Pergamon

Tetrahedron 55 (1999) 6623–6634

TETRAHEDRON

An N-Terminal Method for Peptide Solubilisation

Darren R. Englebretsen* and George T. Robillard

Biochemistry Department, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Nijenborgh 4, Groningen 9747AG, The Netherlands

Received 4 September 1998; revised 22 March 1999; accepted 31 March 1999

Abstract: Peptide-constructs of the form (solubilising peptide)-[$NH(CH_2)_2SO_2(CH_2)_2O$-CO]-(insoluble peptide) were synthesised by Boc SPPS. The HPLC-purified peptide-constructs were cleaved with aqueous base to liberate the insoluble peptides as precipitates.

Keywords: Peptides and polypeptides; Solid phase synthesis; Cleavage reactions.

INTRODUCTION

In previous papers we described SPPS strategies for rendering hydrophobic peptides soluble by linking them at their C-terminus, via an orthogonally cleavable linkage, to hydrophilic solubilising peptides.[1-3] One deficiency of the C-terminal solubilisation method was that peptide amides were not readily obtainable. We therefore investigated a complementary N-terminal peptide solubilisation method. N-terminal peptide solubilisation required an N-terminal linkage which would be stable to all conditions of peptide synthesis, cleavage, and purification, and yet later be readily cleavable. The linker compound 2-[{(N-*t*-Boc)-2-aminoethyl}sulphonyl]ethyl *p*-nitrophenyl carbonate **6** appeared to have potential in such an application.[4] However, use of this linker in subsequent SPPS of the type of N-terminally-linked peptide-construct shown in Figure 1a has not, to our knowledge, been reported.

0040-4020/99/$ - see front matter
PII: S0040-4020(99)00309-9

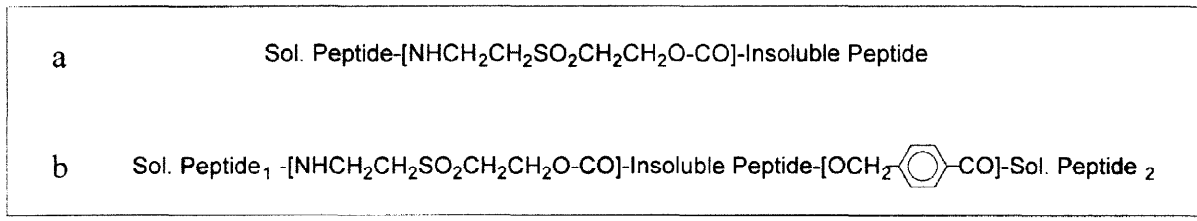
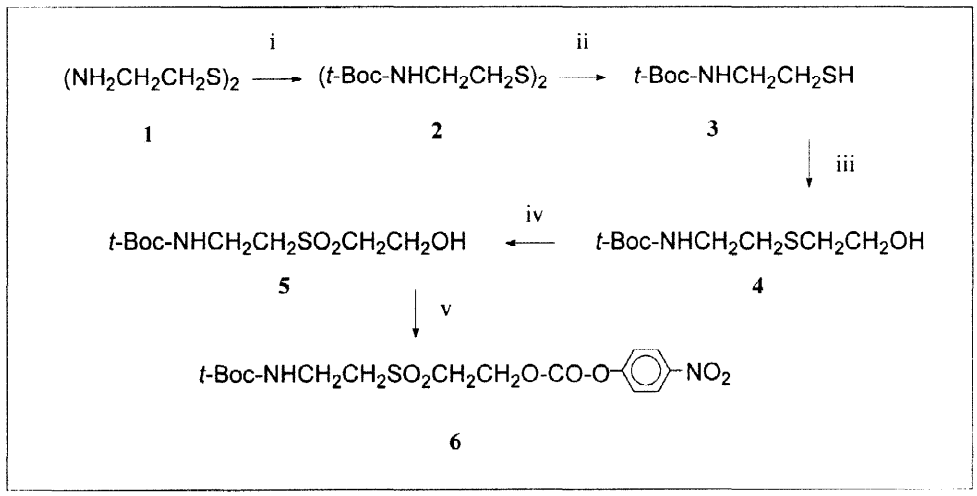


Figure 1. a) N-terminally, and b) N- and C-terminally solubilised peptides.

Urethane linkages based on **6** are cleaved by aqueous base,[4-6] i.e. the same conditions used to cleave the base-labile glycolamide ester[1] or 4-hydroxymethylbenzoic acid-derived [4-Hmb][2,3] ester linkages used in the C-terminal solubilisation methods. Identical cleavage conditions would allow long hydrophobic peptides to be synthesised with both N- and C- terminal solubilising peptides, giving peptide-constructs of the type shown in Figure 1b. Following HPLC purification the N- and C-terminal solubilising peptides of such a construct could be cleaved by a single treatment with aqueous base. In this paper we present an alternative synthesis of linker **6**, and describe its use in Boc SPPS of solubilised peptide-constructs of the types shown in Figure 1.

RESULTS AND DISCUSSION

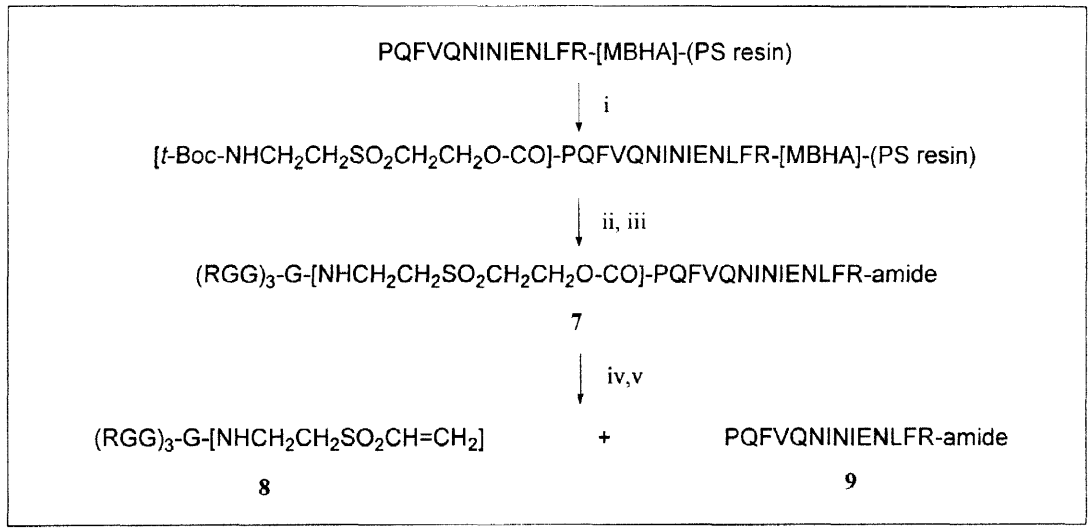
Linker compound **6** was synthesised as shown in Scheme 1. N-*t*-Boc cystamine **2** was prepared using a literature procedure[7] (yield of **2** 99.6% based on cystamine **1**). N-*t*-Boc cystamine **2** was reduced to N-*t*-Boc



Scheme 1. Synthesis of the linker 2-[{(N-*t*-Boc)-2-aminoethyl}sulphonyl]ethyl *p*-nitrophenyl carbonate **6**. i) di-*tert*-butyl pyrocarbonate; ii) tri-*n*-butylphosphine; iii) 2-chloroethanol / NaOH; iv) H_2O_2; v) 4-nitrophenyl chloroformate / pyridine.

cysteamine **3** using tri-*n*-butylphosphine, rather than the more expensive dithiothreitol (DTT).[7] Reaction of **3** with 2-chloroethanol by a slightly modified literature method[8] gave 2-[S-{(N-*t*-Boc)-2-aminoethyl}]mercapto-ethanol **4**. Hydrogen peroxide oxidation of **4** in the presence of a sodium tungstate catalyst[5,6] gave 2-[{(N-*t*-Boc)-2-aminoethyl}sulphonyl]ethanol **5**. Finally, reaction of **5** with 4-nitrophenyl chloroformate yielded 2-[{(N-*t*-Boc)-2-aminoethyl}sulphonyl]ethyl *p*-nitrophenyl carbonate **6**.[4-6] The overall yield of linker **6** was 45% based on cystamine **1**, which may be compared to the 29% overall yield of an alternative synthetic route to **6**.[4] In the final step (Scheme 1, v) TLC analysis after four days reaction showed a ratio of starting material **5** to product **6** of approximately 1:4. Further optimisation of this final step should result in greater yields of **6**.

Peptides were synthesised by manual Boc SPPS methods.[9] Linker **6** was reacted with the N-terminal amino groups of resin-bound synthetic peptides, the Boc group of the linker was removed, and a second, solubilising peptide was synthesised to give resin-bound peptide-constructs of the form $Peptide_1$-[Ntl]-$Peptide_2$-(Resin). We used the abbreviation [Ntl] (for N-terminal linker) to signify the -[$NHCH_2CH_2SO_2CH_2CH_2OCO$]-linkage between the two peptides. Peptide-resins were cleaved using liquid HF with *p*-cresol and *p*-thiocresol as scavengers, and worked-up using normal procedures. The N-terminal peptide solubilisation strategy, using $CP10^{42-55}$-amide as an example, is shown in Scheme 2.



Scheme 2. Strategy for N-terminal solubilisation of hydrophobic and poorly soluble peptides using $CP10^{42-55}$-amide (PQFVQNINIENLFR-amide) as an example. i) 2-[{(N-*t*-Boc)-2-aminoethyl}sulphonyl]-ethyl *p*-nitrophenyl carbonate **(6)** in 5% DIEA / DMF; ii) Boc SPPS; iii) HF cleavage; iv) HPLC purification; v) 0.1 M NaOH.

The first test peptide studied was PQFVQNINIENLFR-amide ($CP10^{42-55}$-amide).[1,10] Although apparently a relatively simple sequence, in practice this synthetic peptide has been difficult to purify owing to its tendency to form insoluble intermolecular aggregates. $CP10^{42-55}$-[MBHA]-resin was reacted with linker **6**, and after Boc removal the solubilising tail peptide $(RGG)_3$-G was synthesised. An HPLC chromatogram of crude cleaved **7** (Figure 2a) showed two major components. The mass of peak 1 (2779.0 Da) coincided with that calculated for $(RGG)_3$-G-[Ntl]-$CP10^{42-55}$-amide **7** (2779.1 Da, MH+), while peak 2 was a side product of mass 2836 Da (+57 Da). An additional mass of +56 Da usually corresponds to a peptide *t*-butyl adduct. Initial studies of peak 2 have shown that the extra mass of 57 Da is located within the $CP10^{42-55}$ portion of peptide-construct **7** (data not shown). If the extra mass of 57 Da in HPLC peak 2 is due to a *t*-butyl adduct, it is likely to have accumulated during the repetitive Boc deprotection steps, as the N-terminal Boc group was removed before the peptide was cleaved from the resin. Investigations into the exact nature of the +57 Da adduct are continuing.

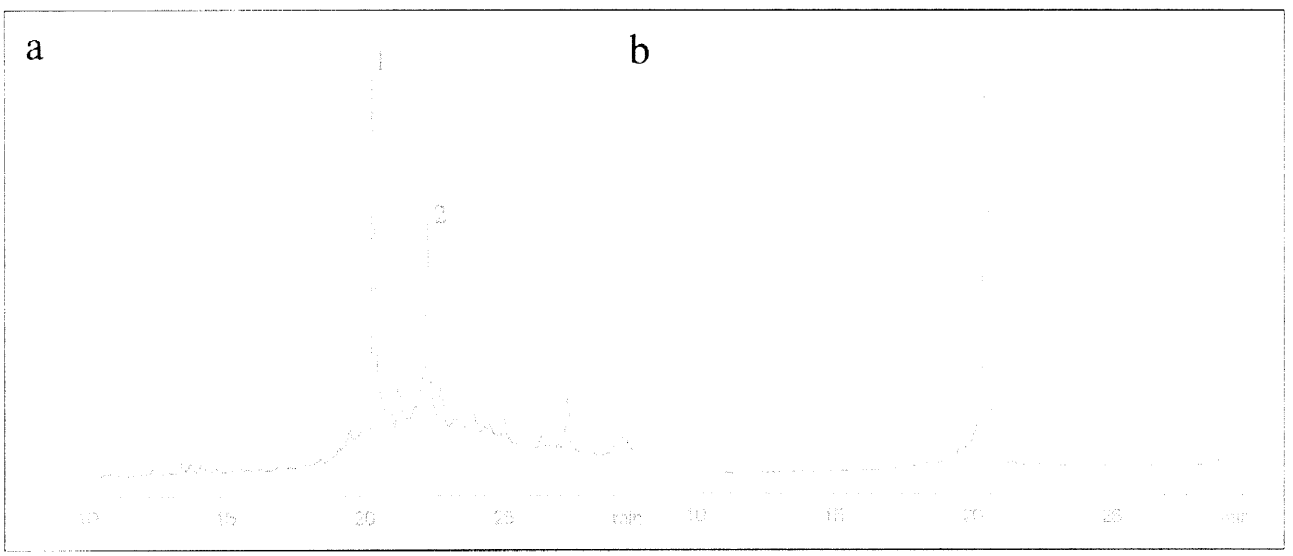


Figure 2. HPLC chromatograms of a) crude $(RGG)_3$-G-[Ntl]-$CP10^{42-55}$-amide **7**; b) HPLC purified **7**.

Peptide-construct **7** was purified by standard HPLC methods (Figure 2b). The [Ntl] linkage of **7** (2 mg/ml) was cleaved by 0.1 M NaOH within 60 seconds. After centrifugation of the subsequently acidified solution mass spectral analysis of the supernatant showed $(RGG)_3$-G-[$NHCH_2CH_2SO_2CH{=}CH_2$] **8** to be present (found 1003.7 Da , calc. MH+ 1003.9 Da), whereas $CP10^{42-55}$-amide **9** was not detected (1732 Da, Figure 3a). A study of cleavage of the [Ntl] linkage of **7** (2 mg/ml) at lower NaOH concentrations showed only partial cleavage with 0.01 M NaOH over 120 seconds, and almost no cleavage with 0.001 M NaOH over the same time.

The gel-like precipitate of $CP10^{42-55}$-amide **9**, resulting from cleavage with 0.1 M NaOH, was washed several times with water, and dissolved in acetic acid for mass analysis (Figure 3b: found 1731.7 Da, calc. MH+ 1731.9 Da). Mass analysis showed that the precipitate of **9** was highly homogeneous.

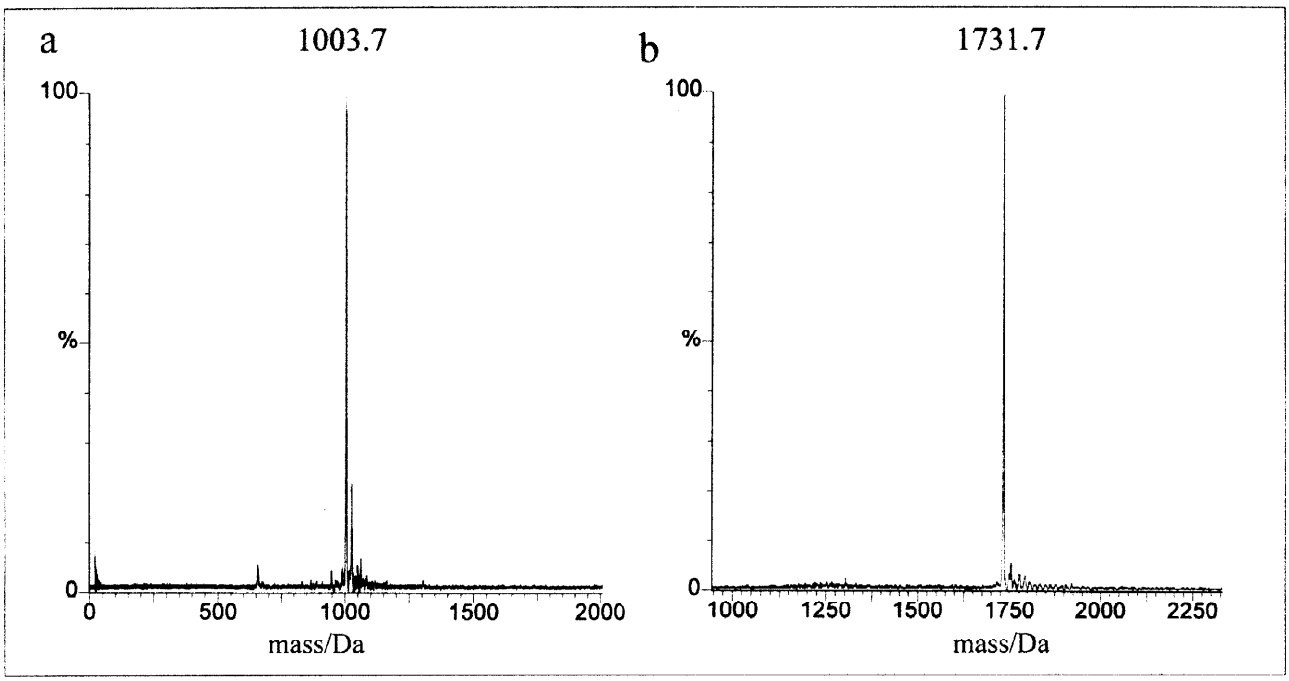


Figure 3. MALDI-TOF mass spectra of : a) supernatant from NaOH cleavage of $(RGG)_3$-G-[Ntl]-$CP10^{42-55}$-amide **7** showing soluble cleavage product $(RGG)_3$-G-[$NHCH_2CH_2SO_2CH{=}CH_2$] **8** (calc. mass MH+ 1003.9 Da); b) precipitate of $CP10^{42-55}$-amide **9** dissolved in acetic acid (calc. mass MH+ 1731.9 Da).

A second peptide to be synthesised was the N- and C- terminally solubilised peptide-construct $(RGG)_3$-G-[Ntl]-Ala_{12}-[4-Hmb]-$GG(RG)_4G$ **11**. This example was chosen to investigate use of the N-terminal linkage in combination with the acid-stable, base-labile ester based on 4-hydroxymethylbenzoic acid (4-Hmb linker) used in the C-terminal solubilising method.[2,3] The course of the synthesis was monitored by cleavage of the peptide-resin at intermediate stages, with analysis by HPLC and MALDI-TOF mass spectrometry. Mass spectral analysis of A-[4-Hmb]-$GG(RG)_4G$, cleaved at such an intermediate stage, showed a main product of the expected mass (found 1247.1 Da, calc. MH+ 1248.3 Da) with only very low levels of unwanted deletion peptides (data not shown). This analysis showed that all steps up to this point had gone in high yield. It was found that by coupling 4-hydroxymethylbenzoic acid as its NHS active ester, di-addition of the 4-Hmb linker was avoided, a problem noted when HBTU was used to couple this linker.[2]

An HPLC chromatogram of A_{12}-[4-Hmb]-GG$(RG)_4$G **10** showed that the intermediate peptide was of acceptable purity, with the major product eluting at 12.7 minutes having a mass of 2029.4 Da (Figure 4a, calc. for **10** MH+ 2030.2 Da). An HPLC chromatogram of crude cleaved $(RGG)_3$-G-[Ntl]-Ala_{12}-[4-Hmb]-GG$(RG)_4$G **11** is given as Figure 4b. The mass of the major peak eluting at 15.0 minutes (3075.8 Da) was as calculated for **11** (MH+ 3077.3 Da).

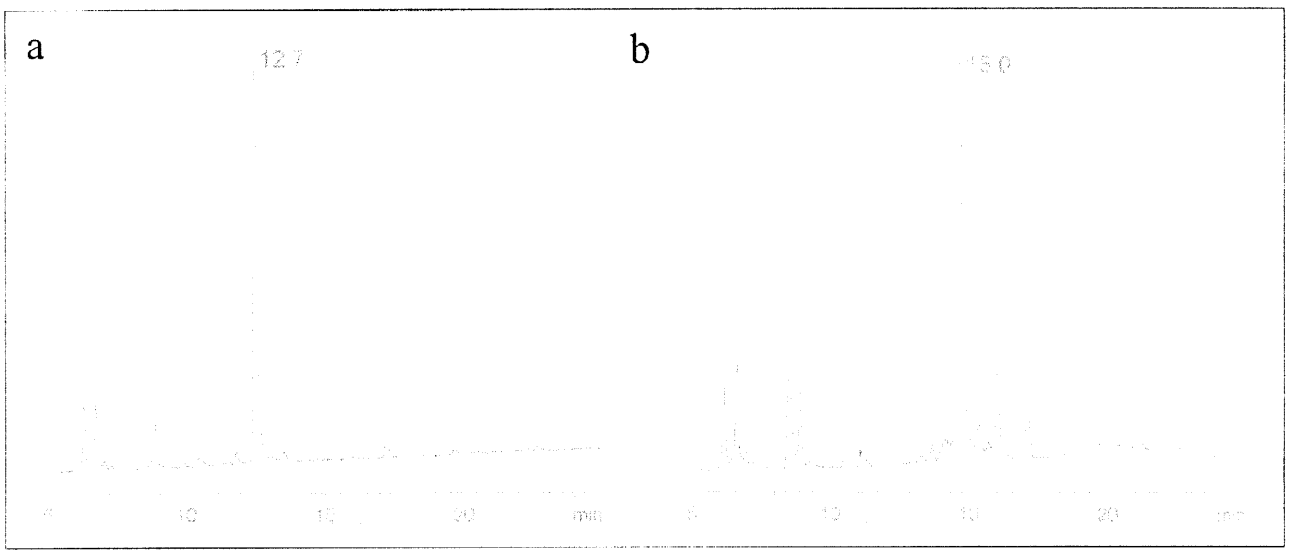


Figure 4. HPLC chromatograms of a) crude A_{12}-[4-Hmb]-GG$(RG)_4$G **10**; b) crude $(RGG)_3$-G-[Ntl]-Ala_{12}-[4-Hmb]-GG$(RG)_4$G **11**.

HPLC-purified **11** (2 mg/ml) was cleaved with 0.1 M NaOH for 60 seconds, acidified with acetic acid, and centrifuged. The mass of the precipitate which formed (872.0 Da) corresponded to Ala_{12} **12** (calc. MH+ 872.2 Da). Peptides with masses of 1002.6 Da and 1177.0 Da were present in the supernatant, and these corresponded to the expected soluble cleavage products $(RGG)_3$-G-[$NHCH_2CH_2SO_2CH{=}CH_2$] **13** (calc. MH+ 1003.9 Da), and [$HOCH_2$-C_6H_4-CO]-GG$(RG)_4$G **14** (calc. MH+ 1177.3 Da) respectively. No uncleaved **11** was found by both HPLC and MALDI-TOF analysis of the supernatant, showing complete cleavage of the 4-Hmb and [Ntl] linkages within 60 seconds using 0.1 M NaOH.

HPLC and MALDI-TOF analyses of the supernatant from a solution of **11** (2 mg/ml) cleaved using 0.01 M NaOH for 120 seconds showed masses corresponding to the expected cleavage products **13** and **14**, but in addition a product with mass 2030.5 Da was present. This mass corresponded to A_{12}-[4-Hmb]-GG$(RG)_4$G **10** (calc. MH+ 2030.2 Da), and indicated incomplete cleavage of the 4-Hmb ester. Despite incomplete cleavage of the 4-Hmb linkage it must be noted that the [Ntl] linkage of **11** was completely cleaved in 120 seconds using 0.01 M NaOH.

In the NaOH cleavage of the [Ntl] linkages of peptide-constructs **7** and **11** it was found that **7** required treatment with 0.1 M NaOH for 60 seconds for complete cleavage of the [Ntl] linkage. On the other hand the [Ntl] linkage of **11** was cleaved in 60 seconds with 0.01 M NaOH, although 0.1 M NaOH was required for complete cleavage of the 4-Hmb ester of **11**. This data suggests dependence of the rate of cleavage of the [Ntl] linkage on the N-terminal amino acid. As a general rule we conclude that the [Ntl] linkage should be cleaved with 0.1 M NaOH for at least 60 seconds.

After cleavage of the C- and/or N-terminal solubilising peptides the target hydrophobic peptides were obtained as gel-like precipitates. Mass analysis of the precipitate of $CP10^{42-55}$-amide **9** (Figure 3b) showed it to be homogeneous. Analysis of Ala_{12} **12** was complicated by its extremely poor solubility, however mass analysis of a suspension of the Ala_{12} precipitate in the MALDI-TOF matrix solution did show an Ala_{12} ion to be present, albeit at low intensity (data not shown). Since the precipitates of the desired hydrophobic peptides were derived from homogeneous purified peptide-constructs they were expected to be of good purity after cleavage of the 4-Hmb and/or [Ntl] linkages.

CONCLUSIONS

We have reported an N-terminal Boc SPPS peptide solubilising strategy which is compatible with modern optimised Boc SPPS methods,[9] and can be used in combination with our previous C-terminal solubilisation method.[1-3] A strength of this strategy is that the water-soluble peptide-constructs could be purified by standard HPLC methods, a distinct advantage over specialised, peptide-specific HPLC methods used to purify hydrophobic peptides.[11] Further uses of this strategy include: i) "rescuing" a resin-bound peptide which proves, against prior expectations, to be poorly soluble ($CP10^{42-55}$-amide is a good example of such a peptide); ii) use as a method for attaching a removable peptide "tail" to the N-terminus of another synthetic peptide (e.g. to attach a His_6 tag for Ni-Agarose affinity purification); and iii) solubilising peptide components for subsequent synthesis of long hydrophobic peptides by chemical ligation - such an arrangement is compatible with thioether ligation.[3,12] Other applications of this peptide modification strategy are also under investigation.

EXPERIMENTAL

Thin layer chromatography was carried out using Merck Kieselgel 60/ Kieselgur F_{254} plates. Solvent systems (all v/v) were: System 1, butanol:acetic acid:water 4:1:1; System 2, hexane:ethyl acetate 4:1; System 3, hexane:ethyl acetate 1:4; System 4, hexane:ethyl acetate:acetic acid 50:50:1; System 5, hexane:ethyl acetate 1:1. Plates were visualised with either ninhydrin spray (1% w/v in ethanol) followed by heating at 100°C or iodine vapour. NMR spectra were recorded using a Varian Gemini 200 (200 MHz) instrument. Perkin Elmer

model 841 scanning (compounds **4** and **5**) or Mattson Instruments model 4020 FT-IR (compound **6**, KBr disc) spectrophotometers were used to record IR spectra.

N-*t*-Boc cysteamine (3). N-*t*-Boc cystamine **2** (7.175 g, 20.4 mmole), synthesised using a literature method (yield 99.6% based on cystamine **1**, TLC: System 1 R_f **1** = 0, R_f **2** = 0.97),[7] was suspended in a mixture of 100 ml isopropanol and 100 ml 0.5 M $NaHCO_3$, and tri-*n*-butyl phosphine (5.3 ml, 21.3 mmole) was added with stirring. After 50 minutes, TLC of the cloudy solution (System 2, R_f **2** = 0.36, R_f N-*t*-Boc-cysteamine **3** = 0.61) showed complete reduction of **2** to **3**. Isopropanol was removed *in vacuo*, 50 ml water and 50 ml saturated NaCl solution were added, and the solution was extracted twice with 100 ml aliquots of ethyl acetate. The combined organic layers were back-extracted extensively with 1 M HCl (10 × 200ml) to remove tri-*n*-butyl phosphine oxide, dried with Na_2SO_4, and ethyl acetate was removed *in vacuo*. The resulting oil, containing traces of ethyl acetate, was subjected to a stream of dry nitrogen until constant weight. The N-*t*-Boc-cysteamine **3** which resulted, a clear colorless oil (6.92 g, 36.4 mmole, 89% [93% purity]), was devoid of the characteristic odour of tri-*n*-butyl phosphine, however NMR analysis showed the presence of 7% tri-*n*-butyl phosphine oxide as an impurity. This harmless impurity was carried through in the subsequent reactions. ^{1}H NMR ($CDCl_3$): δ 4.92 (br m, 1 H, ex D_2O), 3.29 (m, 2 H), 2.63 (m, 2 H), 1.44 (s, 9 H), 1.34 (t, 1 H, ex D_2O).

2-[S-{(N-*t*-Boc)-2-aminoethyl}] mercaptoethanol (4). A solution of sodium hydroxide (1.60 g, 40.0 mmole in 3 ml water, then mixed into 30 ml ethanol) was added, with stirring, to a solution of N-*t*-Boc-cysteamine **3** (6.92 g 39.1 mmoles) and 2.70 ml 2-chloroethanol (40.3 mmole) in 20 ml ethanol. The initially colorless solution went yellow on adding the NaOH solution, and a white precipitate of NaCl formed within five minutes. After stirring for 20 minutes TLC (System 2, R_f **3** = 0.61, R_f product **4** = 0.11) showed that reaction was complete. Ethanol was removed *in vacuo*, water (100 ml) was added and the aqueous solution was extracted with two 100 ml aliquots of ethyl acetate. The combined organic layers were dried with Na_2SO_4, and the ethyl acetate was removed *in vacuo* to give 2-[S-{(N-*t*-Boc)-2-aminoethyl}]mercaptoethanol **4** as an oil. The clear colourless oil was subjected to a stream of nitrogen to remove residual ethyl acetate, and then dried under vacuum overnight until the weight was constant. NMR analysis showed the presence of tri-*n*-butyl phosphine oxide impurity as 7% of the mass of the product (8.13 g, 34.2 mmole, 87% [93% purity]). ^{1}H NMR ($CDCl_3$): δ 4.91 (br m, 1 H, ex D_2O), 3.74 (t, 2 H, J=5.9 Hz), 3.32 (m, 2 H), 2.74 (t, 2 H, J=5.9 Hz), 2.65 (t, 2 H, J=6.6 Hz), 2.23 (br s, 1 H, ex D_2O), 1.44 (s, 9H). IR: 3354 cm^{-1}, 2977 cm^{-1}, 1701 cm^{-1}, 1521 cm^{-1}, 1274 cm^{-1}, 1250 cm^{-1}, 1164 cm^{-1}, 1045 cm^{-1}. Elemental analysis: Calculated for 2-[S-{(N-*t*-Boc)-2-aminoethyl}]mercaptoethanol. 0.07 tri-*n*-butyl phosphine oxide ($C_9H_{19}NO_3S$. $0.07C_{12}H_{27}PO$): C, 49.98; H, 8.84; N, 5.93; S, 13.54. Found: C, 49.67; H, 8.92; N, 5.68; S, 13.39.

2-[{(N-*t*-Boc)-2-aminoethyl}sulphonyl]ethanol (5). A catalytic amount of sodium tungstate (120 mg) dissolved in 20 ml water was added to a solution of oil **4** (8.13 g, 34.2 mmole) in 50 ml ethanol. The mixture was stirred while 3.8 ml 30% H_2O_2, sufficient to oxidise the thioether to the sulphoxide, was added dropwise over five minutes. TLC (System 3, R_f **4** = 0.69, reaction R_f = 0.46) showed the disappearance of the starting compound **4**. Another 3.8 ml H_2O_2 was added over five minutes as above, and TLC now showed a single spot of product **5** (R_f = 0.54). After two hours stirring 0.50 g $NaHSO_4$ in 80 ml water was added to decompose any remaining H_2O_2. Ethanol was removed *in vacuo* and the solution was extracted with three 100 ml aliquots of ethyl acetate. The combined organic layers were back-extracted twice with 25 ml saturated NaCl solution, dried with Na_2SO_4, and then dried overnight over 4Å molecular sieves. Ethyl acetate was removed *in vacuo* and the clear colourless oil was dried to constant weight with a flow of nitrogen to give 2-[{(N-*t*-Boc)-2-aminoethyl}sulphonyl]ethanol **5** containing 7% tri-*n*-butyl phosphine oxide (8.32 g, 30.6 mmole, 83% [93% purity]). ^{1}H NMR ($CDCl_3$): δ 5.26 (br m, 1 H, ex D_2O), 4.10 (t, 2 H, J=5.1 Hz), 3.64 (m, 2 H), 3.33 (t, 2 H, J=6.1 Hz), 3.24 (t, 2 H, J=5.1 Hz), 2.66 (br s, 1 H, ex D_2O), 1.43 (s, 9H). IR: 3359 cm^{-1}, 2979 cm^{-1}, 1714 cm^{-1}, 1520 cm^{-1}, 1285 cm^{-1}, 1254 cm^{-1}, 1168 cm^{-1}, 1122 cm^{-1}, 1045 cm^{-1}. Elemental analysis: Calculated for 2-[{(N-*t*-Boc)-2-aminoethyl}sulphonyl]ethanol. 0.07 tri-*n*-butyl phosphine oxide ($C_9H_{19}NO_5S$. $0.07C_{12}H_{27}PO$): C, 44.02; H, 7.79; N, 5.22; S, 11.93. Found: C, 43.83; H, 8.01; N, 4.94; S, 12.18.

2-[{(N-*t*-Boc)-2-aminoethyl}sulphonyl]ethyl *p*-nitrophenyl carbonate (6). Compound **5** 8.32 g (30.6 mmole) was dissolved in 50 ml dry pyridine at 0°C, and 4-nitrophenyl chloroformate (6.70 g, 33.2 mmole) was added over two minutes with stirring. After two hours at 0°C the solution was brought to room temperature. The course of the reaction was followed by TLC (System 4, R_f **5** = 0.13, R_f product **6** = 0.48). After stirring overnight the reaction was incomplete. Extra 4-nitrophenyl chloroformate (3.30 g, 16.4 mmole) was added and stirring was continued. After four days pyridine was removed *in vacuo* to give a yellow oil, which was dissolved in 100 ml ethyl acetate and extracted with 100 ml 1 M HCl. The HCl solution was back-extracted with 100 ml ethyl acetate, the organic layers were combined, extracted twice with 25 ml saturated NaCl solution, and dried over Na_2SO_4. Ethyl acetate was removed *in vacuo* yielding a yellow oil. Ether (50 ml) was added to the oil and the solution was left to stand overnight. A precipitate which formed was collected, washed with a little ether, and dried under vacuum (9.54 g). TLC of the solid (System 5) showed two spots R_f = 0.16 (**5**) and 0.55 (**6**). The solid was dissolved in 20 ml ethyl acetate with heating and 30 ml hexane was slowly added. After cooling to room temperature and standing for five hours the resulting precipitate, a white solid, was collected and washed twice with 8 ml hexane:ethyl acetate 1:1. The weight of vacuum-dried product 2-[{(N-*t*-Boc)-2-aminoethyl}sulphonyl]ethyl *p*-nitrophenyl carbonate **6** was 7.69 g, (18.4 mmoles, 56%). Melting point 117-119°C (dec., uncorr.). The overall yield of **6** was 45% based on cystamine **1**. ^{1}H NMR ($CDCl_3$): δ 8.29 (d, 2H, J=8.5 Hz), 7.40 (d, 2H, J=8.5 Hz), 5.15 (br m, 1H), 4.73 (t, 2H, J=5.2 Hz), 3.68 (m, 2H), 3.46 (t,

2H, J=5.2 Hz) 3.34 (t, 2H, J= 5.5 Hz), 1.43 (s, 9H). IR: 3376 cm^{-1}, 2292 cm^{-1}, 1765 cm^{-1}, 1682 cm^{-1}, 1534 cm^{-1}, 1265 cm^{-1}, 1221 cm^{-1}, 1196 cm^{-1}, 1134 cm^{-1}, 1057 cm^{-1}. Elemental analysis: Calculated for 2-[{(N-*t*-Boc)-2-aminoethyl}sulphonyl]ethyl *p*-nitrophenyl carbonate ($C_{16}H_{22}N_2O_9S$): C, 45.93; H 5.26; N, 6.70; S, 7.66. Found: C, 45.97; H, 5.31; N, 6.65; S, 7.44.

Solid phase peptide synthesis. Peptides were synthesised by manual Boc SPPS with *in-situ* neutralisation.[9] Side chain protecting groups were Asn (Xan), Arg (Tos), Gln (Xan) and Glu (OBzl). Dried peptide-resins were cleaved over 90 minutes at 0°C using HF/*p*-thiocresol/*p*-cresol 18:1:1 v:v:v. After removing HF *in vacuo*, the peptides were precipitated with 50 ml ether, stirred for 5 minutes at 0°C, filtered off, washed with 2 × 25 ml ether, dissolved in 1 ml TFA, diluted with 20 ml water, and lyophilised. Lyophilised peptides were analysed by HPLC using a Vydac 4.6 × 250 mm C4 column, with a gradient of 0-60%B over 30 minutes at 1 ml/min (A = 0.1% TFA; B = 0.1% TFA, 10% water, 90% acetonitrile) with monitoring at 214 nm. Mass spectra were recorded with a Micromass VG TofSpec E MALDI-TOF mass spectrometer using a saturated solution of α-cyano-4-hydroxycinnamic acid in a 1:2 v/v mixture of HPLC solvents A and B as matrix.

$(RGG)_3$-G-[Ntl]-PQFVQNINIENLFR-amide (7). PQFVQNINIENLFR-amide ($CP10^{42-55}$-amide) was synthesised on a 0.50 mmole scale using NovaBiochem MBHA resin, amine substitution 0.42 mmole/g. Boc-amino acids (2 mmole) were single-coupled except for Ile^{50} and Gln^{43}, which were double coupled after ninhydrin assay indicated poor couplings. Following Boc removal, neutralisation (20% diisopropylethylamine (DIEA)/DMF), and washing with DMF, linker **6** (2 mmole dissolved in 3 ml NMP with 350 µl DIEA) was coupled to 0.33 mmole of the PQFVQNINIENLFR-[MBHA]-resin for 2 hours. The solubilising peptide $(RGG)_3$-G was synthesised onto the linker to give Boc-$(RGG)_3$-G-[Ntl]-PQFVQNINIENLFR-[MBHA]-resin. The Boc group was removed with TFA, the peptide-resin neutralised with 20% DIEA/DMF, washed with DMF followed by DCM, and dried under vacuum. Following HF cleavage and work-up the crude lyophilised $(RGG)_3$-G-[Ntl]-PQFVQNINIENLFR-amide **7** was analysed by HPLC and mass spectrometry, then purified by HPLC (47mg dissolved in 7 ml water, Vydac C4 25 × 250 mm, 0-60% B / 60 min at 8 ml/min) to give 10.8 mg **7**. The purified peptide construct **7** was analysed by HPLC, mass spectrometry and amino acid analysis. Mass: found 2779.0 Da, calc. (MH+) 2779.1 Da. Amino acid analysis (error ± 10%): Found (Expected): Asx 3.0 (3), Glx 2.8 (3), Gly 6.6 (7), Arg 3.8 (4), Val 1.1 (1), Phe 2.2 (2), Ile 2.3 (2), Leu 1.3 (1), Pro 1.1 (1).

Base cleavage of $(RGG)_3$-G-[Ntl]- PQFVQNINIENLFR-amide (7). To solutions of HPLC-purified **7** in water (2.2 mg/ml, 189.5 µl) were added 20.5 µl of either 1.0 M, 0.1 M, or 0.01 M NaOH (final [NaOH] 0.1 M, 0.01 M and 0.001 M respectively, peptide concentration 2.0 mg/ml). Sixty seconds after base addition

the solutions were acidified with 10 µl acetic acid and analysed by HPLC and mass spectrometry. Cleavage using 0.01 and 0.001 M NaOH was also studied after 120 seconds. After cleavage using 0.1 M NaOH the centrifuged precipitate of $CP10^{42-55}$-amide **9** was washed several times with water, with centrifugation after each wash. The precipitate was dissolved in acetic acid and analysed by mass spectrometry. Mass: found 1731.7 Da, calc. (MH+) 1731.9 Da.

$(RGG)_3$-G-[Ntl]-Ala_{12}-[4-Hmb]-GG$(RG)_4$G (11). GG$(RG)_4$G-[PAM]-Polystyrene was synthesised using 1.0 mmole ABI Boc-Gly-[PAM] resin, substitution 0.77 mmole/g, with 2.0 mmole of each Boc-amino acid single coupled. The linker 4-hydroxymethylbenzoic acid was activated as its N-hydroxysuccinimide (NHS) ester (3 mmole) and coupled to 0.67 mmole of the peptide-resin overnight (ninhydrin test negative). Boc-Ala anhydride (1.5 mmole, 2.2 eq) was coupled for 2 hours in the presence of dimethylaminopyridine (DMAP, 0.25 eq over resin-bound hydroxyl groups). Following DMF wash and Boc removal a sample of the A-[4-Hmb]-GG$(RG)_4$G-[PAM]-resin was HF cleaved for mass analysis. SPPS of Ala_{12} was continued using 0.17 mmole of A-[4-Hmb]-GG$(RG)_4$G-[PAM]-resin, with 1 mmole (6 eq) Boc-Ala per single 10 minute coupling. A sample of the de-Boc'd A_{12}-[4-Hmb]-GG$(RG)_4$G-[PAM]-resin was HF cleaved for HPLC and mass analysis. The de-Boc'd peptide-resin was washed with 5% DIEA/DMF, then 1 mmole linker **6** and 1 ml 5% DIEA/DMF were added to give a slurry. Ninhydrin test of the resin after one hour was faintly positive for unreacted amine, and so it was washed with 5% DIEA / DMF and **6** (0.5 mmole) was recoupled over another hour. Because the ninhydrin test was still faintly positive after the second coupling of **6**, the resin was capped for 10 minutes with acetic anhydride/DIEA in DMF (100µl/100µl/2ml). The solubilising peptide $(RGG)_3$-G was synthesised onto the linker (6 eq Boc-amino acid per single 10 minute coupling). After HF cleavage and workup the crude lyophilised $(RGG)_3$-G-[Ntl]-Ala_{12}-[4-Hmb]-GG$(RG)_4$G **11** was analysed by mass spectrometry and HPLC, then purified by HPLC (50 mg dissolved in 7 ml water, Vydac C4 25 × 250 mm, 0-40% B / 40 min at 8 ml/min) to give 9.0 mg **11**. Purified peptide-construct **11** was analysed by HPLC, mass spectrometry and amino acid analysis. Mass: found 3075.8 Da, calc. (MH+) 3077.3 Da. Amino acid analysis (error ± 10%): Found (Expected): Gly 14.6 (14), Ala 11.9 (12), Arg 7.0 (7).

Base cleavage of $(RGG)_3$-G-[Ntl]-Ala_{12}-[4-Hmb]-GG$(RG)_4$G (11). Solutions of **11** (2.2 mg/ml, 225 µl) were treated with 25 µl 1.0 or 0.10 M NaOH (final concentrations: **11** 2 mg/ml, NaOH 0.1 M and 0.01 M respectively). After 60 seconds (0.1 and 0.01 M NaOH) or 120 seconds (0.01 M NaOH only) the solutions were acidified with acetic acid (10 µl), centrifuged, and the supernatants analysed by mass spectrometry and HPLC. In the cleavage with 0.1 M NaOH the supernatant was removed after centrifugation and the precipitate of Ala_{12} was washed three times with water. The centrifuged precipitate was suspended in 20 µl of the MALDI-TOF matrix solution and analysed by mass spectrometry. Mass: found 872.0 Da, calc. (MH+) 872.2 Da.

REFERENCES

1. Englebretsen, D.R. and Alewood, P.F. *Tetrahedron Lett.*, **1996**, 46, 8431-8434.
2. Choma, C.T., Robillard, G.T. and Englebretsen, D.R. *Tetrahedron Lett.*, **1998**, *39*, 2417-2420.
3. Englebretsen, D.R., Choma, C.T. and Robillard, G.T. *Tetrahedron Lett.*, **1998**, *39*, 4929-4932.
4. Canne, L.E., Winston, R.L. and Kent, S.B.H. *Tetrahedron Lett.*, **1997**, *38*, 3361-3364.
5. Tesser, G.I. and Balvert-Geers, I.C. *Int. J. Peptide Protein Res.*, **1975**, *7*, 295-305.
6. Funakoshi, S., Fukuda, H. and Fujii, N. *Proc. Natl. Acd. Sci. USA*, **1991**, *88*, 6981-6985.
7. Winger, T.M., Ludovice, P.J. and Chaikof, E.L. *Bioconjugate Chem.*, **1995**, *6*, 323-326.
8. Lawson, D.D., Getz, H.R. and Miller, D.A. *J. Org. Chem.*, **1961**, *26*, 615-616.
9. Schnölzer, M., Alewood, P., Jones, A., Alewood, D. and Kent, S.B.H. *Int. J. Peptide Protein Res.*, **1992**, *40*, 180-193.
10. Lackman, M., Rajasekariah, P., Iismaa, S.E., Jones, G., Cornish, C.J., Hu, S., Simpson, R.J., Moritz, R.L. and Geczy, C.L. *J. Immunol.*, **1993**, *7*, 2981-2991.
11. Lew, S. and London, E. *Anal. Biochemistry*, **1997**, *251*, 113-116.
12. Englebretsen, D.R., Garnham, B.G., Bergman, D.A. and Alewood, P.F. *Tetrahedron Lett.*, **1995**, *48*, 8871-8874.