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Solid-Phase Synthesis of Peptide and Glycopeptide Thioesters through Side-Chain-Anchoring Strategies

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Abstract: An efficient new strategy for the synthesis of peptide and glycopeptide thioesters is described. The method relies on the side-chain immobilization of a variety of Fmoc-amino acids, protected at their C-termini, on solid supports. Once anchored, peptides were constructed using solid-phase peptide synthesis according to the Fmoc protocol. After unmasking the C-terminal carboxylate, either thiols or amino acid thioesters were coupled to afford, after cleavage, peptide and glycopeptide thioesters in high yields. Using this method a significant proportion of the proteinogenic amino acids could be incorporated as C-terminal amino acid residues, therefore providing access to a large number of potential targets that can serve as acyl donors in subsequent ligation reactions. The utility of this methodology was exemplified in the synthesis of a 28 amino acid glycopeptide thioester, which was further elaborated to an N-terminal fragment of the glycoprotein erythropoietin (EPO) by native chemical ligation.

Keywords: glycopeptides · glycoproteins · ligation · peptide thioesters · solid-phase synthesis

Introduction

More than 50% of all human proteins are glycosylated, a modification known to influence protein folding, solubility and biological half-life.[1,2] Glycosylation is implicated in a variety of biological recognition events such as cell adhesion, cell differentiation, and cell growth.[3–5] Aberrant glycosylation of proteins is known to perturb intracellular recognition, and is associated with several serious illnesses including autoimmune diseases, infectious diseases and cancer.[5] Study of these effects requires pure samples of the relevant glycoproteins, but in nature they are typically expressed as heterogeneous mixtures of glycoforms in which identical protein backbones bear a variety of different glycans. This has greatly impeded the field of glycobiology due to the difficulty of elucidating the exact role of a specific glycan. Access to homogeneous glycoproteins is also important from a drug discovery perspective since many of these biomolecules represent potential therapeutic leads.[6,7] Generally, the isolation of single glycoforms from natural sources is not possible, and the current belief is that the high demand for homogeneous glycopeptides and glycoproteins can only be met by chemical intervention.[8–11] Of the methods available for the synthesis of glycoproteins, chemical ligation is amongst the most promising. Current technologies include native chemical ligation (NCL), sugar-assisted ligation (SAL) and the traceless Staudinger ligation.[12–20] All of these techniques rely on peptide or glycopeptide thioesters as acyl donors, and therefore expedient access to these entities is necessary if the ligation-based assembly of glycopeptides and glycoproteins is to be practical. Unglycosylated peptide thioesters are traditionally synthesized on solid support using the *tert*-butyloxycarbonyl (Boc)-strategy.[21,22] The major drawbacks of this method include the requirement for TFA during repetitive Boc-deprotection steps and the use of highly acidic conditions for example, triflic acid or HF for release of the fully assembled peptide from the resin. Unfortunately, glycopeptide thioesters cannot be synthesized in this way due to the inherent instability of glycosidic linkages to strongly acidic conditions.[23] These difficulties have led to the search for efficient and mild Fmoc-based strategies for their construction.[24] Hence, many research groups chose to synthesize protected glycopeptide thioesters containing a free C-terminus which can be derivatized to the thioester in solution.[25–27] Variants of this solution phase strategy include

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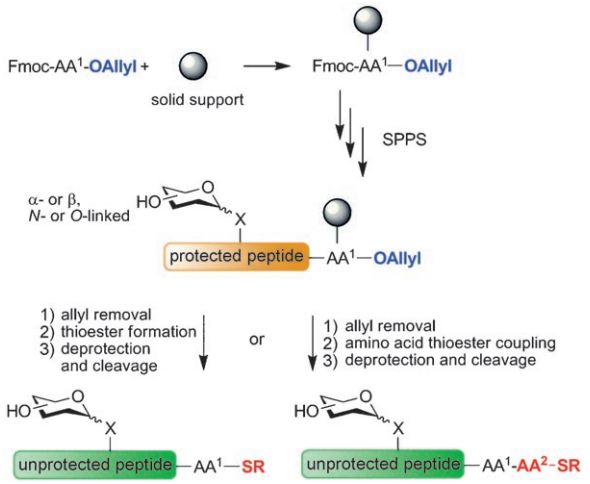
the synthesis of masked glycopeptide esters which can rearrange via O→S acyl shifts to generate the desired glycopeptide thioester.[28] However, the necessity to perform reactions in solution, in conjunction with the poor solubility characteristics of protected peptides, has hampered applications of such a strategy to longer sequences. Alternative approaches which may be amenable to the synthesis of glycopeptide thioesters include Fmoc removal using non-nucleophilic base cocktails, Lewis acid-catalyzed release, racemization-free photochemical approaches, and oxidation-based safety catch strategies.[29–36] The most common and widely used approach for obtaining glycopeptide thioesters involves synthesis on sulfamylbutyryl resin using an activation and thiol release strategy.[37–40] Based on this technique, a variant of the O-linked glycoprotein diptericin ε and a fragment of the N-linked glycoprotein RNase B have been successfully synthesized.[41,42] However, over-acylation and epimerization during resin loading are major shortcomings of this method, and the use of capping steps throughout the synthesis has been reported to reduce yields considerably.[42] In our hands, alkylation of the sulfonamide prior to thiol release also resulted in derivatization of the peptide backbone. The recent development of a self-purification feature of this method has allowed for the production of peptide thioesters in higher purity and with fewer truncated by-products, however, this variant eliminates the potential for sequential ligations since the N-terminus is tied up in the self-purification process.[43] Alternate strategies for the synthesis of peptide thioesters rely on attachment of the growing peptide to an appropriate handle through a backbone amide.[44,45] In one of these, the backbone amide linker (BAL) strategy, a protected C-terminal carboxyl group can be elongated with an amino acid thioester after the peptide has been fully assembled. However, currently only a limited set of amino acids have been shown to be amenable to backbone anchoring, and to the best of our knowledge this method has yet to be implemented in the synthesis of glycopeptide thioesters.

An efficient method for the Fmoc-based synthesis of peptide thioesters involves immobilization of the C-terminal amino acid residue through its side chain.[25,46–48] In this manner, Fmoc-Glu-OAllyl, Fmoc-Asp-OAllyl and Fmoc-Lys-OAllyl have been immobilized onto Wang- or Rink amide resin.[49] After the peptide is assembled on the resin, removal of the C-terminal allyl group, on-resin thioester formation and subsequent cleavage yields the corresponding glutamine, asparagine and lysine thioesters.[50] This method has recently been applied to the synthesis of a 36-amino acid glycopeptide thioester by our laboratory, which was subsequently ligated to a glycopeptide fragment using SAL to afford the antibacterial glycoprotein diptericin ε.[17] To date this approach has been restricted to the construction of peptide thioesters bearing a limited set of amino acids on the C-terminus. Herein we demonstrate that a much larger number of amino acids can serve as an anchoring point for the solid-phase peptide synthesis (SPPS) of peptide and glycopeptide thioesters on a range of resins. The scope of the method is significantly broadened by elongation of the immobilized peptides with a variety of amino acid thioesters in the N- to C-terminal direction (Scheme 1). As a demonstration of the applicability of the side chain anchoring strategy to the construction of more complex targets, a 28-amino acid glycopeptide thioester was successfully synthesized and ligated to form a fragment of the therapeutic glycoprotein human erythropoietin (EPO).



Scheme 1. General strategy for the solid-phase synthesis of peptide and glycopeptide thioesters using a side chain anchoring strategy (AA^1 = anchorable amino acid; AA^2 = any proteinogenic amino acid).

Results and Discussion

Our initial goal was to synthesize peptide thioesters by immobilizing the side chains of aspartic acid and glutamic acid to a solid support. To this end, commercially available Fmoc-Asp-OAllyl and Fmoc-Glu-OAllyl were loaded onto Rink amide resin using PyBOP and *N*-methylmorpholine in DMF for two hours.[49] Pre-loadings proved to be high yielding in all cases as determined by deprotection of the Fmoc group with 10% piperidine/DMF and measurement of the Fmoc-piperidine adduct at 302 nm. Peptide elongation according to the established Fmoc SPPS protocol, followed by the selective removal of the allyl ester using $[Pd(PPh_3)_4]$ and phenylsilane in dichloromethane, gave a free carboxylic acid at the C-terminus to which the thiol ethyl 3-mercaptopropionate could be directly coupled (Scheme 2A). Conditions for on-resin thioesterification have been described by Miranda and co-workers, who showed that DMF/CH_2Cl_2 solvent mixtures in combination with high DIC and HOBt concentrations (>0.5 M) and the use of excess thiol derivative (0.5–0.6 M) reduced C-terminal epimerization to ≈1%.[49] This protocol was therefore used to generate thioesters in our studies. Cleavage of the peptide thioester from the solid support by treatment with TFA/thioanisole/triisopropylsilane/water (85:5:5:5 by volume) proceeded with concomitant amino acid side chain deprotection and gave the desired C-terminal glutamine and asparagine peptide thioesters in 96

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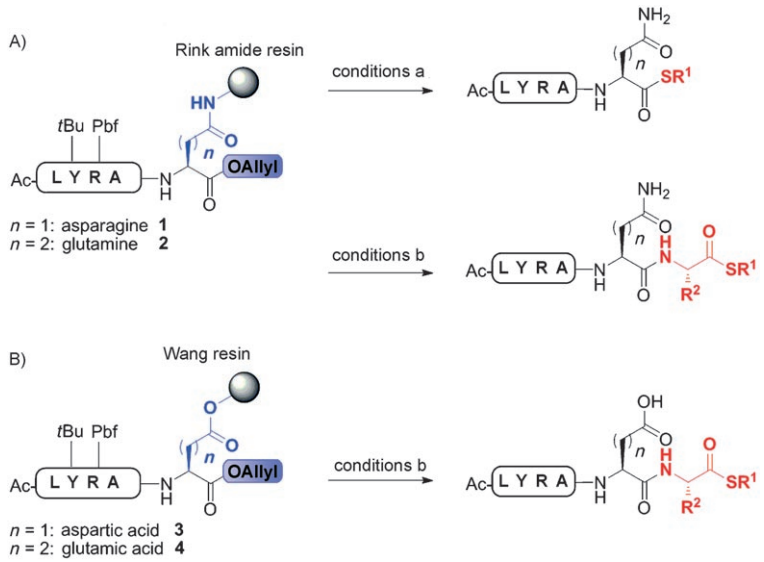

Scheme 2. Synthesis of peptide thioesters by immobilizing the side chains of A) aspartic acid ($n=1$) and glutamic acid ($n=2$) onto Rink amide resin and B) aspartic acid ($n=1$) and glutamic acid ($n=2$) onto Wang resin, followed by C-terminal deprotection and thiol or amino acid thioester coupling (conditions a: 1) $[Pd(PPh_3)_4]$, PhSiH, CH_2Cl_2; 2) R^1SH, DIC, HOBt, DIEA, CH_2Cl_2, DMF; 3) TFA/TIS/TA/H_2O (85:5:5:5); conditions b: 1) $[Pd(PPh_3)_4]$, PhSiH, CH_2Cl_2, 2) H_2N-CHR^2-$COSR^1$·HCl, HATU, DIEA, CH_2Cl_2, 3) TFA/TIS/TA/H_2O (85:5:5:5); $R^1=-(CH_2)_2COOEt$, R^2 = amino acid side chain, DIC = *N*,*N*-diisopropylcarbodiimide, HOBt = 1-hydroxybenzotriazole, Pbf = 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfanyl; HATU = *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; DIEA = *N*,*N*-diisopropylethylamine; TFA = trifluoroacetic acid; TIS = triisopropylsilane; TA = thioanisole).

and 95% yield, respectively, after HPLC purification (Table 1, entries 1 and 2).

We next examined the possibility of extending the scope of the side chain anchoring strategy, such that it could serve as a general strategy for the production of a larger range of thioester targets. Barany and co-workers have previously used amino acid thioesters as coupling partners to the C-terminus of solid-phase bound peptides to access thioesters without inducing epimerization.[44] Encouraged by this report, we sought to carry out a similar strategy using side chain anchored peptide **1**. To this end, glycine, alanine, phenylalanine and methionine thioesters were first synthesized in solution. This entailed DIC- and HOBt-mediated coupling of the appropriate Boc-protected amino acids with ethyl 3-mercaptopropionate, followed by acid-catalyzed Boc removal to afford the desired amino acid thioesters **5**–**8** in good yields (Scheme 3). Glycine was chosen as its thioesters are known to be very efficient acyl donors in ligation reactions, alanine is an example of an amino acid carrying an alkyl chain, phenylalanine is a representative aromatic amino acid and methionine contains a heteroatom in the side chain but is unable to serve as an anchor. These four amino acids cannot be easily immobilized onto solid supports and therefore their corresponding C-terminal thioesters are inaccessible by the method previously described.

Palladium-catalyzed allyl deprotection of solid-supported peptide **1** was followed by HATU-mediated coupling of amino acid thioesters **5**–**8**.[44] Figure 1A shows a representative analytical HPLC trace of the crude peptide thioester Ac-LYRANG-SR after deprotection and cleavage from the solid support. The trace is predominantly a single peak which was confirmed to be the desired product by MALDI-TOF mass spectrometry (Figure 1B). After purification by preparative HPLC, the peptide thioester was obtained in quantitative yield. We were delighted to find that the efficiency of generating these C-terminally extended peptide thioesters using the HATU-mediated coupling method was not limited to extension with glycine thioester **5**, as similar yields were also obtained for the other three thioesters **6**–**8** (Table 1, entries 8–10) and for the corresponding thioesters derived from solid-supported peptide **2** (Scheme 2A) where glutamic acid served as anchor (Table 1, entries 3–6).

At this stage, we were interested in whether other resins could be used for immobilization of amino acid side chains. To this end, Fmoc-Asp-OAllyl and Fmoc-Glu-OAllyl were loaded onto Wang resin using the symmetrical anhydride method.[51] Subsequent peptide synthesis and allyl deprotection were conducted in a similar fashion to that described for Rink amide resin (Scheme 2B). We chose not to attempt direct thioesterification of the C-terminus due to the reported formation of five- and six-membered cyclic anhydrides for peptide thioesters containing C-terminal aspartic and glutamic acid residues.[52] Anhydride formation would not be expected for thioesters extended at the C-terminus. Indeed, HATU-mediated C-terminal coupling of glycine, alanine, methionine and phenylalanine thioesters **5**–**8** gave, after cleavage from the resin and purification by preparative HPLC, the corresponding peptide thioesters in excellent yields (88%–quant.) (Table 1, entries 11–18).

In order to further broaden the scope of this approach, our attention now turned to the immobilization of other amino acid side chains using an alternative resin. There are several commercially available resins commonly employed in solid-phase organic synthesis, which can theoretically be used for the immobilization of amino acids. The resin chosen must allow for the construction of amino acid-resin linkages which are stable to the conditions of standard Fmoc SPPS, but cleave when treated with acid. Bromo-(4-methoxyphenyl)methyl polystyrene fulfilled these requirements and has previously been used for the immobilization of alcohols, amines and thiols.[53] We therefore explored the possibility of using this resin for the side chain anchoring of

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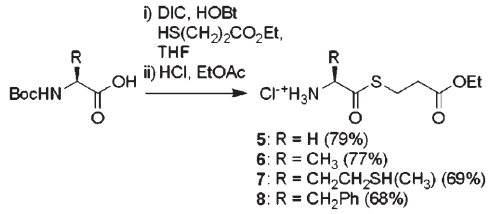
Table 1. Scope of the side chain anchoring strategy for the synthesis of peptide thioesters using glutamic acid and aspartic acid bound to Rink amide and Wang resin (R = S-$(CH_2)_2$COOEt).

Entry	Resin	Amino acid anchor AA^1	C-terminal extension AA^2	Isolated yield [%]
1	Rink amide	Gln	–	96
2	Rink amide	Asn	–	95
3	Rink amide	Gln	Gly	quant.
4	Rink amide	Gln	Ala	quant.
5	Rink amide	Gln	Phe	quant.
6	Rink amide	Gln	Met	quant.
7	Rink amide	Asn	Gly	quant.
8	Rink amide	Asn	Ala	quant.
9	Rink amide	Asn	Phe	96
10	Rink amide	Asn	Met	90
11	Wang	Glu	Gly	95
12	Wang	Glu	Ala	quant.
13	Wang	Glu	Phe	quant.
14	Wang	Glu	Met	99
15	Wang	Asp	Gly	quant.
16	Wang	Asp	Ala	97
17	Wang	Asp	Phe	90
18	Wang	Asp	Met	88



Scheme 3. Synthesis of amino acid thioesters.

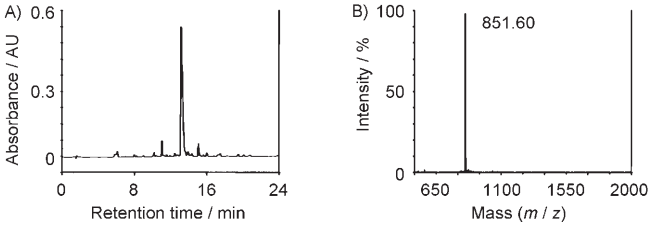
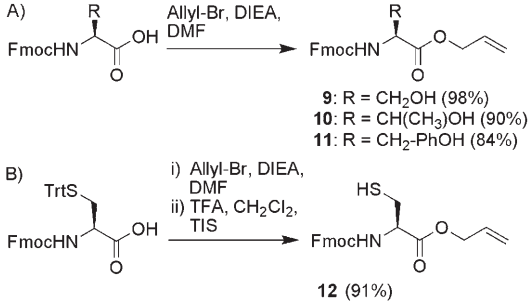


Figure 1. A) Crude analytical HPLC trace of Ac-LYRANG-SR after cleavage from Rink amide resin and B) MALDI-TOF mass spectrum of the desired product (R = S-$(CH_2)_2$COOEt; $[M+H]^+_{calcd}$ = 851.99; matrix = α-cyano-4-hydroxycinnamic acid).

the amino acids serine, threonine, tyrosine and cysteine. These constructs could then be used in the solid-phase synthesis of peptide thioesters using our method. In order to achieve this goal, we first had to generate the desired amino acid coupling partners containing free side chains and an allyl-protected C-terminus. Their synthesis was carried out in solution by treating Fmoc-Ser-OH, Fmoc-Thr-OH, and Fmoc-Tyr-OH with allyl bromide in the presence of diisopropylethylamine, to give the desired allyl esters in good yields (Scheme 4A). Fmoc-Cys-OAllyl was synthesized from Fmoc-Cys(Trt)-OH in two steps, via allyl ester formation followed by trityl deprotection to liberate the thiol side chain (Scheme 4B).



Scheme 4. Synthesis of A) Fmoc-Ser-OAllyl, Fmoc-Thr-OAllyl and Fmoc-Tyr-OAllyl and B) Fmoc-Cys-OAllyl.

Pre-loading of bromo-(4-methoxyphenyl)methyl resin was conducted by reacting derivatized amino acids **9**–**12** in the presence of *N*,*N*-diisopropylethylamine for 18–48 h to give final resin loadings of 0.79–1.28 mmol g^{-1} (depending on the steric bulk of the amino acid side chain). Reactions were carried out in the absence of light to prevent any potential photochemical side reactions on the resin.[53] Elongation of peptides was achieved following the standard Fmoc protocol to give fully assembled constructs **13**–**16** (Scheme 5), followed by allyl deprotection by treatment with $[Pd(PPh_3)_4]$ and phenylsilane in dichloromethane to give the free carboxylic acid. At this stage, peptide thioesters could be generated by direct DIC/HOBt-mediated coupling of ethyl 3-mercaptopropionate as previously described for Rink amide resin (Scheme 2A). Acid-catalyzed side chain deprotection and cleavage from the resin gave the desired thioesters in good yields in all cases. As a representative example, Figure 2 shows the crude analytical HPLC trace of Ac-LYRAC-SR. In contrast to the crude trace of Ac-LYRANG-SR (Figure 1A), there are two products produced in the synthesis. The first (peak a, Figure 2A) accounts for ≈25% and corresponds to the peptide acid Ac-LYRAC-OH, whereas the second accounts for ≈75% (peak b, Figure 2A) and corresponds to the desired peptide thioester Ac-LYRAC-S$(CH_2)_2CO_2$Et. Unfortunately, extending reaction times in an attempt to convert all of the free acid to the desired thioester did not increase the yield substantially. However, under the conditions described here, the C-termi-

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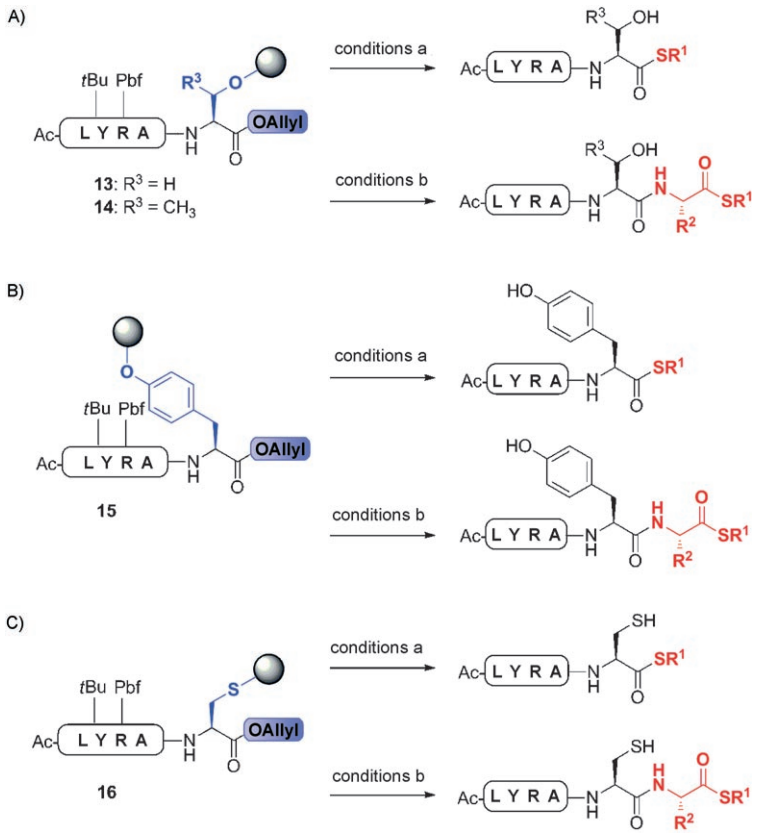


Scheme 5. Synthesis of peptide thioesters by side chain anchoring of A) serine and threonine B) tyrosine and C) cysteine onto bromo-(4-methoxyphenyl)methyl resin, C-terminal deprotection and thiol or amino acid thioester coupling (conditions a: 1) $[Pd(PPh_3)_4]$, PhSiH, CH_2Cl_2; 2) R^1SH, DIC, HOBt, DIEA, CH_2Cl_2, DMF; 3) TFA/TIS/TA/H_2O (85:5:5:5); conditions b: 1) $[Pd(PPh_3)_4]$, PhSiH, CH_2Cl_2, 2) H_2N-CHR^2-$COSR^1$·HCl, HATU, DIEA, CH_2Cl_2, 3) TFA/TIS/TA/H_2O (85:5:5:5); R^1= -$(CH_2)_2COOEt$; R^2=amino acid side chain; R^3=H or Me.

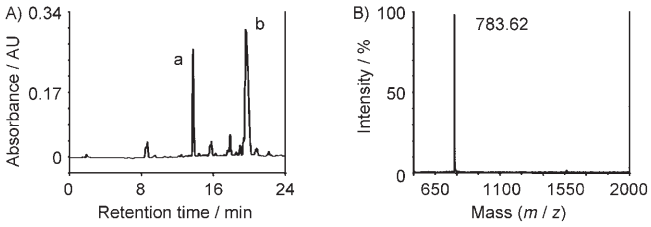


Figure 2. A) Crude analytical HPLC trace of direct thioester formation from **16**. Peak a=Ac-LYRAC-OH, peak b=Ac-LYRAC-SR and B) MALDI-TOF mass spectrum of peak b (R=S-$(CH_2)_2COOEt$; $[M+H]^+_{calcd}$=783.98; matrix=2′,4′,6′-trihydroxyacetophenone).

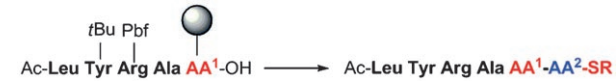
nal cysteine thioester was isolated in 70% yield after purification by preparative HPLC (Table 2, entry 4).

The corresponding C-terminal serine, threonine and tyrosine peptide thioesters were also obtained in good yields ranging from 51–74% (Table 2, entries 1–3). In a similar manner to Wang and Rink amide immobilized peptides **1**–**4**, amino acid thioesters **5**–**8** could be coupled to the free acid of **13**–**16** using the HATU-mediated coupling reaction. Using this method, C-terminal glycine, alanine, methionine and phenylalanine thioesters were obtained in good yields (55–91%; Table 2, entries 5–20).

The application of the side chain anchoring approach to the synthesis of glycopeptide thioesters was next examined. These are currently difficult to obtain, and a general strategy for their synthesis would be extremely useful. Accordingly, bromo-(4-methoxyphenyl)methyl resin was first derivatized via the side chain of Fmoc-Ser-OAllyl (**9**). Standard Fmoc-strategy SPPS was used to construct peptide **17**, incorporating the glycosylated serine residue as the N-terminal residue. Allyl deprotection of **17** was followed by coupling of methionine thioester **7** or phenylalanine thioester **8**. Subsequent deprotection and cleavage from the resin gave the desired C-terminal methionine- and phenylalanine-containing glycopeptide thioesters in 66 and 73% isolated yield, respectively, after HPLC purification (Table 3, entries 1 and 2).

Having demonstrated that the side chain anchoring strat-

Table 2. Scope of the side chain anchoring strategy using serine, threonine, tyrosine and cysteine bound to bromo-(4-methoxyphenyl)methyl resin (R=S-$(CH_2)_2COOEt$).



Entry	Amino acid anchor AA^1	Added amino acid AA^2	Isolated yield [%]
1	Ser	–	70
2	Thr	–	51
3	Tyr	–	74
4	Cys	–	70
5	Ser	Gly	69
6	Ser	Ala	68
7	Ser	Phe	75
8	Ser	Met	55
9	Thr	Gly	81
10	Thr	Ala	80
11	Thr	Phe	90
12	Thr	Met	67
13	Tyr	Gly	58
14	Tyr	Ala	71
15	Tyr	Phe	65
16	Tyr	Met	80
17	Cys	Gly	56
18	Cys	Ala	64
19	Cys	Phe	55
20	Cys	Met	54

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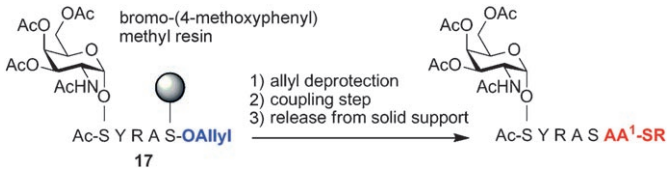
Table 3. Synthesis of glycopeptide thioesters using the side chain anchoring strategy.



Entry	Glycosylated amino acid	C-terminal amino acid AA[1]	Isolated yield [%]
1	Ser	Met	67
2	Ser	Phe	73

egy was amenable to the synthesis of glycopeptide thioesters, our next goal was to apply this method to the synthesis of a biologically relevant target. Construction of homogeneous glycoproteins is an important yet challenging endeavor, and to date the total synthesis of a target containing a native protein sequence has yet to be achieved. We chose to synthesize a fragment of the therapeutic glycoprotein erythropoietin (EPO). EPO, a 166-residue glycoprotein hormone possessing four glycosylation sites (three N-linked and one O-linked) is produced in kidney cells and is responsible for the regulation of red blood cell production.[54] Clinically, it is obtained as a mixture of glycoforms by over-expression in mammalian cells, and used for the treatment of anemia associated with a number of illnesses.[7] Current efforts are focused on the total chemical synthesis of single glycoforms of this medicinally significant glycoprotein for detailed biological studies.[55–57] We embarked on the synthesis of a 40-mer N-terminal fragment of EPO, containing two of the four glycosylation sites (both N-linked glycans). This would serve as a building block for the total synthesis of this target and as an elegant demonstration of the side chain anchoring strategy for the synthesis of large peptide and glycopeptide thioesters. The synthesis of the desired glycopeptide thioester fragment (EPO 1–28) was performed on bromo-(4-methoxyphenyl)methyl resin. Pre-loading of the resin with Fmoc-Ser-OAllyl (**9**) and peptide elongation was achieved as described previously. Fmoc-Asn(Ac_3AcNH-β-Glc)-OH was coupled as the third amino acid, however, only one equivalent relative to the resin was used to minimize waste of this precious building block. Although the reaction time was extended to six hours, this coupling proceeded in only 60% yield. To mitigate further loss of loading, all subsequent amino acids were doubly coupled. After coupling Boc-Ala-OH as the final amino acid residue, removal of the C-terminal allyl group gave access to the free carboxylic acid which was elongated in the N- to C-terminal direction by incorporating glycine thioester **5** under the HATU-mediated coupling conditions previously described. Treatment of the resin with TFA/thioanisole/triisopropylsilane/water (85:5:5:5 by volume) removed the side chain protecting groups, the N-terminal Boc group and cleaved the crude glycopeptide thioester **19** from the resin. After purification by HPLC, **19** was successfully isolated. The 12-mer glycopeptide **18** was synthesized on Wang resin using standard Fmoc SPPS. Coupling of the N-linked glycosyl amino acid building block Fmoc-Asn(Ac_3AcNH-β-Glc)-OH was performed in a similar fashion to that described for glycopeptide thioester **19** and a similar drop in the loading was observed. The remainder of the synthesis proceeded smoothly without a further drop in loading, and Boc-Cys(Trt)-OH was coupled as the final amino acid residue. Treatment with TFA/thioanisole/triisopropylsilane/water (85:5:5:5 by volume) removed the side chain protecting groups, the N-terminal Boc group, and released the crude product from the resin. After HPLC purification, the desired glycopeptide **18** was obtained in 67% yield relative to the final resin loading. Glycopeptide **18** and glycopeptide thioester **19** were submitted to a native chemical ligation reaction using a ligation buffer consisting of a 4:1 mixture of NMP and 1 M HEPES/6 M guanidine hydrochloride pH 8.5, for 24 h. Purification by semi-preparative HPLC afforded the 40-mer ligation product in 76% yield. Subsequent treatment with a solution of 5% hydrazine containing 60 mM dithiothreitol for 1 h removed the acetate groups from the two glycans to afford the desired 40 amino acid N-terminal fragment of EPO (**20**) in 95% yield (Scheme 6).

Conclusion

In summary, an efficient strategy for the synthesis of peptide and glycopeptide thioesters has been described. The method relies on immobilizing the side chains of a number of amino acids to suitable resins. In total six proteinogenic amino acids were successfully anchored: aspartic acid and glutamic acid to Wang or Rink amide resin (use of Rink amide leads to asparagine and glutamine upon cleavage) and serine, threonine, tyrosine and cysteine to bromo-(4-methoxyphenyl)methyl resin. Pre-loading of allyl protected amino acids and elongation using standard Fmoc-SPPS gave solid-supported peptides in high yields. C-terminal deallylation liberated the C-terminal carboxylic acid, to which thiols could be coupled to produce peptide thioesters containing C-terminal asparagine, glutamine, serine, threonine, tyrosine and cysteine in high yields after resin cleavage. In addition to the amino acids studied here, lysine,[49] arginine, histidine and tryptophan could, in theory, also be immobilized to solid supports. These strategies are currently being pursued by our laboratory and will allow for the anchoring of all proteinogenic amino acids containing reactive side chains. In addition to direct coupling of thiols, and in order to broaden the scope of the method, amino acid thioesters were also coupled to the C-terminus to afford, after deprotection and cleavage from the resin, a variety of peptide thioesters in an efficient manner. Glycopeptide thioesters were also constructed using this strategy. Four amino acid thioesters (Gly, Ala, Phe, Met) were used in these C-terminal extension studies, and it is envisaged that the remaining four unanchorable amino acids (Ile, Leu, Pro and Val) could be synthesized as their thioesters and incorporated in a similar

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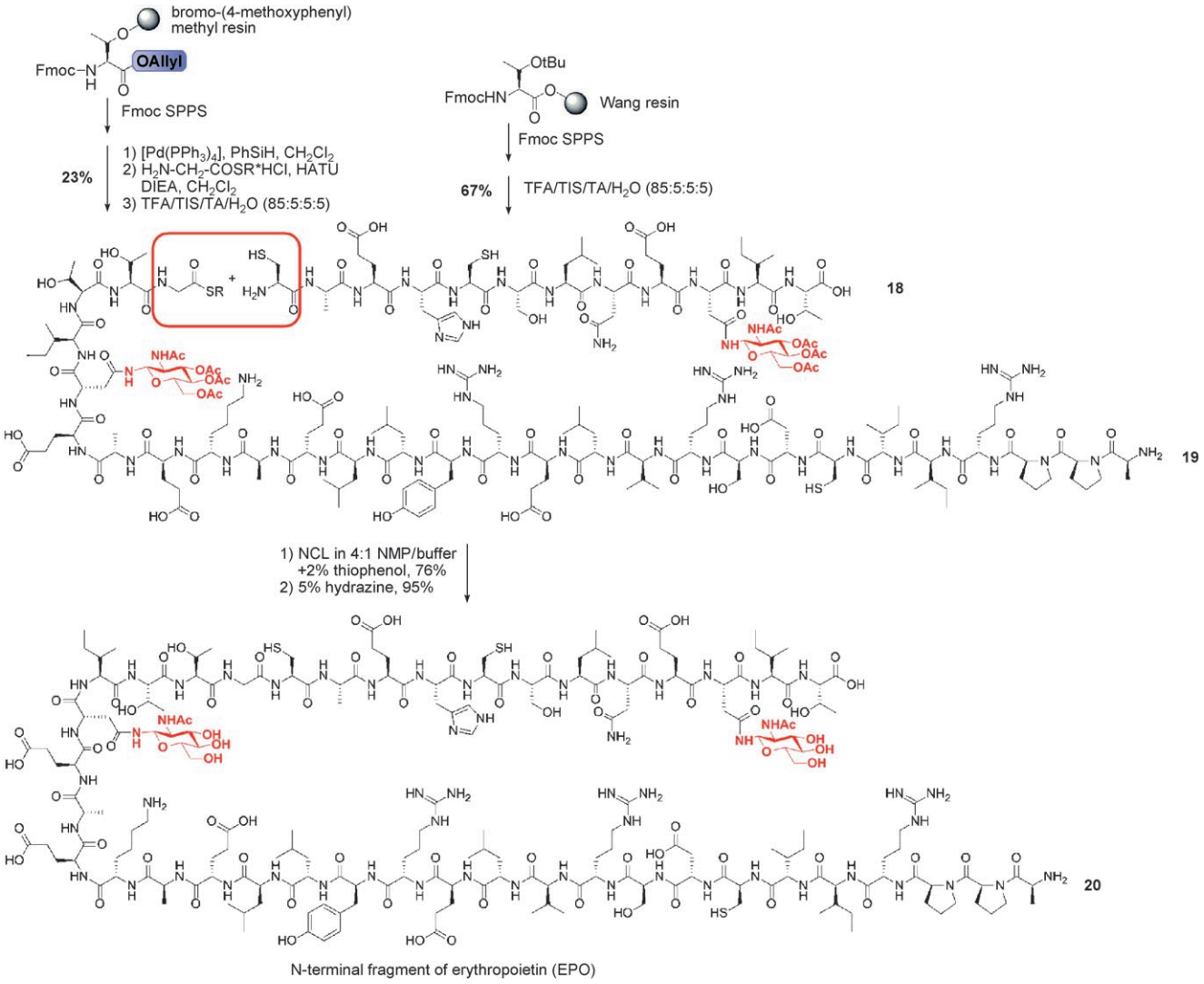

Scheme 6. Synthesis of the N-terminal fragment of the glycoprotein hormone erythropoietin (Fmoc = 9-fluorenylmethoxycarbonyl; NMP = 1-methyl-2-pyrrolidinone; buffer = 1 M HEPES, 6 M Gn·HCl pH 8.5; $[M+H]^+_{calcd} = 4881.41$; matrix = sinapinic acid).

fashion. Combined, direct coupling of a thiol or amino acid thioester to the C-terminus of side chain anchored peptides represents a general strategy for the synthesis of peptide and glycopeptide thioesters. As such, the method should allow access to a large majority of potential targets amenable to subsequent ligation reactions for the construction of proteins and glycoproteins. The method was successfully applied to the synthesis of a glycopeptide thioester fragment of the therapeutic glycoprotein EPO, to exemplify its utility in the construction of complex targets. Subsequent ligation to a glycopeptide by native chemical ligation gave a 40 amino acid homogeneous N-terminal fragment of EPO bearing two of the four glycosylation sites. Current research in our laboratory is focused on using the side chain anchoring strategy in combination with NCL and SAL to produce other peptide and glycopeptide fragments of EPO, with a view to the total synthesis of a homogeneous sample of this therapeutic glycoprotein.

Experimental Section

General: ^{1}H NMR and ^{13}C NMR were recorded on a Bruker DRX-600 spectrometer equipped with a CryoProbe operating at 600 MHz and 150 MHz respectively. Coupling constants (J) are reported in Hertz (Hz), and chemical shifts are reported in parts per million (ppm) relative to tetramethylsilane (TMS, 0.00 ppm). MALDI-TOF mass spectra were measured on a Voyager-DE Pro biospectrometry workstation by PerSeptive Biosystems. Analytical HPLC was run on a Hitachi (D-7000 HPLC system) instrument using an analytical column (Waters "XBridge BEH 130 C18", 150×4.6 mm, 5 μm particle size or Grace Vydac "Protein & Peptide C18", 150×4.6 mm, 10 μm particle size, flow rate 1.5 mL min^{-1}, 50 °C). Semi preparative HPLC was run on a Hitachi (D-7000 HPLC system) instrument using a semi preparative column (Grace Vydac "Protein & Peptide C18", 250×10 mm, 10–15 μm particle size, flow rate 4 mL min^{-1}). Preparative HPLC was run on a Hitachi (D-7000 HPLC system) instrument using a preparative Column (Grace Vydac "Protein & Peptide C18", 250×22 mm, 10–15 μm particle size, flow rate 8 mL/min). Detection of the signal was achieved with either photodiode array or UV detector at a wavelength of $\lambda = 280$ nm (detection of Tyr). For the purification and analytical traces of EPO-fragment glycopeptides,

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detection was performed at $\lambda = 230$ nm. Additional analytical and experimental details are available in the Supporting Information.

Materials: Water was taken from a Milli-Q ultra pure water purification system (Millipore corp.). DMF was purchased as peptide synthesis grade from Alfa Aesar. Commercial reagents were purchased from Sigma-Aldrich or Acros Organics and were used without further purification. Anhydrous-grade solvents were purchased from Sigma-Aldrich and were used directly. Resins, protected amino acids and PyBOP were purchased from Novabiochem. Deuterated solvents were purchased from Cambridge Isotope Laboratories Inc.

Side-chain anchoring onto Rink amide resin: Rink amide resin (100–200 mesh; 1% DVB) (290 mg, loading = 0.69 mmol g^{-1}, 200 μmol) was initially washed with DMF (5×5 mL), CH_2Cl_2 (5×5 mL) and DMF (5×5 mL), treated with DMF/piperidine (9:1 by volume) (2×5 min) and washed with DMF (5×5 mL), CH_2Cl_2 (5×5 mL) and DMF (5×5 mL). Fmoc-Glu-OAllyl or Fmoc-Asp-OAllyl (800 μmol) in dry DMF (4 mL) containing PyBOP (416 mg, 800 μmol) and NMM (176 μL, 1600 μmol) was preactivated for 4 min and then added to the resin. After two hours of shaking, the resin was washed with DMF (5×5 mL), CH_2Cl_2 (5×5 mL) and DMF (5×5 mL), treated with Ac_2O/pyridine (1:9 by volume) for 10 min and then washed with DMF (5×5 mL), CH_2Cl_2 (5×5 mL) and DMF (5×5 mL). Treatment of the resin with 10% piperidine/DMF (2×5 min) and measurement of the resulting fulvene–piperidine adduct at $\lambda = 302$ nm showed that the yield of the side chain anchoring was quantitative.

Side-chain anchoring onto Wang resin: Wang resin LL (100–200 mesh) (454 mg, loading = 0.44 mmol g^{-1}, 200 μmol) was initially washed with DMF (5×5 mL), CH_2Cl_2 (5×5 mL) and DMF (5×5 mL), and then allowed to swell in DMF (5 mL) for 30 min. Fmoc-Glu-OAllyl or Fmoc-Asp-OAllyl (2 mmol, 10 equiv) were dissolved in dry CH_2Cl_2 (15 mL). DIC (155 μL, 1 mmol) in dry CH_2Cl_2 (5 mL) was added to the above amino acid solution at 0°C and the reaction stirred for 20 min, before the solvent was removed in vacuo. The residue was redissolved in DMF (5 mL) before adding to the resin. A solution of DMAP (2.5 mg, 20 μmol) in DMF (1 mL) was added and the resin shaken for 1 h before washing with DMF (5×5 mL), CH_2Cl_2 (5×5 mL) and DMF (5×5 mL). Capping with Ac_2O/pyridine (1:9 by volume) for 10 min was followed by resin washing with DMF (5×5 mL), CH_2Cl_2 (5×5 mL) and DMF (5×5 mL). Treatment of the resin with 10% piperidine/DMF (2×5 min) and measurement of the resulting fulvene–piperidine adduct at $\lambda = 302$ nm showed that the yield of the side chain anchoring was quantitative.

Side-chain anchoring onto bromo-(4-methoxyphenyl)methylpolystyrene: Bromo-(4-methoxyphenyl)methylpolystyrene resin (174 mg, loading = 2.3 mmol g^{-1}, 400 μmol) was initially washed with DMF (5×5 mL) and CH_2Cl_2 (10×5 mL) and the resin swelled with CH_2Cl_2 (5 mL) for 1 h with the exclusion of light. A solution of the C-terminal allyl-protected Fmoc-amino acids **7**–**10** (1200 μmol), DIEA (397 μL, 2400 μmol) in CH_2Cl_2 (4 mL) was added to the resin, which was shaken for 12–48 h at RT with the exclusion of light. The resin was washed with CH_2Cl_2 (5×5 mL) and DMF (10×5 mL). Treatment of the resin with 10% piperidine/DMF (2×5 min) and measurement of the resulting fulvene–piperidine adduct at $\lambda = 302$ nm was used to determine the loading. NB: The commercially available resin had a very high loading, therefore pre-loadings of the resins were conducted for times that allowed for the resin to be loaded less than 1.4 mmol g^{-1} to prevent resin crowding and product aggregation. Final loadings: Fmoc-Ser-OAllyl **7** (18 h): loading = 1.28 mmol g^{-1}; Fmoc-Thr-OAllyl **8** (48 h): loading = 0.79 mmol g^{-1}; Fmoc-Tyr-OAllyl **9** (46 h): loading = 1.02 mmol g^{-1}; Fmoc-Cys-OAllyl **10** (32 h): loading = 0.90 mmol g^{-1}.

General procedures for SPPS of peptides and glycopeptides following the Fmoc strategy (Iterative peptide assembly)

Deprotection: The resin was treated with 10% piperidine/DMF (2×5 min) and subsequently washed (5×DMF, 5× CH_2Cl_2, 5×DMF).

Amino acid coupling: A preactivated solution of 4 equiv protected amino acid (final concentration 0.1 M in DMF) using 4 equiv PyBOP and 8 equiv NMM was added to the resin. After 30 min, the resin was washed with DMF (5×), CH_2Cl_2 (5×) and DMF (5×).

Capping: Acetic anhydride/pyridine (1:9 by volume) was added to the resin. After 5 min the resin was washed with DMF (5×), CH_2Cl_2 (5×) and DMF (5×).

Final N-terminal deprotection: When the peptide was fully assembled, the N-terminal Fmoc group was removed by treatment with 10% piperidine/DMF (2×5 min) and the loading was determined by measuring the absorbance of the resulting fulvene–piperidine adduct at $\lambda = 302$ nm. After acetylating the N-terminus with acetic anhydride/pyridine (1:9 by volume) for 15 min, the resin was washed with DMF (10×) and CH_2Cl_2 (10×) and then dried in vacuo.

General procedure for the direct thioesterification on solid support: 25 μmol of resin was swollen in dry CH_2Cl_2 (5 mL) for 30 min, followed by the addition of a solution of $[Pd(PPh_3)_4]$ (25 mg, 22 μmol) and Ph_3SiH (123 μL, 1 mmol) in dry CH_2Cl_2 (2 mL). The resin was shaken for 1 h and the procedure was repeated. Afterwards, the resin was washed with CH_2Cl_2 (10×5 mL), DMF (5×5 mL) and CH_2Cl_2 (5×5 mL). A solution of ethyl 3-mercaptopropionate (77 μL, 600 μmol), anhydrous HOBt (101 mg, 750 μmol), DIEA (161 μL, 938 μmol) and DIC (116 μL, 750 μmol) in CH_2Cl_2/DMF (1.5 mL, 4:1 by volume) was added and the resin was shaken for 1 h. This thioesterification step was repeated before washing the resin with CH_2Cl_2 (5×5 mL), DMF (5×5 mL) and CH_2Cl_2 (10×5 mL).

Cleavage: A mixture of TFA/thioanisole/triisopropylsilane/water (85:5:5:5 by volume) was added. After 2 h, the resin was washed with TFA (4×4 mL).

Work-up: The combined solutions were concentrated in vacuo. The residue was dissolved in water containing 30% MeCN + 0.1% TFA, purified by preparative HPLC and analyzed by MALDI-TOF/MS (matrix: α-cyano-4-hydroxycinnamic acid).

General procedure for the C-terminal introduction of amino acid thioesters: 25 μmol of resin were swollen in dry CH_2Cl_2 (5 mL) for 30 min, followed by the addition of a solution of $[Pd(PPh_3)_4]$ (25 mg, 22 μmol) and Ph_3SiH (123 μL, 1 mmol) in dry CH_2Cl_2 (2 mL). The resin was shaken for 1 h and the procedure was repeated. The resin was subsequently washed with CH_2Cl_2 (10×5 mL), DMF (5×5 mL) and CH_2Cl_2 (5×5 mL). A solution of amino acid thioesters **5**–**8** (250 μmol) and DIEA (86 μL, 500 μmol) in dry CH_2Cl_2 (1 mL) was added to the resin. HATU (95 mg, 250 μmol) was then added in solid form and the resin shaken for 1 h. The coupling procedure was repeated and the resin washed with CH_2Cl_2 (5×5 mL), DMF (5×5 mL) and CH_2Cl_2 (10×5 mL).

Cleavage: A mixture of TFA/thioanisole/triisopropylsilane/water (85:5:5:5 by volume) was added. After 2 h, the resin was washed with TFA (4×4 mL).

Work-up: The combined solutions were concentrated in vacuo. The residue was dissolved in water containing 30% MeCN + 0.1% TFA, purified by preparative HPLC and analyzed by MALDI-TOF/MS (matrix: α-cyano-4-hydroxycinnamic acid).

General procedure for the synthesis of amino acid thioesters: Boc-protected amino acids (6.00 mmol) were dissolved in anhydrous THF (20 mL) and cooled to 0°C. Ethyl 3-mercaptopropionate (0.77 mL, 6.00 mmol) was added dropwise, followed by HOBt (1.10 g, 7.20 mmol). The solution was stirred at 0°C for 15 min before the dropwise addition of DIC (1.11 mL, 7.20 mmol). The reaction was stirred at RT for 15 h before the urea by-product was removed by filtering through a plug of Celite. The solvent was removed in vacuo and the resulting residue redissolved in ethyl acetate (30 mL). The solution was washed with a 4% $NaHCO_3$ solution (3×30 mL), 1 M HCl (3×30 mL), brine (2×30 mL), dried (Na_2SO_4) and the solvent removed in vacuo. Purification was achieved by column chromatography (hexane/ethyl acetate 3:1) to give the desired Boc-protected amino acid thioesters. The resulting thioester was dissolved in ethyl acetate (15 mL) and the solution cooled to 0°C before saturating with $HCl_{(g)}$. The reaction was stirred at RT for 2 h, before the solvent was removed in vacuo. The resulting residue was re-dissolved in ethyl acetate and the solvent removed in vacuo (3×20 mL) and gave the desired amino acid thioesters as white solids.

Ethyl 3-(2-aminoacetylthio)propanoate hydrochloride (5): Yield = 1.09 g, 79% over two steps; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 1.33 (t, J =

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7.0 Hz, 3H, CH_3), 2.77 (t, J=7.0 Hz, 2H, CH_2), 3.35 (t, J=7.0 Hz, 2H, CH_2), 4.18 (s, 2H, CH_2), 4.22 ppm (q, J=7.0 Hz, 2H, CH_2); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 14.5, 25.1, 31.5, 48.3, 62.0, 173.0, 194.2 ppm; HRMS (ESI-TOF): m/z: calcd for $C_7H_{14}NO_3S$: 192.0685; found: 192.0685 $[M+H]^+$.

(*S*)-Ethyl 3-(2-aminopropanoylthio)propanoate hydrochloride (6): Yield = 1.12 g, 77% over two steps; ^{1}H NMR (600 MHz, $CDCl_3$): δ = 1.24 (t, J=6.6 Hz, 3H, CH_3), 1.56 (d, J=7.2 Hz, 3H, CH_3), 2.68 (t, J=6.6 Hz, 2H, CH_2), 3.25 (td, J=2.4, 6.6 Hz, 2H, CH_2), 4.14 (q, J=7.2, 2H, CH_2), 4.28 ppm (q, J=7.2 Hz, 1H, CH); ^{13}C NMR (150 MHz, $CDCl_3$): δ = 14.5, 17.6, 25.2, 34.8, 56.4, 62.0, 172.9, 198.2 ppm; HRMS (ESI-TOF): m/z: calcd for $C_8H_{16}NO_3S$: 206.0845; found: 206.0845 $[M+H]^+$.

(*S*)-Ethyl 3-(2-amino-4-(methylthio)butanoylthio)propanoate hydrochloride (7): Yield = 1.25 g, 69% over two steps; ^{1}H NMR (600 MHz, $CDCl_3$): δ = 1.25 (t, J=7.2 Hz, 3H, CH_3), 2.12 (s, 3H, CH_3), 2.17 (m, C*H*H), 2.25 (m, CH*H*), 2.65 (t, J=7.2 Hz, 2H, CH_2), 2.70 (td, J=1.2, 6.6 Hz, 2H, CH_2), 3.22–3.32 (m, 2H, CH_2), 4.14 (q, J=7.2, 2H, CH_2), 4.38 ppm (m, 1H, CH); ^{13}C NMR (150 MHz, $CDCl_3$): δ = 14.6, 15.0, 25.5, 29.8, 32.0, 34.7, 59.3, 62.0, 172.9, 197.3 ppm; HRMS (ESI-TOF): m/z: calcd for $C_{10}H_{21}NO_3S_2$: 266.0885; found: 266.0953 $[M+H]^+$.

(*S*)-Ethyl 3-(2-amino-3-phenylpropanoylthio)propanoate hydrochloride (8): Yield = 1.30 g, 68% over two steps; ^{1}H NMR (600 MHz, $CDCl_3$): δ = 1.24 (t, J=7.2 Hz, 3H, CH_3), 2.62 (m, 2H, CH_2), 3.16 (m, 2H, CH_2), 3.23 (m, 2H, CH_2), 4.13 (q, J=7.2 Hz, 2H, CH_2), 4.47 (t, J=7.2 Hz, 1H, CH), 7.27–7.37 ppm (m, 5H); ^{13}C NMR (150 MHz, $CDCl_3$): δ = 14.6, 23.4, 34.7, 38.7, 61.3, 62.0, 129.1, 130.2, 130.7, 134.9, 172.9, 197.2 ppm; HRMS (ESI-TOF): m/z: calcd for $C_{14}H_{20}NO_3S$: 282.1158; found: 282.1158 $[M+H]^+$.

General procedure for the synthesis of Fmoc-protected amino acid allyl esters: Fmoc-protected amino acids (11.7 mmol) were dissolved in anhydrous DMF (75 mL) and cooled to 0°C. *N*,*N*-diisopropylethylamine (4.08 mL, 23.4 mmol) and allyl bromide (2.02 mL, 23.4 mmol) were added dropwise and the reaction stirred at RT for 16 h, before diluting with ethyl acetate (200 mL). The reaction was washed with water (4× 200 mL), brine (200 mL), dried (Na_2SO_4) and the solvent removed in vacuo. Purification was achieved by column chromatography (hexane/ethyl acetate 3:1) to afford the desired amino acid allyl esters as white solids.

(*S*)-Allyl 2-(((9*H*-fluoren-9-yl)methoxy)carbonylamino)-3-hydroxypropanoate (9): Yield = 4.20 g, 98%; R_F (hexane/ethyl acetate 3:1) = 0.06; ^{1}H NMR (600 MHz, $CDCl_3$): δ = 3.91 (d, J=10.2 Hz, 1H, CH), 4.00 (d, J=10.2 Hz, 1H, CH), 4.21 (t, J=7.2 Hz, 1H, CH), 4.29 (m, 3H), 4.68 (d, J=4.8 Hz, 2H, CH_2), 5.24 (d, J=10.2 Hz, 1H, CH), 5.33 (d, J=17.4 Hz, 1H, CH), 5.84 (d, J=7.2 Hz, 1H, CH), 5.90 (m, 1H, CH), 7.30 (t, J=7.2 Hz, 2H, 2×CH), 7.39 (t, J=7.2 Hz, 2H, 2×CH), 7.60 (t, J=6.0 Hz, 2H, 2×CH), 7.76 ppm (d, J=7.2 Hz, 2H, 2×CH); ^{13}C NMR (150 MHz, $CDCl_3$): δ = 47.1, 56.1, 63.2, 66.3, 67.2, 118.9, 119.9, 125.0, 127.0, 127.7, 131.3, 141.3, 143.6, 156.2, 170.2 ppm; HRMS (ESI-TOF): m/z: calcd for $C_{21}H_{21}NO_5$: 368.1492; found: 368.1506 $[M+H]^+$.

(2*S*,3*R*)-Allyl 2-(((9*H*-fluoren-9-yl)methoxy)carbonylamino)-3-hydroxybutanoate (10): Yield = 4.00 g, 90%; R_F (hexane/ethyl acetate 3:1) = 0.12; ^{1}H NMR (600 MHz, $CDCl_3$): δ = 1.26 (d, J=6.0 Hz, 3H, CH_3), 4.24 (t, J=7.2 Hz, 1H, CH), 4.39 (m, 4H, 2×CH_2), 4.68 (d, J=5.4 Hz, 2H, CH_2), 5.25 (d, J=10.8 Hz, 1H, CH), 5.34 (d, J=17.4 Hz, 1H, OH), 5.65 (d, J=8.4 Hz, 1H, CH), 5.91 (m, 1H, CH), 7.30 (t, J=7.2 Hz, 2H, 2×CH), 7.39 (t, J=7.2 Hz, 2H, 2×CH), 7.60 (t, J=6.0 Hz, 2H, 2×CH), 7.76 ppm (d, J=7.2 Hz, 2H, 2×CH); ^{13}C NMR (150 MHz, $CDCl_3$): δ = 19.9, 47.1, 59.1, 66.2, 67.2, 68.0, 119.0, 120.0, 125.1, 127.1, 127.7, 131.4, 141.3, 143.7, 156.7, 170.8 ppm; HRMS (ESI-TOF): m/z: calcd for $C_{22}H_{23}NO_5$: 382.1649; found: 382.1651 $[M+H]^+$.

(*S*)-Allyl 2-(((9*H*-fluoren-9-yl)methoxy)carbonylamino)-3-(4-hydroxyphenyl) propanoate (11): Yield = 4.37 g, 84%; R_F (hexane/ethyl acetate 3:1) = 0.15; ^{1}H NMR (600 MHz, $CDCl_3$): δ = 3.01 (dd, J=6.0, 14.4 Hz, 1H, CH), 3.07 (dd, J=6.0, 14.4 Hz, 1H, CH), 4.20 (t, J=6.6 Hz, 1H, CH), 4.35 (dd, J=7.2, 10.8 Hz, 1H, CH), 4.42 (dd, J=7.2, 10.8 Hz, 1H, CH), 4.62 (d, J=6.0 Hz, 2H, CH_2), 4.65 (m, 1H, CH), 5.25 (d, J=10.8 Hz, 1H, CH), 5.34 (d, J=9.0 Hz, 1H, OH), 5.91 (m, 1H, CH), 6.71 (d, J=8.4 Hz, 2H, CH_2), 6.94 (d, J=8.4 Hz, 2H, CH_2), 7.30 (td, J=1.2, 7.2 Hz, 2H, CH_2), 7.39 (t, J=7.2 Hz, 2H, CH_2), 7.55 (t, J=6.0 Hz, 2H, CH_2), 7.75 ppm (d, J=7.8 Hz, 2H, CH_2); ^{13}C NMR (150 MHz, $CDCl_3$): δ = 37.4, 47.1, 55.0, 66.2, 67.0, 115.5, 119.2, 119.9, 125.0, 127.0, 127.2, 127.7, 130.5, 131.3, 141.3, 143.7, 155.1, 155.7, 171.4 ppm; HRMS (ESI-TOF): m/z: calcd for $C_{27}H_{25}NO_5$: 444.1805; found: 444.1800 $[M+H]^+$.

(*R*)-Allyl 2-(((9*H*-fluoren-9-yl)methoxy)carbonylamino)-3-mercaptopropanoate (12): Fmoc-Cys(Trt)-OH (6.9 g, 11.7 mmol) was dissolved in anhydrous DMF (75 mL) and cooled to 0°C. *N*,*N*-diisopropylethylamine (4.08 mL, 23.4 mmol) and allyl bromide (2.02 mL, 23.4 mmol) were added dropwise and the reaction stirred at RT for 16 h, before diluting with ethyl acetate (200 mL). The reaction was washed with water (4× 200 mL), brine (200 mL), dried (Na_2SO_4) and the solvent removed in vacuo. The resulting residue was dissolved in TFA/CH_2Cl_2 (50 mL, 9:1 by volume). Triisopropylsilane (10 mL) was added dropwise and the reaction stirred at RT for 1 h. The solvent was removed in vacuo and the product purified by column chromatography (hexane/ethyl acetate 3:1) to afford Fmoc-Cys-OAllyl as a white solid. Yield = 4.1 g, 91% over two steps; R_F (hexane/ethyl acetate 3:1) = 0.30; ^{1}H NMR (600 MHz, $CDCl_3$): δ = 3.00 (m, 2H, CH_2), 4.24 (t, J=6.8 Hz, 1H, CH), 4.43 (m, 2H, CH_2), 4.69 (m, 3H, CH_2 + CH), 5.29 (d, J=10.2 Hz, 1H, CH), 5.35 (d, J=17.4 Hz, 1H, CH), 5.72 (d, J=7.2 Hz, 1H, NH), 5.92 (m, 1H, CH), 7.32 (t, J=7.2 Hz, 2H, 2×CH), 7.40 (t, J=7.2 Hz, 2H, 2×CH), 7.61 (d, J=6.6 Hz, 2H, 2× CH), 7.77 ppm (d, J=7.8 Hz, 2H, 2×CH); ^{13}C NMR (150 MHz, $CDCl_3$): δ = 27.1, 47.1, 55.2, 66.4, 67.0, 119.4, 120.0, 125.0, 127.0, 127.7, 131.2, 141.3, 143.6, 155.6, 169.6 ppm; HRMS (ESI-TOF): m/z: calcd for $C_{21}H_{22}NO_4S$: 384.1264; found: 384.1268 $[M+H]^+$.

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[1] R. Apweiler, H. Hermjakob, N. Sharon, *Biochim. Biophys. Acta Gen. Subj.* **1999**, *1473*, 4–8.
[2] R. G. Spiro, *Glycobiology* **2002**, *12*, 43R–56R.
[3] R. A. Dwek, *Chem. Rev.* **1996**, *96*, 683–720.
[4] A. Varki, *Glycobiology* **1993**, *3*, 97–130.
[5] D. H. Dube, C. R. Bertozzi, *Nat. Rev. Drug Discovery* **2005**, *4*, 477–488.
[6] G. Walsh, R. Jefferis, *Nat. Biotechnol.* **2006**, *24*, 1241–1252.
[7] T. B. Drueke, F. Locatelli, N. Clyne, K. Eckardt, I. C. Macdougall, D. Tsakiris, H. Burger, A. Scherhag, *New Engl. J. Med.* **2006**, *355*, 2071–2084.
[8] B. G. Davis, *Chem. Rev.* **2002**, *102*, 579–601.
[9] M. R. Pratt, C. R. Bertozzi, *Chem. Soc. Rev.* **2005**, *34*, 58–68.
[10] L. Liu, C. S. Bennett, C. H. Wong, *Chem. Commun.* **2006**, 21–33.
[11] A. Brik, S. Ficht, C. H. Wong, *Curr. Opin. Chem. Biol.* **2006**, *10*, 638–644.
[12] T. Wieland, E. Bokelmann, L. Bauer, H. U. Lang, H. Lau, *Justus Liebigs Ann. Chem.* **1953**, *583*, 129–149.
[13] P. E. Dawson, T. W. Muir, I. Clarklewis, S. B. H. Kent, *Science* **1994**, *266*, 776–779.
[14] P. E. Dawson, S. B. H. Kent, *Annu. Rev. Biochem.* **2000**, *69*, 923–960.
[15] A. Brik, Y. Y. Yang, S. Ficht, C. H. Wong, *J. Am. Chem. Soc.* **2006**, *128*, 5626–5627.
[16] A. Brik, S. Ficht, Y. Y. Yang, C. S. Bennett, C. H. Wong, *J. Am. Chem. Soc.* **2006**, *128*, 15026–15033.
[17] Y. Y. Yang, S. Ficht, A. Brik, C. H. Wong, *J. Am. Chem. Soc.* **2007**, *129*, 7690–7701.
[18] S. Ficht, R. J. Payne, A. Brik, C. H. Wong, *Angew. Chem.* **2007**, *119*, 6079–6083; *Angew. Chem. Int. Ed.* **2007**, *46*, 5975–5979.

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[19] R. J. Payne, S. Ficht, S. Tang, A. Brik, Y. Y. Yang, D. A. Case, C. H. Wong, *J. Am. Chem. Soc.* **2007**, *129*, 13527–13536.
[20] E. Saxon, J. I. Armstrong, C. R. Bertozzi, *Org. Lett.* **2000**, *2*, 2141–2143.
[21] J. M. Stewart, *Methods Enzymol.* **1997**, *289*, 29–44.
[22] L. E. Canne, S. M. Walker, S. B. H. Kent, *Tetrahedron Lett.* **1995**, *36*, 1217–1220.
[23] J. Kihlberg, M. Elofsson, L. A. Salvador, *Methods Enzymol.* **1997**, *289*, 221–245.
[24] D. A. Wellings, E. Atherton, *Methods Enzymol.* **1997**, *289*, 44–67.
[25] S. Biancalana, D. Hudson, M. F. Songster, S. A. Thompson, *Lett. Pept. Sci.* **2001**, *7(5)*, 291–297.
[26] R. von Eggelkraut-Gottanka, A. Klose, A. G. Beck-Sickinger, M. Beyermann, *Tetrahedron Lett.* **2003**, *44*, 3551–3554.
[27] S. Futaki, K. Tatsuto, Y. Shiraishi, Y. Sugiura, *Biopolymers* **2004**, *76*, 98–109.
[28] J. D. Warren, J. S. Miller, S. J. Keding, S. J. Danishefsky, *J. Am. Chem. Soc.* **2004**, *126*, 6576–6578.
[29] A. B. Clippingdale, C. J. Barrow, J. D. Wade, *J. Pept. Sci.* **2000**, *6*, 225–234.
[30] X. Z. Bu, G. Y. Xie, C. W. Law, Z. H. Guo, *Tetrahedron Lett.* **2002**, *43*, 2419–2422.
[31] X. Q. Li, T. Kawakami, S. Aimoto, *Tetrahedron Lett.* **1998**, *39*, 8669–8672.
[32] M. Lumbierres, J. M. Palomo, G. Kragol, S. Roehrs, O. Muller, H. Waldmann, *Chem. Eur. J.* **2005**, *11*, 7405–7415.
[33] D. Swinnen, D. Hilvert, *Org. Lett.* **2000**, *2*, 2439–2442.
[34] A. Sewing, D. Hilvert, *Angew. Chem.* **2001**, *113*, 3503–3505; *Angew. Chem. Int. Ed.* **2001**, *40*, 3395–3396.
[35] T. J. Hogenauer, Q. Wang, A. K. Sanki, A. J. Gammon, C. H. L. Chu, C. M. Kaneshiro, Y. Kajihara, K. Michael, *Org. Biomol. Chem.* **2007**, *5*, 759–762.
[36] J. A. Camarero, B. J. Hackel, J. J. de Yoreo, A. R. Mitchell, *J. Org. Chem.* **2004**, *69*, 4145–4151.
[37] G. W. Kenner, J. R. McDermot Jr. , R. C. Sheppard, *J. Chem. Soc. D* **1971**, 636–637.
[38] R. Ingenito, E. Bianchi, D. Fattori, A. Pessi, *J. Am. Chem. Soc.* **1999**, *121*, 11369–11374.
[39] R. Quaderer, D. Hilvert, *Org. Lett.* **2001**, *3*, 3181–3184.
[40] N. Ollivier, J. B. Behr, O. El-Mahdi, A. Blanpain, O. Melnyk, *Org. Lett.* **2005**, *7*, 2647–2650.
[41] Y. Shin, K. A. Winans, B. J. Backes, S. B. H. Kent, J. A. Ellman, C. R. Bertozzi, *J. Am. Chem. Soc.* **1999**, *121*, 11684–11689.
[42] S. Mezzato, M. Schaffrath, C. Unverzagt, *Angew. Chem.* **2005**, *117*, 1677–1681; *Angew. Chem. Int. Ed.* **2005**, *44*, 1650–1654.
[43] F. Mende, O. Seitz, *Angew. Chem.* **2007**, *119*, 4661–4665; *Angew. Chem. Int. Ed.* **2007**, *46*, 4577–4580.
[44] J. Alsina, T. S. Yokum, F. Albericio, G. Barany, *J. Org. Chem.* **1999**, *64*, 8761–8769.
[45] J. Brask, F. Albericio, K. J. Jensen, *Org. Lett.* **2003**, *5*, 2951–2953.
[46] A. Ishii, H. Hojo, Y. Nakahara, Y. Ito, Y. Nakaharai, *Biosci. Biotechnol. Biochem.* **2002**, *66*, 225–232.
[47] J. Tulla-Puche, G. Barany, *J. Org. Chem.* **2004**, *69*, 4101–4107.
[48] L. Z. Yan, P. Edwards, D. Flora, J. P. Mayer, *Tetrahedron Lett.* **2004**, *45*, 923–925.
[49] P. Wang, L. P. Miranda, *Int. J. Pept. Res. Therap.* **2005**, *11*, 117–123.
[50] Aspartic and glutamic acid thioesters can also be obtained, however, they are not synthetically useful due to the rapid formation of cyclic anhydrides under ligation conditions: M. Villain, H. Gaertner, P. Botti, *Eur. J. Org. Chem.* **2003**, 3267–3272. Lysine thioesters have been reported to form cyclic lactams: M. C. De Koning, D. V. Filippov, G. A. van der Marel, J. H. van Boom, M. Overhand, *Eur. J. Org. Chem.* **2004**, 850–857.
[51] F. Albericio, L. A. Carpino, *Methods Enzymol.* **1997**, *289*, 104–126.
[52] M. Villain, H. Gaertner, P. Botti, *Eur. J. Org. Chem.* **2003**, 3267–3272.
[53] Novabiochem Catalog 2006/2007.
[54] W. Jelkmann, *Physiol. Rev.* **1992**, *72*, 449–489.
[55] B. Wu, J. H. Chen, J. D. Warren, G. Chen, Z. H. Hua, S. J. Danishefsky, *Angew. Chem.* **2006**, *118*, 4222–4231; *Angew. Chem. Int. Ed.* **2006**, *45*, 4116–4125.
[56] B. Wu, Z. P. Tan, G. Chen, J. H. Chen, Z. H. Hua, Q. Wan, K. Ranganathan, S. J. Danishefsky, *Tetrahedron Lett.* **2006**, *47*, 8009–8011.
[57] J. H. Chen, G. Chen, B. Wu, Q. Wan, Z. P. Tan, Z. H. Hua, S. J. Danishefsky, *Tetrahedron Lett.* **2006**, *47*, 8013–8016.

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