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Automatic procedures for the synthesis of difficult peptides using oxyma as activating reagent: A comparative study on the use of bases and on different deprotection and agitation conditions

A. Caporale[a,b], N. Doti[a,b,*], A. Monti[a,c], A. Sandomenico[a,b], M. Ruvo[a,b,*]

[a] IBB-CNR, Via Mezzocannone 16, 80134 Napoli, Italy
[b] CIRPeB, Via Mezzocannone 16, 80134 Napoli, Italy
[c] DiSTABiF, Università degli Studi della Campania "Luigi Vanvitelli", Via Vivaldi 43, 81100, Caserta, Italy

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ABSTRACT

Solid-Phase Peptide Synthesis (SPPS) is a rapid and efficient methodology for the chemical synthesis of peptides and small proteins. However, the assembly of peptide sequences classified as "difficult" poses severe synthetic problems in SPPS for the occurrence of extensive aggregation of growing peptide chains which often leads to synthesis failure. In this framework, we have investigated the impact of different synthetic procedures on the yield and final purity of three well-known "difficult peptides" prepared using oxyma as additive for the coupling steps. In particular, we have comparatively investigated the use of piperidine and morpholine/DBU as deprotection reagents, the addition of DIPEA, collidine and N-methylmorpholine as bases to the coupling reagent. Moreover, the effect of different agitation modalities during the acylation reactions has been investigated. Data obtained represent a step forward in optimizing strategies for the synthesis of "difficult peptides".

1. Introduction

The Solid-Phase Peptide Synthesis (SPPS) is the most common strategy for the synthesis of biopolymers in high yield and purity [1]. Since the invention of SPPS, synthetic procedures have been continuously improved and refined by the introduction of ever more efficient orthogonal protecting groups [2,3], new functionalized solid supports [4], effective coupling reagents [5,6] and combinations of solvents and reagents [7] to overcome difficulties associated to aggregation and poor yields. These improvements have enabled the access to the step-wise preparation of virtually polypeptides up to about 100 residues [8–11]. However, certain fragments, known as "difficult peptides", still require laborious procedures which still lead to poor yields and purities [1]. Difficult peptides are generally characterized by strong inter- and intra-molecular β-sheet interactions that promote the formation of aggregates [7,12–14]. The tendency of a peptide chain to aggregate during synthesis is translated into a number of side products deriving from recurrent incomplete aminoacylations and slow or incomplete removal of the temporary protecting group [15,16]. Several strategies aimed at disrupting the inter- and intra-chain associations have been described. Most of them are based on the use of backbone amide protecting groups, such as pseudoproline, of substituted benzyl groups or of depsipeptides, that exploit O—N intramolecular acyl migration mechanisms [17–19]. Unless required by specific post-synthesis manipulations and introduction of base-sensitive chemical groups such as thioesters for native chemical ligation [20], SPPS protocols use of the 9-fluorenylmethoxycarbonyl (Fmoc)-based strategy, which mostly relies on the activation of carboxyl groups by phosphonium, oxonium and iminium-derived coupling reagents and removal of the protecting group by strong bases [5,19]. Fmoc is usually deblocked with piperidine at various concentrations [15]. However, many other reagents have been developed and tested to improve synthesis yields when needed [18–21]. To minimize base-catalyzed side reactions, Fmoc deprotection can be also achieved using weaker bases (e.g. morpholine) [22,23].

Oxyma in combination with other coupling reagents has been reported as an effective coupling option for its remarkable capacity to suppress racemization and the impressive coupling efficiency in both automated and manual synthesis [24,25]. Recently, we showed that the use of oxyma/DIC as second coupling reagent in double coupling settings with HATU/collidine, significantly improves the yield during the synthesis of difficult peptides, making easier their characterization and purification [26]. Oxyma/DIC is one of the less expensive in the plentiful landscape of coupling reagents. Several other studies also showed that the addition of bases to coupling reagents might improve the yield

* Corresponding authors at: IBB-CNR, Via Mezzocannone 16, 80134 Napoli, Italy.
E-mail addresses: nunzianna.doti@cnr.it (N. Doti), menotti.ruvo@unina.it (M. Ruvo).

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during the assembly of such complex molecules [27].

On these bases we wanted to further explore the effectiveness of oxyma/DIC as coupling reagent and the combined use of different organic bases and of different deprotecting reagents on the final yields and purity of the resulting molecules. Also the contribution of different modalities of agitation has been investigated by performing the synthesis under continuous or pulsed vortexing as this would also influence the final outcome of automated syntheses.

As deprotecting reagents we have compared the commonly used piperidine in DMF to a mixture of morpholine/DBU in the same solvent, since DBU is reported to have disaggregating effects on growing polypeptide chains [28].

As tertiary organic bases, we have compared the effects of DIPEA, NMM and collidine, since they possess very different basicity (pKa) because of the different capacity in steric shielding of amine function [29,30]. DIPEA (known also as Hünig's base) is an aliphatic amine with a pKa of 10.10 and provides a good activation for the class of phosphonium- or uronium-based coupling reagents. NMM, which belongs to the morpholine class, is substantially less basic than DIPEA (pKa 7.38). Collidine, instead, belongs to the pyridine class, has a pKa similar to that of NMM (pKa 7.43), but shows enhanced C−H abstraction rates compared to other bases. Collidine is also reported to improve preactivation of the carboxylic group in DMF, whereas DIPEA, NMM or non-hindered pyridine bases may even inhibit this step [29].

To further try to reduce aggregation, we finally evaluated the effect of continuous or pulsed mechanical vortexing during the acylation reactions. While this point might appear as a minor issue, the use of continuous vortexing on automatic synthesizers might result in noisy procedures and also lead to life-shortening of mechanical parts.

Peptides used as models for this study are: Aβ[10–26], IAPP[8–18] and Aib-Enkephaline (Aib-Enk) (Table 1). The syntheses of Aβ[10–26] and IAPP[8–18] by SPPS are reported as challenging because of their hydrophobicity and high tendency to aggregate during chain assembly and to form fibrils in solution [31–33]. The Aib-Enk fragment has been chosen, as further model, since it is commonly used for evaluating new exploratory protocols aimed at improving the yield of difficult couplings [24,26].

2. Experimental

2.1. Materials

Chemicals and amino acid derivatives were purchased from Sigma-Aldrich (Milano, Italy), GL Biochem (Shanghai, China) Ltd, Iris Biotech GmbH (Marktredwitz, Germany), or Carlo Erba Reagents (Cornaredo, Italy). In detail, diisopropylcarbodiimide (DIC), ethyl cyanohydroxyiminoacetate (oxyma), morpholine/1,5-diazabiciclo[5.4.0]undec-5-ene (DBU) and the resin were from Iris Biotech GmbH; N,N-Diisopropylethylamine (DIPEA), collidine, N-methylmorpholine (NMM), morpholine and piperidine were from Sigma-Aldrich; Acetonitrile (ACN), N-dimetilformamide (DMF) was from Carlo Erba Reagents. HPLC analyses were performed on a WATERS Alliance e2695 (WATERS, Milano, Italy) equipped with a WATERS 2998 PDA detector. ONYX monolithic C18 columns (50 × 2.0 mm ID) were from Phenomenex (Casalecchio sul Reno, Italy) They were used at 0.6 mL/min. Purifications were performed on a preparative WATERS 2545 Quaternary Gradient Module HPLC supplied with a WATERS 2489 UV/visible Detector; the XBRIDGE Prep BEH130 OBDTM C18 column (5 µm, 50 × 19 mm ID) was from WATERS and was used at 10 mL/min. LC-ESI-TOF-MS analyses were performed with an Agilent 1290 Infinity LC System coupled to an Agilent 6230 time-of-flight (TOF) MS System (Agilent Technologies, Cernusco Sul Naviglio, Italy). The liquid chromatograph Agilent 1290 LC module was also coupled with a photodiode array detector (PDA). For LC–MS determination a C18 Waters XBridge (3 µm, 4.6 × 50 mm) columns operated at 0.200 mL/min was used. For analytical determination, binary solvents were: Buffer A: 0.05% TFA in H_2O; Buffer B: 0.05% TFA in CH_3CN. For preparative HPLC separations binary solvents were: Buffer A: 0.1% TFA in H_2O and Buffer B: 0.1% TFA in CH_3CN. All purifications were performed on crude materials by collecting the peaks corresponding to the target product under identical conditions and always within the same time window.

Table 1
Amino acid sequence of model peptides. Amino acidic regions predicted as difficult sequences, either for the presence of sterically hindered residues or for a strong aggregation propensity are reported in bold.

Entry	Sterically Hindered	Aggregation in solution[a]	Aggregation during synthesis[b]
Aβ[10–26]		YEVHHQK**LVFFAE**DVGS	YEVH**HQKLVFFA**EDVGS
IAPP[8–18]		ATQRL**ANFLVH**	ATQRL**ANFLVH**
Aib-Enk	Y**AibAib**FL		

[a]The aggregation propensity in solution has been predicted using "AGGRESCAN".
[b]Difficult couplings have been predicted by "Peptide Companion" software. For details see Supplementary Material.

2.2. Prediction of peptide aggregation features

For Aβ[10–26] and IAPP[8–18] we evaluated the aggregation potential and the difficulty in the coupling steps by AGGRESCAN and Peptide Companion prediction tools [34,35]. For additional details see the Supplementary data.

2.3. Peptide synthesis procedures

Synthetic procedures used in this work are summarized in Table 2. C-terminal amidated peptides were synthesized using a Fmoc-Rink-Amide-PEG200-HypoGel (n = 5) (HypoGel® 200 RAM, loading 0.56 mmol/g, 40 mg, supplied by Iris Biotech GmbH, Marktredwitz, Germany). For all the syntheses we used oxyma and DIC (oxyma/DIC) as the common activating reagents, and the PEG-based Fmoc-Rink-Amide-PEG200-HypoGel resin, since the presence of PEG linkers has been indicated as the most advantageous for the assembly of aggregating peptides [36,37].

We used a fully automated, computer controlled multiple peptide synthesizer Syro I from MultiSynTech GmbH (Witten, Germany) equipped with a type One U-Typ Reactor Block at 24 positions with 5 mL reactors (PP-reactors 5 mL with TF frit, Cod. V050TF062,

Table 2
Description of experimental conditions applied during the synthesis of peptides. Protocols were named depending on coupling conditions. Capital letters are uniformed to legends of figures.

Conditions applied during the synthesis of peptides		
Deprotection	Coupling Protocols:	Reaction
*Pip 40% **5 min***	***A-c** Without base*	*Continuos **(c)** Vortex*
*Pip 20% **15 min***	***B-c** Collidine*	
	***C-c** NMM*	
	***D-c** DIPEA*	
*Pip 40% **5 min***	***A-d** Without base*	*Discontinuos **(d)** Vortex*
*Pip 20% **15 min***	***B-d** Collidine*	
	***C-d** NMM*	
	***D-d** DIPEA*	
*Mor 40% + DBU 5% **5 min***	***E-c** Without base*	*Continuos **(c)** Vortex*
*Mor 20% + DBU 2.5% **15 min***	***F-c** Collidine*	
	***G-c** NMM*	
	***H-c** DIPEA*	
*Mor 40% + DBU 5% **5 min***	***E-d** Without base*	*Discontinuos **(d)** Vortex*
*Mor 20% + DBU 2.5% **15 min***	***F-d** Collidine*	
	***G-d** NMM*	
	***H-d** DIPEA*	