Merck Biosciences

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# Novabiochem®

innovations 1/06

## Synthesis of thymosin-$\alpha_1$ using pseudoproline dipeptides

Thymosin-$\alpha_1$, Talpha1, is a linear, N-terminally acetylated, 28-residue peptide hormone (Figure 1) [1]. It is secreted by thymus and is primarily involved in stimulation of the thymus-dependent immune system [2]. Synthetic Talpha1 is licensed under the name of Zadaxin for the treatment of HBV and hepatitis and is also being developed in combination with cytokines to treat cancer and to reduce side effects during cytotoxic drug therapy [3].

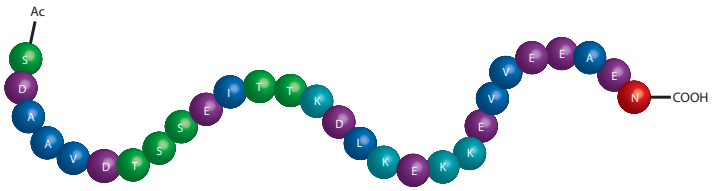


*Fig. 1: Schematic representation of Talpha1.*

For large scale GMP production of Talpha1, convergent, rather than step-wise solid phase synthesis, is the most appropriate approach as the process is easier to control, validate and document, and the purity of intermediates can be more easily characterized. However, the preparation of Talpha1 is particularly problematic as the sequence contains a number of difficult couplings and has the propensity to aggregate during chain extension. In this Innovation, we examine the convergent synthesis of this peptide, and explore how pseudoproline dipeptide substitution can be used to improve synthetic efficiency.

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"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. 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!"

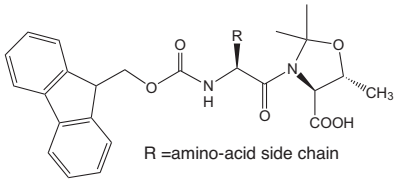
Yingwei He, Protein Chemistry Dept., Abgent, San Diego, CA.

"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.

# Synthetic Strategy

**Fmoc–Aaa–Thr($\Psi^{Me,Me}$pro)-OH**



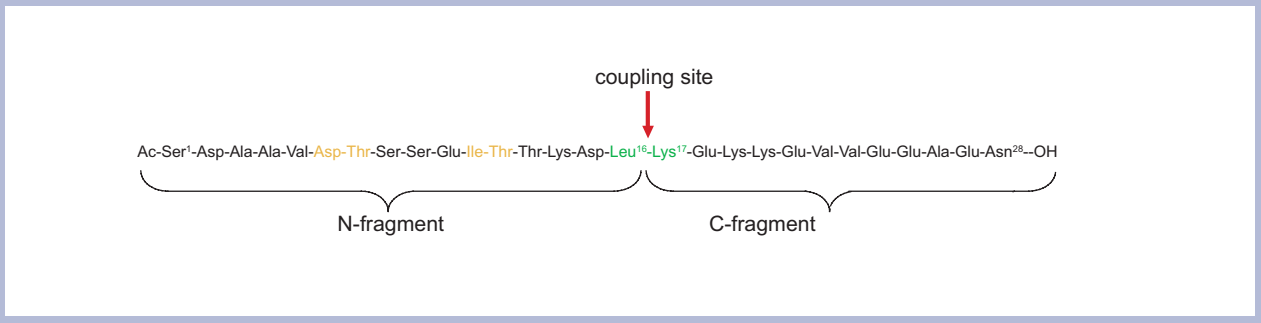

The strategy for the synthesis of Talpha1 is shown in Figure 2. The peptide was prepared from two fragments formed by dividing the peptide between residues 16 and 17 (Figure 3). This site was chosen as preliminary studies have shown the assembly of the C-terminal 12 residues to be particularly difficult, and therefore it seemed judicious to tackle this region separately.

The synthesis of the N-terminal fragment was initially carried out on Fmoc-Leu-HMPB-BHA resin (0.51 mmole/g) using 4-fold excesses of standard Fmoc-amino acids activated with PyBOP®/NMM. Following treatment of an aliquot of peptidyl resin with 95% TFA, the peptide was recovered in very low purity (Figure 4a). LC-MS analysis revealed the major impurities to be peptides missing Ser, Thr, Asp and Ala residues. Repeating the synthesis with introduction of Asp[6]-Thr[7] using Fmoc-Asp(OtBu)-Thr($\Psi^{Me,Me}$pro)-OH (Figure 4b) led to minor improvements in efficiency. The product obtained when Ile-Thr were incorporated using Fmoc-Ile-Thr($\Psi^{Me,Me}$pro) was of much higher quality but contaminated by a peptide missing a Ser residue (Figure 4c). When the synthesis was repeated using both pseudoproline dipeptides, the desired product was obtained in excellent purity (Figure 4d). The residual resin was then treated with 1% TFA in DCM to obtained the required fully protected peptide fragment.

Initial optimization of the synthesis of the C-terminal fragment was carried out on Fmoc-Asn-2-chlorotrityl resin. LC-MS analysis of the material obtained after cleavage with 95% TFA showed the product to be contaminated with by-products arising from incomplete Fmoc removal (Figure 4e). The peptide was then resynthesized on Fmoc-Asp(Sieber Amide resin)-OtBu in order to obtain the protected peptide t-butyl ester. Increasing the piperidine treatment to 13 minutes resulted in a significant improvement in product quality, as indicated by LC-MS analysis (Figure 4f) of the product obtained by cleavage of an aliquot of resin with 95% TFA. The residual peptidyl resin was treated with 1% TFA in DCM to afford the fully protected C-terminal fragment in good purity. The N-terminal Fmoc group was removed in solution with diethylamine in DMF/MeCN to yield the required C-terminal fragment. Work is ongoing to optimize the condensation of these fragments.


*Fig. 3: Strategy for the synthesis of Talpha1.*

*Fig. 3: Primary sequence of Talpha1. Divide point for fragment synthesis is indicated.*



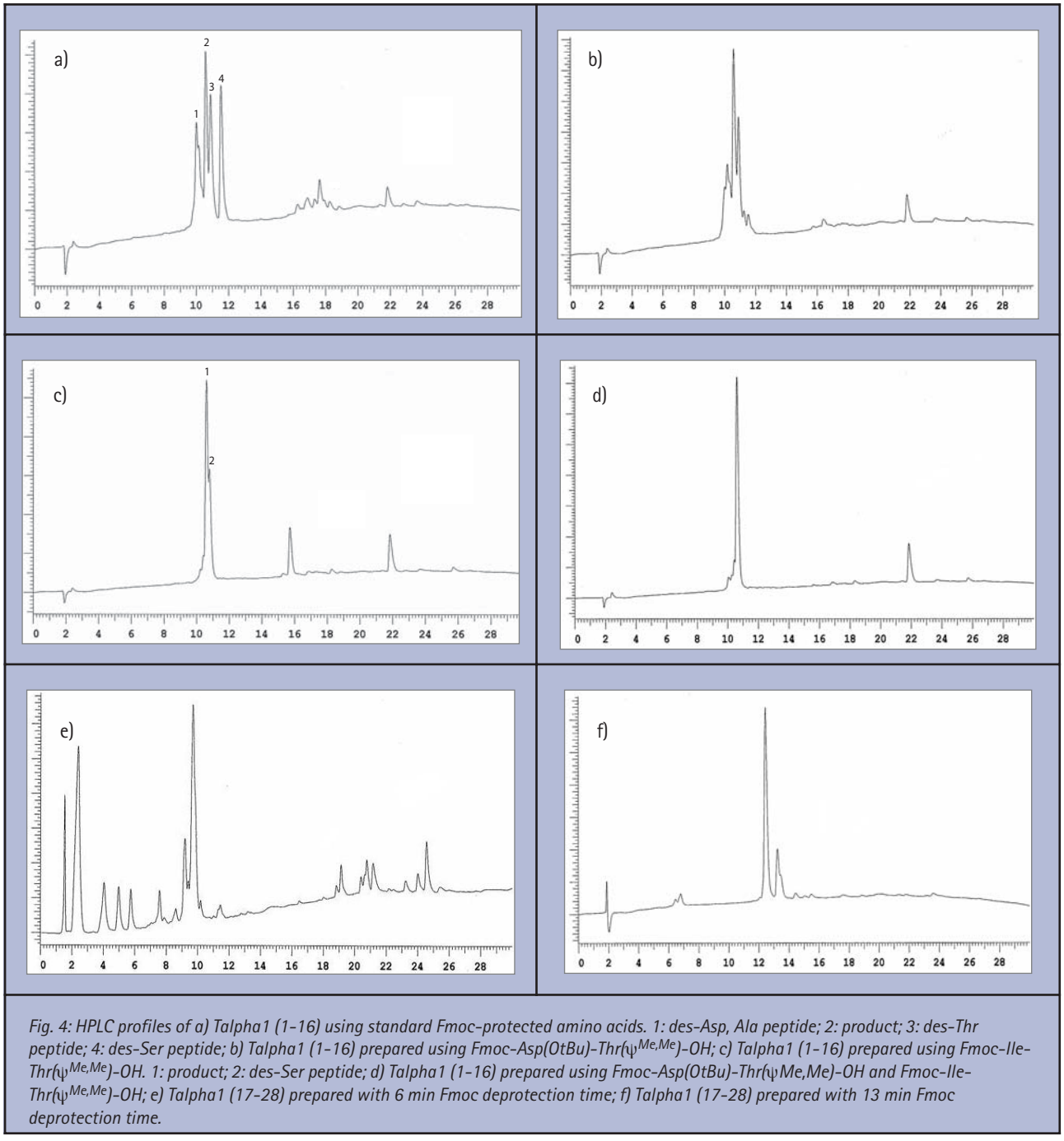




*Fig. 4: HPLC profiles of a) Talpha1 (1-16) using standard Fmoc-protected amino acids. 1: des-Asp, Ala peptide; 2: product; 3: des-Thr peptide; 4: des-Ser peptide; b) Talpha1 (1-16) prepared using Fmoc-Asp(OtBu)-Thr(ψ$^{Me,Me}$)-OH; c) Talpha1 (1-16) prepared using Fmoc-Ile-Thr(ψ$^{Me,Me}$)-OH. 1: product; 2: des-Ser peptide; d) Talpha1 (1-16) prepared using Fmoc-Asp(OtBu)-Thr(ψMe,Me)-OH and Fmoc-Ile-Thr(ψ$^{Me,Me}$)-OH; e) Talpha1 (17-28) prepared with 6 min Fmoc deprotection time; f) Talpha1 (17-28) prepared with 13 min Fmoc deprotection time.*

# Ordering information

| | | |
|---|---|---|
| 05-20-1000 | Fmoc-Ala-Ser($\Psi^{Me,Me}$pro)-OH | 1 g<br>5 g |
| 05-20-1005 | Fmoc-Ala-Thr($\Psi^{Me,Me}$pro)-OH | 1 g<br>5 g |
| 05-20-1010 | Fmoc-Asn(Trt)-Ser($\Psi^{Me,Me}$pro)-OH | 1 g<br>5 g |
| 05-20-1008 | Fmoc-Asn(Trt)-Thr($\Psi^{Me,Me}$pro)-OH | 1 g<br>5 g |
| 05-20-1011 | Fmoc-Asp(OtBu)-Ser($\Psi^{Me,Me}$pro)-OH | 1 g<br>5 g |
| 05-20-1126 | Fmoc-Asp(OtBu)-Thr($\Psi^{Me,Me}$pro)-OH | 1 g<br>5 g |
| 05-20-1115 | Fmoc-Gln(Trt)-Ser($\Psi^{Me,Me}$pro)-OH | 1 g<br>5 g |
| 05-20-1125 | Fmoc-Gln(Trt)-Thr($\Psi^{Me,Me}$pro)-OH | 1 g<br>5 g |
| 05-20-1002 | Fmoc-Glu(OtBu)-Ser($\Psi^{Me,Me}$pro)-OH | 1 g<br>5 g |
| 05-20-1122 | Fmoc-Glu(OtBu)-Thr($\Psi^{Me,Me}$pro)-OH | 1 g<br>5 g |
| 05-20-1127 | Fmoc-Gly-Ser($\Psi^{Me,Me}$pro)-OH | 1 g<br>5 g |
| 05-20-1124 | Fmoc-Gly-Thr($\Psi^{Me,Me}$pro)-OH | 1 g<br>5 g |
| 05-20-1119 | Fmoc-Ile-Ser($\Psi^{Me,Me}$pro)-OH | 1 g<br>5 g |
| 05-20-1118 | Fmoc-Ile-Thr($\Psi^{Me,Me}$pro)-OH | 1 g<br>5 g |
| 05-20-1004 | Fmoc-Leu-Ser($\Psi^{Me,Me}$pro)-OH | 1 g<br>5 g |
| 05-20-1009 | Fmoc-Leu-Thr($\Psi^{Me,Me}$pro)-OH | 1 g<br>5 g |
| 05-20-1003 | Fmoc-Lys(Boc)-Ser($\Psi^{Me,Me}$pro)-OH | 1 g<br>5 g |
| 05-20-1116 | Fmoc-Lys(Boc)-Thr($\Psi^{Me,Me}$pro)-OH | 1 g<br>5 g |
| 05-20-1121 | Fmoc-Phe-Ser($\Psi^{Me,Me}$pro)-OH | 1 g<br>5 g |
| 05-20-1128 | Fmoc-Phe-Thr($\Psi^{Me,Me}$pro)-OH | 1 g<br>5 g |
| 05-20-1012 | Fmoc-Ser(tBu)-Ser($\Psi^{Me,Me}$pro)-OH | 1 g<br>5 g |
| 05-20-1117 | Fmoc-Ser(tBu)-Thr($\Psi^{Me,Me}$pro)-OH | 1 g<br>5 g |
| 05-20-1130 | Fmoc-Trp(Boc)-Ser($\Psi^{Me,Me}$pro)-OH | 1 g<br>5 g |
| 05-20-1013 | Fmoc-Trp(Boc)-Thr($\Psi^{Me,Me}$pro)-OH | 1 g<br>5 g |
| 05-20-1014 | Fmoc-Tyr(tBu)-Ser($\Psi^{Me,Me}$pro)-OH | 1 g<br>5 g |
| 05-20-1007 | Fmoc-Tyr(tBu)-Thr($\Psi^{Me,Me}$pro)-OH | 1 g<br>5 g |
| 05-20-1001 | Fmoc-Val-Ser($\Psi^{Me,Me}$pro)-OH | 1 g<br>5 g |
| 05-20-1006 | Fmoc-Val-Thr($\Psi^{Me,Me}$pro)-OH | 1 g<br>5 g |

Novabiochem® resins

| | | |
|---|---|---|
| 01-64-0059 | Sieber Amide resin | 1 g<br>5 g<br>25 g |
| 04-12-2202 | Fmoc-Leu-HMPB-BHA resin | 1 g<br>5 g |

# References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2. A. Billich (2002) *Curr. Opin. Investig. Drug*, **3**, 198.
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4. C. Toniolo, et al. (1987) *Int. J. Peptide Protein Res.*, **30**, 232.





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