Novabiochem®
innovations 6/04

Epimerization-free fragment condensation with pseudoproline (oxazolidine) dipeptides

Convergent solution and solid phase synthesis methods are the favored strategies for large scale GMP production of peptides [1]. Here peptides are constructed by the systematic joining together of fully protected fragments that have been previously assembled by solution or solid phase methods. Whilst in normal step-wise synthesis, racemization (or enantiomerization [2]) is generally not considered an issue, in fragment condensation the prevention of epimerization is of paramount concern. This is because, in contrast to urethane-protected amino acids, peptides easily form chirally labile oxazolones upon C-terminal carboxyl activation that participate in amide bond formation (Fig. 1) [3].

The normal strategy for overcoming this problem is to design syntheses in such a way that, wherever possible, N-terminal fragments are selected which contain either a C-terminal Gly or Pro residue, as the former is achiral and the latter does not readily form oxazolones. However, depending on the sequence this is not always possible, and other fragments containing more enantiomerization-prone amino acids at their C-terminus must then be chosen.

T. Wöhr, *et al.* [4] have observed that peptides containing C-terminal pseudoproline dipeptide residues can be coupled without stereomutation. As pseudoproline residues are simply masked serine or threonine, this provides a means for peptides containing these amino acids at their C-termini to be coupled without loss of chiral integrity, thereby doubling the number of sites in any given peptide available for epimerization-free fragment condensation.

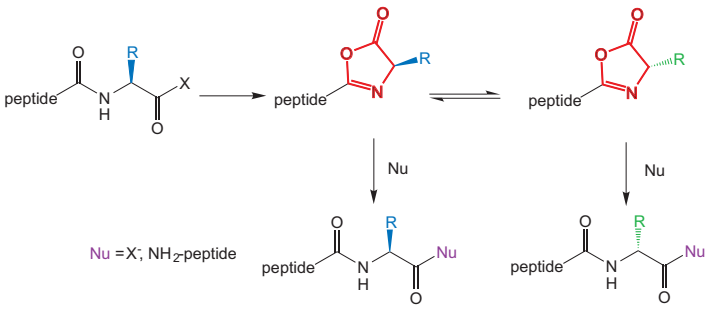


Fig. 1: Mechanism of peptide epimerization by oxazolone formation.

"In our hands, pseudoproline derivatives have proven very effective - particularly in the synthesis of peptides with difficult and long sequences. Using pseudoprolines, we saved time and money for repeat synthesis of failed sequences. We now routinely use pseudoproline derivatives for our peptide synthesis. I would highly recommend using them for peptide synthesis in the manufacturing industry as well. I am glad Novabiochem took the lead in manufacturing pseudoprolines in bulk".

Ved Srivastava, Amylin Pharmaceuticals Inc, San Diego, CA

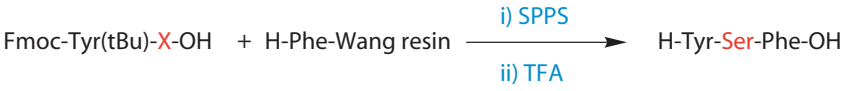
"Pseudoproline dipeptides have greatly increased our success rate for synthesizing both long and difficult peptides. If we are able to integrate pseudoprolines into our syntheses, we can easily machine-synthesize peptides up to 80 amino acids in length. Routine use of pseudoprolines in our peptide syntheses has considerably increased the yield and purity, as well as decreased the number of failed syntheses. They are wonderful products!"

Yingwei He, Protein Chemistry Dept., Abgent, San Diego, CA.

"Biomol started incorporating pseudoproline derivatives into its everyday schedules for routine peptide synthesis some eight years ago. Over the intervening years, the use of these reagents on a routine basis has led to a dramatic reduction in the necessity for repeat synthesis. When coupled with an undoubted improvement in the yield and purity of crude peptides obtained, this has meant considerable financial savings in terms of both synthesis and purification costs. We are firmly of the opinion that the benefits of incorporation of pseudoproline analogs into peptide synthesis protocols is fully justifiable on both scientific and commercial grounds and is to be recommended on a routine basis."

Paul Sheppard, Biomol International Lp, Exeter, UK.

Enantiomerization studies



X= Ser(tBu), Ser($\Psi^{Me,Me}$pro)

To confirm the resistance of C-terminal pseudoproline residues to enantiomerization during peptide bond formation, the model peptides Fmoc-Tyr(tBu)-Ser(tBu)-OH **1,** Fmoc-Tyr(tBu)-D-Ser(tBu)-OH **2** and Fmoc-Tyr(tBu)-Ser($\Psi^{Me,Me}$pro)-OH **3** were coupled to H-Phe-Wang resin under a range of conditions, the products cleaved with TFA, and the extent of epimerization assessed by HPLC. A typical result using PyBOP® activation is shown in Figure 2. No detectable epimerization was observed with the peptide prepared using a pseudoproline dipeptide, whereas in the one made with Ser(tBu), the serine was nearly totally racemized. Similar results were obtained with other coupling methods (Table 1).

Table 1: Studies comparing the enantiomerization of C-terminal Ser residue when protected as a pseudoproline or t-Bu ether.

Experiment	Peptide	Conditions	Solvent	% L-Ser	% D-Ser
1	1	PyBOP/DIPEA	DMF	65	35
2	2	PyBOP/DIPEA	DMF	36	64
3	3	PyBOP/DIPEA	DMF	100	0
4	1	PyBOP/collidine	DMF	68	32
5	1	HATU/DIPEA	DMF	70	30
6	1	HCTU/DIPEA	DMF	60	40

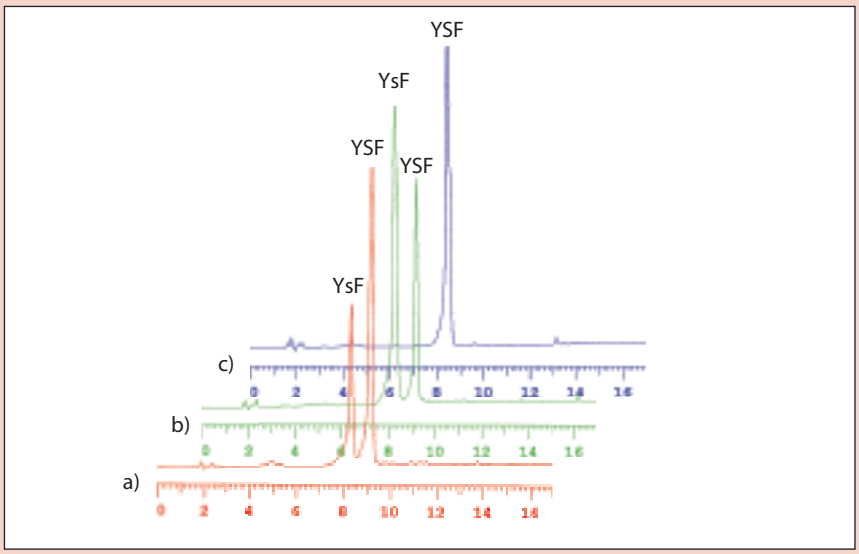
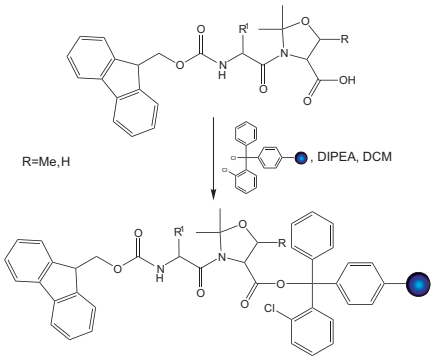


Fig. 2: HPLC profiles of a) Experiment 2; b) Experiment 1; c) Experiment 3.

Fragment condensation using pseudoproline dipeptides



The optimal support for the preparation of protected fragments containing C-terminal pseudoproline dipeptides is 2-chlorotrityl chloride resin. It has a high substitution, is loaded under conditions which do not require carboxyl activation, and peptides can be released under conditions which do not effect the pseudoproline ring. Attachment of the appropriate pseudoproline dipeptide to this resin is carried out using Method 1. Cleavage of fully protected peptides can be effected by treatment with 0.5 % TFA in DCM according to Method 2.

Method 1: Loading 2-chlorotrityl resin with pseudoproline dipeptides

NOTE: it is important to dry all solvents, reagents, and glassware before use.

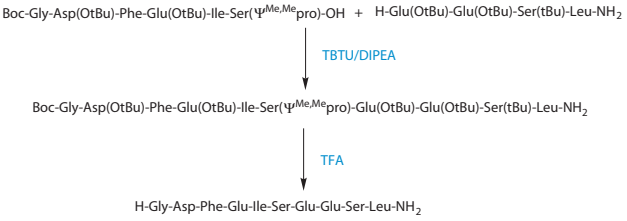
1. Dissolve the pseudoproline dipeptide (0.6 - 1.2 eq. relative to the resin for 2-chlorotrityl chloride resin) and DIPEA (4 eq. relative to pseudoproline dipeptide) in dry DCM (approx. 10 ml per gram of resin) containing, if necessary, a small amount of dry DMF or THF (just enough to facilitate dissolution of the pseudoproline dipeptide).
2. Add this to the pre-swollen resin and stir o/n. At the end of this time, wash the resin with 3x DCM/MeOH/DIPEA (17:2:1), 3x DCM; 2x DMF, 2x DCM. Dry *in vacuo* over KOH.

Pseudoproline dipeptides are best dried before use by repeated evaporation from dioxane; determine loading using Method 3-6, page 3.4 of 2004/5 catalog.

Method 2: Cleavage of protected peptides from 2-chlorotrityl chloride resin.

1. Pre-swell the dry resin (1 g) with DCM in a sealable sintered glass funnel and remove excess DCM.
2. Add 0.5 % TFA in dry DCM (10 ml), seal funnel and shake for 2 min. Filter solution by applying nitrogen pressure into a flask containing 10% pyridine in methanol (2 ml).
3. Repeat step 2 up to 10 times, wash the residual protected peptide from the resin with 3 x 30 ml DCM, 3 x 30 ml MeOH, 2 x 30 ml DCM, 3 x 30 ml MeOH, and check filtrates by TLC or HPLC.
4. Combine filtrates which contain product and evaporate under reduced pressure to 5% of the volume. Add water (40 ml) to the residue and cool mixture with ice to aid precipitation of the product.
5. Isolate product by filtration through a sintered glass funnel. Wash product three times with fresh water. Dry sample in a desiccator under vacuum over KOH, and later over P_2O_5.

Synthesis of Fibrinogen A related peptide



Application 1: Synthesis of H-Gly-Asp-Phe-Glu-Ile-Ser-Glu-Glu-Ser-Leu-NH_2

2-Chlorotrityl chloride resin was loaded with Fmoc-Ile-Ser($\Psi^{Me,Me}$pro)-OH as described in Method 1, to give a resin with a substitution of 0.26 mmole/g, as determined by the Fmoc UV assay. Boc-Gly-Asp(OtBu)-Phe-Glu(OtBu)-Ile-Ser($\Psi^{Me,Me}$pro)-2-ClTrt resin was assembled on this support by standard Fmoc chemistry, using 2-fold excesses of Fmoc-amino acids activated with PyBOP (1 eq.) and DIPEA (4 eq.), and DBU/piperidine/DMF (2:2:96) for Fmoc removal. A coupling time of 60 min was used throughout. Cleavage of the protected peptide from the resin was carried out by treatment of the peptidyl resin with 0.5 % TFA in DCM, as described in Method 2, to yield the product with the HPLC profile shown in Figure 3.

The C-terminal component, H-Glu(OtBu)-Glu(OtBu)-Ser(tBu)-Leu-NH_2, was prepared on Sieber amide resin in a similar manner except 1 % TFA in DCM was used to effect cleavage. The HPLC profile of the crude product is shown in Figure 3.

The two fragments were dissolved in DMF and coupled using TBTU (1.2 eq) and DIPEA (4 eq). The reaction was complete after 4 h as determined by HPLC, so the solvent was evaporated, and the protected peptide precipitated with water. The product was isolated by filtration, washed with water and dried in vacuo over P_2O_5. The peptide was then treated with TFA/water/TIS (95:2.5:2.5) for 3 h. After this time, the TFA was removed by evaporation, and the product precipitated with ether. The crude decapeptide was characterized by HPLC (Figure 4) and LC-MS.

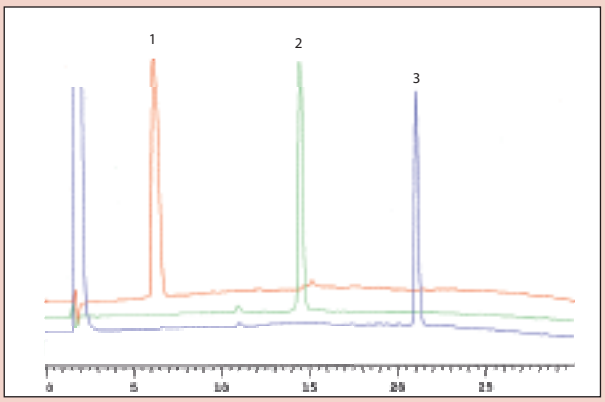


Fig. 3: HPLC profile of 1) C-terminal fragment; 2) N-terminal fragment; 3) full length protected peptide.

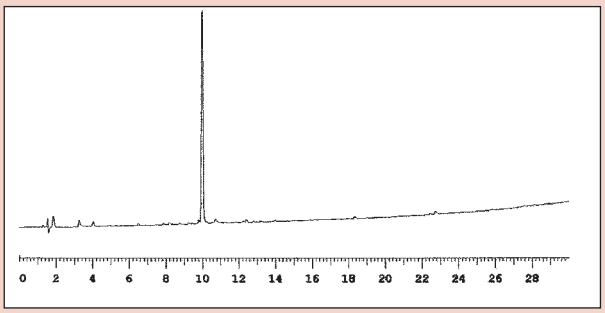


Fig. 4: HPLC profile of crude product after TFA cleavage.

Ordering information

05-20-1000	Fmoc-Ala-Ser($\Psi^{Me,Me}$pro)-OH	1 g 5 g
05-20-1005	Fmoc-Ala-Thr($\Psi^{Me,Me}$pro)-OH	1 g 5 g
05-20-1010	Fmoc-Asn(Trt)-Ser($\Psi^{Me,Me}$pro)-OH	1 g 5 g
05-20-1008	Fmoc-Asn(Trt)-Thr($\Psi^{Me,Me}$pro)-OH	1 g 5 g
05-20-1011	Fmoc-Asp(OtBu)-Ser($\Psi^{Me,Me}$pro)-OH	1 g 5 g
05-20-1126	Fmoc-Asp(OtBu)-Thr($\Psi^{Me,Me}$pro)-OH	1 g 5 g
05-20-1115	Fmoc-Gln(Trt)-Ser($\Psi^{Me,Me}$pro)-OH	1 g 5 g
05-20-1125	Fmoc-Gln(Trt)-Thr($\Psi^{Me,Me}$pro)-OH	1 g 5 g
05-20-1002	Fmoc-Glu(OtBu)-Ser($\Psi^{Me,Me}$pro)-OH	1 g 5 g
05-20-1122	Fmoc-Glu(OtBu)-Thr($\Psi^{Me,Me}$pro)-OH	1 g 5 g
05-20-1127	Fmoc-Gly-Ser($\Psi^{Me,Me}$pro)-OH	1 g 5 g
05-20-1124	Fmoc-Gly-Thr($\Psi^{Me,Me}$pro)-OH	1 g 5 g
05-20-1119	Fmoc-Ile-Ser($\Psi^{Me,Me}$pro)-OH	1 g 5 g
05-20-1118	Fmoc-Ile-Thr($\Psi^{Me,Me}$pro)-OH	1 g 5 g
05-20-1004	Fmoc-Leu-Ser($\Psi^{Me,Me}$pro)-OH	1 g 5 g
05-20-1009	Fmoc-Leu-Thr($\Psi^{Me,Me}$pro)-OH	1 g 5 g
05-20-1003	Fmoc-Lys(Boc)-Ser($\Psi^{Me,Me}$pro)-OH	1 g 5 g
05-20-1116	Fmoc-Lys(Boc)-Thr($\Psi^{Me,Me}$pro)-OH	1 g 5 g
05-20-1121	Fmoc-Phe-Ser($\Psi^{Me,Me}$pro)-OH	1 g 5 g
05-20-1128	Fmoc-Phe-Thr($\Psi^{Me,Me}$pro)-OH	1 g 5 g
05-20-1012	Fmoc-Ser(tBu)-Ser($\Psi^{Me,Me}$pro)-OH	1 g 5 g
05-20-1117	Fmoc-Ser(tBu)-Thr($\Psi^{Me,Me}$pro)-OH	1 g 5 g
05-20-1130	Fmoc-Trp(Boc)-Ser($\Psi^{Me,Me}$pro)-OH	1 g 5 g
05-20-1013	Fmoc-Trp(Boc)-Thr($\Psi^{Me,Me}$pro)-OH	1 g 5 g
05-20-1014	Fmoc-Tyr(tBu)-Ser($\Psi^{Me,Me}$pro)-OH	1 g 5 g
05-20-1007	Fmoc-Tyr(tBu)-Thr($\Psi^{Me,Me}$pro)-OH	1 g 5 g
05-20-1001	Fmoc-Val-Ser($\Psi^{Me,Me}$pro)-OH	1 g 5 g
05-20-1006	Fmoc-Val-Thr($\Psi^{Me,Me}$pro)-OH	1 g 5 g
01-64-0021	2-Chlorotrityl chloride resin (100 - 200) mesh, 1% DVB	1 g 5 g 25 g
01-64-0059	Sieber amide resin	1 g 5 g 25 g

References

1. P. D. White & W. C. Chan in "Fmoc solid phase peptides sythesis: a practical approach", W. C. Chan & P. D. White (Eds.), Oxford University Press, Oxford, 2000, pp. 31.
2. L. N. Benoiton (1994) *Int. J. Peptide Protein Res.*, **44**, 399.
3. B. L. Bray (2003) *Nat. Rev. Drug. Discov.*, **2**, 587.
4. T. Wöhr, et al. (1996) *J. Am. Chem. Soc.*, **118**, 9218.

Merck Biosciences AG · Switzerland
Weidenmattweg 4
4448 Läufelfingen
Phone +41 (62) 285 2525
Fax +41 (62) 285 2520

www.novabiochem.com

NVI604-NP