Novabiochem®
Innovations: 2/11

Step-wise Fmoc SPPS of ubiquitin probes

Ubiquitin (Ub) is a small 76 residue protein that is found in most tissues of eukaryotic organisms. The principal role of ubiquitin is to tag proteins for destruction by the proteasome, although it is also implicated in DNA repair, and protein localization and transcription [1]. Ubiquitinylation is mediated by three enzymes, E1, E2, E3 and results in acylation of protein amino groups by the C-terminal carboxylate of ubiquitin. The initial step is formation of a thioester between this carboxyl group and the active site cysteine residue of E1. This complex acts as the ubiquitin donor to the other members of the enzyme cascade.

Ubiquitin is able to conjugate to itself through any of its seven lysine residues to form polymers. Lysine-48 linked ubiquitin polymers target proteins to the 2S6 proteasome for proteolysis.

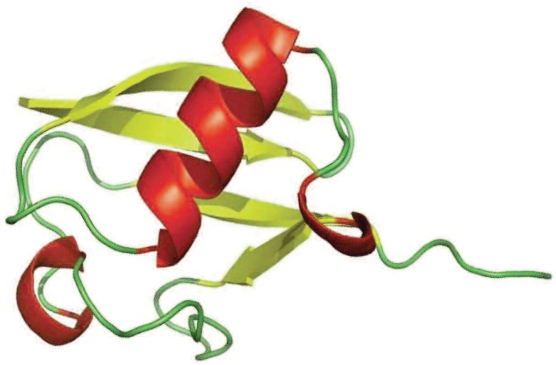


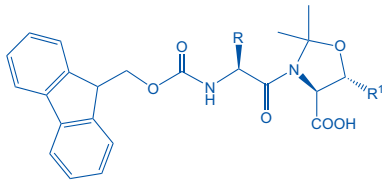
Fig. 1: Crystal structure of ubiquitin [2].

This innovation describes the facile Fmoc SPPS synthesis of native, *N*- and *C*-terminally labeled, and thiolysine mutants of ubiquitin, expedited through the judicious use of pseudoproline and Dmb-dipeptides [3]. These proteins should prove invaluable as tools for studying the function of ubiquitin and the role of polyUb topoisomers. The results shown here were kindly provided by Dr. Huib Ovaa of the Netherlands Cancer Institute and CSO of UbiQ Bio BV.

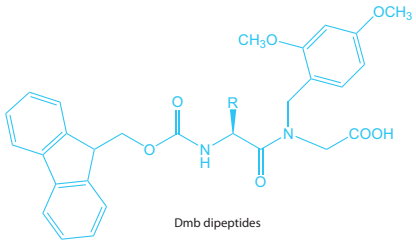
Synthesis design & general procedures

Synthesis design

Pseudoproline dipeptides & Fmoc–Aaa–(Dmb)Gly–OH



Pseudoproline dipeptides



Dmb dipeptides

Pseudoproline and Dmb dipeptides were incorporated into Ub at the sites indicated in Figure 2, according to the Novabiochem® guidelines for insertion of structure breaking derivatives. Four pseudoproline dipeptides and two Dmb dipeptides were utilized. Fmoc-Asp(OtBu)-(Dmb)Gly-OH served also to protect the growing peptide from piperidine-mediated aspartimide formation.

- Optimal results are obtained if the Dmb/pseudoproline derivatives are spaced 5-6 residues apart throughout the sequence.
- The optimum separation between a Dmb/pseudoproline and a Pro residue is 5-6 amino acid residues.
- The minimum separation between a Dmb/pseudoproline and another Dmb/pseudoproline derivative or Pro residue is 2 residues.
- Aim to insert a Dmb/pseudoproline before regions of hydrophobic residues.

General synthetic procedures [3]

Peptide synthesis was carried out using a Syro II MultiSynTech automated peptide synthesizer at a 25 µmol scale on either pre-loaded Wang (0.2 mmol/g) or NovaSyn®TGT (0.2 mmol/g) resins. Couplings were performed using 4-fold excess of Fmoc-amino acids activated with PyBOP®/DIPEA (1:2) in NMP. For the first 30 cycles a coupling time of 45 minutes was used, thereafter this was extended to 1 hour. Pro^{37} and Thr^{12} were double coupled for 90 mins. A 5 minute capping step was performed after each coupling reaction using acetic anhydride/DIPEA/HOBt (0.5 M: 0.125 M:0.015 M) in NMP. Fmoc removal was effected by treating the resin with 20% piperidine in NMP.

The peptides were cleaved from the resin by treatment with TFA/water/phenol/TIPS (90.5:5:2.5:2) for 3 h, and isolated by centrifugation followed by precipitation with cold ether/pentane (3:1).

Purification & folding [3]

Crude Ub was dissolved in a minimum volume of warm DMSO and then diluted with 50 mM NaOAc pH 4.5 such that the final DMSO concentration was 2-10%. The folded peptide was purified by cation exchange chromatography on a MonoS column using a 0 to 1 M NaCl gradient in 50 mM acetate buffer, pH 4.5. Fractions containing product were identified by LC-MS, pooled and then were desalted using a 3 kDa spin column prior to lyophilization.

Fig. 2: Primary sequence of Ubiquitin.

Sites of pseudoproline dipeptide substitutions are highlighted in dark blue; Dmb dipeptides are highlighted in gray.

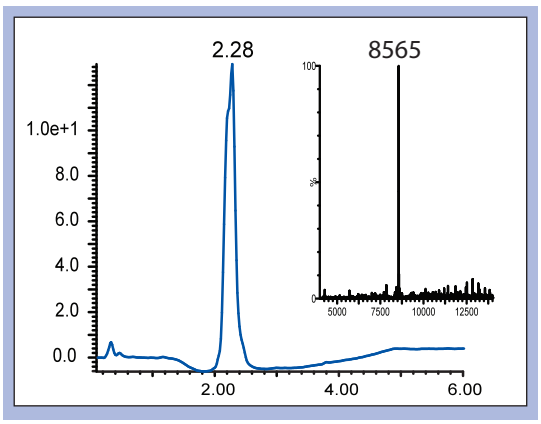
a) H-Met-Gln-Ile-Phe-Val-Lys-Thr-**Leu-Thr**-Gly-Lys-Thr-**Ile-Thr**-Leu-Glu-Val-Glu-Pro-Ser-Asp-Thr-Ile-Glu-Asn-Val-Lys-Ala-Lys-Ile-Gln-Asp-Lys-Glu-Gly-Ile-Pro-Pro-Asp-Gln-Gln-Arg-Leu-Ile-Phe-Ala-Gly-Lys-Gln-Leu-Glu-Asp-Gly-Arg-Thr-**Leu-Ser**-Asp-Tyr-Asn-Ile-Gln-Lys-Glu-**Ser-Thr**-Leu-His-Leu-Val-Leu-Arg-Leu-Arg-Gly-Gly-OH

Synthesis of ubiquitin probes

Native Ubiquitin

Native Ub was prepared according to the general method employing pseudoproline and Dmb dipeptides [3]. A control synthesis not utilizing these derivatives did not produce the desired peptide. Incorporation of $S^{65}T^{66}$ using standard Fmoc amino acids afforded Ub but in poor yield. Omission of any other pseudoproline dipeptides resulted in a failed assembly. Ub was obtained in 14% after refolding and purification. The HPLC chromatograph and EC-MS spectrum of crude Ub obtained is shown in Figure 3.

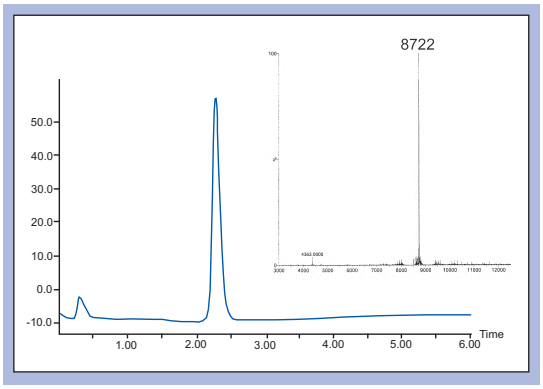
Fig. 3: HPLC profile and ES-MS spectrum of crude ubiquitin.



Ubiquitin amidomethylcoumarin (UbAMC)

UbAMC was prepared as an activity reporter for deubiquitinating enzymes. Ub (1-75) was assembled on Fmoc-Gly-NovaSyn®TGT resin according to the general method, except the N-terminal Met residue was incorporated using a Boc-protected derivative. Cleavage of fully protected Ub was effected by treatment with 25% HFIP in DCM. Remarkably, this 75 residue fully protected peptide was soluble in DCM, enabling its facile coupling to H-Gly-AMC with PyBOP/TEA. The excellent solubility of this protected fragment is almost certainly due to solubility enhancing affects of the backbone protecting groups (pseudoproline and Dmb). Cleavage, refolding and purification were carried out according to the general procedure to afford UbAMC in 6% yield (Figure 4).

Fig. 4: HPLC profile and ES-MS spectrum of crude UBAMC.



Diubiquitin

Ub topoisomers were prepared by the native chemical ligation of Ub thioester with δ-thiolysine-containing Ub mutants (Figure 5) [3]. Ub thioester was generated by treatment of Ub with mercaptoethanesulfonic acid sodium salt (MESNa) in the presence of the enzyme E1. Seven mutants of Ub were prepared substituting methyl disulfide protected δ-thioLys in place of each of the Lys residue. Gly^{76} was substituted with Val to prevent processing by the E1 enzyme. Ligation of these to the Ub thioester and subsequent radical desulfurization with 2,2-azobis(2-methylpropion-amidine (V-50) afforded the desired diubiquitins. Figure 6 shows the HPLC chromatograph and ES-MS spectrum obtained for K^{27}-linked diubiquitin.

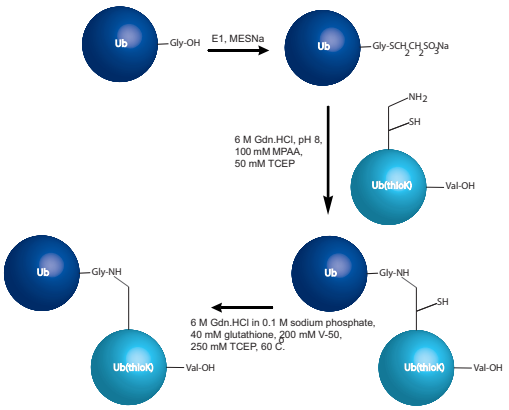


Fig. 5: Synthesis of diubiquitins.

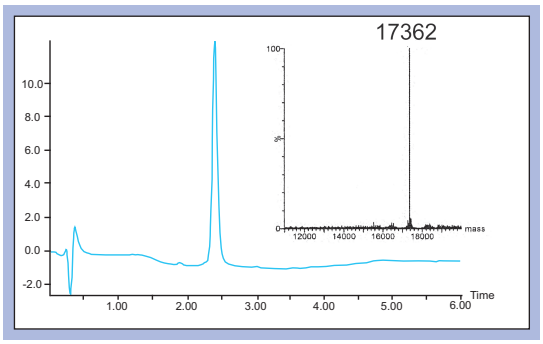


Fig. 6: HPLC profile and ES-MS spectrum of K^{27}-linked diubiquitin.

Conclusions

- The use of pseudoproline and Dmb dipeptides has enabled the synthesis of ubiquitin in high yield and purity.
- This approach has enabled the rapid and efficient parallel synthesis of many analogs of ubiquitin incorporating tags, labels, and N- and C-terminal modifications.
- Fully protected ubiquitin with pseudoproline and Dmb backbone amide protection exhibits remarkable solubility in organic solvents.

Ordering information

852175	Fmoc-Ala-Ser($\Psi^{Me,Me}$pro)-OH	1 g 5 g
852180	Fmoc-Ala-Thr($\Psi^{Me,Me}$pro)-OH	1 g 5 g
852185	Fmoc-Asn(Trt)-Ser($\Psi^{Me,Me}$pro)-OH	1 g 5 g
852183	Fmoc-Asn(Trt)-Thr($\Psi^{Me,Me}$pro)-OH	1 g 5 g
852186	Fmoc-Asp(OtBu)-Ser($\Psi^{Me,Me}$pro)-OH	1 g 5 g
852199	Fmoc-Asp(OtBu)-Thr($\Psi^{Me,Me}$pro)-OH	1 g 5 g
852190	Fmoc-Gln(Trt)-Ser($\Psi^{Me,Me}$pro)-OH	1 g 5 g
852198	Fmoc-Gln(Trt)-Thr($\Psi^{Me,Me}$pro)-OH	1 g 5 g
852177	Fmoc-Glu(OtBu)-Ser($\Psi^{Me,Me}$pro)-OH	1 g 5 g
852196	Fmoc-Glu(OtBu)-Thr($\Psi^{Me,Me}$pro)-OH	1 g 5 g
852200	Fmoc-Gly-Ser($\Psi^{Me,Me}$pro)-OH	1 g 5 g
852197	Fmoc-Gly-Thr($\Psi^{Me,Me}$pro)-OH	1 g 5 g
852194	Fmoc-Ile-Ser($\Psi^{Me,Me}$pro)-OH	1 g 5 g
852193	Fmoc-Ile-Thr($\Psi^{Me,Me}$pro)-OH	1 g 5 g
852179	Fmoc-Leu-Ser($\Psi^{Me,Me}$pro)-OH	1 g 5 g
852184	Fmoc-Leu-Thr($\Psi^{Me,Me}$pro)-OH	1 g 5 g
852178	Fmoc-Lys(Boc)-Ser($\Psi^{Me,Me}$pro)-OH	1 g 5 g
852191	Fmoc-Lys(Boc)-Thr($\Psi^{Me,Me}$pro)-OH	1 g 5 g
852195	Fmoc-Phe-Ser($\Psi^{Me,Me}$pro)-OH	1 g 5 g
852201	Fmoc-Phe-Thr($\Psi^{Me,Me}$pro)-OH	1 g 5 g
852187	Fmoc-Ser(tBu)-Ser($\Psi^{Me,Me}$pro)-OH	1 g 5 g
852192	Fmoc-Ser(tBu)-Thr($\Psi^{Me,Me}$pro)-OH	1 g 5 g
852202	Fmoc-Trp(Boc)-Ser($\Psi^{Me,Me}$pro)-OH	1 g 5 g
852188	Fmoc-Trp(Boc)-Thr($\Psi^{Me,Me}$pro)-OH	1 g 5 g
852189	Fmoc-Tyr(tBu)-Ser($\Psi^{Me,Me}$pro)-OH	1 g 5 g
852182	Fmoc-Tyr(tBu)-Thr($\Psi^{Me,Me}$pro)-OH	1 g 5 g
852176	Fmoc-Val-Ser($\Psi^{Me,Me}$pro)-OH	1 g 5 g
852181	Fmoc-Val-Thr($\Psi^{Me,Me}$pro)-OH	1 g 5 g
852108	Fmoc-Ala-(Dmb)Gly-OH	1 g 5 g
852115	Fmoc-Asp(OtBu)-(Dmb)Gly-OH	1 g 5 g
852109	Fmoc-Gly-(Dmb)Gly-OH	1 g 5 g
852114	Fmoc-Ile-(Dmb)Gly-OH	1 g 5 g
852121	Fmoc-Leu-(Dmb)Gly-OH	1 g 5 g
852116	Fmoc-Val-(Dmb)Gly-OH	1 g 5 g

References

1. M, Hochstrasser, et al. (2009) *Nature*, **458**, 422.
2. S. Vijay-Kumar (1987) *J. Mol.Biol.* **194**, 531. (PDB ID: 1UBQ)
3. F. El Oualid, et al. (2010) *Angew. Chem. Int. Ed.*, **49**, 10149.