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On the use of *N*-dicyclopropylmethyl aspartyl-glycine synthone for backbone amide protection

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To prevent aspartimide formation and related side products in Asp-Xaa, particularly Asp-Gly-containing peptides, usually the 2-hydroxy-4-methoxybenzyl (Hmb) backbone amide protection is applied for peptide synthesis according to the Fmoc-protocols. In the present study, the usefulness of the recently proposed acid-labile dicyclopropylmethyl (Dcpm) protectant was analyzed. Despite the significant steric hindrance of this bulky group, *N*-terminal H-(Dcpm)Gly-peptides are quantitatively acylated by potent acylating agents, and alternatively the dipeptide Fmoc-Asp(OtBu)-(Dcpm)Gly-OH derivative can be used as a building block. In contrast to the Hmb group, Dcpm is inert toward acylations, but is readily removed in the acid deprotection and resin-cleavage step.

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Keywords: aspartimide formation; Asp-Gly sequence motif; backbone amide protection; acid-labile dicyclopropylmethyl group

Introduction

Even by the use of the Fmoc/tBu strategy for peptide synthesis, aspartimide formation, particularly in the sequence motifs Asp-Gly or Asn-Gly, represents a serious side reaction [1–5]. Indeed, the repetitive piperidine treatment deprotonates the dipeptide amide, which then attacks the side-chain ester or amide, leading to aspartimides. These are prone to base-catalyzed epimerization and to the nucleophilic ring opening, generating mixtures of α- and β-piperidides, aspartyl- and isoaspartyl peptides as well as the corresponding diastereomers. To prevent such aspartimide side reaction as well as to increase the solubility of resin-linked peptides of 'difficult sequences' by suppressing β-sheet formation [6], the Hmb [7–10] and, more recently, the Dmb group [10–12] have been proposed for protection of the peptide amide function (Figure 1). Because of the significantly reduced reactivity of the secondary amines of H-(Hmb)Gly-peptides and H-(Dmb)Gly-peptides, generally the commercially available dipeptide building blocks Fmoc-Asp(OtBu)-(Hmb)Gly-OH and Fmoc-Asp(OtBu)-(Dmb)Gly-OH [13] are applied. Removal of both these amide protectants can be problematic as prolonged TFA treatments are required [14,15]. Accordingly, new groups such as Dmab [16], EDOTn [14] and MIM [14] have been proposed for the backbone amide protection (Figure 1). An alternative approach aimed at preventing aspartimide formation is based on a different protection of the critical Asp side-chain carboxy group as β-3-methylpent-3-yl (Mpe) ester [17].

The Dcpm group, which was previously recommended for the protection of carboxy and side-chain amide groups because of its acid lability [18], has recently been proposed to be efficient for backbone amide protection [19,20]. For this purpose, the synthesis of *N*-Dcpm amino acids was elaborated and their expectedly, sterically hindered *N*-acylation analyzed. Relatively unhindered *N*-Dcmp amino acids such as (Dcpm)Ala and (Dcpm)Gly are efficiently acylated as *N*-terminal residues by the use of the potent acylating agent *N*-HATU. The expected solubilizing effect of the Ala derivative in the case of a deca-alanine peptide was confirmed along with prevention of aspartimide formation by (Dcpm)Gly in two model peptides [19].

In order to facilitate incorporation of the (Dcpm)Gly residue into peptides containing Asp-Gly sequences in the present study, the use of the Fmoc-Asp(OtBu)-(Dcpm)Gly-OH dipeptide synthon was compared with the stepwise elongation of resin-bound peptides with the suitably protected (Dcpm)Gly and Asp residues.

Materials and Methods

Fmoc-Asp(OtBu)-(Hmb)Gly-OH and the amino acid derivatives used in peptide synthesis were purchased from Merck Biosciences, Novabiochem, and 2-Cl-trityl-chloride resin (100–200 mesh) from CBL, Patras. The synthesis of the amino acid derivatives H-(Dcpm)Gly-OH, Fmoc-(Dcpm)Gly-OH and Fmoc-(Dcpm)Ala-OH was previously reported [19]; H-(Dcpm)Leu-OH and

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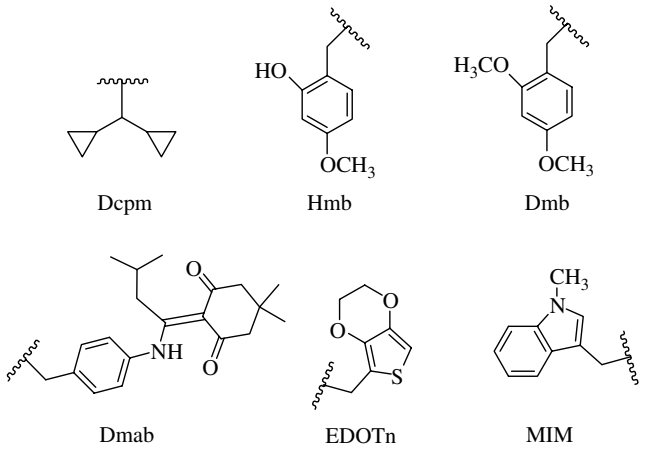

Figure 1. Structures of the backbone amide protectants Dcpm, Hmb, Dmb, Dmab, EDOTn and MIM.

Fmoc-(Dcpm)Leu-OH were synthesized by similar protocols (see Supporting Information). In the preparation of *N*-Fmoc-(Dcpm)amino acids by reaction of (Dcpm)amino acids with Fmoc-Cl in the presence of two equiv DIEA in CH_2Cl_2 according to the reported protocol [19], partial cleavage of the Dcpm group by the liberated HCl could not be avoided completely. On the other hand, acylation with Fmoc-OSu was unsatisfactory due to its lower reactivity compared to the chloride.

Peptide syntheses on solid support were performed using the Fmoc/tBu strategy on an ABI 433A automated peptide synthesizer equipped with UV-detector from Applied Biosystems. Analytical RP-HPLC was carried out on a Shimadzu LC10 system using a Nucleosil 300 C18 column (4.6 × 125 mm, 5 µm) and a linear gradient of 10% B to 100% B over 45 min (A: 2500 ml water, 5 ml TFA; B: 2000 ml acetonitrile, 500 ml water, 5 ml TFA) with spectrometric monitoring at $\lambda = 220$ nm.

MALDI-MS were recorded on a Voyager-DE PRO BioSpectrometry Workstation from Applied Biosystems. Samples were dissolved in 50% aqueous acetonitrile, and α-cyano-4-hydroxycinnamic acid was used as matrix. High-resolution mass spectra were recorded with an ultra performance liquid chromatography (UPLC) LCT Premier XE system from Waters equipped with a ACQUITY ACR UI HSST3 column (100 × 2.1 mm, 1.8 µm) at a temperature of 35 °C. A linear gradient from 5% acetonitrile/95% water to 95% acetonitrile/5% water in 10.5 min was applied with a flow rate of 0.6 ml/min.

NMR spectra were recorded using Bruker 400 MHz and 600 MHz Avance NMR spectrometers. The Dcpm-protected amino acids glycine, alanine and leucine and the dipeptide building block Fmoc-Asp(OtBu)-(Dcpm)Gly-OH were used at a concentration of about 20 mM and the model peptide Mon1 at a concentration of about 2 mM. Chemical shifts were measured using the $CDCl_3$, DMSO-d_6, CD_3CN or D_2O solvent signal (vs tetramethylsilane) for ^1H and ^{13}C signals as a secondary standard. The spectra were processed using the standard Bruker software Topspin.

Synthesis of Fmoc-Asp(OtBu)-(Dcpm)Gly-OH

The dipeptide derivative Fmoc-Asp(OtBu)-(Dcpm)Gly-OH was synthesized by linking H-(Dcpm)Gly-OH (180 mg; 1.065 mmol) onto 500 mg of a 2-Cl-trityl-chloride resin in DMF (3 ml) in the presence of 2 equiv DIEA (362.3 µl; 2.13 mmol). The reaction mixture was stirred for 1.5 h at rt. The resin was capped with MeOH : DIEA (9 : 1 v/v) for 15 min at rt. Then 2.8 equiv Fmoc-Asp(OtBu)-OH (1.23 g; 3 mmol) was coupled with 2.8 equiv *N*-HATU (1.1 g; 3 mmol) and 6 equiv DIEA (1.02 ml; 6 mmol) in DMF (10 ml) at rt overnight. Cleavage of the protected dipeptide acid from the resin by treatment with DCM/TFE/acetic acid (3 : 1 : 1; v/v/v) for 2 h at rt gave 93.5 mg (0.1664 mmol, 16%) of the desired crude dipeptide.

Because of the high acid sensitivity of the Dcpm group, the purification was carried out with 0.025 M ammonium acetate buffer. The product was chromatographed on a Shimadzu LC8 preparative HPLC system using a VYDAC C18 column (250 × 40 mm, 15–20 µm) using a linear gradient of 20% C/80% D to 90% C/10% D over 50 min at a flow rate of 20 ml/min with spectrometric monitoring at $\lambda = 220$ nm (C: 1000 ml 0.025 M ammonium acetate buffer/350 ml methanol; D: acetonitrile). Yield: 27 mg (29% based on the amount of the crude material used for purification); homogeneous on RP-HPLC: R_t 31.73 min (Figure 6); MALDI-TOF-MS: $m/z = 586.10$ $[M + Na]^+$; calcd 585.64; 602.04 $[M + K]^+$; calcd 601.75 (Figure S13, Supporting Information); HR-MS: $m/z = 561.2616$ $[M - H]^-$ and 563.2747 $[M + H]^+$; calcd 562.2679; 1D and 2D NMR spectra confirmed the molecular identity (Table S2 and Figures S16–S19, Supporting Information).

Synthesis of Biotinyl-RQYRLIVHNGYCDGRSERNL

The following side-chain-protecting groups were used for the automated synthesis: 2,2,4,6,7-pentamethyl-dihydro-benzofurane-5-sulfonyl (Arg), *tert*-butyloxycarbonyl (Trp, Lys), *tert*-butyl ether (Thr, Ser, Tyr), *tert*-butyl ester (Asp, Glu) and trityl (Asn, Cys, Gln, His). The syntheses were performed on TentaGel S Ram resin (Rapp Polymere, Germany) using standard protocols and *N*-HBTU coupling steps except for Fmoc-(Dcpm)Gly-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Asp(OtBu)-(Dcpm)Gly-OH (**1**) as well as for Fmoc-Asp(OtBu)-(Hmb)Gly-OH for comparative purposes. These amino acid and dipeptide derivatives were coupled in 2.5 molar excess with *N*-HATU (2.5 equiv) and DIEA (5 equiv) in DMF overnight at rt. Similarly, the final coupling of biotin was also performed with *N*-HATU. Peptides were deprotected and cleaved from the resin with TFA/H_2O/TIPS (95 : 3 : 2) for 3.5 h at rt.

Synthesis without Asp-Gly backbone protection

The crude product was heavily contaminated by the aspartimide side product as shown in the HPLC profile in Figure 2A and in the mass-spectrometric analysis in Figure 3.

Synthesis with Hmb backbone protection

The main component of the crude product (Figure 2B) corresponds to biotinyl-RQYRLIVHNGYCD-(biotinyl-Hmb)GRSERNL according to mass-spectrometric analysis (Figure 4A) and the NMR spectra (Figure 5).

Stepwise synthesis with Dcpm backbone protection

As shown in Figure 2C, the crude product is of relatively good quality. MALDI-TOF-MS confirmed the correct molecular identity (Figure 4B).

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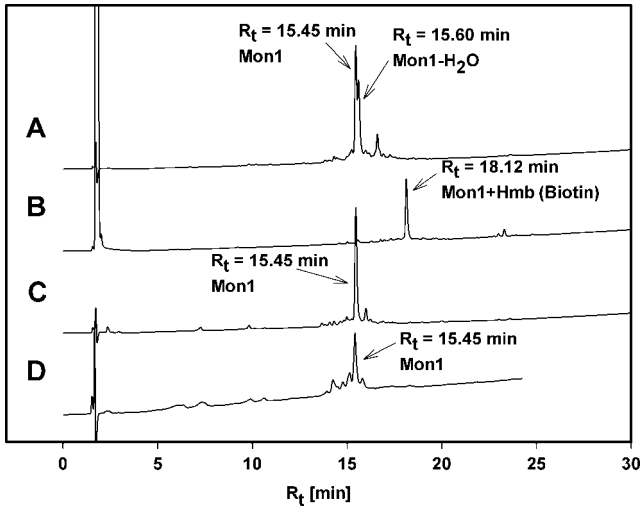

Figure 2. HPLC profile of the crude Mon1 (biotinyl-RQYRLIVHNGYCDG(X)RSERNL) peptides using (**A**) standard Fmoc-chemistry, (**B**) Fmoc-Asp(OtBu)-(Hmb)Gly-OH, (**C**) Fmoc-(Dcpm)Gly-OH, and (**D**) Fmoc-Asp(OtBu)-(Dcpm)Gly-OH.

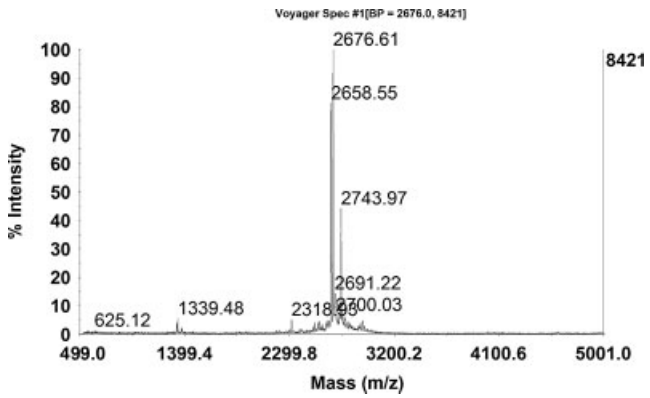

Figure 3. MALDI-TOF-MS of the Mon1 peptide ($[M]_{calc.}$ 2675.06 Da, $[M]_{found}$ 2676.61 Da) contaminated by the aspartimide side product ($[M]_{found}$ 2658.55 Da).

Synthesis with the Fmoc-Asp(OtBu)-(Dcpm)Gly-OH synthon

The synthesis was performed with the crude dipeptide synthon, and HPLC of the resulting crude product showed increased impurities compared to the stepwise synthesis (Figure 2D).

Results and Discussion

In a first standard synthesis of the *N*-biotinylated Mon1 peptide (biotinyl-RQYRLIVHNGYCDGRSERNL), which contains an Asp-Gly sequence, significant formation of the cyclic aspartimide was observed as shown in Figure 2A, which gave low yields of the desired biotinyl peptide. Therefore, as shown in Scheme 1, in a second approach, the well-established Fmoc-Asp(OtBu)-(Hmb)Gly-OH synthon was applied. After biotinylation and final deprotection/resin cleavage with TFA/H_2O/TIPS (95 : 3 : 2), the analysis of the rather good crude product (Figure 2B) by mass spectrometry (Figure 4A) clearly revealed quantitative biotinylation of both the *N*-terminus and the Hmb residue, which in the acylated form is fully stable toward the applied acid deprotection conditions. NMR analysis of the product confirmed the presence of the biotin linked to the *N*-terminal Arg residue as well as to the Hmb residue (Figure 5). For conversion of this peptide derivative to the desired *N*-biotinylated peptide Mon1, saponification of the Hmb ester is required prior to the acid deprotection step.

In a third synthesis, the newly proposed backbone protection with the Fmoc-(Dcpm)Gly-OH derivative [19] was applied (Scheme 1). As expected, the coupling reagent *N*-HBTU had to be changed to the more potent *N*-HATU and the reaction time extended to 12 h to overcome the steric hindrance introduced by the *N*-Dcpm group. The resulting crude product was found to exhibit a remarkable degree of purity as shown in Figure 2C.

In order to examine the usefulness of a dipeptide synthon as recommended for the *N*-Hmb protection, a synthetic route to Fmoc-Asp(OtBu)-(Dcpm)Gly-OH was devised (Scheme 2), which involved linking of the free acid (Dcpm)Gly-OH onto the very acid sensitive 2-Cl-trityl-chloride resin. In the next step, Fmoc-Asp(OtBu)-OH was coupled onto the secondary amino function of the bound glycine residue. After cleavage of the resin, under very mild conditions, with DCM/TFE/acetic acid (3 : 1 : 1; v/v/v) for 2 h at rt, unsatisfactory low yields (16%) of the desired crude dipeptide were obtained.

Purification of the crude product (Figure 6) was carried out with 0.025 M ammonium acetate buffer instead of the commonly used TFA eluents because of the high acid sensitivity of the Dcpm group when attached in the backbone position. The molecular identity of the resulting homogeneous dipeptide synthon was confirmed by MALDI-TOF-MS (Figure S12, Supporting Information), HR-MS via UPLC (Figure S13–S15, Supporting Information) and NMR spectroscopy (Table S2 and Figures S16–S19, Supporting Information).

Using the crude product of the backbone-protected dipeptide synthon for the synthesis of the biotinyl-Mon1 peptide, a crude product was obtained, which exhibited a lower degree of purity than that obtained by stepwise introduction of the Fmoc-(Dcpm)Gly-OH and Fmoc-Asp(OtBu)-OH (Figure 2D). This fact is attributed to the contaminants of the crude dipeptide synthon.

It is reasonable to assume that, with an optimized synthesis of the *N*-Dcpm protected dipeptide, this building block can be used with standard *N*-HBTU coupling protocols for the synthesis of Asp-Gly-containing peptides.

The synthetic route outlined in Scheme 2 should also be applicable for the preparation of other Fmoc-Xaa-(Dcpm)Gly-OH or even Fmoc-Xaa-(Dcpm)Ala-OH dipeptides to be used for enhancing the solubility of 'difficult sequences'.

The NMR spectra of the dipeptide Fmoc-Asp(OtBu)-(Dcpm)Gly-OH were characterized by two complete sets of resonance signals caused by the *cis/trans* isomerization of the peptide bond (Scheme 3). The *cis/trans* ratio is 45/55 in CD_3CN and shifting further to the *trans*-isomer in $CDCl_3$ (35/65) (Table 1). The analogous protons of the two cyclopropyl rings of the *cis*-isomer have identical chemical shifts. This is not the case for the cyclopropyl rings of the *trans*-isomer. This feature results from the unrestricted rotation of the Dcpm group for the *cis*-isomer and the corresponding sterically hindered rotation for the *trans*-isomer. The *cis*- and *trans*-signals undergo shifts as the temperature is raised, but full coalescence is not reached up to 70 °C. Hence, the *cis/trans* isomerization of Dcpm-substituted tertiary amides is similar to that observed with peptides containing proline or *N*-methylated amino acids. In addition to the solvent

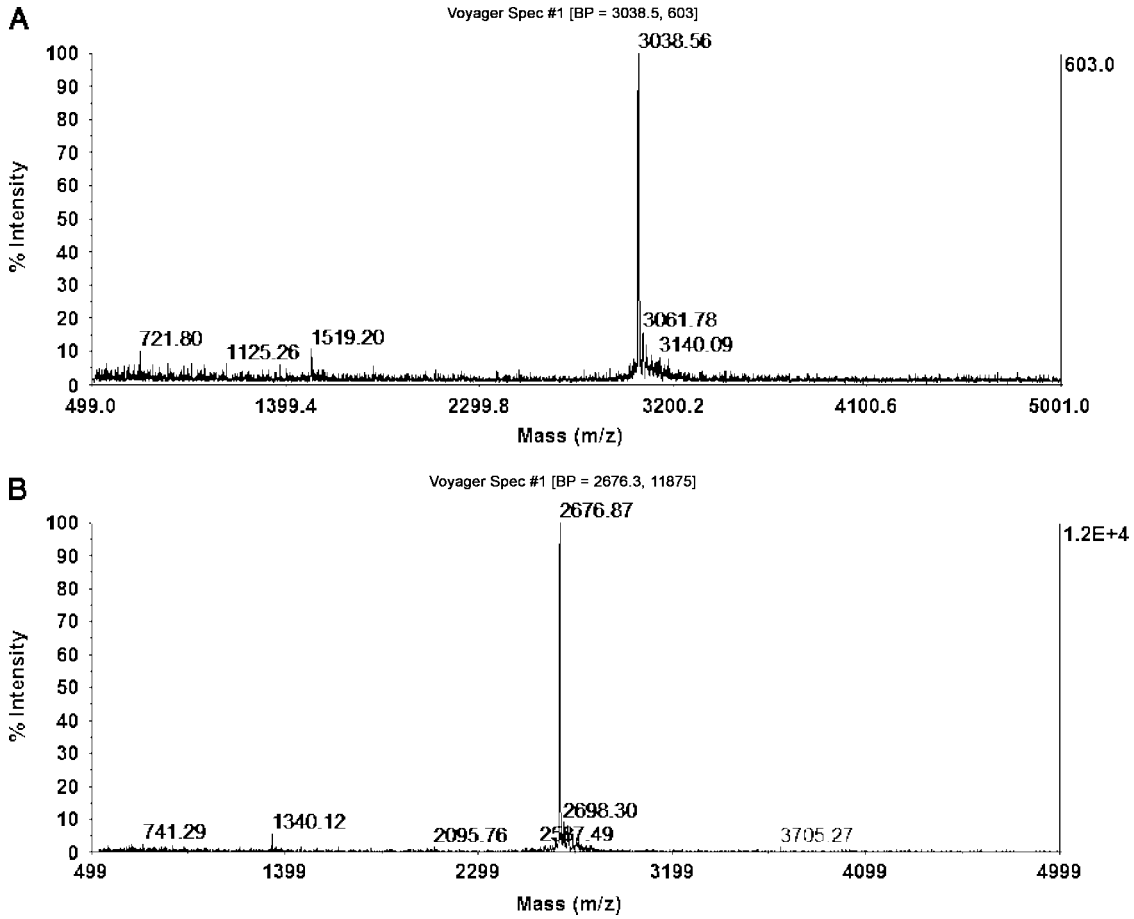

Figure 4. MALDI-TOF-MS of the Mon1 peptide ($[M]_{calc.}$ 2675.06 Da); (**A**) with addition of 362 Da from the biotin-labeled Hmb group ($[M]_{found}$ 3038.56 Da) and (**B**) the correct peptide ($[M + H]_{found}$ 2676.87 Da).

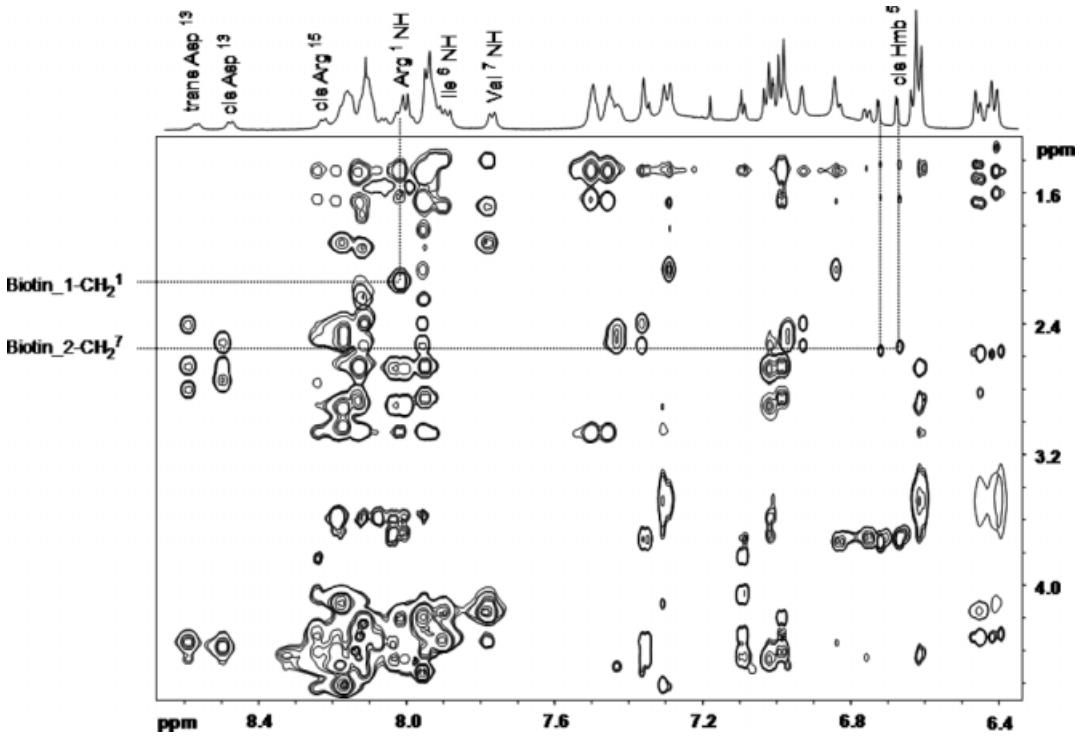

Figure 5. Section of the NOESY spectrum of Mon1 (600 MHz, 298 K, DMSO-*d6*) showing the connection of biotin-1 to Arg-1 and biotin-2 to Hmb.

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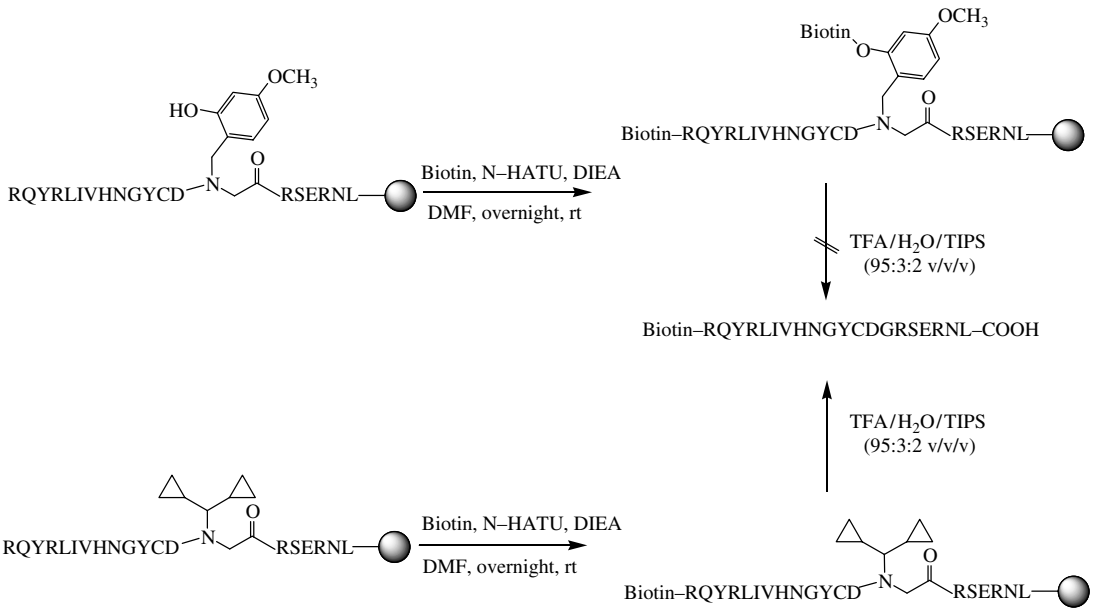
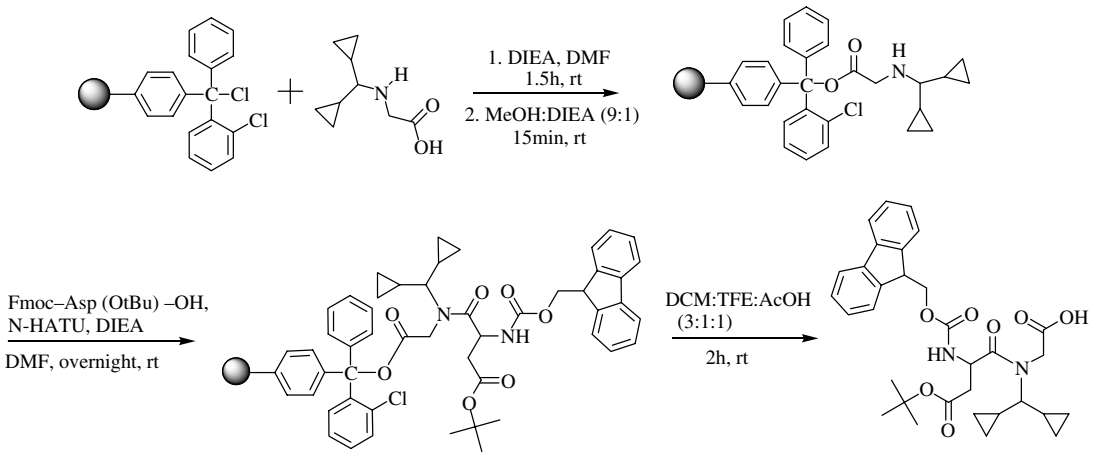


Scheme 1. Synthesis of the biotinylated model peptide Mon1 by using either the Hmb or the Dcpm group for the backbone amide protection of Gly-14.



Scheme 2. Synthesis of Fmoc-Asp(OtBu)-(Dcpm)Gly-OH on solid support.

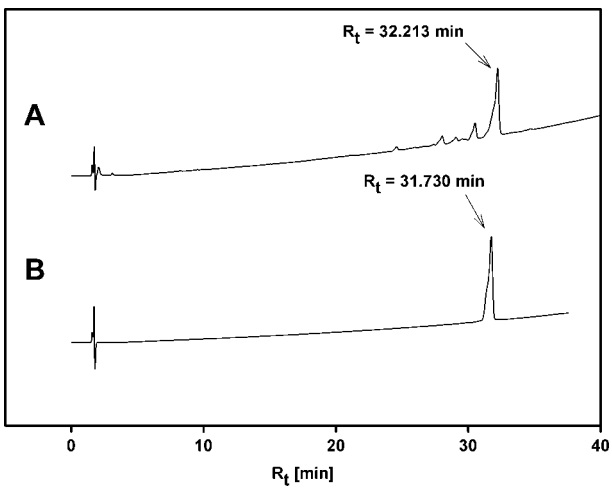
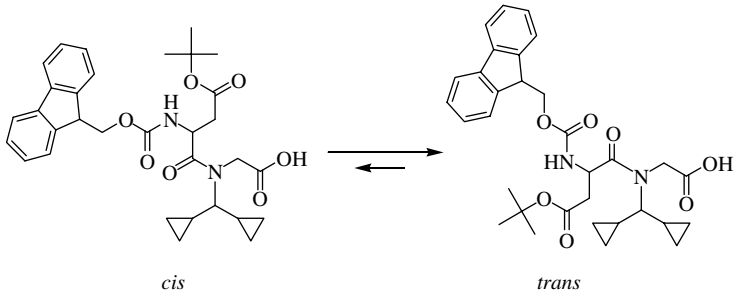


Figure 6. HPLC profiles of (**A**) crude and (**B**) purified Fmoc-Asp(OtBu)-(Dcpm)Gly-OH. For conditions see Section on Materials and Methods.

effect, the amount of *trans*-conformer increases with the size of the amino acid side chain (Table 1). The sterically hindered side chains of Leu and Ala result in restricted rotation of the Dcpm group. This is well evidenced by the separate NMR signals for all cyclopropyl protons of the *trans*-isomer (Table 1, Supporting Information).

Conclusions

The Dcpm group is cleaved even at very low TFA concentrations, e.g. 5% TFA in chloroform in opposition to the Hmb and Dmb groups, which require very strong acidic conditions and long reaction times for the final deblocking step [14,15]. The products of the acidic Dcpm cleavage are readily removed, thus making the dipeptide building block Fmoc-Asp(OtBu)-(Dcpm)Gly-OH a good alternative to the corresponding Hmb- and Dmb-containing dipeptides. For this purpose, further work will be required to optimize the synthesis of the dipeptide synthon.



Scheme 3. *cis/trans* isomerization of Fmoc-Asp(OtBu)-(Dcpm)Gly-OH.

Table 1. Ratios of the *trans*- and *cis*-conformers resulting from the hindered rotation around the tertiary amide bond as measured by ^{1}H NMR spectroscopy

Compound	*trans*	*cis*	Solvent
Fmoc-(Dcpm)Gly-OH	65%	35%	$CDCl_3$
Fmoc-(Dcpm)Ala-OH	75%	25%	$CDCl_3$
Fmoc-(Dcpm)Leu-OH	95%	5%	$CDCl_3$
Fmoc-Asp(OtBu)-(Dcpm)Gly-OH	65%	35%	$CDCl_3$
Fmoc-Asp(OtBu)-(Dcpm)Gly-OH	55%	45%	CD_3CN

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Supporting information

Supporting information may be found in the online version of this article.

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