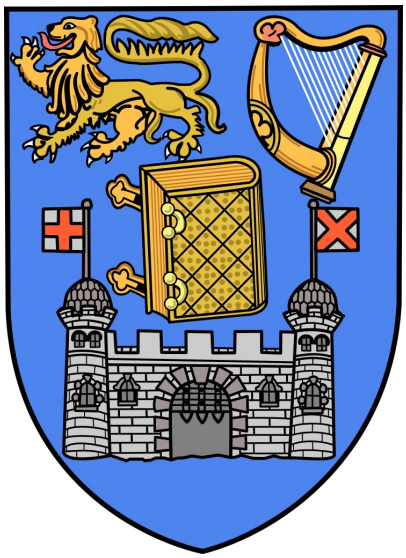
Synthesis and Evaluation of Removable Acyl Transfer Auxiliaries for Extended Chemical Ligation



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Abstract

The synthesis of two ring substituted 2-mercapto-2-phenylethyl acyl transfer auxiliaries is reported. We have demonstrated their utility in extended chemical ligation by assembling a 14 mer model peptide via cysteine-free auxiliary mediated ligation at a Gly-Gly ligation site. Finally we have tuned the auxiliary cleavage conditions to form the desired native peptide in excellent yields.

List of Schemes

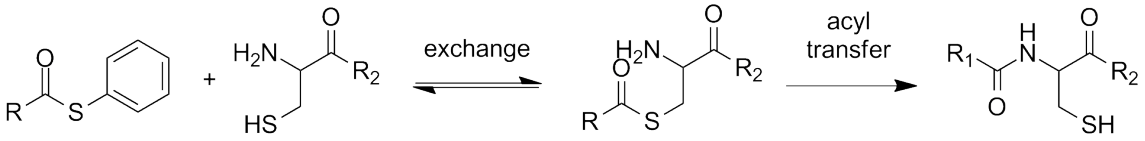
Introduction

Peptides are a ubiquitous class of biological molecules which mediate key processes in living organisms. They frequently display exquisitely selective activity for their targets.[1] This structure-dependent specificity makes them a promising target for drug development. Chemical biology in particular has benefited extensively from the development of techniques allowing access to non-native peptides.[2] Biological approaches such as recombinant peptide expression have provided some access to the material necessary for exploring protein function. However synthetic approaches allowing complete control over peptide sequence and post-translational functionalisation are fundamentally more appealing.

A synthetic approach to peptide synthesis first became practical with the introduction of solid phase peptide synthesis (SPPS) by Merrifield in the late 1960's.[3] This groundbreaking methodology allows for the construction of a native peptide backbone in a sequential, stepwise manner. This otherwise excellent technique is hampered by a limitation to sequence lengths of approximately 50 amino acid residues.[4] Beyond this point peptide aggregation and the accumulation of truncated side products begin to stretch the limits of synthetic accessibility.

1.1 Native Chemical Ligation

The development of the Native Chemical Ligation (NCL) approach by Kent has played a major role in overcoming the length restrictions inherent to SPPS. This elegant strategy facilitates the chemoselective ligation of two unprotected peptide fragments with the formation of a native peptide bond.[5]



Scheme 1.1 Native Chemical Ligation

Native Chemical Ligation allows for the coupling of a C-terminal peptide thioester and an N-terminal cysteine containing peptide over two steps. In an initial Capture step the peptide thioester undergoes a bimolecular thioester exchange to form a transient thioester linked ligation product. This intermediate can subsequently undergo an irreversible intramolecular S->N acyl transfer to form a native peptide containing the desired peptide bond.

Native chemical ligation has become an indispensable tool for peptide chemists. The synthesis of biologically important peptides such as the 203 amino acid HIV-1 protease enzyme [6] and the cyclic α-conotoxin MII [7] exemplify this technique.

1.2 Chemical Ligation at Non-Cysteine Sites

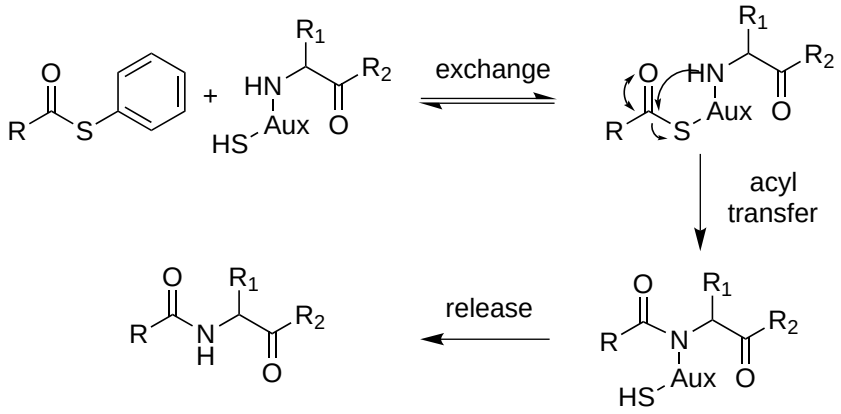
The requirement for a cysteine residue at the ligation site in NCL is a significant limitation. The low abundance of cysteine in many proteins reduces the likelihood of finding a site amenable to NCL in the desired peptide. Extensive research efforts has been directed towards overcoming this restriction and they have been met with some significant success.

1.2.1 Auxiliary Mediated Ligation

Kemp and coworkers focused their attention on an NCL-like intramolecular acyl transfer approach to peptide synthesis. [8] They developed the concept of introducing a removable thiol auxiliary at the N-terminal of the peptide (Scheme 1.2). The use of such a thiol auxiliary allows the excellent chemoselectivity of the NCL thioester exchange Capture step to be retained.

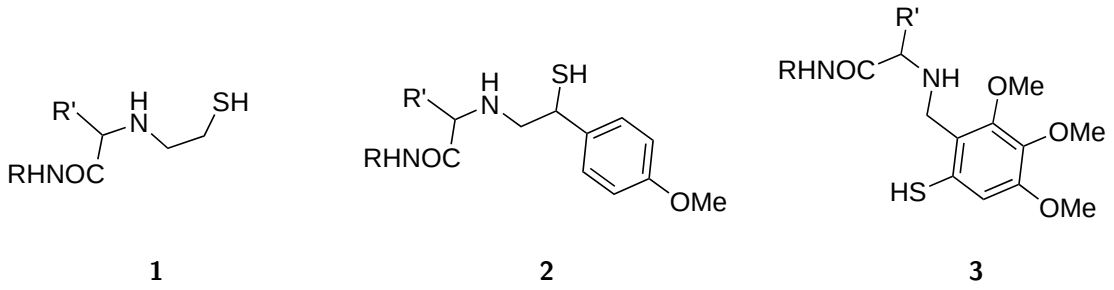
A synthetically useful thiol ligation auxiliary should fulfill a number of criteria. It must be efficiently introducible to the N-terminal peptide, it should be compatible with solid phase peptide synthesis,

it must facilitate ligation rapidly at low peptide concentrations and it must be selectively removable under mild conditions to provide a native peptide.[9]



Scheme 1.2 General mechanism of auxiliary-mediated peptide ligation

The N$^{\alpha}$-2-mercaptoethyl auxiliary **1** demonstrated the potential power of the auxiliary approach to creating amide bonds.[10] This auxiliary underwent rapid ligation and displayed a broad tolerance for many amino acids at the ligation junction. However this original auxiliary **1** was not removable from the ligated peptide.



1 **2** **3**

Fig. 1.1 A selection of previously described ligation auxiliaries

Low and coworkers further proved the utility of this approach with the total synthesis of cytochrome b562. This full-length 106 amino acid peptide was prepared from two peptide fragments via an auxiliary **2** mediated ligation at a His-Gly site.[11] Macmillan and Anderson introduced an improved acid-labile N$^{\alpha}$-2-mercaptobenzyl auxiliary **3**.[12] The trimethoxyphenyl substitution pattern increased the electron density on the ring which enhanced the acid liability of the auxiliary relative

to **2**.

The Danishefsky group employed **3** to good effect in the synthesis of a Human Paratyroid Hormone (hPTH) further demonstrating the synthetic utility of the auxiliary mediated ligation approach.

1.2.2 Desulfurisation Approaches to Cysteine-Free Ligation

An elegant alternative approach has been developed by Yan and Dawson. Their process consists of a standard NCL ligation at cysteine, followed by a desulfurisation step to yield alanine at the ligation site.[13] This extension of the NCL methodology is significant as alanine is rather more abundant than cysteine (6.3 % as opposed to 1.7 %).[14] Yan and Dawson validated their technique with the synthesis of a 56-amino acid streptococcal protein G B1 domain.[13]

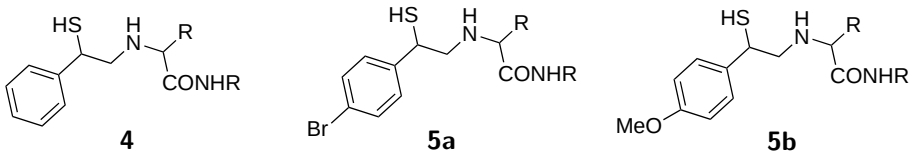
Danishefsky improved on this work by replacing Yan and Dawson's metal based thiol reduction, with a milder radical-mediated desulfurisation.[15] The trialkylphosphine tris(2-carboxyethyl)phosphine (TCEP) promoted desulfurisation greatly simplifies post cleavage peptide isolation by eliminating the problem of peptide adsorption on the metal surface. Danishefsky has demonstrated that the TCEP promoted desulfurisation is highly selective and tolerant of the glycosyl functionalities which are ubiquitous in biological peptides.[15]

1.3 2-mercapto-2-phenyl Auxiliaries

Previous work in this group has demonstrated the ability of the 2-mercapto-2-phenyl based auxiliary **4** to participate in an auxiliary mediated ligation to provide a ligated dipeptide linked by a peptide bond. The group has also demonstrated a novel TCEP promoted cleavage of auxiliary **4** to yield a ligated native peptide.

The first section of this work involves the synthesis of two acyl transfer auxiliaries **5a** and **5b**. It is hoped that a comparative analysis of these auxiliaries will help clarify the steric and electronic factors which influence the peptide ligation step.

With Danishefsky's desulfurisation work in mind, we tentatively propose that a radical-mediated

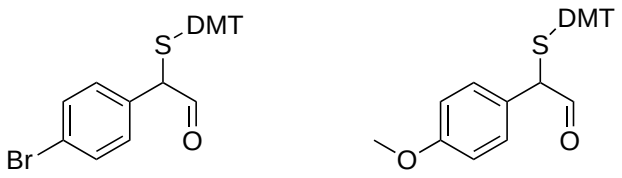


Scheme 1.3 Structure of proposed ligation auxiliaries

processes may be involved in the cleavage of this class of auxiliary. The -I inductive effects of the substituents on the proposed para substituted auxiliaries **5a** and **5b** should help stabilize the envisioned benzylic radical intermediate. As such we were keen to determine the effect that these substituents would have on the auxiliary cleavage process.

Results and Discussion

2.1 Synthesis of 2-mercapto-2-phenylacetaldehyde auxiliaries

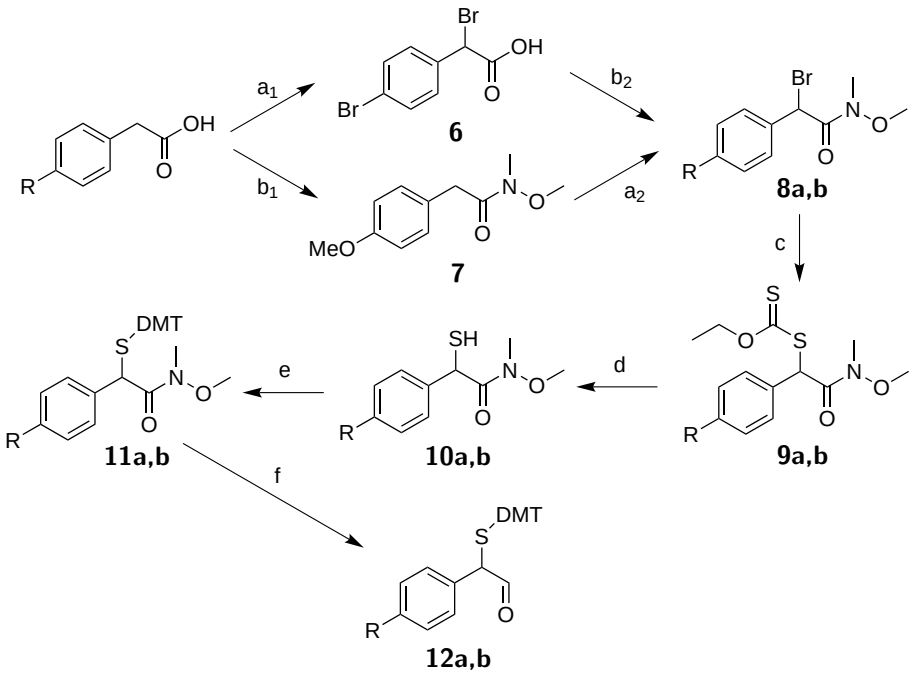


12a **12b**

Scheme 2.1 2-mercapto-2-phenylacetaldehyde ligation auxiliaries

In the initial part of this project we synthesized a set of two S-protected 2-mercapto-2-phenylacetaldehyde auxiliaries **13a** and **12b** starting from commercially available phenylacetic acids via a 6-step route (Scheme 2.2). Both auxiliaries were prepared successfully with good overall yields.

The auxiliaries were synthesised bearing an acid-labile 4,4'-dimethoxytrityl (DMT) thiol protecting group to prevent thioacetal formation. The DMT protecting group was selected as it is rapidly removed under the acidic conditions employed to cleave the auxiliary-peptides from the SPPS support resin.



Scheme 2.2 Key: (a_1) N-bromosuccinimide, irradiation, DCM, (71 %); (a_2) N-bromosuccinimide, AIBN, DCM, (83 % crude); (b_1) N,O-dimethylhydroxylamine, EDC · HCl, Et_3N, dry DCM, (90 % crude); (b_2) N,O-dimethylhydroxylamine, EDC.HCl, Et_3N, dry DCM, (90 % crude); (c) Potassium ethyl xanthate, acetone, (R=Br, 85 %), (R=OMe, 48 % for 2 steps); (d) piperidine, DCM, (R=Br, 41 %), (R=MeO, 73 %); (e) 4,4'-dimethoxytrityl chloride, Et_3N, dry DCM, (R=Br, 84 %), (R=OMe, 79 %); (f) $LiAlH_4$, THF, (R=Br, 60 %), (R=OMe, 78 %);

The initial benzylic bromination of 4-bromophenylacetic proceeded cleanly to provide **6**. However attempts to synthesize 2-bromo-2-(4-methoxyphenyl)acetic acid under identical conditions failed with the formation of a complex mixture of products. Efforts to perform the radical bromination on the 4-methoxyphenylacetic acid substrate under milder conditions (NBS, AIBN, 30 °C) also yielded a complex mixture of products which were not readily purifiable by flash chromatography.

A literature review indicated that 4-methoxyphenylacetic acid can undergo rapid decarboxylation to produce a benzylic radical via an intermediate aromatic radical cation [16]. To avoid this decomposition pathway, the route to **8b** was modified to first form the Weinreb amide **7** as amides cannot the undergo the undesired decarboxylation process. A subsequent AIBN promoted benzylic bromination on the amide afforded **8b** which was used without further purification. The formation

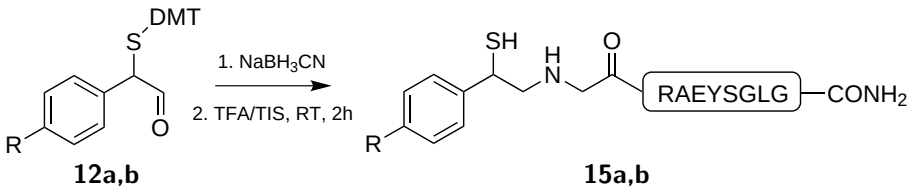
of a Weinreb amide before radical bromination is likely to provide the most consistent results when synthesizing future substituted 2-mercapto-2-phenylacetaldehyde auxiliaries.

The subsequent steps to the protected auxiliaries **12a** and **12b** proceeded smoothly and both compounds were obtained as red crystalline solids in acceptable yields overall (**12a**=12 %, **12b**=18 %).

2.2 Synthesis of the model peptide

The model peptide **14** with sequence H−GRAEYSGLG−NH_2 was synthesised on an automated peptide synthesiser by standard Fmoc/t-Bu based SPPS on Tentagel Rink amide PEG resin. A N-terminal glycine site was selected for our initial research in order to minimize the potential for side chain interface during ligation or auxiliary removal.

2.3 Introduction of ligation auxiliaries to the model peptide



12a,b **15a,b**

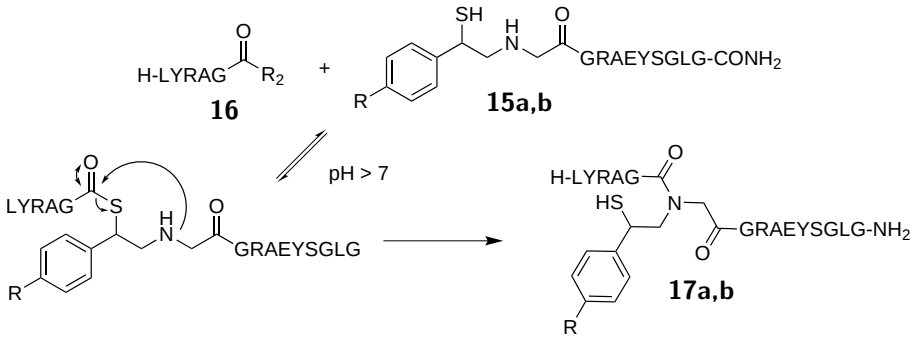
Scheme 2.3 Reductive amination of auxiliary to peptide **14** on solid support.

The auxiliary aldehydes **12a** and **12b** were coupled to the N-terminus of the resin-supported model peptide **14** under unoptimized reductive amination conditions (Scheme 2.3). The auxiliary-peptides were subsequently cleaved from the resin (95:5 TFA/TIS; 2 h), precipitated from cold ether and purified by preparative HPLC.

The desired unprotected auxiliary peptides **15a,b** were isolated as white powders in acceptable yields (**15a**=7.77 %, **15b**=24.4 %) (Fig. A.1). It may be advantageous to introduce the auxiliary to the peptide via a two step approach. The auxiliary would initial be reductively aminated to the desired N-termni amino acid in solution.[17]. The auxiliary-amino acid could then be coupled to the peptide N-terminus under proven peptide coupling conditions.

2.3.1 Peptide Ligation

With the desired auxiliary-peptides in hand, we began to examine their ability to facilitate peptide ligation. We selected an exemplar peptide thioester **16** (H-LYRAG-MPA-G-NH2) for all our ligations. This thioester in used as a model across many NCL studies [18] and consequently it was available within our research group.



Scheme 2.4 Ligation of auxiliary-peptide and thioester.

The ligations of auxiliary-peptides **15a** and **15b** with the alkyl peptide-thioester **16** were carried out under standard NCL conditions (100 mM Na_2HPO_4, pH 7.5, RT) in degassed phosphate ligation buffer. The reducing agent TCEP was added to the ligation buffer at 100 mM concentration to prevent disulfide bond formation. Additionally 3 % thiophenol was introduced to promote the *in situ* conversion of our alkyl thioester to a more reactive aryl thioester [19]. Auxiliary peptide solubility issues were mitigated by the use of denaturating aqueous guanidine as the ligation solvent.

Ligation of Auxiliary Peptide 15a

UPLC analysis shows that the ligation of **15a** and the model thioester occurs rapidly with almost complete consumption of the auxiliary peptide observed within 30 min. A small excess of **15a** was used in this ligation and as a result the excess auxiliary is observable at the 150 min point in Fig. 2.1.

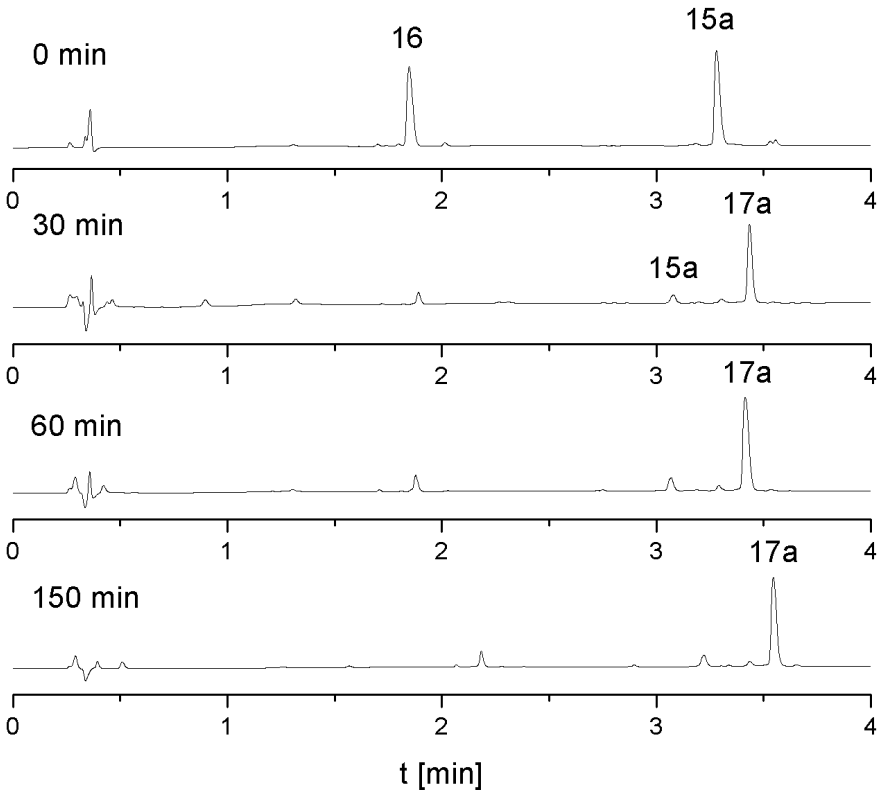


Fig. 2.1 UPLC of the time-course for the ligation of 4-bromophenyl auxiliary-peptide **15a**

Ligation of Auxiliary Peptide 15b

The ligation of auxiliary peptide **15b** again occurred rapidly. The ligation was highly selective under the experimental conditions with complete conversion of **15b** to the desired ligated peptide **17b**. The other peptide fragments in Fig. 2.2 arise from the presence of excess thioester **16** post-ligation. The thiophenol aryl thioester **18** is formed *in situ*. The ligated auxiliary peptide **17b** can undergo a second thioester exchange in the presence of the *in situ* generated thiophenol aryl thioester **18** to form **19**.

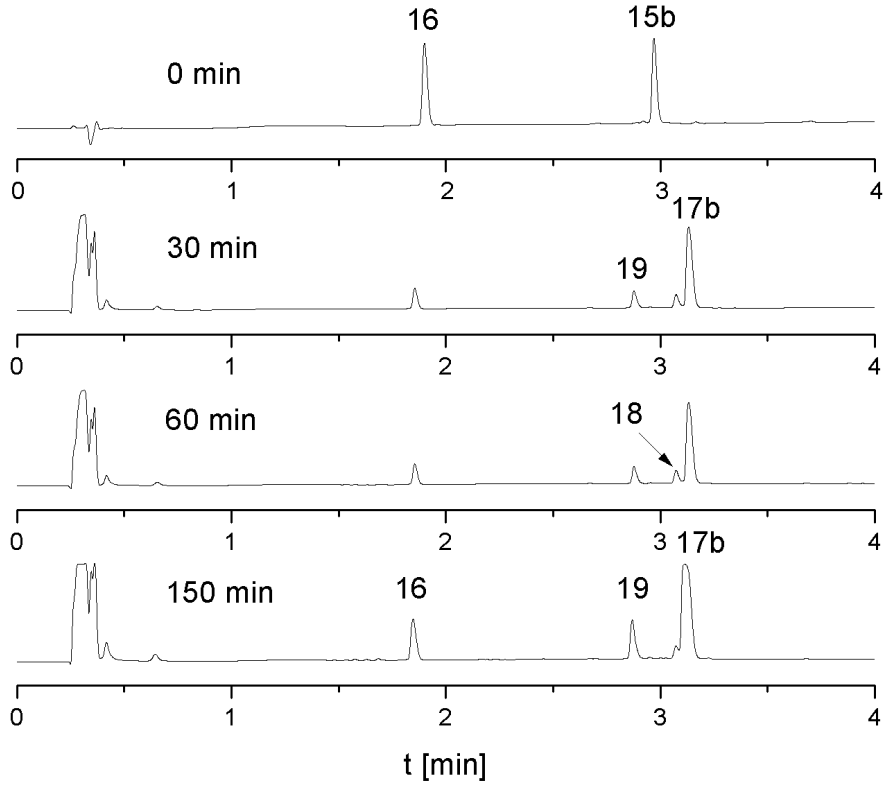


Fig. 2.2 UPLC of the time-course for the ligation of 4-methoxyphenyl auxiliary-peptide **15b**

Both ligation products were purified by preparative HPLC to yield the ligated auxiliary peptides **17a** (83 % yield) and **17b** (87 % yield) as white solids.

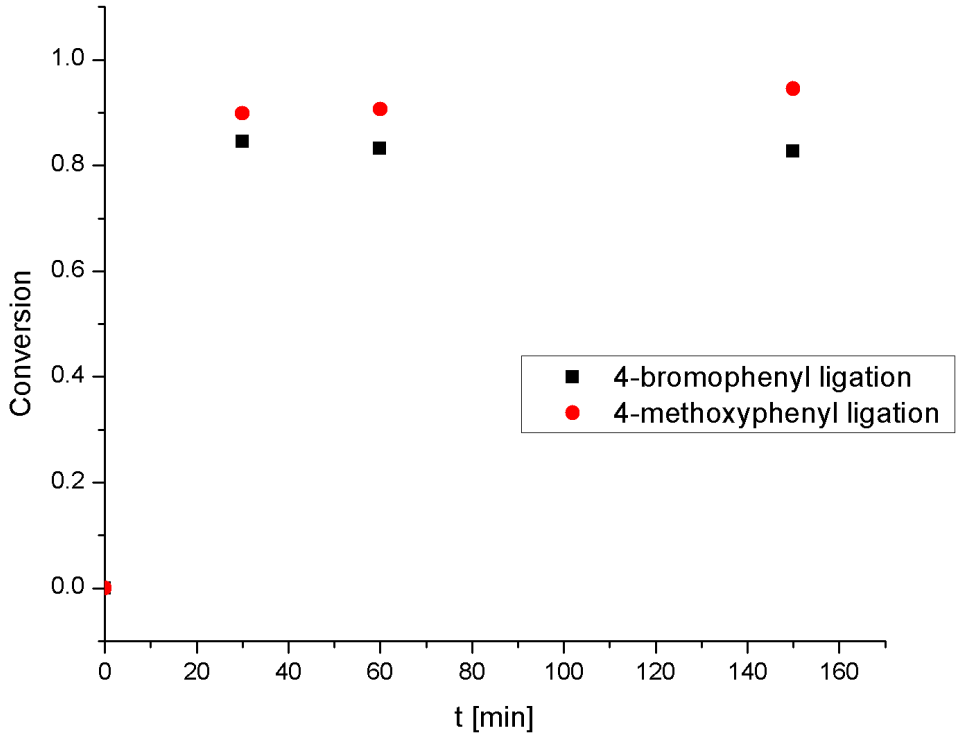


Fig. 2.3 Rate of ligation for both auxiliary peptides at the model Gly-Gly site.

It is clear that both auxiliaries can promote rapid, selective peptide ligation at a Gly-Gly junction. These results compare favorably to those obtained by Macmillan and Anderson with their acid labile auxiliary **3** at the same junction[12].

Further trials at other more challenging ligation sites should be performed before significant comparisons made between the ligation effectiveness of our two auxiliaries.

2.3.2 Auxiliary Cleavage

The ligation auxiliaries must be removable if they are to be synthetically useful for peptide synthesis. To begin, we subjected **17a** to the aqueous TCEP promoted cleavage conditions (100 mM TCEP, 400 mM morpholine, 50 °C) previously examined in our group. We were pleasantly surprised to observe 73 % conversion to the native peptide **20** (H-LYRAGGRAEYSGLG-NH_2) within 24 h. This positive result was marred by the observation of the peptide backbone cleavage product **21** (N-formyl-GRAEYSGLG-NH_2) as a side product (Fig. A.2).

In the hopes of minimizing peptide degradation we reexamined the cleavage of **17a** at room temperature with a lower TCEP concentration (20 mM). However these milder cleavage conditions lead to a large reduction in the rate of auxiliary removal. UPLC analysis indicated just 26 % conversion after 24 h (Fig. 2.4).

At this point, we reflected on Wan and Danishefsky's free radical based, TCEP promoted selective cysteine desulfurization[15]. With that work in mind, we hypothesised that the cleavage may be proceed proceeding via a rate limiting bimolecular TCEP promoted desulfurization step. A subsequent room temperature cleavage in the presence of 400 mM TCEP provided excellent results. UPLC analysis indicated complete consumption of the ligated auxiliary peptide **17a** after 4 h. The native peptide **20** was observed as the almost exclusive peptide product after 24 h (Fig. A.3).

Significantly a fragment of mass 282.19 Da was observed indicating the formation of the phosphoranyl species 3,3',3"-phosphorothionyltripropanoic acid (M_{calc}. 282.03 Da). These results suggest TCEP promoted desulfurization is a rate controlling step during the auxiliary cleavage.

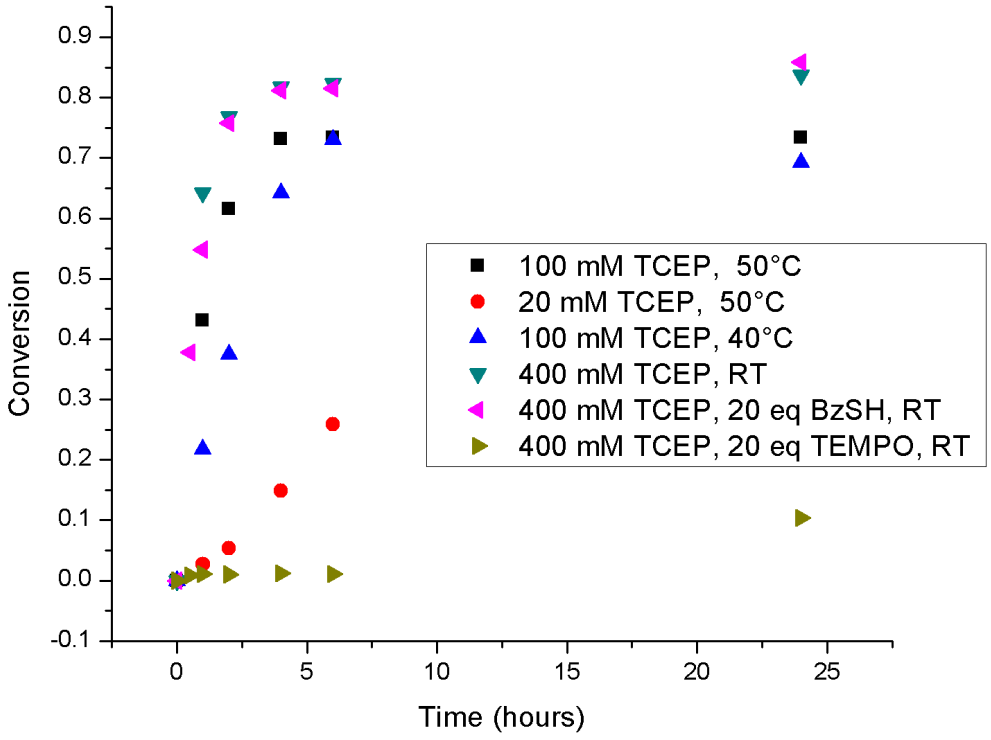


Fig. 2.4 Influence of cleavage conditions on the rate of auxiliary **17a** removal

It is notable that negligible product formation was observed when the stable free radical TEMPO (20 eq.) was added to the cleavage mixture (Fig. 2.4). This result, while not conclusive, does further support our hypothesis that a radical process is in play.

We proceeded to repeat a subset of these successful cleavage experiments on our second auxiliary-peptide **17b**. Again we observed rapid cleavage of our ligation auxiliary to provide the desired peptide **20**. The conversion was found to be somewhat higher with the (4-methoxyphenyl) bearing **17b** as shown in Fig. 2.5.

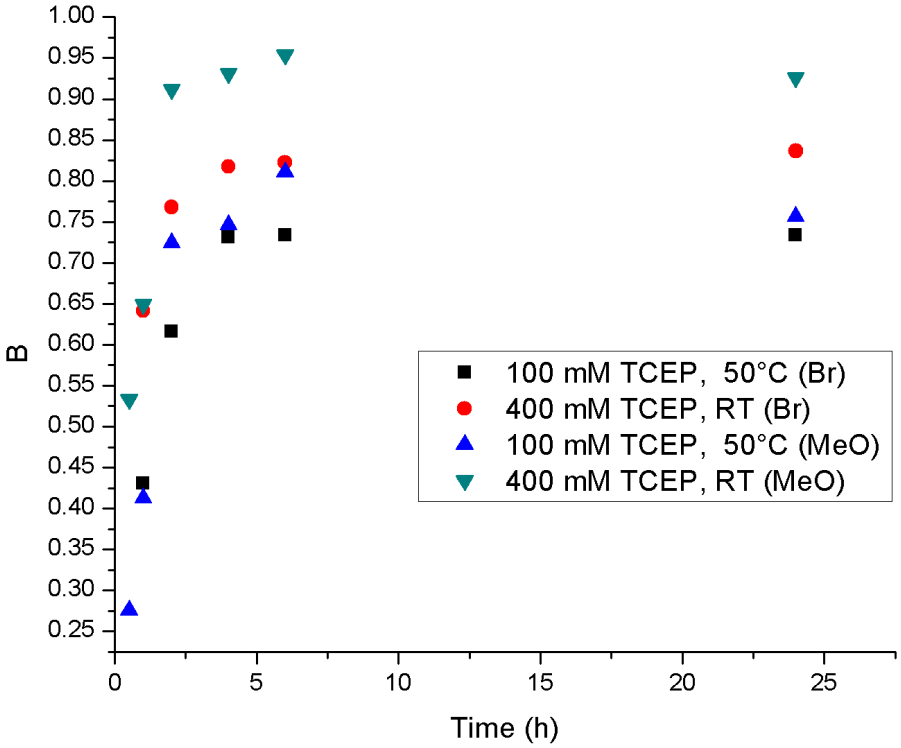


Fig. 2.5 Comparison of the cleavage rate for both auxiliary peptides.

17b was subjected to the standard cleavage conditions (100 mM TCEP, 50 °C) in the presence of the water-soluble radical initiator VA-044 (2eq. and 20 eq.). The radical initator producer a

significant increased in the rate of cleavage (Fig. A.5). However the final yield of the native peptide was impacted by the extensive formation of an N-methylated peptide side product **22** (up to 35 %) and by desulfurisation of the auxiliary to give **23** (Fig. A.4).

The radical initiator may be promoting the formation of a benzyl radical before intermolecular TCEP promoted desulfurization has had an opportunity to occurred. Subsequent C-C bond scission would result in the formation of the observed N-methylated peptide product. We suspect that further work will allow the rates of intermolecular desulfurisation and radical formation to be matched. This may allow us to take advantage of the radical initator provided rate increases while minimising the production of unwanted peptide degradation side products.

Conclusions and Further Work

In this project we successfully synthesized two phenyl substituted ligation auxiliaries via a six-step route from commercially available starting materials. We then demonstrated that both compounds can effectively facilitate auxiliary-mediated ligation at a Gly-Gly site in a model peptide system. Finally we showed that both auxiliaries can be cleaved rapidly under mild conditions. In doing so we synthesized a 14-mer cysteine-free peptide. Our auxiliary system can be removed under neutral conditions which is a major improvement on the HF and TFA conditions required for the removal of the previously reported auxiliaries **2** and **3**.

Future work could focus on tuning the auxiliary substituent groups in an effort to enhance the rate and selectiveness of the cleavage step. It would be illuminating to investigate the effect of substituents with a large -I effect as they should exert a stabilizing effect on the proposed benzylic radical intermediate.

The characteristic isotope mass spectra of bromine may allow for the identification of auxiliary cleavage fragments from the cleavage of auxiliary **15a**. A more complete understanding of the cleavage mechanism will help focus further research on this class of ligation auxiliaries.

Experimental Methods

Materials and Methods

All commercial material (Acros, Fluka, Sigma Aldrich) were used without purification. All solvents were reagent grade or HPLC grade. All reactions were performed under an atmosphere of argon. NMR spectra (^{1}H and ^{13}C) were recorded on a *Bruker Advance II 300* or *Advance II 500*, referenced to TMS or residual solvent. Analytical TLC was performed on E. Merck silica gel 60 F254 plates and flash column chromatography was performed on silica gel 60 from *Macherey Nagel*.

Preparative HPLC separations were performed on an *Agilent 1100 Series* HPLC equipped with a Nucleodur C18 Gravity (5 µm) and Nucleosil 300-7 C4 columns from *Macherey and Nagel* at a flow rate of 15 mL/min. The mobile phase was a binary mixture of A (98.9 % water, 1 % acetonitrile, 0.1 % TFA) and B (98.9 % acetonitrile, 1 % water, 0.1 % TFA) with a gradient of 3 % to 45 % in 30 min.

UPLC analysis was performed on a *Acquity* UPLC from *Waters* and a BEH300 C18 column (50 x 2.1 mm, 1.7 µm with a flow rate of 0.6 mL/min. A gradient of 3 % to 40 % in 6 min unless otherwise noted. The yield of all isolated peptides were determined via absorption measurements at 280 nm on a *NanoDrop 2000* UV-Vis spectrophotometer.

4.1 Synthesis of model peptide H-GRAEYSGLG-NH_2 by Fmoc-SPPS

The model peptide H-GRAEYSGLG-NH_2 **14** was synthesised by standard Fmoc-based solid phase peptide synthesis protocol on an Applied Biosystems Peptide Synthesizer. The peptide was sup-

ported on Tentagel Rink amide resin.

4.2 2-bromo-2-(4-bromophenyl)acetic acid (24)

A suspension of 2-(4-bromophenyl)acetic acid (10.6 g, 46.5 mmol) and N-bromosuccinimide (9.932 g, 55.8 mmol) in CCl_4 was irradiated under refluxed with a 240 W tungsten lamp.

An additional 0.5 eq of NBS was added after 3 h and the reaction was maintained at reflux for a further 3 h, at which point the mixture was cooled, filtered, and the concentrated *in vacuo*. The residual orange solid was purified by flash column chromatography (2:1 cyclohexane:ethyl acetate with 1 % formic acid). Residual formic acid was removed by co-evaporation with ethyl acetate to yield **24** (9.733 g, 33.1 mmol, 71 %) as an off-white crystalline solid.

^{1}H-NMR (500 MHz, $CDCl_3$): δ (ppm) = 7.52 (d, J = 8.6 Hz, 2H, ArH), 7.44 (d, J = 8.5 Hz, 2H, ArH), 5.31 (s, 1H, CBrH). ^{13}C-NMR (125 MHz, $CDCl_3$): δ (ppm) = 172.17, 134.24, 132.37, 130.63, 124.14, 44.97.

4.3 N-methoxy-2-(4-methoxyphenyl)-N-methylacetamide (7)

To a stirred solution of 2-(4-methoxyphenyl)acetic acid (1.662 g, 10 mmol) in dry DCM (50 ml) under argon at 0 °C was added N,O-dimethylhydroxylamine hydrochloride (0.975 g, 10 mmol) and triethylamine (1.4 ml, 10 mmol) followed by 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (2.013 g, 10.5 mmol) in portions over 20 min. The mixture was maintained at 0 °C for 15 min and allowed to rise to room temperature.

After 1 h the reaction mixture was washed with aq. HCl (1 M, 3x50 ml), sat. $NaHCO_3$ (3x50 ml), brine (1x100 ml), dried over $MgSO_4$ and the solvent removed *in vacuo* to yield **7** (1.7061 g, 8.15 mmol, 82 %) as a clear yellow oil which was not further purified.

4.4 2-bromo-2-(4-bromophenyl)-N-methoxy-N-methylacetamide (8a)

To a stirred solution of **6** (4.409 g, 15 mmol) in dry DCM (120 ml) under argon at 0 °C was added N,O-dimethylhydroxylamine hydrochloride (1.463 g, 15 mmol) followed by 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (3.019 g, 15.75 mmol) in portions over 15 min. Triethylamine (3.14 ml, 22.5 mmol) was then added and the mixture was allowed to rise to room temperature after stirring at 0 °C for a further 15 min.

After 1 h the reaction mixture was washed with aq. HCl (1 M, 3x50 ml), sat. $NaHCO_3$ (3x50 ml), brine (1x50 ml) and dried over $MgSO_4$. The solvent was removed *in vacuo* to yield **8a** (4.586 g, 13.6 mmol, 91 % crude yield) as a clear yellow oil which was not further purified.

4.5 2-bromo-2-(4-methoxyphenyl)-N-methoxy-N-methylacetamide (8b)

To a solution of **7** (0.837 g, 4 mmol) in CCl_4 (20 ml) was added N-bromosuccinimide (1.068 g, 6 mmol) and azobisisobutyronitrile (0.067 g, 0.4 mmol). The reaction was stirred overnight at 40 °C, washed with water (2x20 ml), brine (20 ml), dried over $MgSO_4$ and the solvent removed *in vacuo* to yield **8b** (0.962 g, 3.34 mmol, 83 % crude) as a clear yellow oil which was not further purified.

4.6 S-(1-(4-bromophenyl)-2-(methoxy(methyl)amino)-2-oxoethyl) O-ethyl carbonodithioate (9a)

A solution of **8a** (4.586 g, 13.6 mmol) and potassium ethyl xanthate (2.180 g, 13.6 mmol) in acetone (100 ml) was stirred at room temperature for 3 h. The solvent was removed *in vacuo* to yield a yellow oil which was taken up in water, extracted with DCM (2x50 ml), the organics dried over $MgSO_4$ and the solvent removed *in vacuo* to yield a yellow oil which formed a crystalline solid on standing overnight. The solid was purified by column chromatography (4:1 cyclohexane:ethyl acetate) to yield **9a** as a white crystalline solid (4.3523 g, 11.5 mmol, 84.5 %) on standing.

^{1}H-NMR (500 MHz, $CDCl_3$): δ 7.47 (d, J = 8.5 Hz, 2H, ArH) 7.35 (d, J = 8.6, 2H, ArH), 6.04 (s, 1H, CHS), 4.61 (q, J = 7.1 Hz, 2H, OCH2), 3.63 (s, 3H, OCH3), 3.21 (s, 3H, NCH3), 1.40 (t, J =

7.1 Hz, 3H, CCH3).

^{13}C-NMR (125 MHz, $CDCl_3$): δ 132.38, 130.94, 70.77, 61.92, 55.65, 46.24, 33.40, 14.16.

4.7 S-(1-(4-methoxyphenyl)-2-(methoxy(methyl)amino)-2-oxoethyl) O-ethyl carbonodithioate (9b)

To **8b** (0.962 g, 3.33 mmol) in acetone (20 ml) at 0 °C were added potassium ethyl xanthate (0.587 g, 3.66 mmol) in portions over 15 min. The stirred reaction was allowed to rise to R.T., and after 3 h the solvent was removed *in vacuo* to yield a yellow oil. This oil was taken up in DCM, washed with water (2x50 ml), brine and dried over $MgSO_4$ to yield a yellow oil which was purified by flash chromatography (4:1 cyclohexane:ethyl acetate) to yield **9b** (0.5728 g, 1.90 mmol, 48 % overall from **7**) as a yellow oil.

^{1}H-NMR (500 MHz, $CDCl_3$): δ 7.34-7.28 (m, 2H, ArH), 6.82-6.76 (m, 2H, ArH), 5.97 (s, 1H, CHS), 4.54 (q, J = 7.1 Hz, 2H, OCH2), 3.72 (s, 3H, OCH3), 3.52 (s, 3H, NCH3), 3.15 (s, 3H, OCH3), 1.33 (t, J = 7.1 Hz, 3H, CCH3).

^{13}C-NMR (125 MHz, $CDCl_3$): δ 159.67, 130.08, 114.28, 70.08, 61.44, 55.29, 26.92, 13.77.

4.8 General Procedure for 10:

2-(4-methoxyphenyl)-2-mercapto-N-methoxy-N-methylacetamide (10b)

Piperidine (0.2 ml, 1.93 mmol) was added drop-wise to a solution of **9b** (0.5757 g, 1.75 mmol) in dry degassed DCM under argon on a ice/water bath. An additional 0.4 eq of piperidine was added after 1 h.

HCl (1 M, 50 ml) was added after a further hour, the organics separated, washed with HCl (1 M, 2x25 ml), brine, dried over $MgSO_4$, and evaporated *in vacuo*. The residue was purified by flash chromatography (4:1 cyclohexane:ethyl acetate with 1 % formic acid) to yield **10b** (0.3172 g, 1.291 mmol, 73 %) as a yellow oil.

^{1}H-NMR (500 MHz, $CDCl_3$): δ 7.33-7.25 (m, 2H, ArH), 6.83-6.75 (m, 2H, ArH), 5.09 (d, J = 8.7 Hz, 1H, CHS), 3.72 (s, 3H, OCH3), 3.47 (s, 3H, NCH3), 3.14 (s, 3H, OCH3).

^{13}C-NMR (125 MHz, $CDCl_3$): δ 159.13, 128.93, 114.15, 109.98, 61.50, 55.31, 42.21, 32.81.

2-(4-bromophenyl)-2-mercapto-N-methoxy-N-methylacetamide (10a)

Product **10a** obtained as a white crystalline solid (41 %).

4.9 General Procedure for 11:

2-(4-bromophenyl)-N-methoxy-N-methyl-2-(4,4'-dimethoxytritylthio)acetamide (11a)

To a stirred solution of **10a** (1.069 g, 3.68 mmol) in DCM was added 4,4'-dimethoxytrityl chloride (1.372 g, 4.05 mmol) followed by triethylamine (0.56 ml, 4.05 mmol). After 1 h an additional 0.2 eq of both $4,4'-DMT \cdot Cl$ and triethylamine were added. After a further 30 min the organics were washed with water (2x70 ml), dried over $MgSO_4$, and evaporated *in vacuo*. The crude was purified by flash column chromatography (4:1 cyclohexane:ethyl acetate + 0.5 % DMEA) to yield **11a** as a fine white crystalline solid (1.8419 g, 3.1 mmol, 85 %).

^{1}H-NMR (500 MHz, $CDCl_3$): δ Ît 7.34-7.27 (m, 2H, ArH), 7.29-7.04 (m, 8H, ArH), 6.89-6.83 (m, 2H, ArH), 6.80-6.72 (m, 1H, ArH), 6.70-6.63 (m, 4H, ArH), 4.49 (s, 1H, CHS), 3.70 - 3.72 (m, 6H, OCH3), 3.19 (s, 3H, NCH3), 2.96 (s, 3H, OCH3).

^{13}C-NMR (125 MHz, $CDCl_3$): δ 158.17, 131.34, 131.03, 130.95, 130.11, 129.61, 127.83, 113.02, 77.22, 55.27. The DMT protecting group introduced a complex set of signals which were not readily resolvable.

2-(4-methyoxyphenyl)-N-methoxy-N-methyl-2-(4,4'-dimethoxytritylthio)acetamide (11b)

Product **11b** (0.5517 g, 1.02 mmol, 79 %) was obtained as a straw yellow crystalline solid.

^{1}H-NMR (500 MHz, $CDCl_3$): δ 7.36-7.27 (m, 2H, ArH), 7.24-7.20 (m, 4H, ArH), 7.18-7.13 (m, 2H, ArH), 7.13-7.07 (m, 2H, ArH), 7.00-6.91 (m, 2H, ArH), 6.82-6.73 (m, 1H, ArH), 6.72-6.62 (m, 6H,

ArH), 4.51 (s, 1H, CHS), 3.72-3.70 (m, 6H, OCH3), 3.69 (s, 3H, OCH3), 3.13 (s, 3H, NCH3), 2.91 (s, 3H, OCH3).

4.10 General Procedure for 12

2-(4-bromophenyl)-2-(4,4'-dimethoxytritylthio)acetaldehyde (12a)

A solution of $LiAlH_4$ (3 M in THF, 220 µl) was added drop-wise over the course of 10 min to a stirred solution of the amide **25** (1.068 g, 3.68 mmol) in dry THF (30 ml) under argon while cooling in a dry ice/isopropanol bath.

The reaction was quenched after 30 min by addition of $NaHSO_4$ 1 M, 30 ml) drop-wise at first, followed by DCM (100 ml) and water (50 ml. The organics were separated, washed with $NaHSO_4$ solution (1 M, 50 ml) and the combined aqueous washes were back extracted with DCM (20 ml). The combined organics were dried over $MgSO_4$ and the solvent evaporated *in vacuo* to yield a yellow oil which was purified by flash chromatography (4:1 cyclohexane:ethyl acetate with 0.5 % DMEA) to yield the protected auxiliary **12a** (0.2112 g, 0.40 mmol, 61 %) as a red crystalline solid.

2-(4-methoxyphenyl)-2-(4,4'-dimethoxytritylthio)acetaldehyde (12b)

Protected auxiliary **12b** (0.1884 g, 0.389 mmol, 78 %) was obtained as a red crystalline solid.

4.11 General Procedure: Introduction of auxiliary onto model peptide 14 (15)

To auxiliary **12** (15 eq) in 350 µl of an NMP:i-PrOH:AcOH (4:1 and 5 %) solution was added $NaBH_3CN$ (15 eq). This reductive amination mixture was shaken with peptide **14** (7 µmol) on solid support for 6 h. The peptide was subsequently treated with the cleavage mixture (95:5 TFA:TIS; 2 ml) for 2 h, precipitated from cold Et_2O and centrifuged at 20000 RPM for 20 min. The crude was purified by preparative RP-HPLC to give the auxiliary-peptide **15**.

(Br-Aux)-GRAEYSGLG-NH_2 (15a):, observed mass 1124.45 Da, calculated $(M+H)^+$ 1122.4

Da, ε_{280} 1961 M^{-1} cm^{-1}, 7.77 % yield.

(MeO-Aux)-GRAEYSGLG-NH_2 (15b):, observed mass 1074.56 Da, calculated $(M+H)^+$ 1074.5 Da, ε_{280} 2450 M^{-1} cm^{-1}, 24.4 % yield.

4.12 General Procedure: Peptide Ligation

Auxiliary peptide **15** (1 eq) and peptide thioester **16** (1 eq) were dissolved in degassed ligation buffer (10 % CH_3CN, 100 mM TCEP, 100 mM Na2HPO4, 3 % thiophenol) which had been adjusted to pH 7.5 with 2 M NaOH. The reaction mixture was shaken at room temperature until UPLC analysis indicated complete consumption of the auxiliary peptide. The auxiliary dipetide was purified by preparative RP-HPLC.

LYRAG-(Br-Aux)-GRAEYSGLG-NH_2 (17a):, observed mass 842.97 Da, calculated $(M+2H)^{2+}/2$ 841.9 Da, ε_{280} 3241 M^{-1} cm^{-1}, 83 % yield.

LYRAG-(MeO-Aux)-GRAEYSGLG-NH_2 (17b): observed mass 817.98 Da, calculated $(M+2H)^{2+}/2$ 817.9 Da, ε_{280} 3730 M^{-1} cm^{-1}, 83 % yield.

4.13 General Procedure: Auxiliary Cleavage

A solution of the ligated auxiliary peptide **17** (30 nmol) in degassed aqueous cleavage solution (20-400 mM TCEP, 80-1600 mM morpholine, 60 µl) was shaken in a 200 µl Eppendorf tube at the experimental temperature for 24 h. The reaction progress was followed by UPLC analysis. Conversion to the native peptide was evaluated by calculating the relative area of the native peptide peak to the areas of the native peptide and peptide side product peaks. The differing molar absorption coefficients for the products at 280 nm were taken into account.

H-LYRAGGRAEYSGLG-NH_2 (20): The formation of the ligated native peptide was observed after auxiliary cleavage from both **17a** and **5c**. ε_{280} 2560 M^{-1} cm^{-1}.

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Appendix

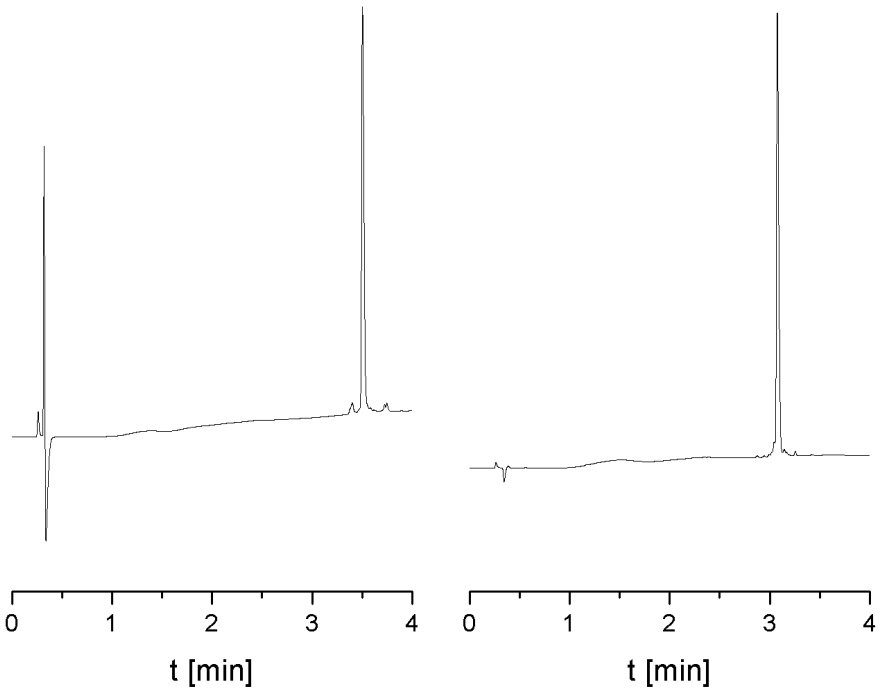

Fig. A.1 UPLC trace of 4-bromophenyl (**15a**) and 4-methoxyphenyl (**15b**) auxiliary peptides

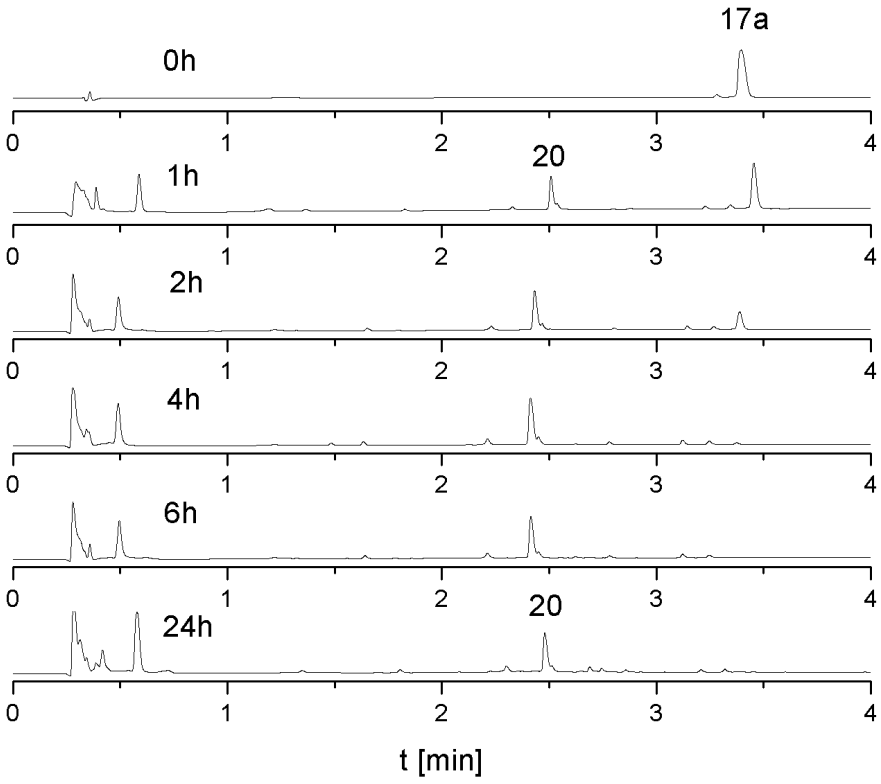

Fig. A.2 UPLC trace for the cleavage of **17a** (100 mM TCEP, 50 °C)

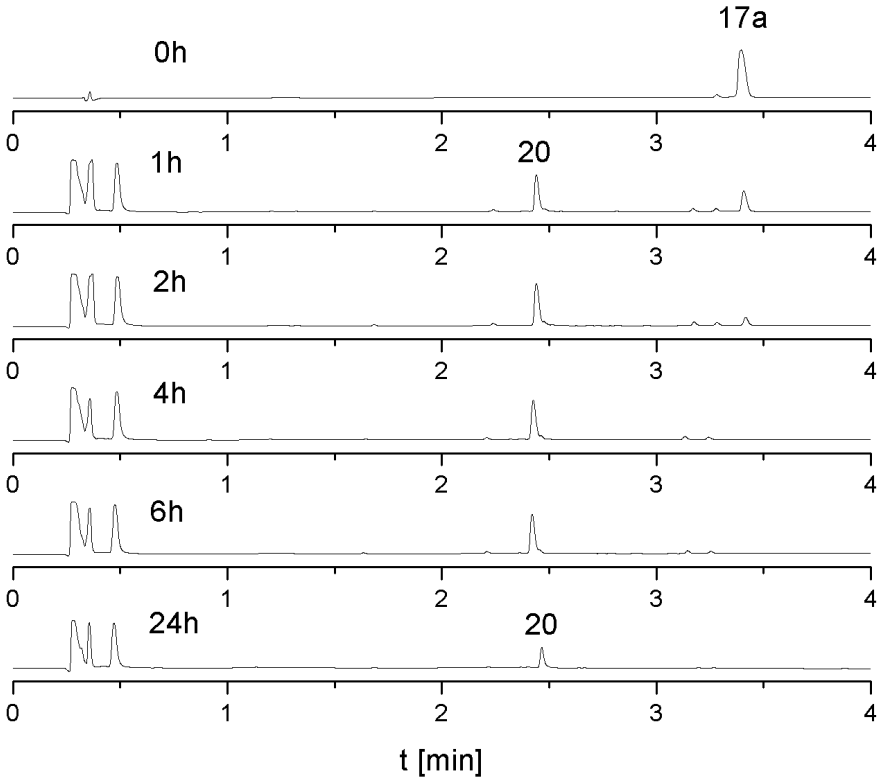

Fig. A.3 UPLC trace for the cleavage of **17a** (400 mM TCEP, R.T.)

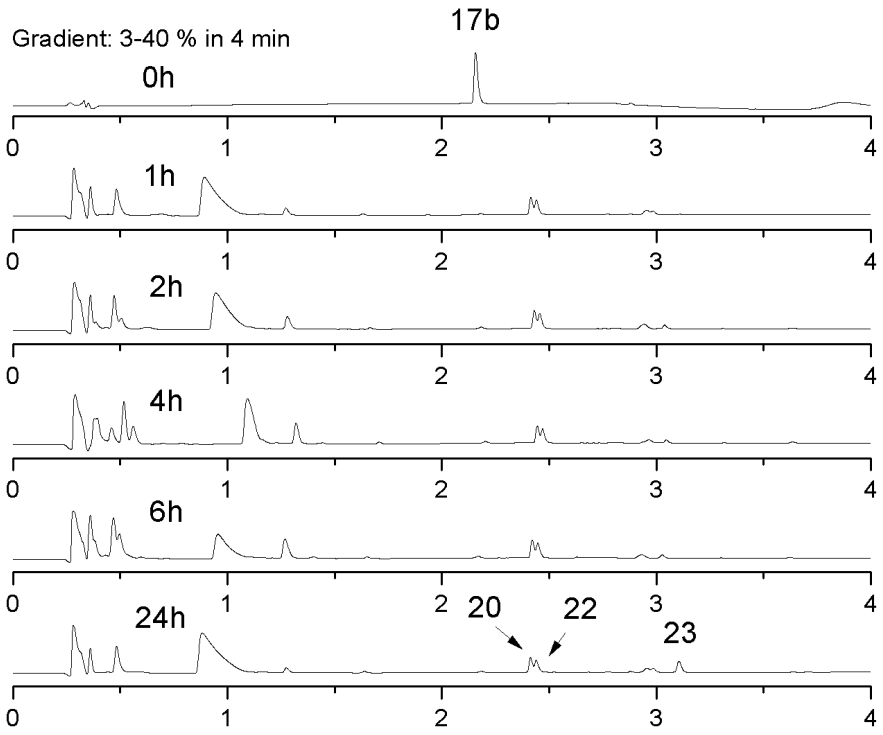

Fig. A.4 Side product formation during cleavage of **17b** in the presence of a radical initator (VA-044 20 eq.)

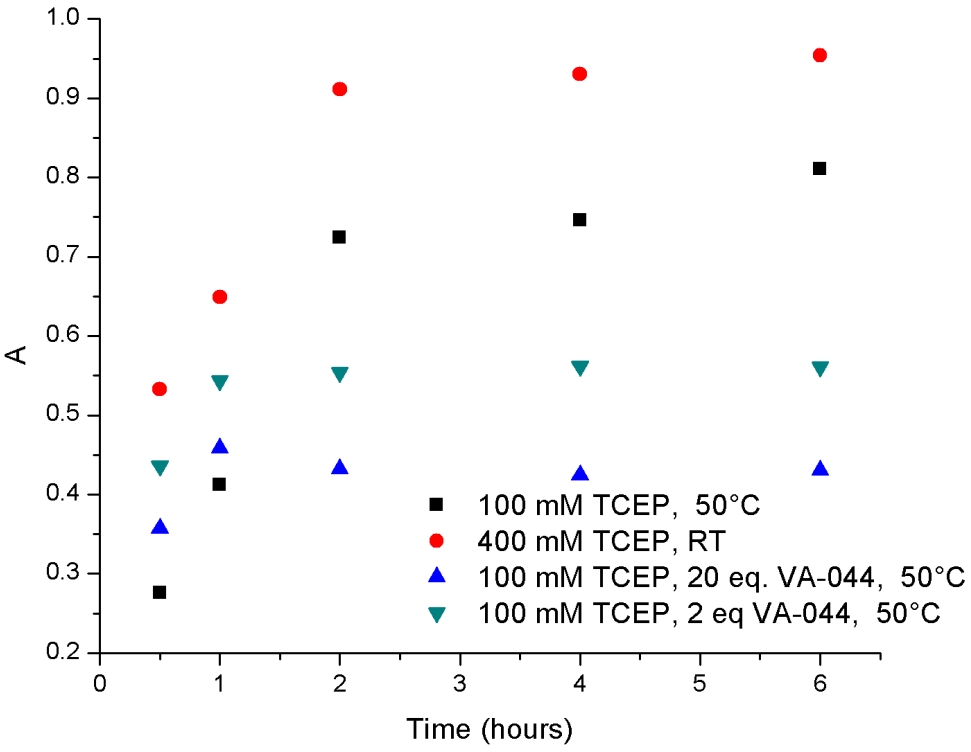

Fig. A.5 Rate of auxiliary peptide **17b** cleavage