Solid-phase peptide synthesis using acetonitrile as a solvent in combination with PEG-based resins

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This manuscript shows that ACN can be an excellent choice for the coupling of hindered amino acids as illustrated by the coupling of Fmoc-amino acids on free amino acids anchored on a BAL synthesis. Furthermore, ACN can be a good alternative for solid-phase peptide synthesis in the absence of DMF (washings, removal of Fmoc, and coupling). Copyright © 2009 European Peptide Society and John Wiley & Sons, Ltd.

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Introduction

There is not a doubt that the solid-phase methodology first described by the Nobel Prize Laureate R. Bruce Merrifield is the method of choice for the synthesis of peptides for both research and active pharmaceutical ingredient (API) production purposes [1–4]. The solid-phase method is based on a rather simple scheme as follows: The growing peptide is first anchored through the carboxylic function of the C-terminal amino acid to a solid support. Next, reagents in order to remove the temporal N-protecting group and incoming protected amino acids in the presence of the coupling reagents are used in excess in order to reach quantitative yield reactions [5]. Excess of reagents and soluble side products are removed by simple washings with solvents. Examples of peptides synthesized via the solid-phase method involve small bicyclic peptides [6,7], large peptides or small proteins [8,9], and multi-kilogram-scale production of APIs [10,11]. Since the first scheme proposed by Merrifield was introduced, there has been a great evolution in the majority of parameters involved in the synthetic process. These parameters have evolved from polystyrene (PS) resins, polyamides, rigid supports, and later to PEG-based resins [12]; from diimides and active esters to onium-based coupling reagents [13] and from a rather stable protecting group such as the bencyloxycarbornyl group (Z) to orthogonal protecting groups [14]. However, little attention has been paid to the solvent. Thus, in his first synthesis Merrifield used DMF first [1], then methylene chloride (DCM), and finally mixtures of DCM–DMF [15]. Although, DCM could be considered an excellent solvent for the activation of the N-protected amino acid, mainly when carbodiimides are used as coupling reagents [13], with the advent of fluorenylmethoxycarbonyl (Fmoc) chemistry [16] and the new era of automatic synthesizers [17], DMF – and in its absence N-methylpyrrolidone (NMP) – remains the main solvent. DCM is not compatible with piperidine, which is used to remove the Fmoc group, because of the formation of piperidine hydrochloride. Moreover, DCM is not a good solvent for the Fmoc-amino acids

or for the urea that is formed when diimides are used. The rather low boiling point of DCM is not always compatible with automatic synthesizers. Finally and even though DCM is included in the Q3C guidance of the US Food and Drug Administration [18], along with DMF and NMP, the fact that DCM is a chloride solvent precludes its use in many geographical areas. In this sense, acetonitrile (ACN) has not been broadly used in solid-phase peptide synthesis. To the best of our knowledge, the literature reports only one example in which ACN was used as a solvent in the coupling step [19]. Herein, the use of ACN for coupling on hindered peptidyl resins and also as the only solvent for the solid-phase synthesis of peptides is investigated.

Experimental Procedures

General

Aminomethyl-ChemMatrix® resin (0.6 mmol/g) was from Matrix Innovation (Canada). Backbone amide linker (BAL)-PEG-PS resin

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(0.3 mmol/g) was from Applied Biosystems (Foster City, CA). Fmoc-amino acids were from Luxembourg Biotech (Tel Aviv, Israel). TFA and ACN (HPLC grade) were purchased from Scharlau (Barcelona, Spain) HPLC was performed on SunFire C_{18} column (4.6 × 100 mm, 3.5 μm).

Swelling Experiments

Resins (200 mg) were placed in a 3-ml syringe, equipped with a 0.45-μm filter, treated with enough solvent to swell the resin, and left to stand for 5 min. The swollen resin was compressed with the piston until no more solvent could be extracted. The piston was pulled slowly until the resin recovered its maximum volume in the syringe and the volume was then read (the void volume of the tip and the syringe was averaged to 0.15 ml). The swelling was calculated according to the following formula:

$$(\text{volume of the swelled resin} + 0.15\ \text{ml})/0.2\ \text{g} = x\ (\text{ml/g})$$

A deviation of less than 10% was noted for each solvent and for each resin.

Peptide synthesis

Manual solid-phase peptide elongation and other solid-phase manipulations were carried out in polypropylene syringes fitted with a polyethylene porous disk using 100 mg of the different resins. Solvents and soluble reagents were removed by suction. Cleavages were carried out with TFA/triisopropylsilane (TIS)/H_2O (95 : 2.5 : 2.5), and peptides were precipitated with cold *tert*-butylmethylether (MTBE). The solvent was decanted and the solid was washed twice with MTBE. The crude peptide was dissolved in acetic acid–H_2O and lyophilized.

Results and Discussion

ACN is also included in the Q3C guidance mentioned above, together with DMF and NMP, and although it shows a dipole moment (3.92 vs 3.82 C·m) and dielectric constant (36.6 vs 38.3) similar to those of DMF, it can be considered more user-friendly than DMF. Thus, its lower boiling point (81 °C vs 153 °C) should favor its recycling and recovery, and its lower viscosity (0.38 vs 0.92 Pa·s) should favor its handling and filtration steps. Furthermore, DMF can decompose into formaldehyde and dimethylamine, which can jeopardize the peptide synthetic process. All these favorable properties should be more important for API production.

The main requirement for a solvent to be used in the solid-phase synthesis of peptides is its capacity to swell the resin. ACN does not swell polystyrene-based resins properly, but it swells much better PEG-based resins. The optimal properties conferred to PEG chains are due to two properties [20]. Hence, and because of the vicinal arrangements of carbon–oxygen bonds throughout the chain, PEG assumes three helical structures with gauche interaction between the polarized bonds. One is largely hydrophobic with oxygen atoms in the interior of the helix, the second one is of intermediate polarity, and the third one is hydrophilic exposing the oxygen atoms to the exterior. Therefore, PEG chains have an amphipathic nature and are well solvated by both polar and non-polar solvents. Additionally, the organized structure of polymer fragments affords a large and highly organized solvent shell and this in turn leads to a high tension of solvation in a cross-linked polymer.

Acylation of BAL-Anchored Amines by the Penultimate Amino Acid

BAL is a linker whereby the growing peptide is anchored through a backbone nitrogen, allowing for considerable flexibility in the management of the *C*-termini [21]. Once the first residue is added, the acylation of the secondary amine is rather demanding due to its steric hindrance (Figure 1).

For the preparation of libraries based on a DKP scaffold, the acylation of the BAL-PEG-PS-resin containing the first residue in the form of its methyl ester was investigated. First of all, a screening of different coupling methods, including [1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo-[4,5-b]pyridinium hexafluorophosphate 3-oxide (HATU) [22], 7-aza-benzotriazol-1-yl-*N*-oxy-tris(pyrrolidino)phosphonium hexafluo-rophosphate (PyAOP) [23] and symmetric anhydride] was performed. Solvents (DMF and DCM–DMF (9 : 1), [21]) were also screened, resulting in PyAOP (two additions of 5 equiv each of solid PyAOP at a 1-h interval for a total 3 h of coupling time) and DCM–DMF (9 : 1), the optimal conditions for unhindered amino acids such as Leu, but clearly poor results for other first residues. Consequently, the concourse of ACN was essayed.

Table 1 clearly shows that in all cases the use of ACN resulted in better yields than when DCM–DMF (9 : 1) was used. Even the most
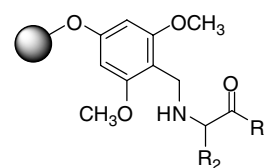

Figure 1. Structure of the BAL resin containing the first anchored residue.

Table 1. Yields of the incorporation of the penultimate residue on H-BAL(Aa-OMe)-PEG-PS-resin[a]

Product	Solvent	First coupling (%)	Second coupling (%)
Fmoc-Phe-BAL(Leu-OMe)-R	ACN	90	100
Fmoc-Phe-BAL(Leu-OMe)-R	DCM–DMF (9 : 1)	36	55
Fmoc-Leu-BAL(Phe-OMe)-R	ACN	38	82
Fmoc-Leu-BAL(Phe-OMe)-R	DCM–DMF (9 : 1)	10	34
Fmoc-His(Trt)-BAL(Phe-OMe)-R	ACN	40	89
Fmoc-His(Trt)-BAL(Phe-OMe)-R	DCM–DMF (9 : 1)	11	23
Fmoc-Phe-BAL[Lys(Alloc)-OMe]-R	ACN	43	68
Fmoc-Phe-BAL[Lys(Alloc)-OMe]-R	DCM–DMF (9 : 1)	29	42

[a] Yields were calculated by quantitative spectrophotometric monitoring following deblocking by treatment with piperidine.
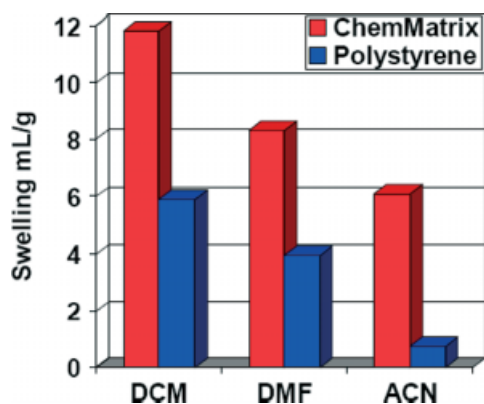
Figure 2. Swelling of amino methyl ChemMatrix® and polystyrene resins in the three key solvents.

hindered His derivative gave an acceptable yield after a second coupling.

Racemization is a key parameter to be studied for the coupling of chiral protected amino acids. First, racemization during the incorporation of the Fmoc-His(Trt)-OH was investigated by gas chromatography (GC)/MS after hydrolysis of the peptide with ^{2}HCl. His is more prone to lose its chiral integrity, due to the assisted basic catalysis performed by the unprotected $^\pi N$ [5]. The analysis showed that the use of DCM–DMF (9:1) led to a 40.0% presence of D-His, while with the use of ACN D-His went down to 28.5%. These results confirm that His undergoes racemization easily.

On the other hand, the investigation of racemization in the Fmoc-Phe-BAL(Leu-OMe)-R case by HPLC, following the preparation of the diastereoisomer analog, showed 12.1% of racemization for ACN and only 1.7% for DCM–DMF (9:1).

Stepwise Synthesis Using a ChemMatrix® Resin

ChemMatrix® is a solid support that contains only highly cross-linked PEG chains through primary ether bonds, which confer high stability and loading [24]. Evidence of the superior performance of this support has been certified by the success of highly structured peptide synthesis such as the HIV protease [9], which contains 99 amino acids, Rantes (1–68) and CCL4-L1 chemokines through the combination with pseudoprolines [25,26], and in the most striking example, the synthesis of β-amyloid (1–42) [24]. Moreover, the

synthesis of other biomolecules, such as oligonucleotides, has been achieved using ChemMatrix resin [27].

The ChemMatrix® resin swells more than polystyrene in all solvents. Thus, in ACN it swells at the same range as polystyrene does in DCM, which is the best solvent for that resin. Therefore, ChemMatrix® should be regarded as very suitable for the solid-phase synthesis of peptides using just ACN as a solvent (Figures 2 and 3).

As model peptides, Leu-enkephalinamide and acyl carrier protein (ACP) (65–74) were prepared on Fmoc-Rink-ChemMatrix resin (0.5 g, 0.62 mmol/g). The synthetic protocol involved was: (i) washings: ACN (4 × 1 min); (ii) Fmoc removal: piperidine–ACN (1 × 2 min + 2 × 10 min); (iii) washings: ACN (4 × 1 min); (iv) coupling: AA (3 equiv), DIPCI (3 equiv), 2-ethyl-cyanoglyoxylate Oxime (Oxyme Pure) [28] (3 equiv) dissolved in 0.3 ml of DMF and 4 ml of ACN; (v) washings: ACN (4 × 1 min). In all cases the ninhydrin test was negative [29]. Cleavage was carried out with TFA/TIS/H_2O (95:2.5:2.5). HPLC analysis of crude peptides shows an excellent yield in both cases (Figure 4). Remarkably, the most demanding ACP (65–74) was obtained with a 95% purity just 3 equiv of the protected amino acid and the coupling reagents.

Racemization was also studied for the coupling of Fmoc-Phe-OH on H-Leu-Rink-resin. After the preparation of the diastereoisomer analog H-YGGfL-NH$_2$, HPLC showed 0.7% of racemization with ACN and 1.1% with DCM–DMF (9:1).

Conclusions

Peptide chemists have been perhaps a little conservative regarding the use of solvents and while other parameters such as resins, coupling reagents, and protecting groups have been investigated, little attention has been paid to the solvent. Herein, it has been demonstrated that ACN can be an excellent choice for the coupling of hindered amino acids as illustrated by the coupling of Fmoc-amino acids on free amino acids anchored on a BAL synthesis. Furthemore, ACN can be a good alternative for solid-phase peptide synthesis in the absence of DMF. Although, racemization is not important for the synthesis of regular peptides, it should be controlled during the incorporation of hindered amino acids. However, racemization can be tolerated because the use of ACN increases the overall yield. The use of PEG-based resins such as the ChemMatrix is mandatory for ACN to work properly as a solvent in the solid-phase synthesis of peptides.
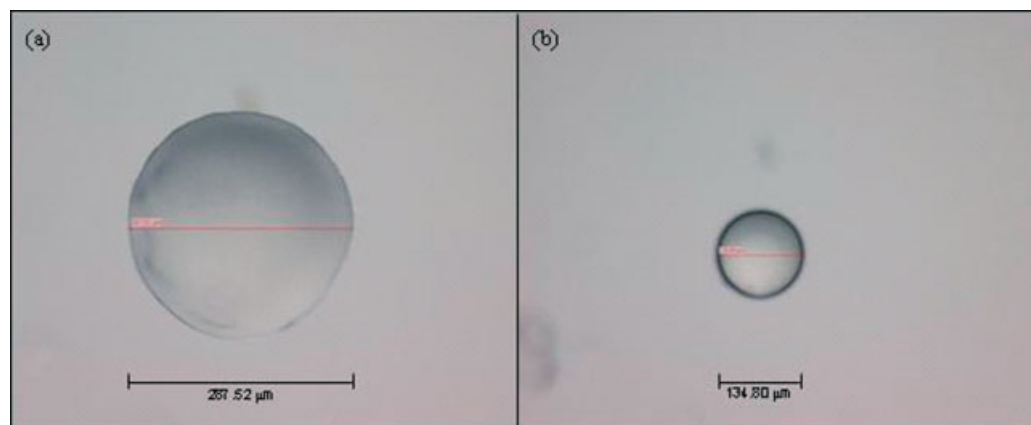

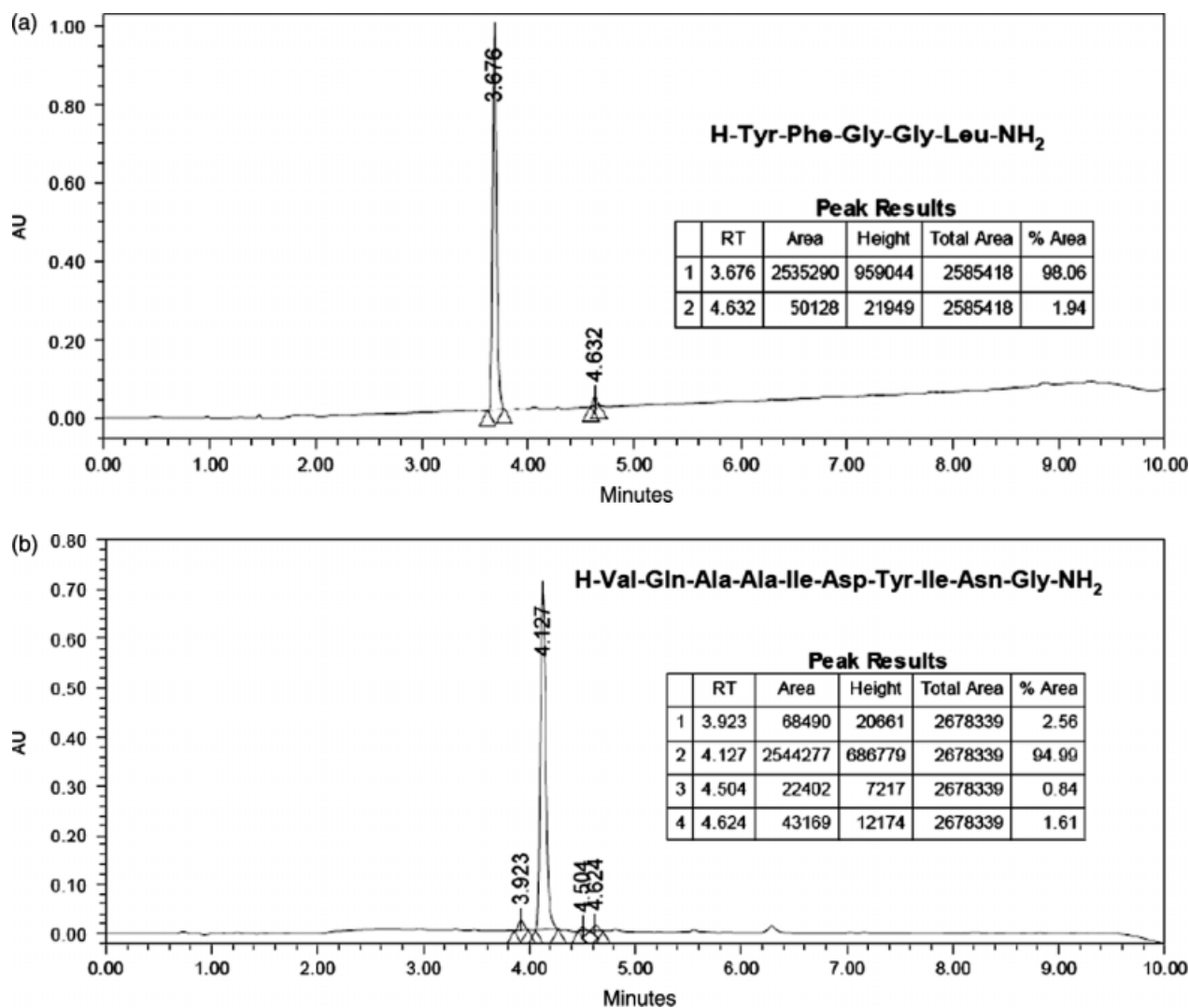**Figure 3.** Beads swelled in ACN of (a) ChemMatrix and (b) polystyrene.

Figure 4. HPLC chromatograms of crude peptides directly after precipitation of the cleavage cocktail. Reversed-phase C-18 columns were used for tha analysis with elution by a linear gradient over 8 min at 0.045% TFA in ACN and 0.036% aqueous TFA from (A) 1 : 19 to 1 : 0 and (B) 1 : 9 to 6 : 4; flow rate 1.0 min^{-1}, detection 220 nm.

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