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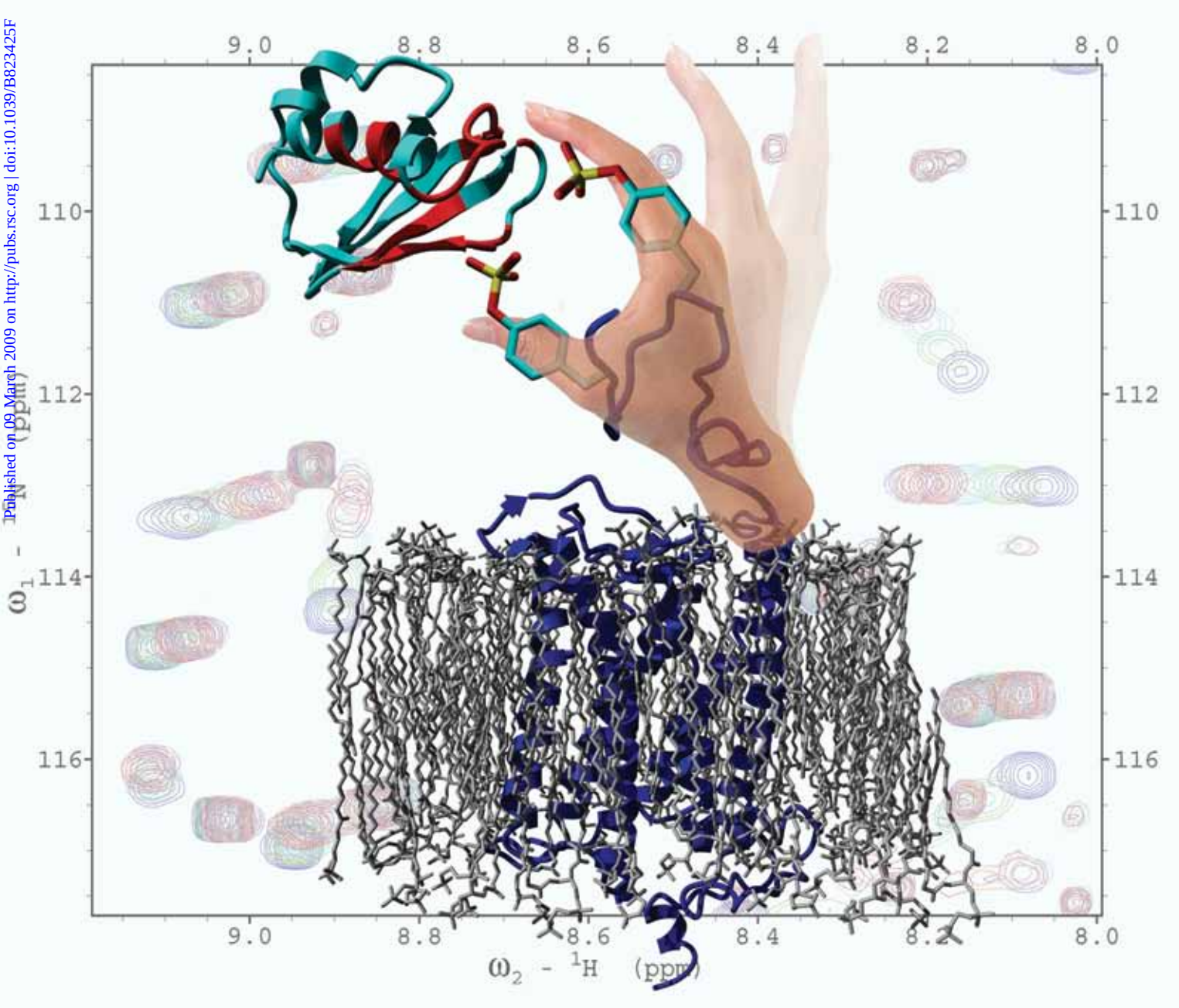

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A general sequence independent solid phase method for the site specific synthesis of multiple sulfated-tyrosine containing peptides†

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In this communication, a new site specific synthesis of highly functionalized and multiple sulfated peptides using convential Fmoc-tBu solid phase peptide synthesis is described.

Although much less known than protein *phosphorylation*, the significance of protein *sulfation* is rapidly gaining momentum.[1]

The importance of sulfation of for example GPCR proteins as a key modulator of extracellular protein–protein interactions requires the availability of methods for the preparation of crucial sulfated peptide parts. However, so far no reliable general sequence independent method is available for the site specific incorporation of especially multiple sulfated tyrosines.[2]

Clearly, the absence of these methods reflects the relative instability of sulfated tyrosine residues as compared to phosphorylated tyrosine residues in peptides and proteins.[3]

In order to circumvent studies of ligands with the entire and complex GPCR-C5a receptor, we needed the sulfated N-terminal peptides as representative mimics of part of this receptor, which contains two sulfated tyrosine residues.

However, upon evaluation of the relatively scarce literature on the synthesis of multiple sulfated peptides,[4] we concluded that (i) a solid phase synthesis according to the Boc-strategy is unfit in view of the acid lability of sulfated tyrosine residues, (ii) evidently, global sulfation using *e.g.* DMF–SO_3 does not lead to sulfation of specific tyrosine residues when more of these are present, (iii) thus far TFA stable sulfated tyrosine building blocks for use in solid phase peptide synthesis (SPPS) are unavailable and (iv) no general and convenient sequence independent SPPS strategy is available for the synthesis of highly functionalized and multiple sulfated peptides using conventional Fmoc-tBu SPPS.[5]

Therefore, we describe in this communication a general sequence independent solid phase method by which a peptide is synthesized according to the Fmoc-tBu-strategy, followed by selective deprotection of the tyrosine residues to be sulfated and introduction of a protected sulfate group. Upon completion of the synthesis of the sulfated peptide, it is cleaved from the resin by acidolysis and protection groups are removed *with the exception of the sulfate protection group* thereby preventing undesired acid induced removal of the sulfate group(s) during this step.

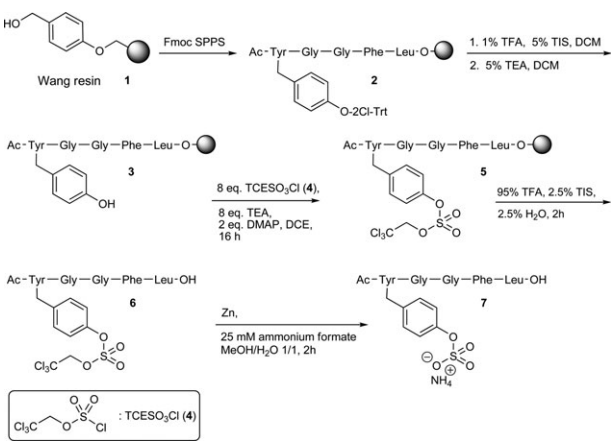
Finally, the sulfate protecting groups were removed in a slightly acidic (pH 6.4) reductive step, leaving the sulfate groups untouched.

Since sulfation is not limited to proteins, but also occurs on bioactive peptides such as neuropeptides, for evaluation of this protocol, we first attempted to synthesize sulfated-Leu-enkephaline **7** (Scheme 1).[6]

In this protocol the tyrosine phenolic OH in **2** was protected with a 2-Cl-Trt-group, which, after liberation with a low percentage of TFA, was sulfated with 2,2,2-trichloroethyl chlorosulfate leading to **5**.[7] After treatment with 95% TFA, Leu-enkephaline **6** containing the protected sulfate moiety was cleaved from the resin. These harsh acidic conditions do not affect the 2,2,2-trichloroethyl sulfate ester,[8] which was successfully removed by a reductive beta-elimination reaction using zinc-dust.[7,9] The sulfated Leu-enkephaline **7** was obtained pure, in an excellent yield of 63% (after HPLC purification).

Encouraged by these results, we embarked on our actual target,[10] that is, synthesis of the three possible sulfated variants of the N-terminal peptide part of the C5a receptor ($C5aR_{7\text{-}28}$), which contains two tyrosine residues.[11]

Thus, the above protocol was used for this significantly more complex peptide, containing more than 70% (16 out of 22) of amino acid residues with a functional side chain, requiring protection. Standard protection groups were used for the side chains, which were all compatible with the strategy



Scheme 1 Solid phase synthesis of sulfated-Leu-enkephalin **7**.

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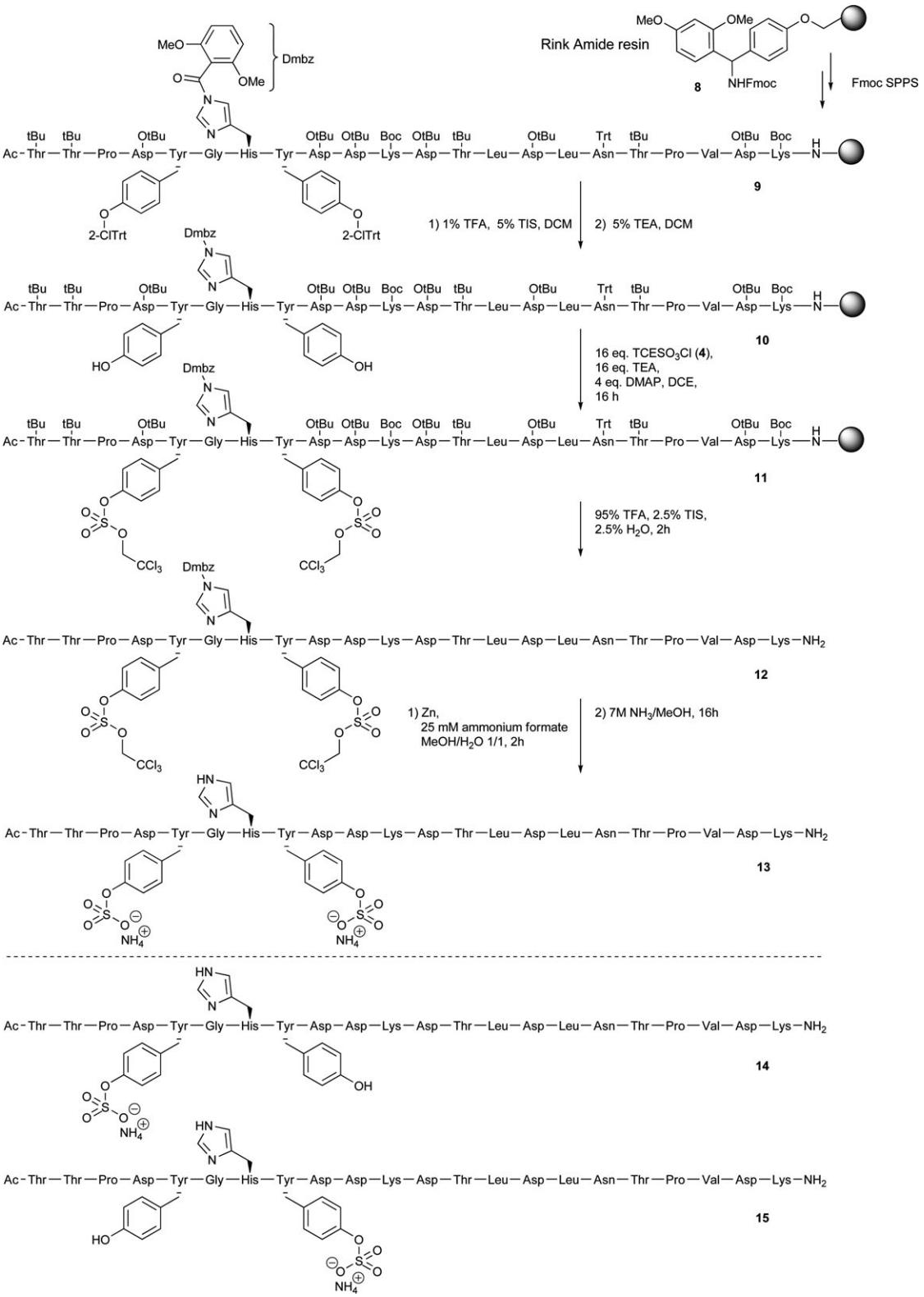
exemplified by the synthesis of sulfated Leu-enkephaline **7** (Scheme 1), with the exception of the Trt-group. Unexpectedly, the Trt-group was removed in part from histidine residue **13** during the selective deprotection of the tyrosine residue(s) to be sulfated.[12] After treatment with the sulfation agent, followed by deprotection of all other side chains, concomitant cleavage from the resin and reductive cleavage of the sulfate ester, this led to undesired sulfation (30–50%) of the histidine residue.

The only way to prevent this side product was to use a histidine residue with another protective group, which should



Scheme 2 Solid phase synthesis of a disulfated C5aR$_{7\text{-}28}$ mimic **13**.

Table 1 Binding of C5aR peptides to CHIPS

Peptide	Position of sulfation	K_d/nM
16: $C5aR_{7\text{-}28}$	—	3200 ± 100
14: $C5aR_{7\text{-}28}(SO_3^-NH_4^+)$	11	460 ± 30
15: $C5aR_{7\text{-}28}(SO_3^-NH_4^+)$	14	37 ± 5.5
13: $C5aR_{7\text{-}28}(SO_3^-NH_4^+)_2$	11 + 14	8.4 ± 1.1

not be acid labile in the stage that the tyrosine residues were selectively deprotected by acid, but also not be acid labile once the sulfate moiety has been introduced and deprotected, since acidolysis in the latter step will also lead to desulfation. Fortuitously, the base-labile dimethoxy benzoyl (Dmbz) group described by Zaramella *et al.* turned out to be a suitable replacement for the Trt-group.[13] This group can be removed by 7 M ammonia in methanol and it was found that these conditions do not affect the sulfate group on a tyrosine residue (see ESI†).

The final stage was now reached for the successful synthesis of three highly functionalized sulfated peptides (Scheme 2 and Table 1). Instead of Fmoc-His(Trt)-OH, now Fmoc-His(Dmbz)-OH was used. After solid phase peptide synthesis for the preparation of **9**, selective deprotection of the tyrosine residues leading to **10**, sulfation affording **11**, deprotection—with the exception of the sulfated tyrosine residues and the histidine residue—and cleavage from the resin to give **12** and removal first of the sulfate 2,2,2-trichloroethyl ester and then of the Dmbz group led to the successful completion of the sulfated peptide **13**. When the same protocol was used and also a tyrosine residue with the standard tBu protecting group was incorporated, site specific sulfation was achieved by selective removal of the 2Cl-Trt-group in the presence of this tBu-group. In this way site specific synthesis of sulfated tyrosine peptides **14** and **15** was realized in addition to the multiple sulfated tyrosine peptide **13** (*vide supra*).

The resulting sulfated peptides were extensively characterized by both NMR spectroscopy and mass spectrometry. In addition, in order to show that these sulfated peptides were perfect mimics of the N-terminal part of the C5a receptor, we carried out isothermal calorimetry (ITC) studies to determine their binding ability to the immune evasive protein CHIPS, which binds very strongly to the complete, sulfated, C5a receptor.[10] It was found that the disulfated C5a receptor mimic **13** bound CHIPS with an affinity that is comparable to that of the C5a receptor itself (Table 1). Upon evaluation of the two mono sulfated C5a receptor mimics **14** and **15** as well as the non-sulfated C5a N-terminal receptor mimic **16**, it was concluded that sulfation is essential for tight binding of a ligand, since the non-sulfated receptor mimic bound CHIPS much less strongly. This underlines the importance of sulfation as a post-translational modification similar to the significance of the phosphorylation post-translational modification. Furthermore, selective sulfation is a means of tuning the binding, since there was a clear difference in binding affinity of mono sulfated peptide **14** and mono sulfated peptide **15**.

To summarize, we present here the first general method for the synthesis of sulfated peptides. This method allows the introduction of multiple sulfate groups in highly functional complex peptides as was illustrated by the synthesis of disulfated C5a receptor mimic **13**. In addition, site specific sulfation was possible as was shown by the preparation of sulfated peptides **14** and **15** having sulfate groups at different positions. The unexpected hurdle of premature cleavage of the commonly used histidine protective group was taken by changing to another convenient protection of the histidine side chain.

In our opinion this method will contribute to open up research to study the importance of the sulfation post-translational modification, which in terms of the functional group appears to be related to phosphorylation, so that one might denote it as an *isosteric version* of the *phosphorylation* post-translational modification, albeit so far much less known and investigated. Finally, we are confident that the presented method is also amenable for the site specific sequence independent synthesis of sulfated serine and threonine containing peptides, which will be reported in due course.

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