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The concept of internal solubilization in peptide synthesis: ethylene glycol-based protecting groups

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This work is dedicated to Professor Kálmán Medzihradszky on the occasion of his 80th birthday

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ABSTRACT

A novel, ethylene glycol-based protecting group is designed and synthesized for use in solid phase peptide synthesis. Ether and ester type protected amino acids are prepared. The acid stability of the new protecting group showed complete Fmoc/*t*-Bu compatibility. The new derivatives are tested in solid phase peptide synthesis, with a 'difficult' sequence to examine the disruption of peptide aggregation.

It was recognized more than 20 years ago that during solid phase peptide synthesis, after reaching a certain peptide region with characteristic sequences, the complete acylation of the free N-terminus is very difficult to achieve.[1,2] Since, these special cases have been known as 'difficult sequences', and are thought to be connected with the association of intermediate resin-bound peptide chains into extended β-sheet type structures. The approaches eliminating the problems can be classified into different categories (a) Applying improved coupling conditions, such as specialized coupling reagents[3–5] and additives or in situ neutralization,[6] (b) applying special resins and linkers,[7,8,4] to improve the synthetic conditions,[9] (c) by substituting the amide hydrogen atom with reversible N-protection with *N*-benzyl or *N*-(2-hydroxy-4-methoxybenzyl) (Hmb) groups[10] as the amide hydrogen is involved in the formation of hydrogen bonds in the aggregating fragments, (d) using building blocks which modulate the conformational and physicochemical properties of peptides (pseudoprolines),[11] (e) using depsipeptides which can be later transformed to peptides.[12] Polyethylene glycol (PEG) chains have been shown to possess β-sheet disintegrating properties.[13] When PEG is attached to the solid phase, the efficiency of a solid phase synthesis improves significantly as long as the growing peptide chain is close (closer than 15 amino acid residues) to the polyethylene glycol-containing matrix.

PEG also increases the solubility of the moieties attached. PEG had been introduced previously as a solubilizing C- and N-terminal protecting group of carrier-bound peptides in SPPS. The attachment of PEG blocks the hydrophobic peptides, increases their solubility, and decreases intermolecular aggregation.[14] Zier et al. attached PEG chains covalently to the usual protecting groups, and used the products in SPPS.[15]

There are two currently applied cases of polyethylene glycol solubilization.

(A) When the solubilizing polyethylene glycol moiety is isolated from the growing peptide chain, it behaves as an intact, external and sterically hindered solubilizing agent. This is 'external' solubilization, which can be observed either with PEG-containing resins or when the PEG chain is attached to the N- or C-terminus of the peptide chain (Fig. 1A).
(B) When the PEG chains are attached somewhere on the side chains or on the backbone of the peptide chain, they achieve 'internal' solubilization, since the PEG chains approaching

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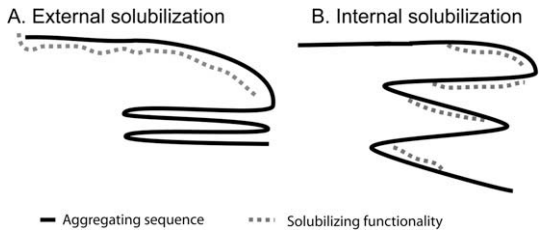

Figure 1. The difference between external and internal solubilization on the same aggregating sequence.

the center to be solubilized are not from an external region, but rather from inside the peptide chain (Fig. 1B). Repetitive use of the solubilizing functionality can provide more specific targeting and disruption of a specific aggregating region, being more effective in solubilizing due to the higher spatial/conformational restrictions.

PEG chains can be inserted into the backbone of the peptide chain, which is expected to show similar solubilizing effects as internal solubilization. This strategy, however, modifies the primary amino acid sequence of the peptide.

It is evident that whereas external solubilization has a finite and diminishing effect on the peptide chain, internal solubilization has the potential to disrupt aggregation throughout the peptide chain. Thus, it is a concept for synthesizing formerly so-called 'difficult sequences' in a more successful manner than with techniques known so far. Repetitive and systematic incorporation of solubilizing moieties into a growing sequence of protected building blocks prevents the formation of aggregated structures, regardless of how long the sequence may be. Figure 1B shows how the solubilizing functionality appears, if PEG-containing groups are attached to the side chains of protected amino acids. Amino acids normally protected by a *t*-butyl in their side chain (Asp, Glu, Ser, Thr, and Tyr) can be modified and protected in their side chains by *para* PEG-substituted benzyl protecting groups.

Zinieris et al. investigated the effect of an ethylene glycol derivative in SPPS. Their observation supports the external and internal solubilization concept.[16] Current techniques, such as using pseudoprolines or isopeptides, need the presence of Ser or Thr in the peptide sequence. However, this might not be sufficient for efficient disintegration. A wider variety of amino acid derivatives can be used, if ethylene glycol moieties are attached through ester or ether type protecting groups. Triethylene glycol monomethyl ether was selected as the solubilizing agent due to its price and availability. To obtain a widely applicable benzyl type protecting group system, triethylene glycol monomethyl ether was attached to the phenolic group of 4-hydroxybenzyl alcohol, a widely available building block, to yield 4-(3,6,9-trioxadecyl)oxybenzyl alcohol (TEGBz-OH, Fig. 2). The derivatives of this activated benzyl alcohol are expected to be susceptible to trifluoroacetic acid cleavage,[17] and therefore the derivatives might be used in Fmoc peptide synthesis.

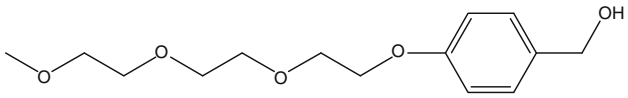

Figure 2. The structure of TEGBz-OH.

Using TEGBz-OH, the derivatives to be employed in the synthesis of Acyl Carrier Protein 65-74 (ACP 65-74, VQAAIDYING-NH_2), a widely applied model peptide, were synthesized.[18,19]

The Fmoc/TEGBz protected amino acids were synthesized from their Boc derivatives. The TEGBz-OH was activated with methanesulfonic chloride, and the mesyl derivative was used in the preparation of ether and ester type derivatives. The products were purified by column chromatography.[20]

The qualitative acid lability of the TEGBz group was investigated. Boc-Asp(OTEGBz)-O^{t}Bu was cleaved using different reagent mixtures. In the presence of hydrogen chloride in dioxane or in diisopropyl ether, the Boc and *tert*-butyl groups were removed selectively, and the H-Asp(OTEGBz)-OH derivative precipitated from the reaction mixture. In 20% TFA/DCM mixture, the Boc and *tert*-butyl groups removal was complete after 15 min. However, H-Asp(OTEGBz)-OH was cleaved further to yield unprotected aspartic acid.

According to our results, the use of the TEGBz protecting group is fully compatible with an Fmoc/*tert*-butyl SPPS strategy, because it can be completely removed with TFA, and has sufficient acid stability to allow its use in the synthesis of protected peptide fragments, for example, on 2-chlorotrityl resin.[21]

The expected internal solubilization effect (Fig. 3) of the side chain protected derivatives was probed during the synthesis of ACP 65-74 (VQAAIDYING-NH_2).

Fmoc-Rink amide resin (0.43 mmol/g) was used in the comparative study. Fmoc-Asp(O^{t}Bu)-OH and Fmoc-Tyr(^{t}Bu)-OH were utilized to compare with the effect of Fmoc-Asp(TEGBz)-OH and Fmoc-Tyr(TEGBz)-OH. The peptide was constructed applying Fmoc-strategy (coupling: Fmoc-AA-OH:HBTU:DIEA = 4:4:8 for 4 min, deprotection: DMF:DBU:piperidine = 96:2:2 (v:v:v) for 6 min).[22] The effect of the protecting groups on the product distribution in the crude peptide is shown in Figure 4 and Table 1.

By using TEGBz building blocks, the overall yield of the target peptide increased, and in particular, the percentage of the target sequence relative to all deletion sequences has significantly increased. Thus, it has been demonstrated that applying internal PEG solvolysis is a working concept, which can be applied especially on difficult sequences.

Our results show that TEGBz side protection is an effective technique to synthesize peptides, which tend to aggregate. As TEGBz derivatives can substitute the currently used *t*-butyl derivatives, they might be used more broadly and have potential to become useful building blocks, even in automated peptide synthesis.

With the combination of the available amino acid derivatives, appropriate incorporation of TEGBz protecting groups at certain amino acids in the peptide sequence may prevent the formation

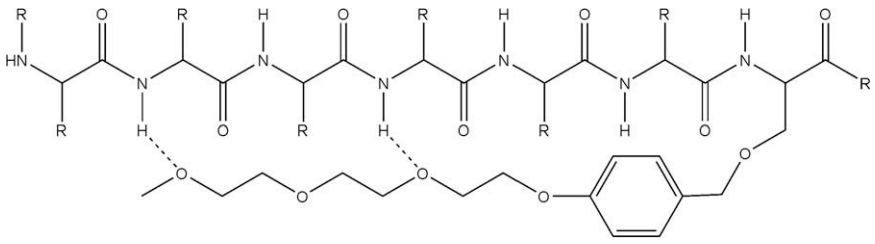

Figure 3. Simplified schematic view of the internal solvation by the TEGBz group.

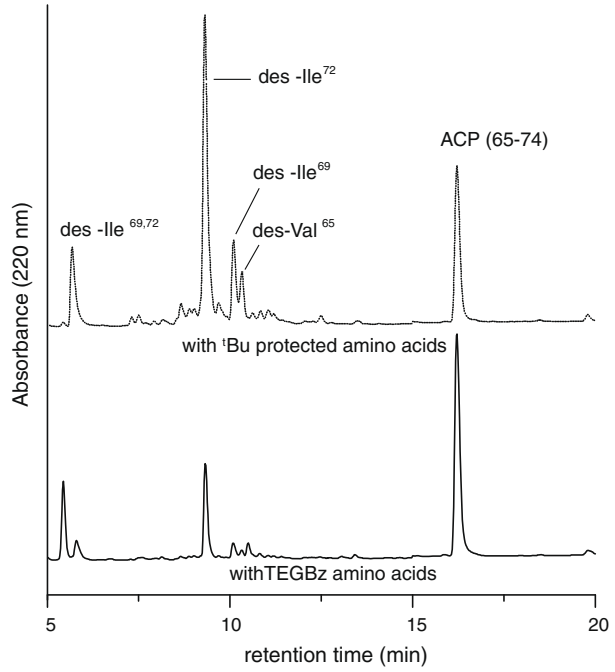

Figure 4. HPLC of the crude ACP 65-74 products. Column: Vydac 218TP 150 × 2.3 mm (3 μm), linear gradient over 15 min from: 5% to 20% acetonitrile (0.036% TFA) then hold in 0.045% TFA containing water at 0.5 ml/min flow rate.

Table 1
Comparison of the composition of the crude peptide mixtures cleaved from the resin (0.43 mmol/g Rink amide resin)

	Crude yield after cleavage (%)	ACP (%)	-Ile^{69}, -Ile^{72} (%)	-Ile^{72} (%)	-Ile^{69} (%)	-Val^{65} (%)
With ^{t}Bu protection	57	30	14	41	6	7
with TEGBz protection	63	62	12	18	3	2

of aggregation in 'difficult' sequences. The synthesis of longer peptides by this technique is required to further evaluate the full potential of this approach. Another field, which needs to be further explored is how the TEGBz protecting group can improve the solubility of protected peptide fragments, and how this technique will further develop the use of the fragment condensation technique, in order to produce longer peptides or even proteins.

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