) (470 mg, 0.25 mmol, 1.0 equiv.) was allowed to swell in DMF (5 mL) for 10 min. The resin was drained and the Fmoc group was removed with a 20% piperidine solution in DMF (2 x 5 mL) for 3 min. The resin was then rinsed with DMF (5 x 5 mL), CH_2Cl_2 (5 x 5 mL) and DMF (5 x 5 mL), and treated with a solution of Fmoc-protected amino acid (0.13 mmol, 0.5 equiv.), PyBOP (68 mg, 0.13 mmol, 0.5 equiv.) and NMM (0.03 mL, 0.25 mmol, 1.0 equiv.) in DMF (2 mL) and the resulting mixture was shaken for 4 h. The resin was then drained and washed with DMF (5 x 5 mL). A solution of acetic anhydride/pyridine (5 mL, 1:9 v/v) was added to the resin and agitated for 5 min. The resin was drained and washed with DMF (5 x 5 mL), CH_2Cl_2 (5 x 5 mL) and DMF (5 x 5 mL).

***Fmoc deprotection*:** A 20% solution of piperidine in DMF (5 mL) was added to the resin and shaken for 3 min. The resin was drained and the step was repeated once before rinsing the resin with DMF (5 x 5 mL), CH_2Cl_2 (5 x 5 mL) and DMF (5 x 5 mL).

***Amino acid coupling*:** A solution of Fmoc-protected amino acid (1.0 mmol, 4.0 equiv.), PyBOP (520 mg, 1 mmol, 4.0 equiv.), and NMM (0.22 ml, 2 mmol, 8.0 equiv.) in DMF (2.5 mL) was added to the resin and the resulting mixture was shaken for 1 h. The resin was then drained and washed with DMF (5 x 5 mL). For the coupling of *O*-allyl and *O*-TBS protected tyrosine (**11** and **12**, respectively), a solution of amino acid (0.38 mmol, 1.5 equiv.), HATU (140 mg, 0.38 mmol 1.5 equiv.) and DIPEA (0.13 mL, 0.75 mmol, 3.0 equiv.) in DMF (4 mL) was added to the resin and the resulting mixture was shaken for 16 h. For the coupling of *o*-nitrobenzyl-protected tyrosine (**10**), a solution **10** (0.50 mmol, 2.0 equiv.), PyBOP (260 mg, 0.50 mmol, 2.0 equiv.), HOBt (65 mg, 0.50 mmol, 2.0 equiv.) and NMM (0.11 mL, 1.0 mmol, 4.0 equiv.) in DMF (5 mL) was added to the resin and the resulting mixture was shaken for 16 h. The resin was subsequently washed with DMF (5 x 5 mL), CH_2Cl_2 (5 x 5 mL) and DMF (5 x 5 mL).

***Capping*:** A solution of acetic anhydride/pyridine (5 mL, 1:9 v/v) was added to the resin and agitated for 3 min. The resin was drained and rinsed with DMF (5 x 5 mL), CH_2Cl_2 (5 x 5 mL) and DMF (5 x 5 mL).

Orthogonal Deprotection Steps and Solid-Phase Sulfation:

Deprotection A. *o-nitrobenzyl removal*: A solution of resin-bound peptide (25 μmol) in DMF (20 mL) was irradiated with UV light (λ = 365 nm, UV reactor) for 24 h. The resin was then drained and washed with DMF (5 x 2 mL), CH_2Cl_2 (5 x 2 mL) and DMF (5 x 2 mL).

Deprotection B. *Allyl ether deprotection:* To the resin-bound peptide (25 μmol) was added a solution of tetrakis(triphenylphosphine)palladium(0) (29 mg, 25 μmol, 1.0 equiv.), triethylsilane (64 mL, 0.4 mmol, 16.0 equiv.) and acetic acid (AcOH, 23 mL, 0.4 mmol, 16.0 equiv.) in CH_2Cl_2 (2.0 mL) and the resulting mixture was agitated for 1 h. The resin was then drained and washed with CH_2Cl_2 (10 x 2 mL), DMF (10 x 2 mL) and CH_2Cl_2 (10 x 2 mL). The procedure was repeated once.

Deprotection C. *TBS ether deprotection:* A 1 M solution of tetrabutylammonium fluoride (TBAF) (0.4 mmol, 16.0 equiv.) in THF (0.4 mL) was placed under vacuum for 1 h to remove the solvent and the resulting white solid was reconstituted in CH_2Cl_2 (2.0 mL) containing AcOH (23 mL, 0.4 mmol, 16.0 equiv.). The resulting solution was added to the resin-bound peptide (25 μmol, 1.0 equiv.) and agitated for 3 h. The resin was drained and washed with CH_2Cl_2 (5 x 2 mL), DMF (5 x 2 mL) and CH_2Cl_2 (10 x 2 mL).

Solid-phase Sulfation with TCE Imidazolium Salt 1: A resin-bound peptide (25 μmol) with deprotected tyrosine(s) was allowed to swell in DMF (5 mL) for 20 min. The resin was drained and a solution of imidazolium-sulfating reagent **1** (8 equiv. per free phenol), Et_3N (8 equiv. per free phenol) and CH_2Cl_2 (4 mL) was added and agitated for 16 h. The resin was then drained and washed with DMF (5 x 2 mL), CH_2Cl_2 (5 x 2 mL) and DMF (5 x 2 mL). The above procedure was repeated again.

Peptide cleavage from the resin: To the resin-bound peptide was added a cleavage cocktail of TFA/triisopropylsilane (TIS)/water (90:5:5 v/v/v, 3 mL) or TFA/thioanisole/tri-isopropylsilane/water (85:5:5:5 v/v/v/v, 3 mL) and the resulting mixture was agitated for 2 h.

The resin was then drained and washed with the cocktail solution (3 x 3 mL). The combined cleavage solution and washes were evaporated under reduced pressure. The crude mixture was resuspended in ice cold Et_2O and centrifuged. The supernatant was carefully removed and the crude peptide solid was dried under vacuum overnight.

Deprotection of trichloroethyl (TCE)-protected sulfopeptide: Deprotection of trichloroethyl-protected sulfopeptides was performed according to the procedure described by Ali *et al*.[43] To a solution of the crude trichloroethyl-protected sulfopeptide (25 μmol) in water/MeOH (1:1, v/v, 8 mL) was added Et_3N (1.88 mmol, 260 μL) and $Pd(OH)_2$ (20 wt% on carbon, 5 mg) and the mixture was stirred under a hydrogen atmosphere for 4 h. The reaction mixture was filtered through a pad of reverse-phase silica and washed with methanol (5 x 5 mL). The combined filtrates were evaporated under reduced pressure and the crude material was purified using preparative reverse-phase HPLC (see Supporting Information). Lyophilization of the appropriate fractions three times (to remove excess NH_4OAc) afforded the desired sulfopeptides **2-9**.

Production of recombinant soluble CD4 (rsCD4):

The CD4deltaTM plasmid expresses recombinant soluble human CD4 lacking the transmembrane domain but which retains the cytoplasmic domain that facilitates solid-phase binding assays (rsCD4, aa 1-395, 421-458). CD4deltaTM was produced by splice overlap extension PCR from the full-length clone of CD4, T4pMV7 (NIH, Bethesda, DC), removing the region encoding the TM domain of CD4. Two intermediate clones were produced. The first clone encoding the CD4 extracellular domain was generated using the **RL1**: *CGG GAA TTC ACA ATG GAC CGG GGA GTC CC* and **RL3**: *G CCT TCG GTG CCG GCA CCT CTG CAC CGG GGT GGA CC* primer pair. The second clone encoding the CD4 cytoplasmic tail with a 6x His tag at the C-terminus was generated using the **RL4:** *GG TCC ACC CCG GTG CAG AGG TGC CGG CAC CGA AGG C* and **RL5***: GGG CTC GAG TCA ATG ATG ATG ATG ATG ATG AGA ACC ACC AAT GGG GCT ACA TGT CTT CTG AA* primer pair. Each clone was created with an overlapping region. The two clones were combined using PCR with the outermost 5' and 3' primers, RL1 and RL5. The construct was then subcloned into the eukaryotic expression vector pcDNA 3.1 Zeocin (Invitrogen, San Diego, CA). CD4deltaTM clones were confirmed by DNA sequencing.

To produce rsCD4, HEK 293T cells were transfected with 70 μg CD4deltaTM plasmid using Lipofectamine 2000 (Invitrogen, San Diego, CA) according to the manufacturer's protocol. Cell culture supernatants containing sCD4 were collected after 48 to 72 h and clarified by centrifugation to remove cellular debris.

Competitive binding assay between peptides 2-9 and anti-gp120 monoclonal antibody 17b:

All incubation steps were carried out using 50 μL solutions for 2 h at room temperature, except where stated otherwise. Every step was followed by four washes with 0.05% (v/v) PBS/Tween (pH 7.4). Microtiter plates (Maxisorp; Nunc) were coated overnight at 4 °C with soluble CD4 diluted in coating buffer (18.2 mM Na_2CO_3, 45 mM $NaHCO_3$, pH 9.6). The wells were blocked for 2 h with 230 μL 3% (w/v) skim milk in PBS (blocking solution). HIV-1_{BaL} gp120, recombinant was then diluted in PBS/Tween 0.05% at 1 μg/mL. Dilutions of synthetic CCR5 sulfopeptides were added to the plates and incubated for 1 h. 17b antibody (SDIX) was then added in the presence of sulfopeptides at a final concentration of 15 nM for 30 min. After washing, plates were incubated with sheep anti-human/HRP 1:2000 in PBS/Tween 0.05% solution. Control samples of CD4 bound alone were detected with mouse anti-CD4 RPA-T4 (Sigma) at 1:1000 and anti-mouse/HRP at 1:2000. Bound enzyme was quantified using the 3,3′5,5′-tetramethylbenzidine/H_2O_2 colorimetric assay (Sigma), which was quenched with 0.5 M H_2SO_4, and A_{450} values were measured. The A_{450} values were corrected for the background absorbance of substrate alone. Data points were analyzed using GraphPad Prism Version 5.0, using a single-site binding equation: $Y = Bmax*X/(K_D + X)$. This equation describes the binding of ligand to a receptor that follows the law of mass action. Bmax represents the maximal binding, and K_D is the concentration of ligand required to reach one-half maximal binding.

Complementation assays:

The production and titration of luciferase reporter viruses, and infection of cells expressing CCR5 mutants have been described previously.[21] Briefly, U87-CD4 cells were transfected with 4 μg of pcDNA3-CCR5 (Wild type-WT) or CCR5 (Δ2-17) using lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. The WT CCR5 plasmid was serially diluted (2-fold) to create populations of cells expressing a range of CCR5. These cells were

used to create a standard curve of CCR5 expression, to which the expression of CCR5 (Δ2-17) could be matched. Expression of CCR5 was determined by flow cytometry 48 h post transfection with the CCR5 specific antibody 2D7 (BD Pharmingen). 48 h post transfection, the cells were either left untreated (WT and Δ2-17) or incubated for 30 min with 100 μM of CCR5 sulfopeptides (Δ2-17 only) prior to infection with 200 $TCID_{50}$ of YU-2 Env pseudotyped luciferase reporter virus. At 72 h post infection, the cells were lysed and luciferase activity was read according to the manufacturer's protocol (Promega). The luciferase activity in CCR5 Δ2-17 cells incubated with CCR5 sulfopeptides was expressed as a percentage of that obtained in cells expressing an equivalent amount of WT CCR5.

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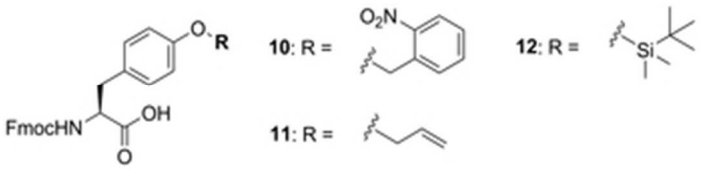

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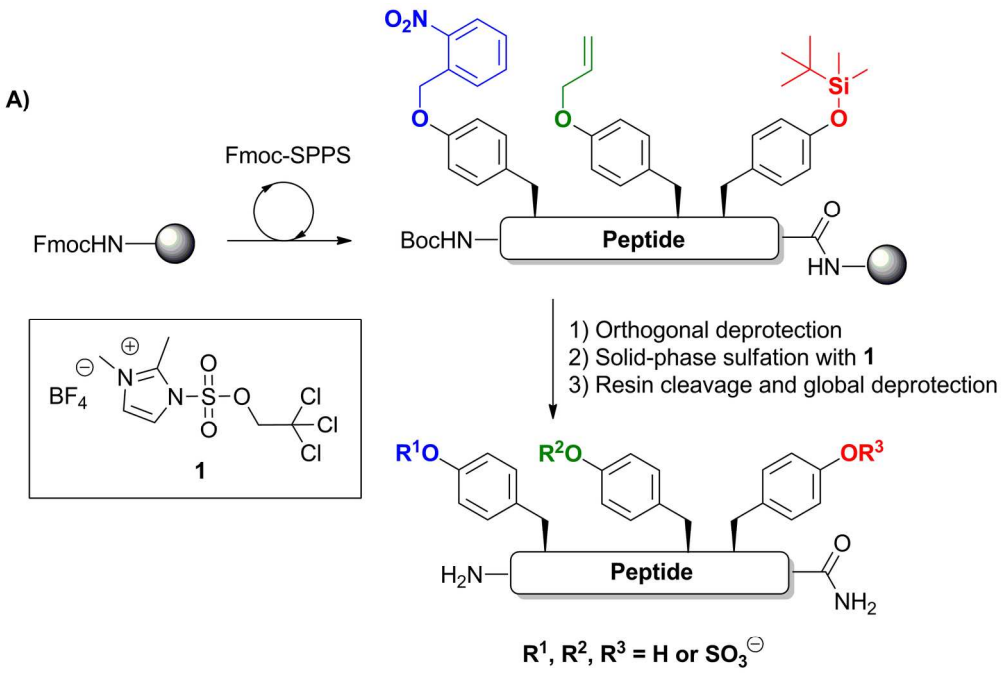

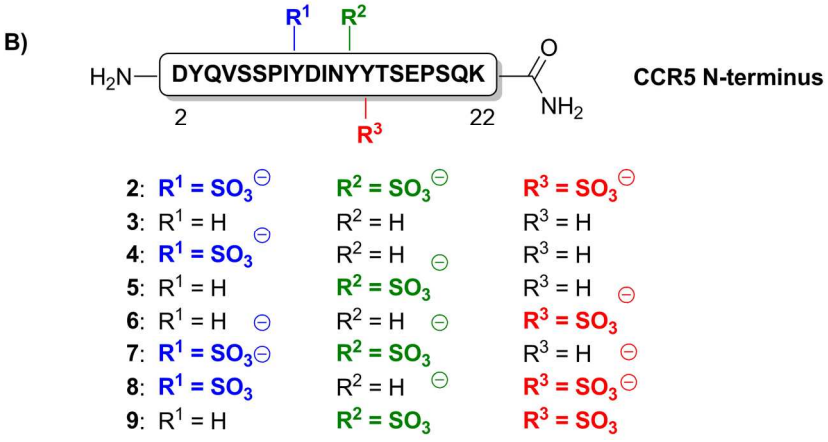

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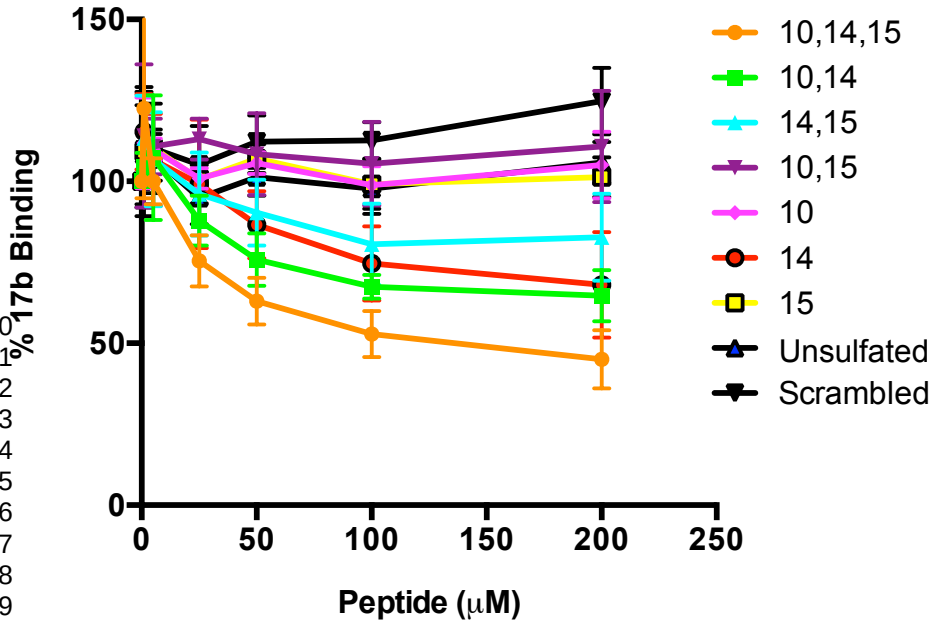

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