Solid-Phase Synthesis of Peptides Containing α,β-Didehydroamino Acids

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α,β-Didehydroamino acids are frequently encountered in natural peptides with important biological activity. Herein, we report a mild and convenient method for the preparation of peptides containing α,β-didehydroamino acids, where solid-phase techniques are used both for elongation of the peptide chain and formation of the double bond. This bond is formed through a β-elimination reaction, using a water soluble carbodiimide as the activating reagent of the hydroxyl function, and catalyzed by CuCl.

Introduction

α,β-Didehydroamino acids (DDAAs) are frequently encountered in naturally occurring peptides with important biological activity.[1] Although DDAAs are also constituents of medium size peptides, such as the antibiotics known as lantibiotics,[2] and of enzymes from plant and bacterial sources,[3] the majority of them have been found in relatively low molecular weight peptides of microbial and fungal metabolite origin,[1c] or from marine organisms.[4] The presence of these residues in peptides confers an increased resistance to enzymatic degradation[5] and restricts the conformational flexibility of both the backbone and the side chain of the didehydro residue.[6] The incorporation of DDAAs into normal bioactive peptides has also become another interesting objective, and consequently the search for suitable methods for the synthesis of peptides containing DDAAs [α,β-didehydropeptides (DDP)] has increased considerably in the last few years.[7]

Even the synthesis of peptides containing DDAAs derived from proteinogenic amino acids, such as ΔAla (from Ser or Cys) or α,β-didehydro-α-aminobutyric acid (Dhb, ΔAbu) (from Thr), by activation of the β-hydroxyl or thiol function and posterior β-elimination, is not a straightforward process. Preparation of the DDAAs with N^{α}-carbamate protecting groups, which can be relatively easily performed from the corresponding N^{α}-carbamate amino acids (Ser, Thr, phenylserine) and activating reagents, such as disuccinimidyl carbonate (DSC)[8] or di-*tert*-butyl dicarbonate (Boc_2O)[9] in the presence of base,[10] are not useful as building blocks for peptide synthesis. Thus, once the N^{α}-protecting group is removed, the weak nucleophilicity of the corresponding enamine function prevents elongation in the C → N direction.[11,12] Alternatively, the synthesis of *N*-acyldidehydroamino acids and their incorporation into the peptide sequence can be a convenient method for preparing DDP.[12,13] Furthermore, the formation of the double bond can be carried out after the peptide sequence has been assembled.[7,14] Usually, all these processes have to be carried out in solution and there are very few reports in the literature describing the solid-phase synthesis of ΔAla-containing peptides.[15]

The present communication reports on a convenient method for the solid-phase synthesis of peptides containing DDAAs, where solid-phase techniques are used for both the elongation of the peptide chain and the formation of the double bond. This method is of general applicability to peptides containing common DDAAs, and is not restricted only to ΔAla-containing peptides.

Results and Discussion

As model targets, the peptides Ac−Tyr−Gly−*Z*-Dhb−Phe−Leu−NH_2 (**1**) and Ac−Val−Phe−*Z*-Dhb−Val−NH_2 (**2**) were chosen, with the idea of starting the study with the most hindered Thr in order to find conditions of general applicability. Ac−Tyr(*t*Bu)−Gly−Thr−Phe−Leu−Amide-Linker-resin and Ac−Val−Phe−Thr−Val−Amide-Linker-resin were prepared using a standard 9-fluorenylmethoxycarbonyl (Fmoc)/*tert*-butyl (*t*Bu) protocol.[16] Aliquots of the first resin were washed with *N,N*-dimethylformamide (DMF) and then used in tests of a variety of reagents for the activation of the hydroxyl function and posterior β-elimination. The course of the reaction was followed by amino acid analysis (AAA) of the hydrolyzed acid because the formation of the *Z*-Dhb residue is accompanied by a decrease of the Thr value in AAA (Table 1).[17]

DSC (entries 1 and 2) has been used for the formation of protected *Z*-Dhb in solution,[8] and a ΔAla-containing peptide in the solid phase.[15a] (Diethylamino)sulfur trifluoride (DAST) (entry 3) was found to convert alcohols into fluorides in solution[18] and has been used for the dehydration of Thr in solution.[19] None of these three experiments led to a significant decrease of the value of Thr in AAA. The experiments corresponding to entries 4 and 5 in Table 1 were carried out in two steps with the idea of forming first the tosyl derivative and then carrying out the β-elimination. HPLC of the cleaved product after the first step showed, in

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Table 1. Study of the formation of Ac−Tyr(*t*Bu)−Gly−*Z*-Dhb−Phe−Leu−Amide-Linker-resin from the corresponding Thr derivative

Entry	Reagents (equiv.)	Solvent	Time (h)	Thr:AA[a]
1	DSC (25) DIEA (25)	DMF	21	0.72
2	DSC (25) DABCO (25)	DMF	21	0.76
3	DAST (50) DIEA(50)	DMF	23	0.61
4	(i) Tos_2O (25)	pyridine/DMF	3.3	0.77
	(ii) DABCO (50)		47	
5	(i) TosCl (25)	pyridine/DMF	3.3	0.83
	(ii) DABCO (50)		47	
6	PPh_3 (50) DEAD (50)	THF	32	0.10
7	EDC (50) CuCl (15)	DCM/DMF (1:1)	24	0.30

[a] AAA gives a value of Thr with respect to the rest of the amino acids (AA). Due to the decomposition of Thr during acid hydrolysis, the value of Thr in the initial peptide respect to the rest of the AAs is 0.78 (average value). Peptide-resins were hydrolyzed in 12 N aqueous HCl−propionic acid (1:1), at 155 °C for 90 min.

Table 2. Study of the formation of Ac−Val−Phe−*Z*-Dhb−Val−Amide-Linker-resin from the corresponding Thr derivative[a]

Entry	Equiv. EDC:CuCl	Solvent	Temp.	Thr:AA[b]
1	100:30	DCM/DMF (1:1)	25 °C	0.37[c]
2[d]	100:30	DCM/DMF (1:1)	25 °C	0.04
3[e]	100:30	DCM/DMF (1:1)	25 °C	0.16
4[f]	100:30	DCM/DMF (1:1)	25 °C	0.24
5	50:15	DCM/DMF (1:1)	25 °C	0.43
6	100:60	DCM/DMF (1:1)	25 °C	0.29
7	200:60	DCM/DMF (1:1)	25 °C	0.31
8	100:30	DCM	25 °C	0.31
9	100:30	DMF	25 °C	0.56
10[g]	100:30	DMF	37 °C	0.56
11[g]	100:30	DMF	60 °C	0.60
12	50:30	DCM/DMF (1:1)	25 °C	0.34
13	100:90	DCM/DMF (1:1)	25 °C	0.31
14	100:60	DCM	25 °C	0.29

[a] The reactions were carried out for 3 days, unless otherwise stated. − [b] See footnote (a) in Table 1. − [c] This experiment was carried in triplicate with values of Thr:AA of 0.37, 0.37, and 0.38, respectively. − [d] The reaction was carried out for 2 weeks. − [e] The reaction was carried out for 6 days with a single addition of reagents. − [f] The reaction was carried out for 6 days, but every day the resin was filtered off and washed with DMF before adding fresh EDC and CuCl. − [g] Due to the low boiling point of DCM, experiments at higher temperature were carried out in DMF.

both cases, a major single peak that co-eluted with the one corresponding to the peptide without the tosyl group.[20] Although the AAA of Thr in the experiment corresponding to entry 6 in Table 1 (Mitsunobu conditions)[21] gave a low number indicating the disappearance of Thr, and the ES-MS showed a small signal corresponding to the desired peptide, the HPLC showed multiple peaks. Finally, 3-(3′-dimethylaminopropyl)-1-ethylcarbodiimide hydrochloride (EDC),[22] which was used for dehydration in solution of a residue of Thr in a fragment of Nisin,[23] and more recently for the preparation of a lantibiotic precursor containing a ΔAla residue,[24] showed a decrease of the value of Thr, a clean reaction by HPLC, and the correct mass in the ES-MS.[25]

With these results in mind, we performed a complete study using EDC and taking as a substrate the sequence corresponding to peptide **2**, where the residue of Thr is next to the hindered Val residue.

The first experiment (entry 1, Table 2) confirmed the results outlined in Table 1 (entry 7). If the same reaction is left for 2 weeks (entry 2) the HPLC shows exclusively the DDP. Experiments corresponding to entries 3 and 4 differ in that in entry 4 after each day (total 6 days) the resin was filtered off and washed with DMF before adding fresh EDC and CuCl. This protocol slows down the reaction. In experiments 5−14 (Table 2), the amount of EDC/CuCl, solvent, and temperature are studied. The conclusions are: (i) a large excess of both reagents is necessary (entry 1 vs. entry 5); (ii) a larger amount of CuCl is more important than a larger amount of EDC (entry 6 vs. entries 1 and 7; and entry 12 vs. entry 5), although the amount of EDC should be clearly superior to that of CuCl (entry 13 vs. entry 6); (iii) DCM is a better solvent than DMF (entry 8 vs. entries 1 and 9); (iv) a higher temperature does not improve yields (entries 10 and 11 vs. entry 9); (v) the conditions of choice are 100 and 60 equiv. of EDC and CuCl, respectively, DCM/DMF or DCM as a solvent, and 25 °C (entries 6 and 14).[26] In all cases the ES-MS spectra showed that the residual Thr residue was left in the form of *O*-alkylisourea. In order both to accelerate and complete the reaction, several experiments similar to entry 6 were carried out in the presence of base (DIEA or DBU).[27] Both amino acid analysis and HPLC showed that the reaction after 3 days has taken place to the same extent as in the absence of base.

The experiment corresponding to entry 2 (Table 2) was repeated on a larger scale (32 μmol),[28] and the crude peptide, obtained by treatment of the peptide resin with TFA/H_2O (95:5), showed (Figure 1) essentially 100% conversion by HPLC. The NMR spectrum of the purified product by medium pressure liquid chromatography showed a single set of signals, confirming that a single isomer was formed. The configuration *Z* of the Dhb residue was assigned based on the chemical shift of the olefinic proton (δ = 6.64) when compared with data published in the literature,[29] and the presence of a NOESY signal between the H of the amide Phe-Dhb and the β-CH_3 of the Dhb. The dehydration reaction was repeated using Ac−Val−Phe−D-*allo*-Thr−Val−Amide-Linker-resin as a substrate and, again, a single product was obtained, which co-eluted in HPLC with the one formed from the Thr peptide and showed a similar NMR spectrum. This suggests that the β-elimination reaction does not follow a E2 mechanism, but rather a monomolecular E1 or $E1_{cb}$ mechanism with the formation of the thermodynamically most stable isomer.[30]

The same method was applied to L-Ser and D,L-phenylserine {PheSer, β-hydroxyphenylalanine [(β-OH)Phe]} containing tetrapeptides to give, with good purity, the Δ-Ala and *Z*-Δ-Phe tetrapeptides, respectively.[31,32] In the second case, a single product was again obtained, which was assigned the configuration *Z* on account of the UV spectra showing maxima at 276 nm and 273 nm with CH_3CN and EtOAc as solvent, respectively.[33] This result

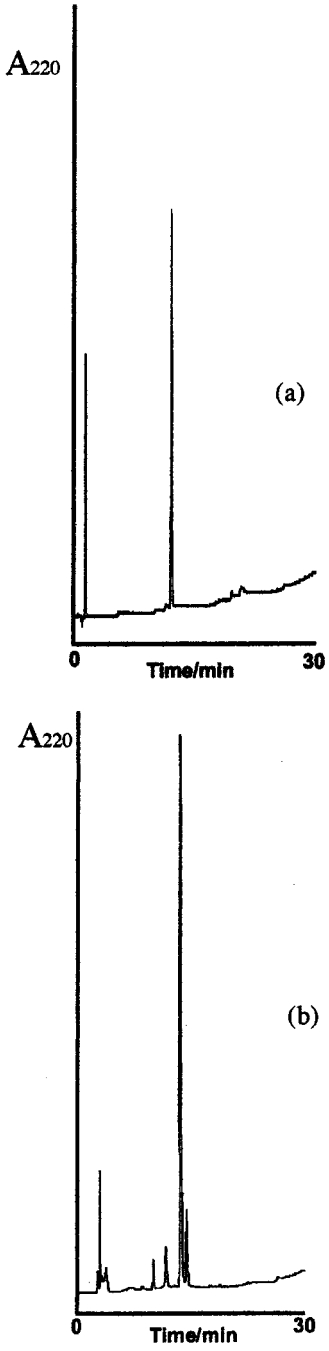

Figure 1. HPLC chromatogram of crude cleavage of Ac−Val−Phe−Thr−Val−NH_2 (a) and Ac−Val−Phe−(*Z*)-Dhb−Val−NH_2 (b); reverse-phase C-18 columns (Kromasil C_{18}, 4.6 × 250 mm, 10 μm, Akzo Nobel) were used for the analysis with elution by a linear gradient over 30 min of 0.036% TFA in ACN and 0.045% TFA in H_2O from 1:9 to 1:0, flow rate 1.0 mL/min

confirms those obtained with Thr derivatives, where only the thermodynamically more stable isomer is obtained.

Conclusions

A mild and convenient method for the solid-phase preparation of peptides containing α,β-didehydroamino acids has been described. The double bond is formed through a β-elimination reaction, using EDC as the activating reagent for the hydroxyl function in the presence of CuCl. This method is of general applicability to peptides containing common DDAA. This method has been applied successfully to the solid-phase synthesis of Kahalalide F, which is a marine cyclic depsipeptide, which contains thirteen amino acids including a (*Z*)-Dhb and 5-methylhexanoic acid at the *N*-terminus.

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[1] For reviews describing the occurrence of peptides containing these amino acids, see: [1a] E. Gross, *Adv. Exp. Med. Biol.* **1976**, *86B*, 131−153. − [1b] U. Schmidt, J. Häusler, E. Öhler, H. Poisel, *Fortschr. Chem. Org.* **1977**, *37*, 251−327. − [1c]K. Noda, Y. Shimohigashi, N. Izumiya, in *The Peptides* (Eds.: E. Gross, J. Meienhofer), Academic Press,: New York, **1983**, Vol. 5, pp. 285−339.

[2] G. Jung, *Angew. Chem. Int. Ed. Engl.* **1991**, *30*, 1051−1068.

[3] [3a] I. L. Givot, T. A. Smith, R. H. Abele, *J. Biol. Chem.* **1969**, *244*, 6341−6353. − [3b] H. R. Hanson, E. A. Havir, *Arch. Biochem. Biophys.* **1970**, *141*, 1−17.

[4] M. T. Hamann, P. J. Scheuer, *J. Am. Chem. Soc.* **1993**, *115*, 5825−5826.

[5] Y. Shimohigashi, M. L. English, C. H. Stammer, T. Costa, *Biochem. Biophys. Res. Commun.* **1982**, *104*, 583−590.

[6] R. Jain, V. S. Chauan, *Biopolymers (Peptide Science)* **1996**, *40*, 105−119.

[7] U. Schmidt, A. Lieberknecht, J. Wild. *Synthesis* **1988**, pp. 159−172.

[8] H. Ogura, O. Sato, K. Takeda, *Tetrahedron Lett.* **1981**, *22*, 4817−4818.

[9] P. M. T. Ferreira, H. L. S. Maia, L. S. Monteiro, *Tetrahedron Lett.* **1998**, *39*, 9575−9578.

[10] While the first method is compatible with both Fmoc and Boc protection and renders the succinimidyl ester of the protected DDAA, the latter is only compatible with Boc protection and gives the N^{α}-$(Boc)_2$-DDAA-O-*t*Bu.

[11] [11a] T. Moriya, N. Yoneda, M. Miyoshi, K. Matsumoto, *J. Org. Chem.* **1982**, *47*, 94−98. − [11b] C. Shin, Y. Yonezawa, T. Yamada, *Chem. Pharm. Bull.* **1984**, *32*, 3934−3944.

[12] The elongation in the N → C direction is also a challenging process. In our hands, the best results have been obtained when coupling reagents based in 1-hydroxy-azabenzotriazole (HOAt) are used. Under these conditions, protected peptides bearing a *C*-terminal DDAA have been quantitatively incorporated into peptide resins (unpublished results).

[13] J. P. Gamet, R. Jacquier, J. Verducci, *Tetrahedron* **1984**, *40*, 1995−2001.

[14] D. H. Rich, in *Specificity in Plant Diseases* (Eds.: R. K. S. Woods, A. Granti), Plenum, New York, **1976**, pp. 169−183.

[15] [15a] A. Polinsky, M. G. Cooney, A. Toy-Palmer, G. Ösapay, M. Goodman, *J. Med. Chem.* **1992**, *35*, 4185−4194. − [15b] C. Blettner, M. Bradley, *Tetrahedron Lett.* **1994**, *35*, 467−470. − [15c] M. Yamada, T. Miyajima, H. Horikawa, *Tetrahedron Lett.* **1998**, *39*, 289−292.

[16] Fmoc-*p*-[*R,S*]-*α*-[1-(9*H*-fluoren-9-yl)-methoxyformamido]-2,4-dimethoxybenzyl]-phenoxyacetamido-polyethylene glycol-polystyrene resin (Fmoc-AM-PEG-PS-resin, 0.19 mmol/g, PerSeptive Biosystems Inc.) was washed with DMF (5 × 0.5 min), and Fmoc removal was accomplished with piperidine/DMF (1:4, 1 × 10 min, 2 × 5 min), followed by washing with DMF (5 × 0.5 min). Fmoc-amino acids (5 equiv.) were activated with *N,N′*-diisoproylcarbodiimide (DIPCDI) (5 equiv.) in the presence of 1-hydroxybenzotriazole (HOBt) (5 equiv.) in DMF, and

the coupling carried out for 2 h at 25 °C. The β-hydroxyl of the Thr was unprotected. Final acetylation was carried out with Ac_2O/DCM (1:9, 10 min). In all cases, the ninhydrin test (E. Kaiser, R. L. Colescott, C. D. Bosinger, P. Cook, *Anal. Biochem.* **1970**, *34*, 595−598) carried out after the coupling was negative. After all acylations, the resin was washed with DCM (5 × 0.5 min).

[17] Although this method is probably not the most accurate for estimating the degree of conversion, it is the most convenient for these preliminary studies where a large number of samples need to be checked.

[18] [18a] S. P. Halasz, O. Glemser, *Chem. Ber.* **1970**, *103*, 594−602. − [18b] M. Hudlicky, *Org. Reactions* **1988**, *35*, 513−553.

[19] L. Somekh, A. Shanzer, *J. Org. Chem.* **1983**, *48*, 907−908.

[20] This method has been succesfully described for the dehydration in solution of a Thr-containing peptide: K. W. Li, J. Wu, W. Xing, J. A. Simon, *J. Am. Chem. Soc.* **1996**, *118*, 7237−7238.

[21] Mitsunobu conditions (O. Mitsunobu, *Synthesis* **1981**, pp. 1−28) have been applied to convert protected Thr to its corresponding *Z*-Dhb derivative in solution: H. Wojciechowska, R. Pawlowicz, R. Andruszkiewicz, J. Grzybowska, *Tetrahedron Lett.* **1978**, *50*, 4063−4064; R. J. Cherney, L. Wang, *J. Org. Chem.* **1996**, *61*, 2544−2546.

[22] Another soluble carbodiimide [1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate] has also been used for the dehydration in solution of peptides containing residues of Ala and Cys: M. J. Miller, *J. Org. Chem.* **1980**, *45*, 3131−3132.

[23] K. Fukase, M. Kitazawa, A. Sano, K. Shimbo, S. Horimoto, H. Fujita, A. Kubo, T. Wakamiya, A. Shibe, *Bull. Chem. Soc. Jpn.* **1992**, *65*, 2227−2240.

[24] S. Burrage, T. Raynham, G. Williams, J. W. Essex, C. Allen, M. Cardno, V. Swali, M. Bradley, *Chem. Eur. J.* **2000**, *6*, 1455−1466.

[25] All final peptides were characterized by ES-MS {488.3 $[M + H]^+$ for Ac−Val−Phe−*Z*−Dhb−Val−NH_2; 474.7 $[M + H]^+$ for Ac−Val−Phe−Δ-Ala−Val−NH_2; and 550.2 $[M + H]^+$ for Ac−Val−Phe−Δ-Phe−Val−NH_2] and NMR spectroscopy.

[26] The use of DCM/DMF is more convenient for avoiding evaporation of the solvent during the reaction.

[27] 2−50 equiv. of either DIEA or DBU were added at different stages of the reaction.

[28] The reaction was performed in a 10-mL polypropylene syringe fitted with a polyethylene disc and a septum under N_2 atmosphere. The peptide attached to the resin (208 mg, 32 μmol) was treated with EDC·HCl (606 mg, 3.16 mmol) and CuCl (94 mg, 0.95 mmol) in DCM/DMF (1:1, 5 mL). After 7 days of reaction, the resin was washed with DMF (5 × 10, 0.3 mL), 5% EDTA in methanol/H_2O (4:6) (5 × 10 min), DMF (5 × 10, 0.3 mL), and DCM (5 × 10, 0.3 mL).

[29] D. Srinivasan, R. W. Stephnson, R. K. Olsen, *J. Org. Chem.* **1977**, *42*, 2256−2259.

[30] This result is different from that obtained with DAST in solution, which is reported to behave stereospecifically; see ref. [19].

[31] As a model, the same tetrapeptide Ac−Val−Phe−Xxx−Val-Amide-Linker-resin was used, where Xxx was Ser or (β-OH)Phe.

[32] When Cys was used as residue Xxx, the Δ-Ala containing peptide was not obtained.

[33] Rich and Mathiaparanam have described that UV spectrum of peptides containing the *Z* isomer of Δ-Phe presents a maximum at 276 nm, while the maximum of the *E* isomer is at 282 nm: D. H. Rich, P. Mathiaparanam, *Tetrahedron Lett.* **1974**, *46*, 4037−4040.

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