Solid phase peptide synthesis of 'difficult sequences' using Pyoxim

Erik Holm Nielsen and Troels Skrydstrup

Center of Insoluble Protein Structures, Interdisciplinary Nanoscience Centre, iNANO, Aarhus University

Gustav Wiedsvej 14, 8000 Aarhus C. holm@chem.au.dk

Introduction

Although SPPS has advanced significantly in terms of reagents, solid supports and instrumentation, the peptide chemist is still often faced by challenges, where optimization is required in order to obtain acceptable crude purities. In such optimization processes, the coupling reagent often play a crucial role. During the past five years a range of novel coupling reagents, based on the oxyma auxilarry, has been introduced. One of these, namely Pyoxim, have shown highly effective in our laboratory, especially in the synthesis of peptaibols. Despite the reported efficacy of pyoxim in SPPS, not many publications, describing the utilization of this coupling reagent have appeared since its introduction back in 2010 [1]. In one of these studies [2] the performance of pyoxim did not match similar coupling reagents (Pyclock, COMU), findings which are in stark contrast with our experience with Pyoxim. In the present study we sat out to elucidate the optimal coupling conditions using Pyoxim as coupling reagent in SPPS, along with the related coupling reagents HCTU and Pyclock (fig. 1).

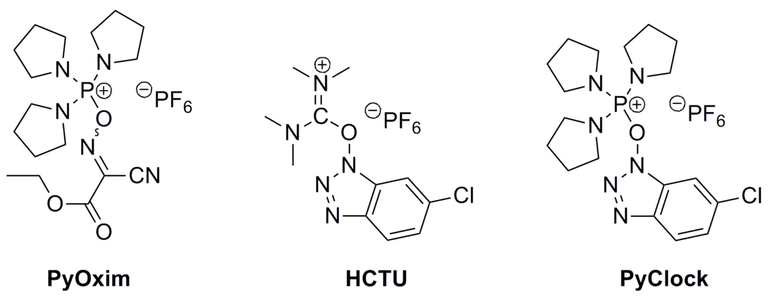


Fig 1. *The coupling reagents used in this study.*

Methods

The ACP$^{70-74}$ peptidyl-resin fragment, H-DYING-Wang-Tg, used in fig. 2, was synthesised as described for (**2**) on a 300μmol scale. The synthesis of the individual entries in table 2 was performed as described for (**1**), however manually, on a 10μmol scale.
The Alamethicin 17-20 fragment used in fig. 3, was synthesised as described for (**3**) on a 200μmol scale. The synthesis of the individual entries in fig. 3 was performed manually on 10μmol scale using the indicated coupling reagent and base (pre-mixed in closed vial), for the indicated time.
The following was performed on a ABI 433A synthesiser.
Synthesis of (1): "Fast cycle" (11mins cycle time) 100μmol scale , Fmoc-Gly-Wang-Tg (0.25mmol/g) - Deprotection: 2x30s with 20% piperidine/DMF. 2x60s couplings using 5 eq. Aa, 5 eq. coupling reagent, 10 eq. Coupling base.
Synthesis of (2): 100μmol scale, Fmoc-Gly-Wang-Tg (0.25mmol/g) - Deprotection: 2x3mins with 20% piperidine/DMF. Coupling 5 eq. Aa, 5 eq. Pyoxim, 10 eq. DIPEA, 8mins, ^{68}U 30mins and ^{67}U 2x30mins.
Synthesis of (3): 100μmol scale, H-Phol-2CT PS (0.44mmol/g) - Deprotection: 2x3mins with 20% piperidine/DMF. Coupling 5 eq. Aa, 5 eq. Pyoxim, 10 eq. DIPEA, 30mins, P^{14} and U^{16} 2x30mins. Acetylation – 10 eq. Ac_2O, 20 eq. DIPEA, 2 ml DMF, 1 hr.
Synthesis of (4): 100μmol scale, Chemmatrix RAM (0.51mmol/g) - Deprotection: 5+10 mins with 2%/20% DBU/piperidine/DMF. Coupling 5 eq. Aa, 5 eq. Pyoxim, 10 eq. DIPEA, 30mins.
Synthesis of (5): 100μmol scale, Fmoc-Asp(tBu)-Trt-Tg (0.17mmol/g) - Deprotection: 5+10 mins with 2%/20% DBU/piperidine/DMF for Tyr^{9} to Asp^{19}, 2x3mins 20% piperidine/DMF for Gln^{1} to His^{8}. Coupling: 5 eq. Aa, 5 eq. Pyoxim, 10 eq. DIPEA for 30mins, Y^{9}, N^{10,} I^{11} and V^{12} 2x60mins.
Cleavage: All peptides were cleaved using TFA/TIPS/H_2O (95/2.5/2.5 - 50μL/μmol) for 1 hr, with the exeption of **(3),** in which case DCM/TFA/TIPS/H_2O (48/48/2/2 - 50μL/μmol) was used for 1 hr. Following concentration of the filtrate by N_2 agitation, the crude peptides were collected by precipitation in ice-cold tBME.
Analysis: RP-HPLC analysis was performed on an Agilent 1260, using a Phenomenex Aeris (4.6x150mm, 2.6μm, 100Å) column, with a gradient elution of Acetonitrile (0.1% TFA) 5.7%/min in Water (0.1% TFA) and detection at 215nm.

Fast cycle

Initially we sought to reproduce the results reported by Chantell *et al*., employing a "fast cycle" approach using HCTU or Pyoxim pre-mixed with base in the synthesis of $^{65-74}$ACP.

Table 1. *"Fast cycle" synthesis of $^{65-74}$ACP[a]*

Coupling reagent	Pre-mixing	Crude Purity (%)
HCTU	Yes	82 (75[b])
PyOxim	Yes	74 (20[b])
PyOxim	No	78

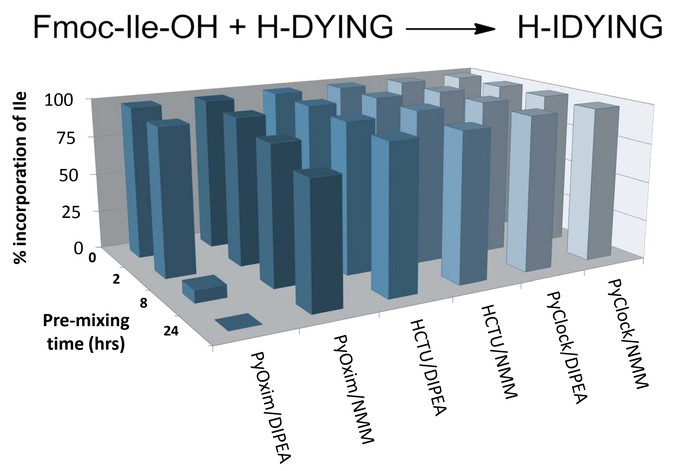
[a] See methods for details. [b] Ref[2]

With HCTU we obtained a crude purity (82%) similar to that previously reported (75%). However, a comparable crude purity was obtained when employing Pyoxim as coupling reagent (74%), an nearly four-fold increase in crude purity, compared to that previously reported (Table 1). When performing the synthesis under identical conditions, but omitting the pre-mixing of Pyoxim and NMM, a comparable crude purity was obtained.

Stability

The stability of coupling reagents has been examined in recent studies [3,4,5] and uromium based coupling reagents was shown to be more stable in solution compared to phosphonium based derivatives (with the exception of COMU). Hence, it seemed obvious to investigate the effect of pre-mixing the coupling reagent and base on the coupling efficacy. The coupling of Fmoc-Ile-OH onto H-DYING-resin was used as a representative example. The coupling reagent was mixed with the coupling base and used in the coupling at indicated time shown in Fig. 2.

Fig 2. *Effect of pre-mixing coupling reagent and base[a]*



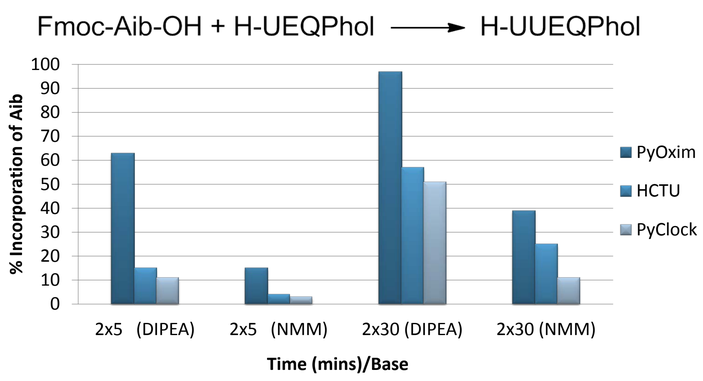
[a] See methods for details

Pyoxim was found to be far more susceptible to degradation compared to HCTU and Pyclock, when pre-mixed with the coupling base. In addition, the stronger base DIPEA (pKa: 10.4) accelerated the degradation of Pyoxim to a much higher degree compared to NMM (pKa: 7.4). Pyclock performed best under the given conditions, and even after 24 hrs in solution with NMM, a reasonable level of coupling efficacy was observed (fig.2).

Challenging Couplings

Next the perfomance of the three coupling reagents in the sterically demanding condensation of two consecutive Aib residues was investigated. The four residue C-terminal part of Alamethicin$^{17-20}$ (H-UEQPhol) was used as a template. Fmoc-Aib-OH was double coupled for the indicated time using either DIPEA or NMM as base (fig.3). It is clear that Pyoxim is far superior to HCTU and Pyclock with regards to the coupling of sterically demanding couplings. In addition, the base strength is crucial, and the stronger base DIPEA outperformed NMM (fig.3).

Fig 3. *Sterically demanding coupling[a]*



[a] See methods for details.

'Difficult sequences'

Finally Pyoxim was used in the synthesis of a range of sequences reported to be 'difficult sequences' (**2-5**)**.** In all cases we were able to obtain similar results, and in the case of **3** and **5** the crude purity was improved 35% and 25%, respectively (table 2)

(1) H-VQAAIDYING-OH
$^{65-74}$ACP

(2) H-VQUUIDYING-OH
$^{65-74}$ACP(U$^{67-68}$)

(3) Ac-UPUAUAQUVUGLUPVUUEQ-Phol
Alamethicin

(4) H-WFTTLISTIM-NH_2
JR-10

(5) H-QAEPDRAHYNIVTFSSKSD-OH
8Qser

Table 2. *Synthesis of "difficult sequences"*

Sequence	Crude Purity (%)[a]	Reported (%)
1	82	75[b]
2	85	83[c]
3	88	53[d]
4	59	62[e]
5	67	43[f]

[a] See methods for conditions. [b] Ref[2]. [c] Ref[1] Manual synthesis - Deprotection: 10mins 20% piperidine/DMF. Coupling: 3eq/2eq/4eq Aa/Pyoxim/DIPEA, 5mins (U^{68} 30mins, U^{67} 2x30mins). [d] Ref[6] MAS – Deprotection 30+180s, Fmoc-Aib-F or HBTU/Aa/DIPEA. [e] Ref[7] MAS - Deprotection: 2+2mins with 40% and 20% piperidine/DMF respectively. Coupling: 5eq. COMU/Aa/DIPEA 5mins. [f] Ref[8] Deprotection 10+20mins with 20%piperidine/DMF. Coupling: HATU/Aa/DIPEA 2x120mins.

Conclusions

- It was shown that pyoxim is more susceptible towards degradation, resulting from pre-mixing coupling reagent and base, compared to Pyclock and HCTU.
- In the coupling of the α,α-disubstituted amino acid Aib onto another Aib residue, Pyoxim was superior to HCTU and Pyclock. In addition, the choice of base is crucial, and the stronger base DIPEA showed to be superior compared to NMM.
- Pyoxim was shown to be as effective as HCTU and Pyclock in the synthesis of $^{65-74}$ACP applying fast cycle times.
- In the synthesis of the "difficult sequences" $^{65-74}$ACP($^{67-68}$U), JR-10, 8Qser and Alamethicin we were able to obtain similar or better results using pyoxim compared to other reported methods comprising the use of HATU, acid fluorides and/or microwave-assisted synthesis.

References

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