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High-throughput synthesis of conopeptides: a safety-catch linker approach enabling disulfide formation in 96-well format

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Abstract: Conotoxins exhibit a high degree of selectivity and potency for a range of pharmacologically relevant targets. The rapid access to libraries of conotoxin analogues, containing multiple intramolecular disulfide bridges for use in drug development, can be a very labor intensive, multi-step task. This work describes a high-throughput method for the synthesis of cystine-bridged conopeptides.

Peptides were assembled on a peptide synthesizer employing the Fmoc solid-phase strategy using a safety-catch amide linker (SCAL). Side-chain protecting groups were removed on solid phase before SCAL activation with ammonium iodide in TFA, finally releasing the peptide into the TFA solution. Disulfide bond formation was performed in the cleavage mixture employing DMSO.

This improved method allows mixtures of oxidized peptides to be obtained in parallel directly from a peptide synthesizer. A single HPLC purification of the resulting crude oxidized material produced peptides of >95% purity. Copyright © 2006 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: conopeptides; solid-phase peptide synthesis; disulfide bonds; safety-catch linker; high-throughput synthesis

INTRODUCTION

Marine natural product bioprospecting has produced a considerable number of drug candidates [1]. These chemical entities have been optimized through an evolutionary selection process to highly potent inhibitors of physiological processes in the prey, predators or competitors of the marine organisms that use them. Since the basic physiological signalling processes are very similar across the phyla, marine natural products represent excellent starting points for drug development. The genus *Conus* (marine cone snails) contains an exceptionally rich source of such highly evolved pharmacologically active compounds. These predatory molluscs are equipped with a venom apparatus that produces a species-specific cocktail of peptides to immobilize their prey.

Similar to spider and scorpion venoms, conotoxins are not only unique owing to their evolutionary-selected high target specificity, but their typically constrained structures also are rigidified into a framework of often multiple cysteine–cysteine disulfide bonds. This disulfide framework gives rise to a series of loops of intervening amino acids that determine activity. Given their characteristic potency, selectivity and chemical stability, venom peptides, especially conotoxins, are exceptionally good tools for drug development [2–5].

Xenome is commercially exploiting these focussed peptide libraries through molecular biological translation of DNA, encoding the venom peptides produced in a species. As a result, Xenome possesses a unique venom peptide library that represents an exceptional discovery platform for novel drug leads [6–14].

The chemical synthesis of thousands of cystine-bridged (Cys-S-S-Cys) venom peptides and synthetic analogues requires high-throughput peptide chemistry. Although the synthesis of peptides today is a highly automated process, the production of conopeptide libraries is still a time consuming procedure, as each individual peptide requires purification at the crude stage, oxidation and a final re-purification step to deliver the often multiple disulfide-bridged conopeptides.

The aim of this work was to develop methods for faster and more efficient production of disulfide-bridged conopeptides and their synthetic analogues.

Moving the oxidation of individual peptides into a 96-well format on a synthesizer was considered the effective approach to improve the efficiency of the process.

The two alternative approaches to achieve this goal were considered: (i) on-resin disulfide bond formation and (ii) disulfide bond formation in the cleavage mixture.

Although there has been some progress in the field of on-resin oxidation [15–18], this approach was not suitable because of a strong sequence dependence to the outcome of these transformations, thereby lacking in broad applicability, and therefore not suitable for library production.

The oxidation of peptides in cleavage mixtures is hampered by the use of scavengers that are designed to trap reactive species and to prevent cysteine-rich peptides from undergoing oxidation. Moreover,

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these scavengers can cause problems in further HPLC purifications, or can result in crude peptides that are unsuitable to be used in high-throughput screening without further purification.

To overcome these problems, a method was devised to separate the side-chain cleavage step from the disulfide bond formation step. This was achieved by adapting and improving a safety-catch amide linker (SCAL) approach, developed by Lebl and coworkers [19,20].

The SCAL (Figure 1) is a safety-catch linker, which in its sulfoxide form (S=O) is stable in TFA. This allows the removal of all side-chain protecting groups and leaves an unprotected 'naked' peptide still attached to the resin, which is then able to undergo extensive resin washes to remove all residual scavenger and cleavage material.

The activation of the linker for the final cleavage is performed by reduction of the sulfoxide moiety to the corresponding thioether (S=O → –S–). This transformation increases the electron density in the aromatic system of the linker and allows the cleavage of the benzydrylamine function with TFA.

The sulfoxide to thioether transformation releases the peptide from the SCAL in the presence of Lewis acid systems such as Me_3SiBr/thioanisole, $SiCl_4$/anisole and Me_3SiCl/triphenylphospine in TFA [19,20,] or hydrogen bromide (0.1%) in acetic acid [21–23].

Although the SCAL strategy adds a further dimension of orthogonality to the solid-phase synthesis of *C*-terminal peptide amides, this approach is not widely used, and no investigation into its application for library production of peptides has been reported. Here, we present a new activation and cleavage method for the SCAL, which is superior to the procedures reported in the literature. The new method enables high-throughput library production of cysteine-rich venom peptides and their synthetic analogues in their oxidized disulfide-folded forms (Figure 2). The crude oxidized conopeptides obtained are free of scavengers, making them potentially suitable for direct biological screening. A single purification step yields high-purity peptides suitable for library development and lead optimization.

To illustrate the features of this method we selected two loop members of the alpha (α-), rho (ρ-) and chi (χ-) class of conopeptides [24–30] as well as a conopressin-analogue (single loop) and synthetic analogues with a 'bookend'-like (CC–CC) pairing of cysteine in their sequence.

Table 1 shows the peptides selected for synthesis, sorted by class, pharmacological target, their disulfide bond framework and the number of amino acids between the cysteine residues.

MATERIALS AND METHODS

Reagents

Protected Fmoc amino acid derivatives were purchased from Auspep P/L (Melbourne, Australia). The following side-chain protected Fmoc amino acids were used: Cys(Trt), His(Trt), Hyp(tBu), Tyr(tBu), Lys(Boc), Trp(Boc), Arg(Pbf), Asn(trt), Asp(OtBu), Glu(OtBu), Gln(Trt), Ser(tBu), Thr(tBu), Tyr(tBu). All other Fmoc amino acids were unprotected. Dimethylformamide (DMF), dichloromethane (DCM), diisopropylethylamine (DIEA) and TFA were all peptide-synthesis grade supplied by Auspep P/L (Melbourne, Australia). 2-(1*H*-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), triisopropyl silane (TIPS), dimethyl sulfide (DMS), HPLC-grade acetonitrile, diethyl ether and methanol were supplied by Sigma Aldrich (Australia). The resin used was a Boc-Leu-PAM resin (0.56 mmol/g) supplied by Peptides International (Louisville, KY-USA). Ethandithiol (EDT) was supplied by Merck (Darmstadt, Germany) and Fmoc-SCAL was purchased from CSPS Pharmaceuticals (San Diego, CA, USA).

Fmoc-SCAL-Leu-Pam resin. Boc-Leu-Pam resin (2 g, 0.56 mmol/g) was conditioned overnight in DMF. The Boc-protecting group was removed by neat TFA (10 ml, 2 × 5 min).

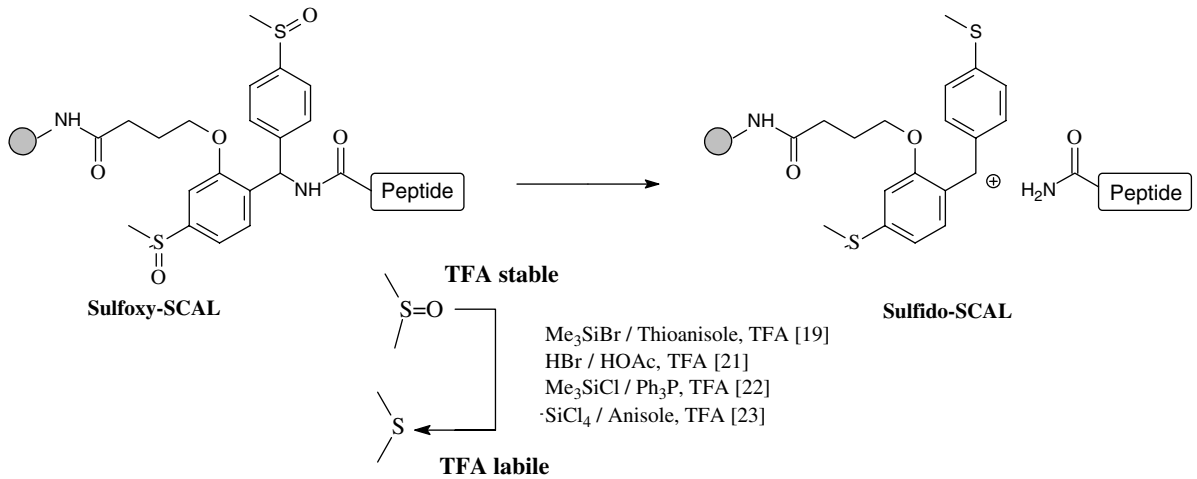

Figure 1 Peptide synthesis on SCAL : Sulfoxide reduction [19,21–23] renders the carboxamide bond of TFA labile and releases the peptide amide.

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Table 1 Conopeptides and their synthetic analogues

Class	ID	Sequence	Target	Source	Framework		Reference
Alpha α	MII	GCCSNPVCHLEHSNLC	nAChR	*C. magnus*	CC–C–C	0-4-7	25
	GI	ECCNPACGRHYSC	nAChR	*C. geographus*	CC–C–C	0-3-5	26
	SI	ICCNPACGPKTSC	nAChR	*C. striatus*	CC–C–C	0-3-5	27
	ImI	GCCSDRRCMVWC	nAChR	*C. imperialis*	CC–C–C	0-4-3	28
Rho ρ	TIA	FNWRCCLIPACRRNHKKFC	α-adrenoreceptor	*C. tulipa*	CC–C–C	0-4-7	13
		FNRCCLIPACRRNHKKFC	—	Synthetic	CC–C–C	0-4-7	
Chi χ	MrIA	NGVCCGYKLCHOC	Norepinephrine-transporter	*C. marmoreus*	CC–C–C	0-4-2	13,29,30
	Xen2174[a]	UGVCCGYKLCHOC	Norepinephrine-transporter	Synthetic	CC–C–C	0-4-2	6,7,14
Other		CCKLMYGCC	—	Synthetic	CC–CC	0-5-0	
		VCCGYKLCC	—	Synthetic	CC–CC	0-4-0	
		VCCGFKLCC	—	Synthetic	CC–CC	0-4-0	
Other	Conopressin S	CFIRNCPRG	Vasopressin agonist	*C. striatus*	C–C	-4-	

[a] Xen2174 is in development for the treatment of severe intractable pain and is currently in phase I/II clinical trials, examining intra-thecal administration in oncology patients experiencing persistent pain [14].

Table 2 LC-MS analysis of peptides obtained from peptide–SCAL–resin cleavage and subsequent DMSO oxidation in cleavage mixture

	Sequence	Resin (mg)	SCAL cleavage (NH_4I/Me_2S/TFA)			SCAL cleavage (NH_4I/Me_2S/TFA) and DMSO oxidation				
			Crude (mg)	Crude-HPLC (%)		Crude oxidized (mg)	Crude oxidized-HPLC (%)			
				M	M + 137		M – 4 Isomer 1	M – 4 Isomer 2	M – 4 Isomer 3	2(M – 4) Dimer
1	FNRCCLIPACRRNHKKFC	35	6.83	35	—	6.07	30	—	—	—
2	GCCSNPVCHLEHSNLC	50	13.7	35	—	9.7	3	22	10	—
3	UGVCCGYKLCHOC	25	6	65	—	4.5	29	22	9	—
4	UGVCCGYKMCHOC	100	23	48	—	17	22	16	—	—
5	ECCNPACGRHYSC	42	8.25	50	—	9.2	4	23	11	—
6	ICCNPACGPKTSC	65	14	64	—	13	13	57	19	—
7	CCKLMYGCC	73	13	80	—	8.3	4	7	73	—
8	VCCGYKLCC	90	1.9	41(M-2)	—	2.4	2	39	10	—
9	VCCGFKLCC	40	3.3	16 + 17(M-2)	—	6.1	8	23	—	—
10	CFIRNCPRG	45	7.4	24 + 28(M-2)	—	12.9	68(M-2)	—	—	17
11	FN**W**RCCLIPACRRNHKKFC	37	1.9	—	—	2.56	—	—	—	—
12	UGVCCGYKLC**W**OC	50	7	36	19	6.7	—	—	—	—
13	UGVCCG**W**KLCHOC	42	25	25	23	—	—	—	—	—
14	GCCSDRRCMV**W**C	45	5	24	20	3.1	—	—	—	—
15	NGVCCGYKLCHOC	120	No workup on reduced stage			7.5[a]/42[b]	11	16	28	
							Peptide recovery (mg)			
							1.17	5.33	7.0	

[a] Crude oxidized yield after linker cleavage with NH_4I/TFA for 4 h and 18 h DMSO oxidation.
[b] Crude oxidized yield after linker cleavage with NH_4I/Me_2S/TFA for 4 h and 18 h DMSO oxidation.

After washing with DMF, the resin was neutralized with a 10% solution of DIEA in DMF (10 ml, 2 × 5 min). The Fmoc-SCAL (1.74 g, 2.7 mmol) was dissolved in 5.4 ml of a solution of HBTU in DMF (0.5 M). After activation with 470 µl of DIEA, the linker mixture was added to the resin and coupled overnight. Washing with DMF and DCM and drying under vacuum delivered the Fmoc-SCAL-Leu-PAM resin (SV = 0.46 mmol/g) used in described experiments.

Peptide Synthesis

The peptides shown in Table 2 were synthesized on an Advanced ChemTech (ACT-396) automated peptide

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synthesizer in a 96-well reaction block. One hundred milligrams of the Fmoc-SCAL-Leu-PAM resin (SV = 0.46 mmol/g) was delivered to each well. Coupling was achieved using Fmoc amino acids dissolved in an equimolar amount of a 0.5 M HBTU solution. Four equivalents of the above solution was dispensed to the resin and activated *in situ* by addition of DIEA (4 equiv.). Fmoc deprotection was achieved by treatment with a solution of 50% piperidine in DMF (1.5 ml, 2 × 5 min).

Side-chain deprotection. After chain assembly and final Fmoc deprotection, the resin was dried and the reaction block transferred onto an ACT-LABTECH shaker. Here, the side-chain cleavage was performed for 3 h in 2.5 ml of TFA/water/EDT/TIPS (87.5 : 5 : 5 : 2.5). The side-chain deprotected peptide–SCAL resins were then washed several times (3 × 3 ml each) starting with TFA, water, methanol, DCM/methanol and DCM and finally dried in a stream of nitrogen.

SCAL activation/cleavage. To the washed and dried petidyl-SCAL-resins, still in the ACT-396 wells, 50 mg of NH_4I, 100 µl of Me_2S and 2.5 ml of neat TFA were added. The cleavage was performed while shaking for 4 h at RT. The addition of Me_2S is optional, but longer cleavage times are required without Me_2S (for details, see, 'Results and Discussion').

Disulfide formation. After SCAL activation/cleavage, the peptide solution was drained into glass vials and split into two fractions (2 × 1.5 ml). To one fraction, DMSO was added to perform oxidation overnight, while the second fraction was left untreated to evaluate the quality of the reduced peptides.

Cold diethyl ether (30 ml) was added to the cleavage mixtures, resulting in the precipitation of the respective reduced or oxidized peptides. The precipitate was collected by centrifugation and subsequently washed with cold diethyl ether (2 × 20 ml) to remove scavengers and linker residues. The final product was dissolved in 50% aqueous acetonitrile (30 ml) and lyophilized to yield a fluffy white solid. The reduced or oxidized crude peptide was examined by reverse-phase HPLC for purity, and the correct molecular weight was confirmed by electrospray ionization mass spectrometry (ESI-MS).

HPLC Analysis

Analytical HPLC runs were performed using a Shimadzu HPLC system with a dual-wavelength UV detector set at 214 nm and 254 nm. A reversed-phase C-18 column (Zorbax 300-SB C18; 4.6 × 50 mm) with a flow rate of 2 ml/min was used. Gradient elution was performed with the following buffer systems: A, 0.05% TFA in water and B, 0.043% TFA in 90% acetonitrile in water, from 0% B to 80% B in 20 min. The crude peptides were purified by semi-preparative HPLC on a Shimadzu HPLC system associated with a reversed-phase C-18 column (Vydac C-18, 25 cm × 10 mm) at a flow rate of 5 ml/min with a 1% gradient of 0–40% B. The purity of the final product was evaluated by analytical HPLC.

ESI-MS

Electrospray mass spectra were collected inline during analytical HPLC runs on an Applied Biosystems quadrupole spectrometer (API-150) operating in the positive ion mode with a declustering potential (DP) of 20V, a focusing potential (FP) of 220 V and a Turbospray heater temperature of 350 °C. Masses between 300 and 1800 amu were detected (step 0.2 amu, dwell 0.3 ms).

RESULTS AND DISCUSSION

Solid-phase Peptide Assembly

Before commencing this work, the performance of the used Fmoc-SCAL-Leu-PAM resin was investigated by manual synthesis of acyl carrier protein ACP (65–74). The achieved coupling yields were quantitatively measured by employing the Ninhydrin test [31]. Coupling yields were greater than 99.5% within 15 min of reaction (results not shown).

The peptides (**1–14**) described in Table 2 were assembled on the Fmoc-SCAL-Leu-PAM resin described above, using the standard assembly protocol as described in 'Material and Methods'.

After side-chain deprotection employing standard cleavage conditions, the side-chain unprotected peptide–SCAL resins were washed several times (3 × 3 ml) starting with TFA, water, methanol, DCM/methanol and DCM, and dried in a stream of nitrogen. This intensive washing was necessary to prevent residual thiol scavengers interfering with the subsequent safety-catch linker activation, peptide oxidation and potential use of the peptides in screening assays.

SCAL cleavage. To evaluate the most suitable cleavage conditions for cysteine-rich conopeptides and their synthetic analogues, methods reported in the literature were revisited. SCAL reduction with subsequent peptide cleavage has been described using 1 M TMSBr/TFA and 0.1% HBr/CH_3COOH activation with subsequent TFA cleavage [18–22]. In our hands, neither cleavage condition (4 h) led to any cleavage of peptides attached via a *C*-terminal cysteine (peptides **1**–**9** and **11**–**14**). Only in the case of the conopressin-analogue (**10**) attached via a *C*-terminal glycine were low yields of peptide obtained. In the search for a more efficient method for the cleavage of the peptides from the SCAL, the sulfoxide reduction system NH_4I/TFA/Me_2S was investigated. This method was originally described for the reduction of methionine sulfoxide [32]. Interestingly, this method was found to be suitable for cleaving all peptides in our test series (**1**–**14**) from the SCAL.

The proposed mechanism [32] of the NH_4I/TFA sulfoxide reduction is shown in Figure 3.

Acid catalysis causes protonation of the sulfoxide, promoting the attack of iodide at the sulfur, forming an iodosulfonium cation. Further acid-catalysed dehydration, followed by reaction of the iodosulfonium cation with iodide, yields the sulfide and iodine as the only by-products. Figure 3 also rationalizes the effect of dimethylsulfide (Me_2S) addition in increasing the reaction rate owing to attack at the iodosulfonium cation affording DMSO.

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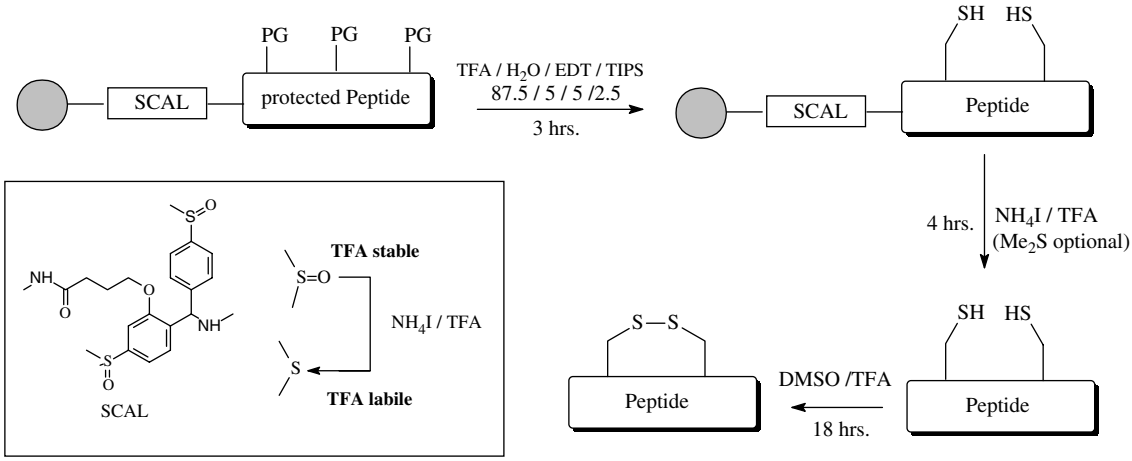

Figure 2 Developed sequential side-chain cleavage, SCAL cleavage and oxidation for high-throughput production of venom peptides.

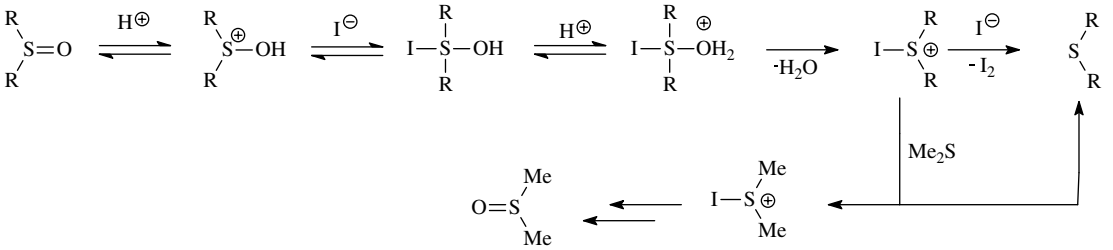

Figure 3 Proposed mechanism [32] for sulfoxide reduction with NH_4I in TFA.

In our experiments, the dry, unprotected peptide–SCAL–resins were treated for 4 h with ammonium iodide (NH_4I, 50 mg) in 2.5 ml TFA and 100 µl of DMS. Under these conditions, the sulfoxide (S=O) side chains of the SCAL were reduced to the thioether (–S–), which enabled the cleavage by TFA and release of the peptide into solution. After 4 h, half the cleavage mixture was treated with diethyl ether to precipitate the crude peptides, which were then lyophilized to yield mainly reduced peptides (see HPLC/MS results, middle column in Table 2). The remaining cleavage mixture was treated as described below.

Peptide Oxidation

In contrast to the observations of Giralt and coworkers [32], the formation of disulfide-bridged peptides under the described conditions (NH_4I/Me_2S/TFA) was observed in only some cases (peptides **8–10**: see HPLC/MS results in Table 2, middle column). An additional oxidation step was therefore introduced into the procedure.

The most effective approach was considered to be the disulfide bond formation with DMSO in TFA, as first described by Fujii and coworkers [33,34], which required the addition of DMSO to the peptide cleavage solution.

In this experiment, the second half of the obtained cleavage mixture (from NH_4I activation/cleavage) of the peptide in TFA (1.5 ml) was treated overnight with DMSO (100 µl) to facilitate the formation of disulfide bonds.

A mixture of the oxidized peptides was then obtained by diethyl ether precipitation and lyophilization from acetonitrile/water (see HPLC-MS results, right column in Table 2).

The crude peptides obtained from the SCAL cleavage with NH_4I/Me_2S/TFA in conjunction with those obtained from cleavage with subsequent DMSO oxidation were analysed by LC-MS. Table 2 summarizes the crude peptide yields and peptide composition from these experiments.

These HPLC/MS results show clearly that the SCAL cleavage with NH_4I/TFA/Me_2S is suitable for the synthesis of conopeptides and their synthetic analogues (peptides **1–14**). The synthesized peptides were obtained as the main product with the correct mass confirmed by ESI-MS. There were no significant side reactions such as oxidation, iodination or hydration observed even with peptides containing sensitive amino acids such as phenylalanine (Phe, F), histidine (His, H), tyrosine (Tyr, Y) and methionine (Met, M) (peptides **1–10**). At this stage of synthesis, peptides **8–10** yielded a mixture of the reduced and partially oxidized peptide, while all the other peptides yielded only the

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reduced peptide. The HPLC/MS analysis of the peptides obtained after sequential linker activation followed by linker cleavage and additional DMSO oxidation is also summarized in Table 2 (right column). As expected, peptides with four cysteine residues that underwent this oxidation step yielded three disulfide-bridged isomers with a mass of M – 4 in relation to the reduced peptide. The nomenclature given to the three disulfide-bridged isomers relates to the order in which the oxidized peptides eluted from the HPLC column using the described method.

The formation of disulfide-bridged peptides is a thermodynamically driven process. Owing to the solvation of amino acids, the dielectric constant (ε) of the solvent plays an important role in the preferred conformation and therefore formation of a certain disulfide-folded isomer. A difference in the relative isomer contributions obtained from oxidations in aqueous solutions ($\varepsilon = 78.5$) *versus* TFA ($\varepsilon = 42.1$) is therefore expected. For example, oxidation of Xen2174 (**3**) in an aqueous solution (1 mg/ml) of 30% DMSO/0.1 M NH_4OAc (pH 6.0) yields a distribution of isomers 1–3 at 15, 57 and 8%.

The crude oxidized peptide (**3**) obtained via SCAL cleavage (NH_4I/TFA) followed by DMSO (4%) oxidation in the cleavage mixture yielded a different isomer distribution, isomers 1–3 at 29, 22 and 9%.

Our peptide library contains novel disulfide-rich peptides. In order to obtain maximum diversity, the aim is not for the selective production of one specific disulfide-bridged isomer, but rather to evaluate the pharmacological properties of all the formed isomers. The developed method is therefore applicable to the drug development process, as it is not strongly differentiating between disulfide-bridged peptides. Some examples of HPLC-ESI-MS traces of obtained oxidized crude peptides are shown in Figure 4.

Peptides containing tryptophan (Trp, W, peptides **11–14**) are not suitable for the developed method. Cleavage of peptide **11** (TIA) did not deliver the expected product. Peptides **12–14** did yield some of the expected reduced peptide, but also showed a large proportion of a peptide adduct with an additional mass of 137 amu. This adduct is most likely related to SCAL fragmentation and the subsequent irreversible alkylation of the sensitive tryptophan residues (Figures 5 and 6).

After DMSO/TFA oxidation of Trp-containing peptides (**11–14**), none of the expected peptide was obtained. This result underlines the high acid lability of unprotected Trp, which undergoes acid-catalysed 2,2′ coupling between unprotected Trp residues, a side reaction that was not minimized with the addition of Me_2S [32].

This finding is strongly supported by comparison of the results obtained for TIA (**11**) and the associated Trp-deletion analogue (**1**). The linker cleavage/oxidation of **11** gave products that could not be characterized; however, the Trp-deletion analogue of **11** yielded the corresponding reduced peptide **1** and the oxidized disulfide-folded analogue as expected.

To evaluate the peptide recoveries obtained from this method, we have assembled libraries of peptides sourced from our venom peptide sequence library. As a representative example, the results obtained for MrIA (**15**) are included in Table 2.

MrIA (**15**) and the library peptides were, after automated assembly using 100 mg of Fmoc-SCAL-Leu-PAM resin (SV = 0.46 mmol/g), treated as described here. The cleavage from the SCAL-resin was performed with NH_4I (50 mg) in TFA (2.5 ml) for 4 h, once under addition of Me_2S (100 µl), and once without Me_2S, followed by DMSO oxidation in the cleavage mixture. Both methods delivered oxidized MrIA with very similar cystine isomer ratios. However, the yield of the oxidized crude peptide differed from 42 mg with Me_2S addition to 7 mg without. The overall cleavage rate increase effect of Me_2S can be explained by a contribution during sulfoxide reduction (Figure 3) but also during carboxamide cleavage due to the highly nucleophilic Me_2S increasing the rate of fission. The choice of cleavage media depends on the intended use of the resultant peptides. If the crude isomer mixtures are to be used directly in pharmacological assays, the cleavage can be performed without Me_2S addition but using longer cleavage times. If an HPLC purification step is performed prior to pharmacological testing, the addition of Me_2S is not problematic and improves cleavage rate and yield. Using the example of MrIA (**15**), good yields of individual isomers 1–3 (1.17/5.33/7.0 mg) were obtained following HPLC purification.

CONCLUSION

In this work, it has been demonstrated that disulfide-bridged conopeptides can be efficiently synthesized by employing a SCAL. The removal of side-chain protecting groups under standard conditions and thorough washes yielded a 'naked' peptide on resin. Release of the peptide into TFA solution was efficiently achieved by NH_4I/TFA linker activation and subsequent cleavage. Addition of Me_2S increased cleavage rate and peptide yield, but it is not necessary if scavenger-free peptides are required. The peptide folding was facilitated in cleavage mixture by DMSO addition, to yield mixtures of oxidized conopeptides, requiring only a single HPLC purification step to achieve purities of >95%.

This broadly applicable method, with the exception of Trp-containing peptides, allows the synthesis of disulfide-folded conopeptides in a 96-well format on a peptide synthesizer. This method thus represents

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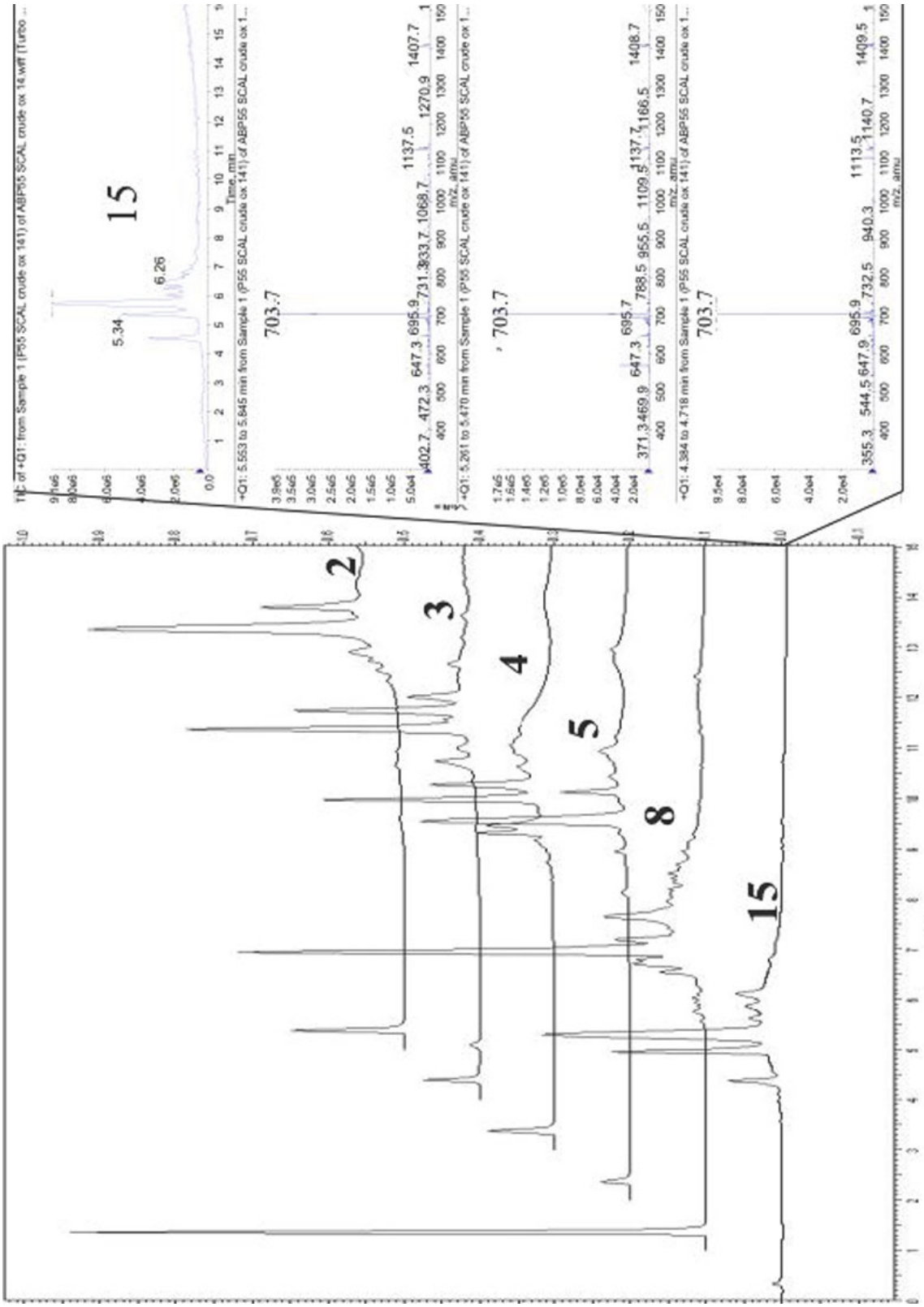

Figure 4 HPLC traces of peptides (**2–5**, **8**, **15**) obtained from sequential side-chain deprotection, SCAL activation/cleavage and subsequent DMSO oxidation. ESI-MS trace of peptide **15** (MrIA) is shown.

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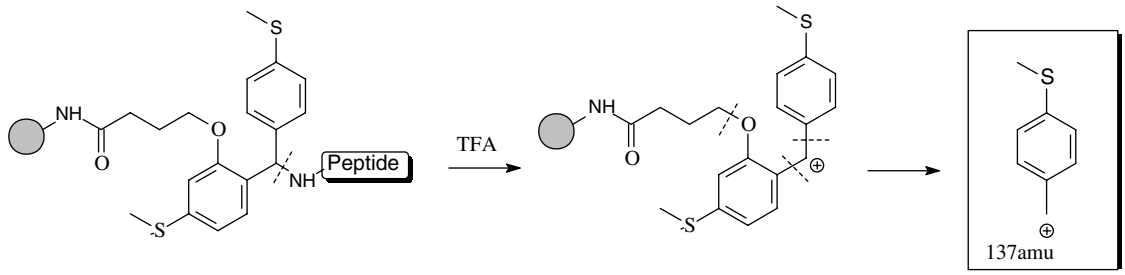

Figure 5 SCAL fragmentation: Dashed lines show the possible cleavage position forming a resonance-stabilized carbocation with a mass of 137 amu leading to alkylation of Trp-containing peptides.

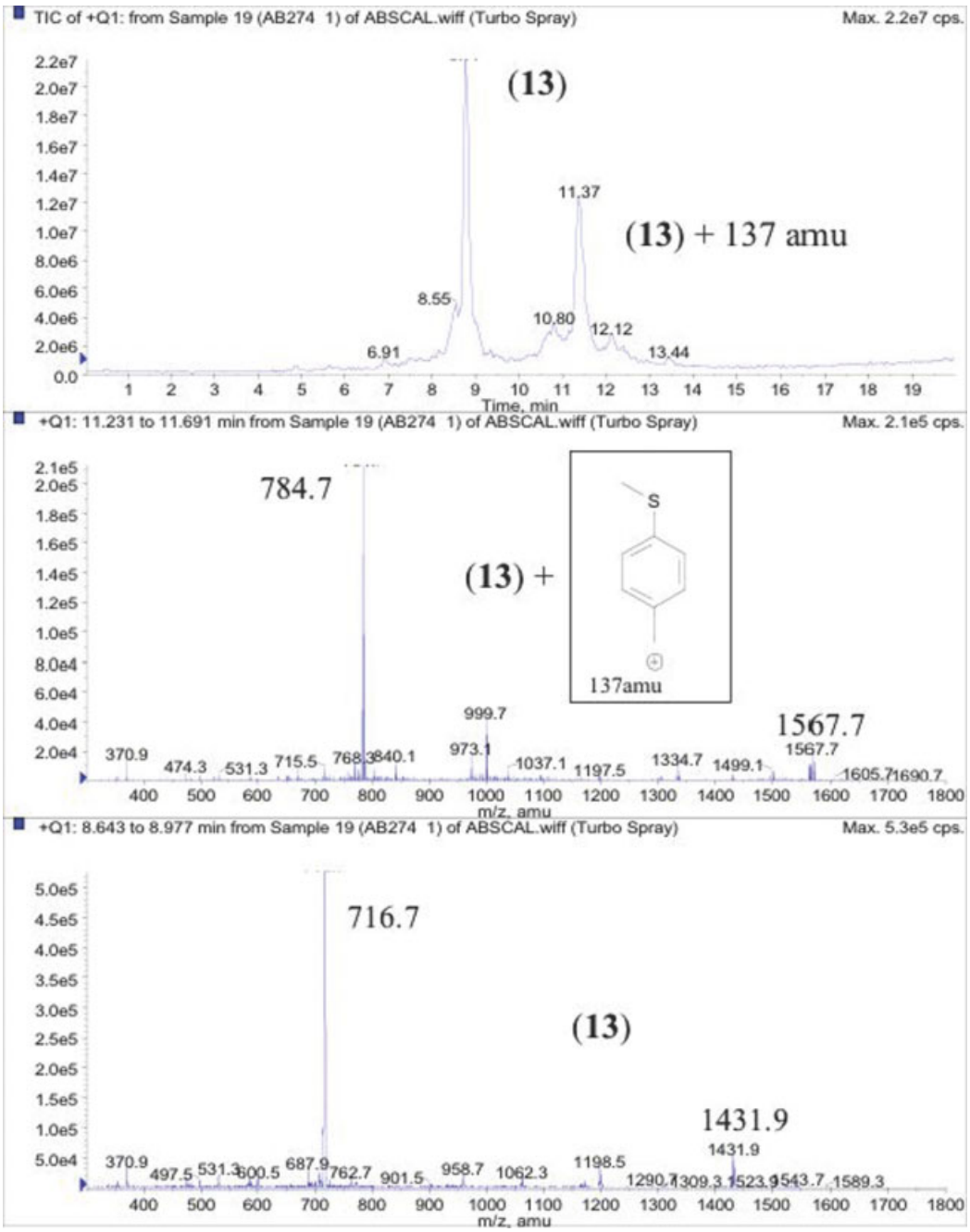

Figure 6 ESI-MS and HPLC profile of the Trp-containing peptide (**13**) obtained after NH_4I/TFA cleavage, showing the alkylated by-product (M + 137).

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an important development towards efficient, high-throughput production of pharmacologically active peptides.

The procedure has been validated for the routine production of venom-peptide libraries.

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