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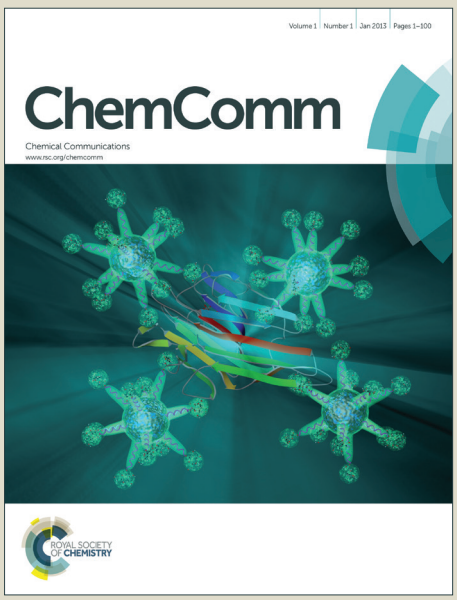

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Examination of mercaptobenzyl sulfonates as catalysts for native chemical ligation: Application to the assembly of a glycosylated Glucagon-Like Peptide 1 (GLP-1) analogue

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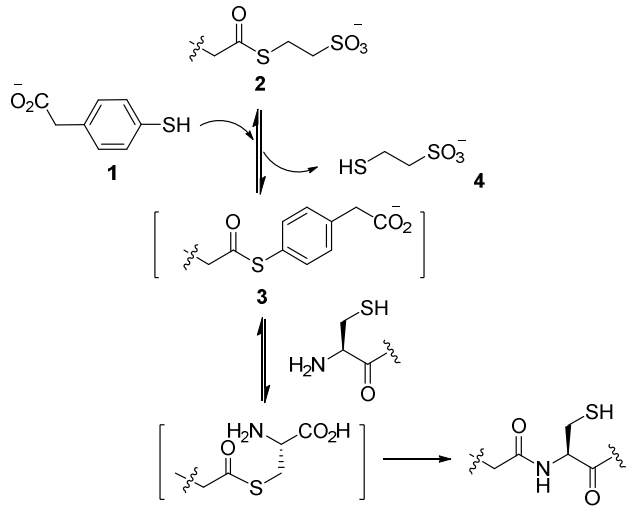
Ben Cowper[a], Tsz Mei Sze[a], Bhavesh Premdjee[a], Aileen F. Bongat White[b], Andrew Hacking[b], and Derek Macmillan[a]*

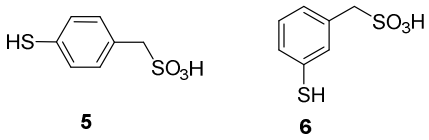
3/4-mercaptobenzyl sulfonates were investigated as aryl thiol catalysts for native chemical ligation (NCL). Whilst catalysing NCL processes at a similar rate to 4-mercaptophenyl acetic acid (MPAA), the increased polarity and solubility of 3-mercaptobenzyl sulfonate in particular may favour its selection as NCL catalyst in many instances.

Native chemical ligation (NCL), where peptide thioesters and cysteinyl peptides combine to form native peptide linkages,[1, 2] has benefited significantly from the use of 4-mercaptophenyl acetic acid (MPAA) as catalyst.[3] MPAA (**1**, scheme 1) is relatively non-malodorous, has good water solubility under most commonly employed NCL reaction conditions, and catalyses NCL through formation of a reactive aryl thioester intermediate (**3**).[4, 5]



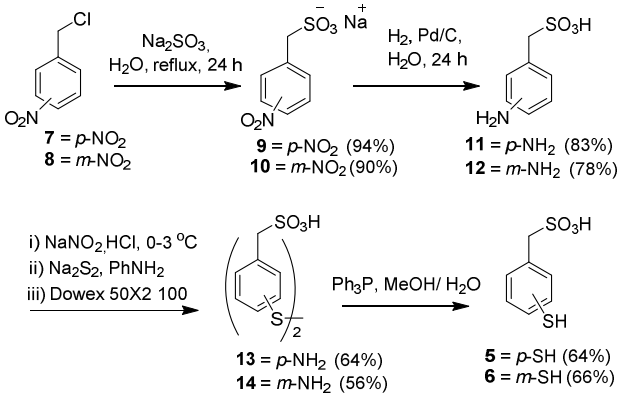
Scheme 1. *In-situ* thiol-thioester exchange during NCL converts the 2-mercaptoethanesulfonate (MESNa) thioester **2** to the highly reactive MPAA thioester **3**.

These advantageous properties have resulted in the near ubiquitous adoption of MPAA in NCL reactions**.** However, MPAA is rather hydrophobic, has poor solubility below pH 7, and it can often co-elute with NCL products during HPLC purification.[6] In contrast sodium 2-mercaptoethane sulfonate (MESNa, **4**), whilst less reactive, is significantly more polar and elutes with buffer components. Consequently, in the absence of established MPAA alternatives, we examined 4-mercaptobenzyl sulfonate (4-MBSA, **5**) and 3-mercaptobenzyl sulfonate (3-MBSA, **6**) as catalysts (scheme 2).[7] Ideally, they would possess the desirable polarity profile of MESNa yet, as aryl thiols, exhibit similar potential for catalysis to MPAA.



Scheme 2. MPAA analogues investigated as NCL catalysts.[7]

5 and **6** were readily prepared on gram scale from the corresponding nitrobenzyl chlorides (**7**/**8**) in four steps (Scheme 3)[8, 9] Notably, throughout the synthesis no flash column chromatography was required.



Scheme 3. Synthesis of mercaptobenzyl sulfonates **5** and **6**.

With **5** and **6** in hand we were pleased to observe that they both eluted 10 minutes earlier than MPAA on a C_{18} analytical reverse-phase column[10] and we next examined application in NCL. To compare their performance as catalysts model ligations were conducted using H-LYRAG-$SCH_2CH_2SO_3Na$ (**15**) and H-CRAFS-OH (**16**) over a pH6-pH8 range (Figure 1).[11] The thioester component was conveniently prepared from H–LYRAGC-OH (**17**) in near quantitative yield via *N*→*S* acyl shift.[12-17] NCL progress was then monitored by analytical reverse-phase HPLC and confirmed by LC-MS.

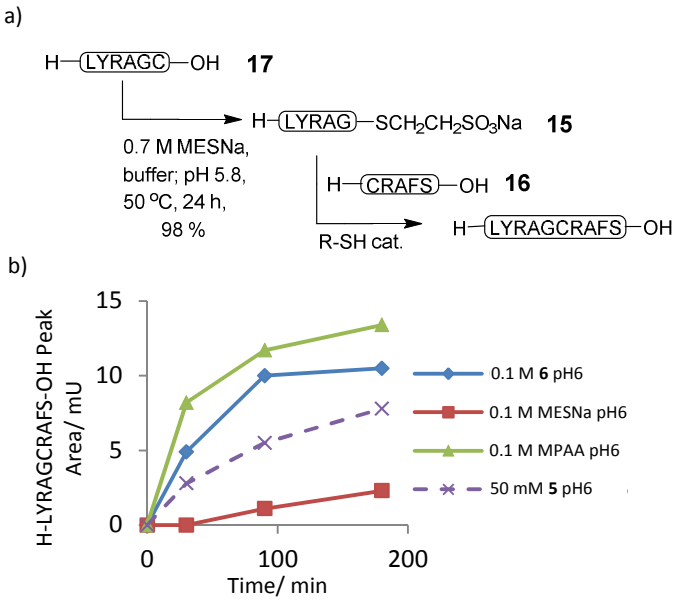
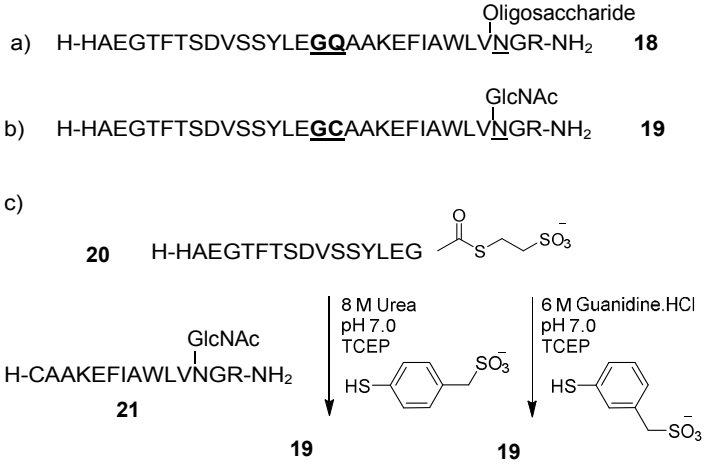


Figure 1. a) Preparation of LYRAG thioester **15** and reaction with **16** in NCL reactions in the presence of thiol catalyst. b) **5** and **6** are compared with MPAA as NCL catalysts at pH 6.[18]

At this stage the anticipated instability of 4-MBSA (**5**) became evident.[19] Whilst preparing 0.25 M aqueous stock solutions of 4-MBSA for immediate use at 50 mM final concentration in NCL reactions allowed us to evaluate it as a catalyst, at higher concentrations 4-MBSA rapidly precipitated and/or polymerised. Consequently no reliable kinetic data could be obtained for the **5**-mediated ligation between **15** and **16** at typical working concentrations (0.1-0.2 M) at pH 6 or above.

Interestingly **5** also precipitated from all solutions prepared in 6 M guanidine hydrochloride, a common NCL component yet did not precipitate when identical solutions were prepared in 8 M urea, allowing 4-MBSA mediated ligation reactions between peptides of low solubility to take place. We took advantage of this to assemble a glycosylated Glucagon-Like Peptide-1 (GLP-1) analogue (Scheme 4). GLP-1 (residues 7-36) has gained attention as a therapeutic for the treatment of type 2 diabetes and affects glucose control by stimulating insulin secretion. Although GLP-1 is not naturally glycosylated, a recent study showed that the addition of carbohydrate moieties at N34 (**18**, Scheme 4a) prolonged the half-life of the peptide in vivo, thus increasing its potential for therapeutic applications.[20] We envisaged that GLP-1 analogues of **18** could be reached *via* simply glycosylated **19** (Scheme 4b). Following straightforward production of NCL components **20** and **21** we successfully assembled **19** in 8 M Urea using 50 mM 4-MBSA as catalyst (Scheme 4c). In this case we observed similar catalytic performance to 50 mM MPAA.[21] However, the poor stability of **5** at neutral pH led us to focus on 3-MBSA (**6**) since 1 M solutions were fully soluble at pH7 and compatible with 6 M guanidine hydrochloride (Figure 2).



Scheme 4 a) Sequence of glycosylated GLP-1(7-36) **18**. b) Simply glycosylated analogue **19**. c) Preparation of **19** from thioester **20** and cysteinyl glycopeptide **21** employing 50 mM **5** or 0.1 M **6**.

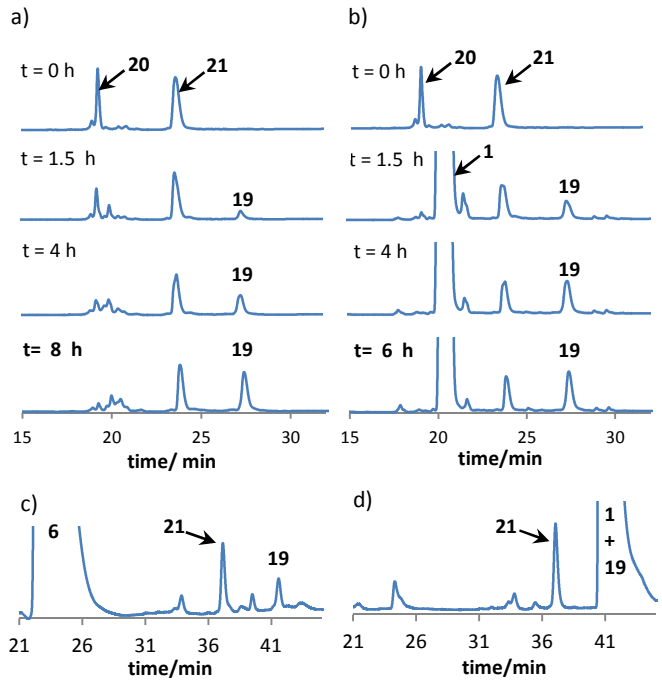
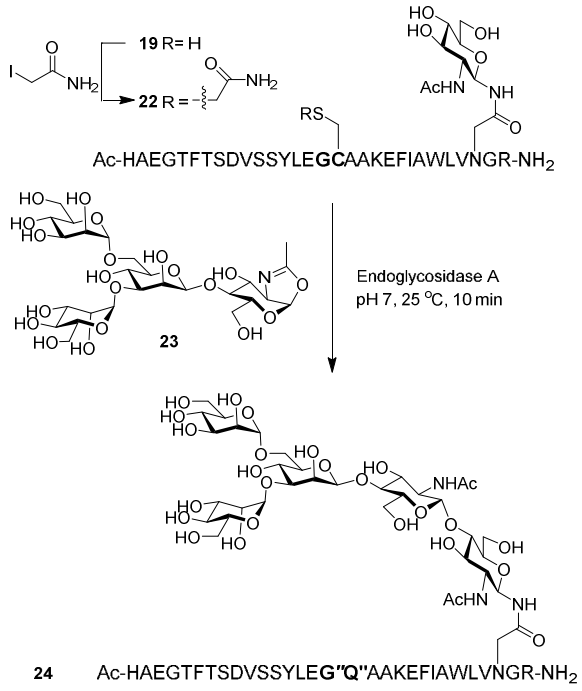


Figure 2. Analytical HPLC analysis of a) 3-MBSA and b) MPAA catalysed ligation between **20** and excess **21** (0.82 mM **20**, 1.4 mM **21**, 0.1 M catalyst, 6 M guanidine hydrochloride, 0.1 M Na phosphate buffer; pH 7, 35 mM TCEP). c-d) Comparison of semi-preparative HPLC purification for c) the 3-MBSA catalysed reaction and d) the MPAA catalysed reaction.

The 0.1 M 3-MBSA catalysed reaction was successful but appeared to proceed significantly more slowly than the corresponding MPAA catalysed process (Figure 2a and 2b), requiring an extra 2 h reaction time to reach a similar level of conversion. It is clear from Figure 2a that a more significant

quantity of the initial MESNa thioester (**20**) is still present in the 3-MBSA catalysed process after 1.5 h indicating that the thiol-thioester exchange is slower for 3-MBSA than for MPAA (Figure 2b). It is likely that the 3-MBSA catalysed reaction can be accelerated further by employing the higher concentrations (0.2 M) that are optimal for MPAA catalysis, and are more comparable with employing the pre-formed thioester.[3] Despite the slightly slower reaction rate when using 3-MBSA, its enhanced polarity enabled straightforward purification of the ligation product whereas MPAA co-eluted with it.

After isolation of **19** the Gln→Cys mutation at the Gly-Cys ligation site was simply carboxamidomethylated to restore a pseudo-glutamine ("Q") residue at this position (scheme 5). The *N*-acetyl glucosamine unit was finally extended to the native N-glycoprotein pentasaccharide core structure upon exposure to oligosaccharyl oxazoline (**22**) in the presence of Endoglycosidase A.[22, 23]



Scheme 5. Thiol capping and Endoglycosidase A mediated elaboration of **19**.

Conclusions

Overall, the results demonstrate that compounds based on the mercaptobenzyl sulfonate scaffold can accelerate NCL reactions at a comparable rate to MPAA. 3-MBSA (**6**) served as the more stable catalyst and, owing to the increased polarity conferred by the sulfonate group, facilitated straightforward ligation of two GLP-1 fragments, and isolation of the product. MPAA can be significantly removed from ligation reaction upon acidification and repeated extraction of the reaction mixture,[24,25] or by using MPAA hydrazide analogues which can be captured on suitably functionalised solid supports.[26, 27] However it is hoped that the additional flexibility provided by 3-MBSA may allow purification of peptide products without these additional handling steps. Furthermore the high solubility of these aryl thiols, and further analogues such as mercaptobenzyl phosphonates, at low pH may additionally find application in processes relevant to *N*→*S* acyl transfer and peptide transamidation reactions where MPAA has already been employed advantageously, despite its low solubility under these conditions.[17, 28]

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[a]Department of Chemistry, University College London, 20 Gordon Street, London, WC1H 0AJ, UK.
[b]Dextra Laboratories Ltd., Science and Technology Centre, Earley Gate, Whiteknights Road, Reading, RG6 6BZ, UK.

Electronic Supplementary Information (ESI) available: experimental procedures for the synthesis of **5** and **6**. See DOI: 10.1039/c000000x/

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