Novabiochem®
Innovations: 4/11

NEW Tools for native chemical ligation

The sulfonamide method as described by Pessi and co-workers [1] remains the most frequently used approach to generating peptide thioesters by Fmoc for use in native chemical ligation (NCL) reactions [2]. It involves displacement of the peptide fragment with a thiol from an alkylated peptidyl-sulfamylbutyryl resin (Figure 1). The sulfonamide method, whilst popular, has been plagued by notoriously low yields. These originate from three sources:

1) incomplete acylation of the resin-bound sulfonamide with the C-terminal residue;

2) incomplete alkylation of the sulfonamide, which is usually performed blind and not optimized;

3) incomplete thiolysis, due to the inherent low reactivity of the activated sulfonamide towards thiols and poor solvation of the resin-bound protected peptide.

In this Innovation, the development of a novel dual linker strategy is described [3], involving anchoring of the sulfonamide linker to a standard acid-labile resin. This approach overcomes the limitations of the sulfonamide method and provides a simple and robust strategy for Fmoc SPPS-based NCL (Figure 2).

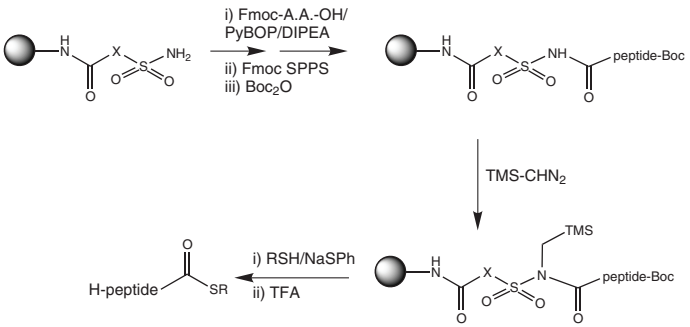


Fig. 1: Synthesis of thioesters using sulfamyl linkers.

Double linker strategy

Features

- Loading of the sulfamylbutyryl linker with the C-terminal residue prior to its attachment to the solid phase overcomes issues with incomplete loading, enantiomerization and double addition that can occur during on-resin linker functionalization.
- By using Rink amide or Sieber amide resin as the solid support for the pre-loaded linker, the *N*-peptidylsulfonamide can be released from the resin by TFA treatment, enabling the progress of the synthesis and extent of methylation to be easily checked by LC-MS.
- The unprotected *N*-peptidyl-N-methyl-sulfonamide that is released from the resin by TFA treatment can be used directly in NCL reactions without prior conversion to a thioester, thereby eliminating the poor yields associated with on-resin thiolysis.

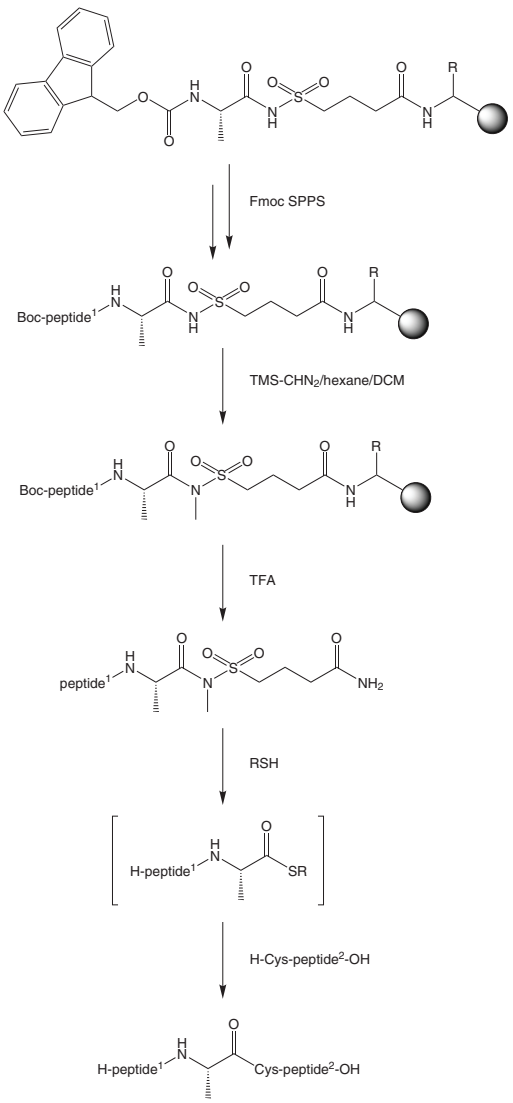


Fig. 2: Double linker strategy for NCL by Fmoc SPPS.

Model synthesis

To evaluate the double linker approach, *N*-peptidyl-*N*-methylsulfonamide **1** (H-Ala-Tyr-Arg-Ala-Ala-N(Me)$SO_2CH_2CH_2CH_2CONH_2$) was prepared to test its utility as surrogate thioester in model NCL reactions. To this end, Fmoc-Ala-sulfamylbutyric acid was prepared and coupled to Sieber Amide resin. (Rink Amide AM resin works equally well for this application.) Peptide chain extension was performed using standard Fmoc synthesis protocols, except the *C*-terminal residue was incorporated as a Boc-protected amino acid.

Methylation was effected by treatment with 2M trimethylsilyl diazomethane (TMS-CHN_2) in hexane/DCM (1:1). The progress of the reaction was checked by treating small sample of resin with TFA/TES/water/EDT (94.5:2.5:2.5:0.5) for 1.5 hours and analysing the cleaved peptide by LC-MS (Figure 3). The reaction was complete in 18 hours. Treatment of the bulk resin with TFA as described previously afforded crude peptide **1**, which was purified by RP-HPLC prior to its use in model ligation reactions.

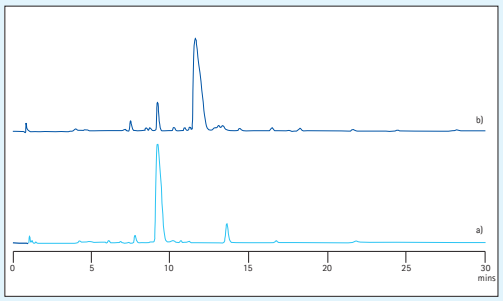


Fig. 3: HPLC profiles of a) crude peptide 1 prior to methylation and b) after methylation.

A ligation reaction was conducted in 1M GnHCl, 60 mM 4-mercaptophenylacetic acid (MPAA), 0.2 M pH 7.5 phosphate buffer using peptide **1** as the latent thioester and H-Cys-Phe-Ala-Pro-Leu-Val-OH **2** as the *N*-cysteinyl component. Ligation between peptides **1** and **2** was found to occur at a moderate rate (Figure 4).

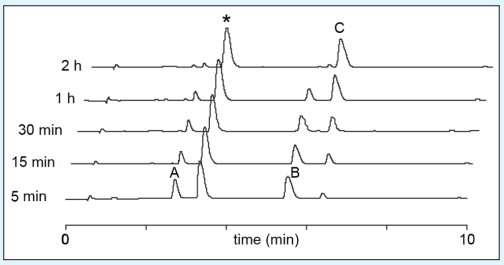
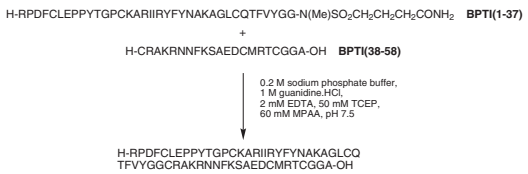


Fig. 4: Ligation of peptides 1 (A) & 2 (B); *MPAA; H-Ala-Tyr-Arg-Ala-Ala-Cys-Phe-Ala-Pro-Leu-Val-OH (C)

Synthesis of BPTI

Following the success of the double linker strategy in the synthesis of a small model peptide, the preparation of BPTI was then undertaken to provide a more challenging and realistic test of the method. BPTI is a 58 residue, small protein containing a full range of side chain functional groups. Its length is more typical of peptides used for NCL. The strategy of Lu and co-workers [4] was selected for the preparation of BPTI, involving the ligation of two fragments: BPTI(1-37) and BPTI (38-58) (Figure 5).

Fig. 5: Synthesis of BPTI.



BPTI(38-58) was assembled on Fmoc-Ala-Wang resin using standard HOBt/DIPCI activation (Application 1).

Application 1: Synthesis of BPTI (38-58)

BPTI(38-58) was assembled using a CS Bio 336 automated synthesizer on Fmoc-Ala-Wang resin using 5-fold excesses of Fmoc-amino acids activated with DIPCDI/HOBt in DMF. A coupling time of 45 min was used throughout. Fmoc removal was effected by three 5 min treatments of 20% piperidine in DMF. Cleavage of the peptide from the resin with concommitant side-chain deprotection was achieved by treatment with TFA/TES/water/EDT (94.5:2.5:2.5:0.5) for 1.5 h. The crude peptide was analyzed (Figure 6) and purified by RP-HPLC.

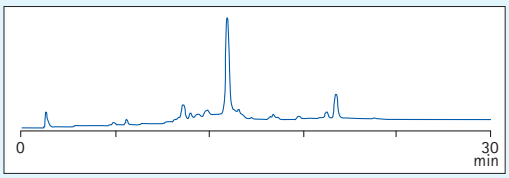


Fig. 6: HPLC profile of BPTI (38-58).

BPTI(1-37) was assembled in a similar manner on Fmoc-Gly-sulfamylbutyryl Sieber Amide resin. Methylation of the linker was effected by treatment with 2M TMS-CHN_2 in hexane/DCM (1:1) for 18 hours. TFA cleavage afforded the deprotected *N*-BPTI(1-37)-*N*-methylsulfonamide. Following purification by RP-HPLC, the two fragments were ligated (Fig. 2) using low millimolar fragment concentration. The reaction was almost complete in 8 h, a time comparable to that observed by Lu and co-workers [4] with classical NCL using a preformed thioester.

Application 2: Synthesis of BPTI (1-37)

BPTI(1-37) was assembled using a CS Bio 336 automated synthesizer on Fmoc-Gly-sulfamylbutyryl Sieber Amide resin using 5-fold excesses of Fmoc-amino acids activated with DIPCDI/HOBt in DMF. A coupling time of 45 min was used throughout. Fmoc removal was effected by three 5 min treatments of 20% piperidine in DMF. A small sample of resin was treated with TFA/TES/water/EDT (94.5:2.5:2.5:0.5) for 1.5 h and the purity of the isolated product checked by RP-HPLC (Figure 7a). The bulk of the resin was then treated overnight with TMS-CHN_2 in hexane/DCM (1:1). *N*-BPTI(1-37)-*N*-methylsulfamylbutyramide was cleaved from the resin by treatment with TFA as described above and the isolated product analyzed by RP-HPLC (Figure 7b). The crude peptide was purified by RP-HPLC.

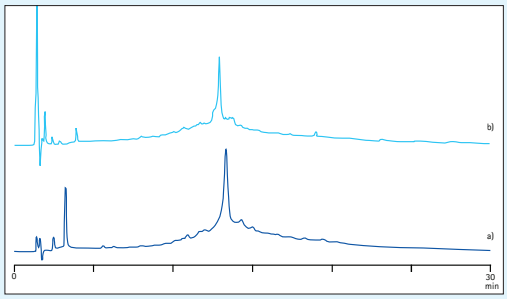


Fig. 7: HPLC profiles of a) *N*-BPTI(1-37)-sulfamyl-butyramide & b) *N*-BPTI(1-37)-*N*-methylsulfamyl-butyramide.

Application 3: Preparation of BPTI (1-58)

N-BPTI(1-37)-*N*-methylsulfamylbutyramide (10 mg, 2 µmol) and BPTI(38-58) (5.8 mg, 2 µmol) were dissolved in 1 mL of degassed 0.2 M sodium phosphate buffer pH 7.5, containing 6 M guanidine·HCl, 2 mM EDTA, 50 mM TCEP, 60 mM MPAA. The solution was heated at 40 °C. The reaction was allowed to stir under Ar for 8 h. HPLC purification yielded linear BPTI (2.6 mg, 16% yield). The product was characterized by MALDI-TOF MS in positive linear mode using CHCA matrix: m/z= 6517.9 [M+H]+ (average isotope composition), calc: 6517.6. Figure 8 shows the monitoring by HPLC of a preliminary sample scale ligation reaction.

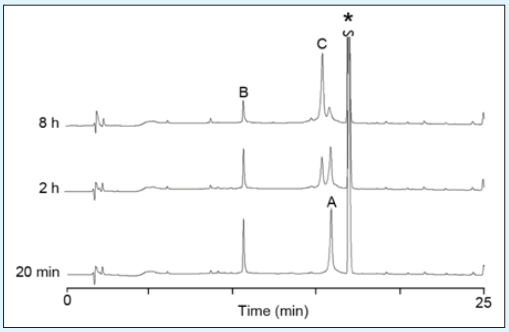


Fig. 8: HPLC profiles of monitoring of a preliminary ligation reaction. *N*-BPTI(1-37)-N-methylsulfamyl-butyramide (A), BPTI(38-58) (B); BPTI(1-58) (C); *MPAA.

Conclusion

The double linker approach described here provides a simple and scalable method for peptide ligation *via* Fmoc SPPS, which overcomes many of the limitations of the existing sulfamylbutyryl method. This development should prove to be a valuable new tool for the synthesis of the chemically defined post-translationally modified proteins.

Ordering Information

In the experiments described overleaf, Sieber Amide resin was used to support the pre-loaded sulfamylbutyryl linker. However, for the commercial products, Rink Amide AM resin was used as it is more cost effective.

Prod. No.	Product name	Pk	Price
855147	4-Sulfamylbutyryl Rink Amide AM resin	1 g	POA
NEW		5 g	POA
856191	Fmoc-Ala-4-Sulfamylbutyryl Rink Amide AM resin	1 g	POA
NEW		5 g	POA
856192	Fmoc-Gly-4-Sulfamylbutyryl Rink Amide AM resin	1 g	POA
NEW		5 g	POA

References

1. R. Ingenito, et al. (1999) *J. Am. Chem. Soc.*, **121**, 11369.
2. S. B. Kent (2009) *Chem. Soc. Rev.*, **38**, 338.
3. F. Burlina, et al. (2012) *Chem. Commun.*, DOI:10.1039/C2CC15911B.
4. W. Lu, et al. (1998) *FEBS Lett*, **429**, 31.