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Cyclohexyloxycarbonyl Based Orthogonal Solid Phase Peptide Synthesis in Boc Chemistry

Gábor Mezö*, Nikolett Mihala, György Kóczán, Ferenc Hudecz

Research Group of Peptide Chemistry, Hungarian Academy of Sciences, Eötvös L. University, H-1518 Budapest 112, POB 32, Hungary

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Abstract: Application of *N*-cyclohexyloxycarbonyl (Choc) protection in Boc chemistry on solid phase provides a new possibility for the preparation of protected peptide fragments. A Choc/OcHex protection scheme allows also the assembly of cyclic lactam peptides linked to the resin through the C-terminus. Choc protection is stable under the 1M TMSOTf-thioanisole/TFA cleavage condition at 0°C, but it is removable by anhydrous HF. We have utilized cyclohexyloxycarbonyl as an orthogonal protecting group for the synthesis of a i) bicyclic epitope peptide of glycoprotein D of HSV 1 on BHA resin and ii) fully protected hexapeptide involved in protein transport on Merrifield resin.

INTRODUCTION

There is an increasing interest in the application of orthogonal protecting groups in solid phase peptide synthesis. Preparation of protected peptide fragments for convergent solid phase peptide synthesis as well as cyclization of resin bound peptides needs selectively removable new orthogonal protection schemes. A number of methods have been described for the synthesis of protected[2] or cyclic lactam[3] peptides on solid phase, but generally these procedures require special resins or combination of special protecting groups that result in rather expensive syntheses. For the most common cyclization with Boc[1] strategy, OFm ester protection is used with OBzl or OcHex esters as orthogonal blocking groups.[4] However, during the removal of fluorenylmethyl group by 20% piperidine/DMF from Asp we have observed the formation of an Asp-piperidide adduct.[5]

We found earlier that ω–cyclohexyl esters of aspartic acid and glutamic acid are stable at 0°C in 1M TMSOTf-thioanisole/TFA mixture introduced by Yajima et al.[6] This observation was utilized for the synthesis of partially protected peptide fragments of glycoprotein D from Herpes simplex virus (H-SALLE(OcHex)NPVG-NH_2 and Fmoc-SALLQD(OcHex)PVG-OH). These semiprotected peptides were used succesfully for fragment condensation in solution as well as on solid phase.[7] We have also realized that in 1M TMSOTf-thioanisole/TFA mixture the cleavage of peptide-BHA resin bond did not occur. Based on this finding we have introduced a new cyclization method for peptides linked to the resin through their C-terminus using benzyl and cyclohexyl to form ester of the side chain carboxyl groups.[5] In this experiment we applied an Fmoc/ClZ protecting scheme for N^{α}- and N^{ε}-amino groups. Prior to the HF-cleavage, the Fmoc protecting group has to be removed. In order to avoid this step, Fmoc was replaced by a cyclohexyloxycarbonyl (Choc) group that is stable under the 1M TMSOTf-thioanisol/TFA cleavage condition but removable with anhydrous HF.

The Choc group was first described by McKay and Albertson[8] and used as acid labile N^{α}-amino protection for Gly, DL-Ala, DL-Phe and DL-Met. Comparison with other acid sensitive amino protecting groups (Boc, Z, methoxybenzyloxycarbonyl, cyclopentyloxycarbonyl) indicated no advantages under the studied conditions (HBr in nitromethane or in acetic acid). Therefore the utilisation of cyclohexyloxycarbonyl group has been discontinued. Recently the use of Choc group has been proposed as N^{in}-protecting moiety for the synthesis of Trp containing peptides by the Boc-strategy.[9]

* mezo@para.chem.elte.hu

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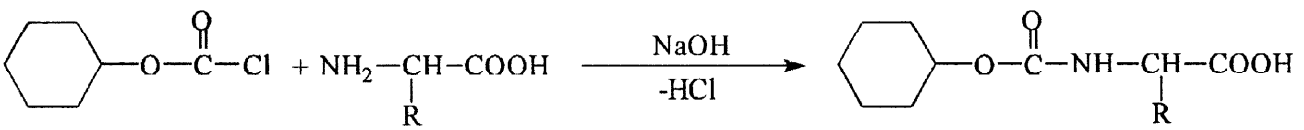
We report here the improved synthesis of cyclohexyloxycarbonyl chloride, and of *N*-Choc-amino acid derivatives. Choc-based orthogonal solid phase strategy has been used for the preparation of a bicyclo epitope peptide of glycoprotein D of HSV 1 on BHA resin and of a fully protected hexapeptide involved in protein transport on Merrifield resin.

RESULTS AND DISCUSSION

Synthesis of Cyclohexyloxycarbonyl Amino Acid Derivatives

Cyclohexyloxycarbonyl chloride (**1**) prepared from cyclohexanol and phosgene[10] reacts with α- or ε-amino group of free or semiprotected amino acids (**2**) in alkaline solution as depicted in Scheme 1.



1. **2.** **3.**

Scheme 1. Synthesis of Choc-amino acid derivatives

Yield and characteristics are presented in Table 1. Some of the cyclohexyloxycarbonyl amino acid derivatives (**3**) like Choc-Leu, Choc-Pro, Choc-Val, Choc-Met and Choc-Thr have low melting point value, and can be best crystallized as DCHA salt. The Boc-Lys(Choc)-OH was prepared from the Cu-complex of lysine that was treated with Choc-Cl in dioxane-2M NaOH mixture adopting the synthesis condition of Lys(Z).[11] The ε–cyclohexyloxycarbonyl lysine was first liberated from its copper complex by treatment with thioacetamide[12] then the Boc protecting group was introduced to the α-amino group of the purified lysine derivative using $(Boc)_2O$.[13]

The N^{α}- and/or N^{ε}-cyclohexyloxycarbonyl amino acids prepared were characterized by melting point, retention factor of tlc, optical rotation and elemental composition (Table 1). The purity of the compounds was greater than 99% based on the RP-HPLC chromatogram (λ=220nm).

Optical Purity of Choc-Amino Acids

The optical purity of amino acid derivatives was demonstrated on four samples. Choc-Ala-OH, Choc-D-Ala-OH, Choc-Ser-OH and Choc-D-Ser-OH were used for this study. Amino acid derivatives were hydrolyzed in 6M HCl at 110°C for 24h, like in the case of amino acid analysis. After evaporation and neutralization, the samples were derivatized by Marfey's reagent[14,15] and run on RP-HPLC using a Hypersyl ODS C_{18} column (150x4.6 mm). Under isocratic conditions with 0.1M sodium acetate buffer (pH4)-methanol-acetonitrile 82:6:12 (v/v/v) as eluent, a single peak corresponding to the amino acid derivative was detected (Figure 1.). These results suggest that the Choc-amino acids were produced with high optical purity.

Racemization-free Coupling of Choc-Amino Acid Derivatives

In order to study racemization of Choc-amino acids during coupling in the SPPS, Choc-Ala-OH was attached to Ser(Bzl)-PAM resin and Choc-Ser(Bzl)-OH was coupled to Leu-Merrifield resin by DCC/HOBt method. The protected dipeptide-resins were hydrolyzed as described above and the samples were derivatized with Marfey's reagent. The results of RP-HPLC analysis performed under optimized isocratic conditions[16] [0.1M sodium acetate buffer-methanol-acetonitrile 82:12:6 (v/v/v)] are summarized in Figure 2. The absence of D-Ala (at 65 min) (a) or D-Ser (at 17 min) (b) derivatives in the chromatograms suggests that there was no racemization of Choc-Ala-OH or Choc-Ser(Bzl)-OH during the coupling reaction studied.

Table 1. Characteristics of Cyclohexyloxycarbonyl-Amino Acid Derivatives

Amino acid derivatives	Mol. formula (Mw.)	Yield [%]	Mp [°C]	$[\alpha]_{468}$	tlc [Rf]	Elemental analysis calc.[%]; found[%] C	H	N
Choc-Ala-OH	$C_{10}H_{17}NO_4$ (215.25)	60	78-79	-26.8[a]	0.72[c]	55.80	7.96	6.51
					0.30[d]	55.69	7.92	6.45
Choc-D-Ala-OH	$C_{10}H_{17}NO_4$ (215.25)	58	80-81	+26.9[a]	0.72[c]	55.80	7.96	6.51
					0.30[d]	55.72	7.99	6.47
Choc-Asn-OH	$C_{11}H_{18}N_2O_5$ (258.27)	88	172-174	+4.5[b]	0.12[c]	51.12	7.03	10.85
					0.25[d]	51.19	6.97	10.81
Choc-Gly-OH[8]	$C_9H_{15}NO_4$ (201.22)	83	97-98		0.60[c]	53.72	7.51	6.96
					0.21[d]	53.76	7.46	6.87
Choc-Ile-OH · DCHA	$C_{25}H_{46}N_2O_4$ (438.66)	72	110-112	-0.7[b]	0.80[c]	68.45	10.57	6.39
					0.41[d]	68.16	10.41	6.39
Choc-Leu-OH · DCHA	$C_{25}H_{46}N_2O_4$ (438.66)	83	102-104	-14.8[a]	0.77[c]	68.45	10.57	6.39
					0.38[d]	68.42	10.57	6.60
Boc-Lys(*Choc*)-OH	$C_{18}H_{32}N_2O_6$ (372.47)	76	76-78	-7.4[a]	0.79[c]	58.04	8.66	7.52
					0.41[d]	57.96	8.60	7.47
Choc-Lys(*Choc*)-OH	$C_{20}H_{34}N_2O_6$ (398.52)	80	89-90	-7.6[b]	0.64[c]	60.28	8.60	7.03
					0.40[d]	60.19	8.62	7.00
H-Lys(*Choc*)-OH	$C_{13}H_{24}N_2O_4$ (272.35)	56	171-173	+20.6[a]	0.54[e]	57.33	8.88	10.28
					0.59[f]	57.27	8.81	10.24
Choc-Met-OH · DCHA	$C_{12}H_{21}NO_4S$ (265.37)	74	119-120	-13.4[a]	0.69[c]	52.34	7.68	5.08
					0.31[d]	52.38	7.79	5.04
Choc-Phe-OH	$C_{16}H_{21}NO_4$ (291.35)	40	68-70	-2.8[b]	0.74[c]	65.91	7.27	4.81
					0.34[d]	65.60	7.23	4.82
Choc-Pro-OH · DCHA	$C_{24}H_{42}N_2O_4$ (422.62)	62	151-152	-35.9[a]	0.59[c]	68.21	10.02	6.63
					0.31[d]	68.21	10.10	6.63
Choc-Ser-OH	$C_{10}H_{17}NO_5$ (231.66)	64	78-80	+1.7[b]	0.19[c]	51.85	7.40	6.05
					0.25[d]	51.73	7.36	6.06
Choc-Ser(Bzl)-OH	$C_{17}H_{23}NO_5$ (321.39)	90	76-78	+7.3[b]	0.75[c]	63.53	7.21	4.36
					0.37[d]	63.54	7.23	4.21
Choc-D-Ser-OH	$C_{10}H_{17}NO_5$ (231.66)	68	79-81	-1.8[b]	0.19[c]	51.85	7.40	6.05
					0.25[d]	51.70	7.44	5.98
Choc-Thr-OH · DCHA	$C_{23}H_{42}N_2O_5$ (426.60)	72	146-147	-4.5[b]	0.26[c]	64.76	9.92	6.57
					0.28[d]	64.78	10.17	6.61
Choc-Trp-OH · DCHA	$C_{18}H_{22}N_2O_4$ (330.38)	81	69-71	-8.0[b]	0.64[c]	65.44	6.71	8.48
					0.40[d]	65.16	6.74	8.17
Choc-Val-OH · DCHA	$C_{24}H_{44}N_2O_4$ (424.63)	56	134-136	-7.4[a]	0.75[c]	67.88	10.44	6.60
					0.34[d]	67.42	10.45	6.68

[a]c=0.5 in acetic acid; [b]c=2 in acetic acid

[c-f]tlc eluents: ethyl acetate—pyridine—acetic acid—water = 333:20:6:11 (v/v/v/v) (c); *n*-butanol—ammonium hydroxide (25%) = 7:3 (v/v) (d); *n*-butanol—acetic acid—water = 4:1:1 (v/v/v) (e); chloroform—methanol = 1:9 (v/v) (f).

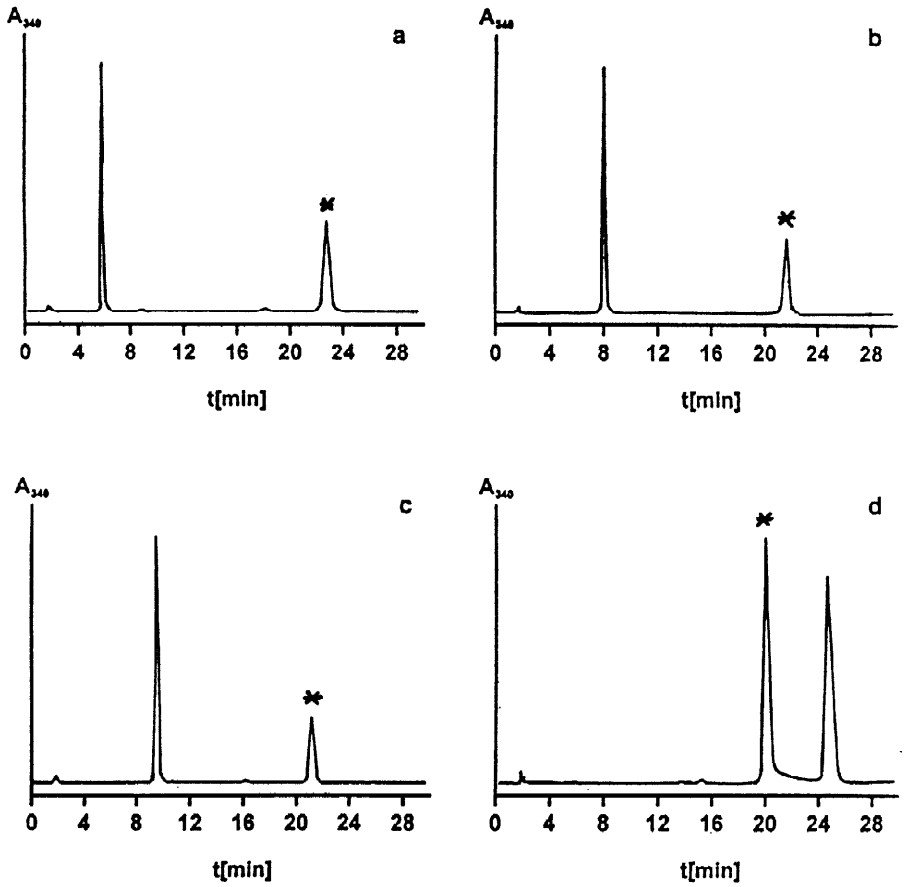


Figure 1. HPLC profile of derivatized Choc-amino acids with Marfey's reagent. (a)Choc-L-Ser-OH; (b) Choc-D-Ser-OH; (c) Choc-L-Ala-OH; (d) Choc-D-Ala-OH (*Hydrolyzed reagent).

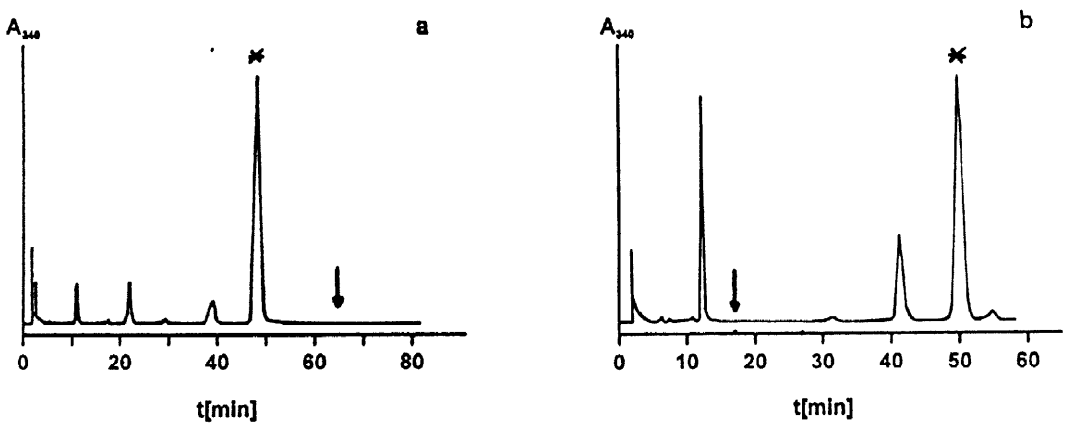


Figure 2. HPLC profile of hydrolyzed and derivatized Choc-dipeptides from (a)Choc-Ala-Ser(Bzl)-PAM and (b) Choc-Ser(Bzl)-Leu-Merrifield resin (*Hydrolyzed reagent).

Stability of Cyclohexyloxycarbonyl Group in Choc-Amino Acid Attached to BHA Resin

Choc-Ala-OH and Choc-Lys(Choc)-OH were coupled to benzhydryl amine resin by the DCC/HOBt method using three-fold molar excess of compounds calculated for the capacity of the resin. After this reaction all amino groups were blocked as demonstrated by the quantitative ninhydrin assay[17]. The Choc-amino acid attached to the resin was first treated with 40% TFA in DCM at RT for 3 h. After this period of time no free amino group was detected and there was no change in nitrogen content of the samples investigated (Table 2). This indicates the lack of fission of the amide bonds. In the next experiment 0.5M or 1M TMSOTf-thioanisole in TFA was added at different concentrations to the Choc-amino acid-resins in the presence of *m*-cresol at 0°C. The reaction was proceeded for 30 or 60 min. The resin was filtered out and neutralized with 10% DIEA in DCM. Prior to the quantitative ninhydrin assay the samples were washed several times with DCM and methanol. Data summarized in Table 2. show no significant changes in the N content of the samples before or after the treatment with TMSOTf. This suggests that no significant split occurs under these conditions between the resin and the Choc-amino acid residue. The cleavage between the Choc group and the amino acid was also investigated. The results of the quantitative ninhydrin assay indicate the presence of a small amount of free amino acid-resin after 30 min of treatment, which represents only 0.23% of the resin capacity. This figure increases by time and TMSOTf concentration, but even after 60 min it is 0.51% in the case of Choc-Ala-BHA sample. These data suggest that the CO-NH bond between the Choc group and the amino acid residue is quite stable.

Table 2. Stability of Choc Protecting Group and Amino Acid-Benzhydrylamine Resin Bond Under Different Cleavage Conditions

		Conditions							
		40% TFA/DCM 3h		0.5M TMSOTf 0.5h		1.0M TMSOTf 0.5h		1.0M TMSOTf 1h	
	N, calc. [%]	NH_2 [μmol/g][b]	N^a [%]	NH_2 [μmol/g][b]	N^a [%]	NH_2 [μmol/g][b]	N^a [%]	NH_2 [μmol/g][b]	N^a [%]
Choc-Ala-BHA	2.05	0	1.92	1.98	2.00	2.56	1.93	4.36	1.83
Choc-Lys(Choc)-BHA	2.79	0	2.70	n.t.	n.t.	n.t.	n.t.	10.2	2.65

[a]As determined by the nitrogen content according to Kjeldahl

[b]As determined by quantitative ninhydrin assay[17]

Synthesis of Cyclic Lactam HSV Epitope Peptides

The nonapeptide SALLEDPVG (276-284) from glycoprotein D of Herpes simplex virus was found to function as epitope that can induce antibody production with virus neutralizing capacity.[18] Cyclic variants of the epitope were designed and prepared for stabilization of the secondary structure. For this reason, lysine residue was incorporated at the C-terminal of the peptide (SALLEDPVGK) and its ε-amino group was utilized for lactam bridge formation with the γ-carboxyl group of glutamic acid at position 280 (Scheme 2). The peptide chain was built up on benzhydryl amine resin by Boc-chemistry considering that the peptide—BHA-resin bond in TMSOTf containing cleavage mixture is stable. Protecting groups from Lys(ClZ) and Glu(OBzl) were removed with 1M TMSOTf-thioanisole/TFA mixture at 0°C during 30 min. After the neutralization with DIEA the cyclization was carried out on the solid support by PyBOP/DIEA. Since the β-COOH of aspartic acid was blocked as cyclohexyl ester and the N-terminus with cyclohexyloxycarbonyl group, these moieties remained intact under the cleavage conditions used. The monocyclic lactam peptide was cleaved from the resin simultaneously with the removal of the cyclohexyl type protecting groups by anhydrous HF.

The bicyclo(1-6, 5-10)SALLEDPVGK peptide was prepared by formation of the second lactam bridge between the N-terminal amino group and the β-carboxyl group of Asp (Scheme 2). This was carried out in DMF with PyBOP reagent in the presence of DIEA. Both the monocyclo(5-10)SALLEDPVGK and bicyclic lactam

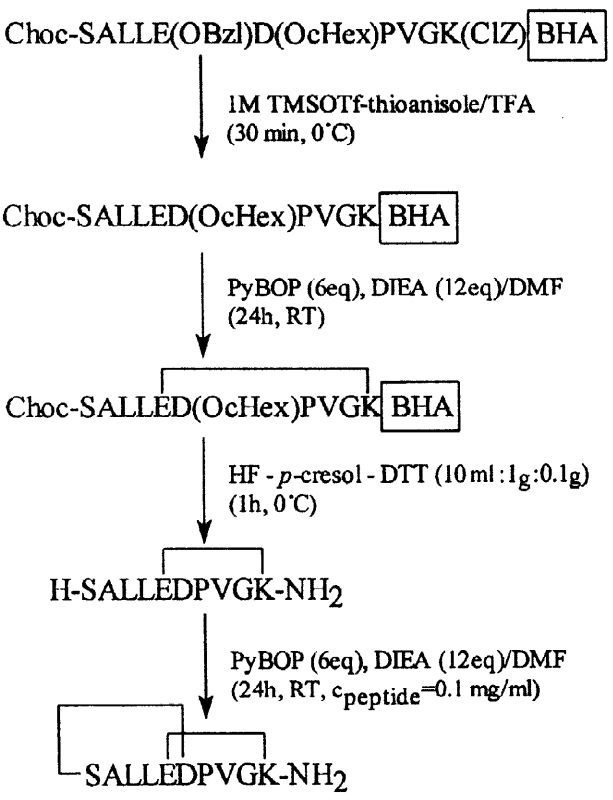
peptides were characterized by amino acid analysis, RP-HPLC and FAB-MS. The HPLC chromatograms of the starting monocyclic peptide and the resulting crude mixture of the bicyclo variant are demonstrated on Figure 3.

These results demonstrate the feasibility of the application of cyclohexyloxycarbonyl group as orthogonal protecting group in Boc strategy.

Synthesis of Fully Protected AVKDEL Signal Peptide

The KDEL sequence in various proteins plays an important role in intramolecular transport processes. Among others, this motif is responsible for retention of heatshock proteins in the endoplasmatic reticulum lumen.[19]

For biological investigation our aim was to synthesise a hexapeptide that represents the N-terminally elongated form of KDEL. The presence of two amino- and three carboxyl groups in this peptide prevents the unambiguous conjugation of the unprotected molecule to carriers to be utilized for further studies. Therefore the synthesis of fully protected derivative of AVKDEL-OH is quite a challenging task. To fulfil this, we have applied the cyclohexyl type protecting groups on Merrifield resin using Boc chemistry. The ω-carboxyl groups of Asp and Glu were protected as cyclohexyl ester, while the ε-amino group of Lys and the α-amino group of Ala were blocked by Choc group. The peptide was built up by the DCC/HOBt coupling method. The fully protected peptide was detached from the resin with 1M TMSOTf-thioanisole/TFA cleavage mixture at 0°C for 1h. The poorly soluble compound was purified by recrystallization and it was characterized by FAB-MS, elemental analysis, melting point determination and thin-layer chromatography.



Scheme 2. Synthesis of bicyclo(1-6, 5-10) SALLEDPVG-NH_2 peptide

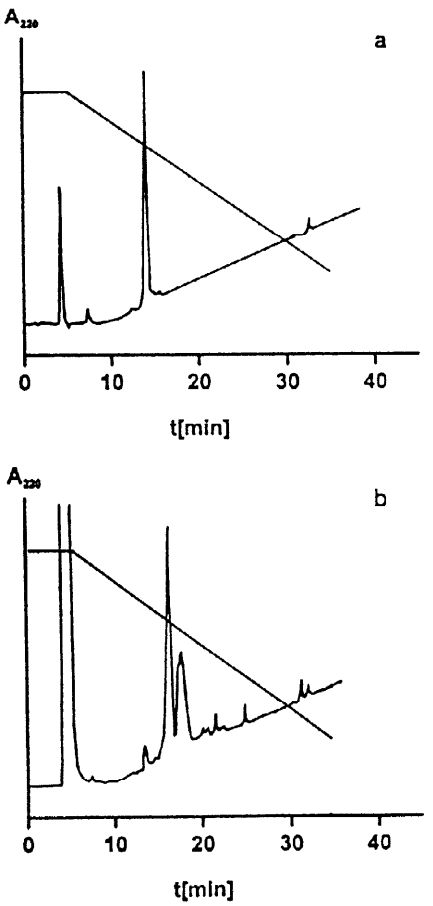


Figure 3. Analytical HPLC chromatograms of (a) purified monocyclo(5-10) and (b) crude bicyclo(1-6, 5-10) SALLEDPVG-NH_2

CONCLUSION

We have demonstrated that Choc-amino acids can be synthesized using Choc-Cl with high yield and optical purity. The racemization free coupling of Choc-amino acids in solid phase synthesis was also verified. Choc group at both on N^{α}- and N^{ε}-amino positions was stable in 1M TMSOTf-thioanisole/TFA at 0°C for 1h. Based on these findings a new synthesis strategy was developed in which Choc group as an orthogonal protecting group with Bzl or ClZ protecting groups was applied. The usefulness of this scheme was documented by the synthesis of bicyclo lactam peptide of HSV glycoprotein D epitope on benzhydrylamine resin. The Choc/OcHex protection scheme also enabled the preparation of fully protected hexapeptide with multiple amino- and carboxyl groups. The Choc-Ala-Val-Lys(Choc)-Asp(OcHex)-Glu(OcHex)-Leu-OH hexapeptide derivative was prepared in good yield.

EXPERIMENTAL

Amino acids were purchased from Reanal (Budapest, Hungary). Cyclohexanol was Aldrich (Budapest, Hungary) product, while phosgene was obtained from Merck (Darmstadt, Germany).

For the peptide synthesis Boc-amino acid derivatives were purchased from Chem-Impex International (Wood Dale, IL, USA), while TFA, TMSOTf, DIEA, DCC, HOBt, PyBOP, HF, thioanisole, *m*- and *p*-cresol were from Fluka (Buchs, Switzerland). BHA and Boc-Leu-Merrifield resins were from Bachem (Bubendorf, Switzerland). Solvents used for syntheses were Chemolab (Budapest,Hungary) or Reanal products, while HPLC grade acetonitrile was purchased from Romil Ltd (Cambrige, UK).

Amino acid derivatives and the fully protected peptide were checked for purity by elemental analysis (Heraeus CHN-O Rapid, Heraeus Gmbh., Germany).

Melting points were determined by VED (GDR) NAGEMA-type hot-stage apparatus. Optical rotation was measured on Polamat A polarimeter (Carl Zeiss Jena, GDR).

The amino acid composition of peptides was determined by amino acid analysis using a Beckman System 6300 (Fullerton, CA, USA) analyser. Prior to the analysis samples were hydrolyzed in 6M HCl in sealed and evacuated tubes at 110 °C for 24h.

Analytical HPLC was performed using Delta Pak C_{18} (3.9x300 mm, 15μm, 300Å) reverse phase column on Knauer apparatus (Knauer, Bad Homburg, Germany). The instrument consisted of two pumps, a Variable Wavelength Monitor, which were functionalized by HPLC Software/Hardware Package Version 2.21 A. Eluent A was 0.1% TFA in d.i. water and eluent B was 0.1% TFA in AcN-water (80:20 v/v). Linear gradient of eluents (0 min 15%B; 5 min 15%B; 35 min 75%B) with 2 ml/min flow rate was used as the mobile phase. Detection was carried out at λ=220nm.

Purification of cyclopeptides was carried out on a Phenomenex, Jupiter C_{18} (10x250 mm, 10μm, 300Å) semipreparative column using the same eluents and gradients with 4 ml/min flow rate.

Fast atom bombardment mass spectometry (FAB-MS) was carried out on a Fisons (UK) VG-ZA-2SEQ tandem mass spectrometer equipped with a Cs^+ ion gun (30 keV). The peptide samples were dissolved in DMSO and mixed with glycerol matrix.

Modified Preparation of Cyclohexyloxycarbonyl Chloride. 1 litre round bottom flask equipped with a gas inlet tube, pressure-equalising dropping funnel and a dry-ice-acetone cooled jacketed Vigreaux condenser was placed in an ice-bath. The exit tube of the condenser was connected to a $CaCl_2$ drying tube and suitable traps for phosgene and HCl. 99g (1 mol) phosgene was washed with linseed oil, concentrated H_2SO_4 and was condensed into the reaction vessel. While stirring, 95 ml (90g, 0.9 mol) cyclohexanol was added from the dropping funnel at such a rate that the mixture was kept in gentle reflux for approx. 2 h. Stirring was continued for one additional hour. The condenser was removed and dry N_2 was bubbled through the mixture for 6 h to drive off the HCl and the excess phosgene. The escaping fumes were treated properly. The resulting oil was used without further purification and stored at 4°C. The Choc-Cl content of the product was determined as follows: a 0.1 ml aliquot

of Choc-Cl is pipetted into 10 ml of 10% NH_3 solution. Cyclohexyl-urethane crystals were filtered off, dried and analysed (Mw:129.23, Mp:111.3-112.3°C[20]).

Yield: 134.5g (92%) Purity: 99% (determined with HPLC analysis of the corresponding urethane) Bp_{2mm}:38-44°C[21], n_D^{20}:1.4587[21], d_{25}:1.122[21].

General Procedure for the Synthesis of Cyclohexyloxycarbonyl Amino Acid Derivatives. A solution of free amino acid or the copper complex of Lys (100 mmol) in 2M NaOH (50 ml) was cooled in an ice-water bath. Under vigorous stirring 110 mmol (17 g, 15.2 ml) cyclohexyloxycarbonyl chloride and 2M NaOH (55 ml) were added dropwise to the reaction mixture (10 min). Then the solution was allowed to warm up to room temperature and it was stirred for 3h. The alkaline solution was extracted four times with ether (30 ml each) and the aqueous layer was acidified to pH 2-3 with 5M HCl under cooling. The oil separated was extracted with ethyl acetate and the organic phase was dried over $MgSO_4$. The solvent was evaporated *in vacuo* and the endproduct was precipitated by *n*-hexane or isolated as DCHA salt after addition of equivalent amount of dicyclohexylamine.

Stability of Cyclohexyloxycarbonyl Group in Choc-Amino Acid Attached to BHA Resin. Three equivalent (2.58 mmol) of Choc-Ala-OH or Choc-Lys(Choc)-OH were used for coupling to 100 mg benzhydryl amine resin (0.86 mmol/g capacity) by the DCC/HOBt method. 20 mg Choc-Ala- or Choc-Lys(Choc)-resin was treated with *a)* 2 ml of 40% TFA in DCM at RT for 3 h, *b)* 2 ml of 1M TMSOTf-thioanisole/TFA mixture in the presence of 40 µl *m*-cresol for 60 min or for *c)* 30 min.

The resin samples were filtered out, neutralized with 10% DIEA in DCM and prior to the quantitative ninhydrin assay they were washed several times with DCM and methanol. The standard quantitative ninhydrine assay was used for the determination of free amino groups.[17]

Study of the Optical Purity and Racemization-free Coupling of Choc-Amino Acid Derivatives. 100mg of Boc-Leu-Merrifield resin (0.71 mmol/g) and 100mg of Boc-Ser(Bzl)-PAM resin were deprotected with 35% TFA/DCM solution (1+20 min) then washed with DCM (5x0.5 min) and neutralized with 10% DIEA/DCM (3x1 min). After washing the resins with DCM (4x0.5 min), 3 eq of Choc-Ser(Bzl)-OH was added to the Leu containing resin and 3 eq of Choc-Ala-OH to the Ser-PAM resin in the presence of 3 eq of DCC and HOBt calculated for the resin capacity in each cases. The coupling was performed in DCM-DMF 4:1 (v/v) mixture. The reaction was continued for 1h, then the resin was washed with DMF and DCM twice for 0.5 min, respectively.

The dried resins were hydrolyzed in 6M HCl in sealed and evacuated tubes at 110°C for 24h. Derivatization of amino acids from acid-free hydrolyzates was performed with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amid (Pierce, Rockford, IL, USA) (Marfey's reagent)[14]. The hydrolyzates prepared from 2.5 µmol of peptide were dissolved in 100 µl of 0.5M sodium bicarbonate solution and mixed with 200 µl of a 1% Marfey's reagent in aceton. After incubation at 40°C for 90 min the cold mixture was neutralized with 25 µl of 2M HCl, and diluted with 20 fold methanol. 20 µl of samples were injected into the HPLC.

Similar procedure was used for the analysis of the optical purity of Choc-amino acids (Choc-Ser-OH, Choc-D-Ser-OH, Choc-Ala-OH, Choc-D-Ala-OH).

Synthesis of Bicyclic Lactam Peptide of HSV gD-1 Epitope. The linear ALLEDPVGK peptide was built up on BHA resin (0.86 mmol/g). First 0.5 mmol Boc-Lys(ClZ)-OH was attached to 1 g resin by DCC/HOBt coupling method. The remaining free amino groups of the resin were blocked by acetylation using 5 equivalent acetic anhydride (calculated for the resin capacity) in the presence of DIEA. The capacity of the resin used for further steps was 0.28 mmol/g. One mmol amino acid derivatives were utilized for the couplings using DCC/HOBt in DCM-DMF 4:1 (v/v). The β-carboxyl group of aspartic acid was blocked by cyclohexyl ester while the side chain of glutamic acid was protected by benzyl ester. Boc group was removed with 35% TFA in

DCM and 10% DIEA in DCM was used for neutralization. The N-terminal Choc-Ser-OH was attached to the peptide chain with one eq PyBOP in the presence of 2 equiv. DIEA in DMF.

ClZ and benzyl groups were removed from the peptide-resin by the treatment with 10 ml 1M TMSOTf-thioanisole/TFA mixture containing 200µl *m*-cresol as scavanger at 0°C for 30 min. Then the resin was filtered out, it was washed twice with TFA, then five times with DCM. The semiprotected peptide bound resin was treated with 10% DIEA/DCM (3x1 min) followed by washing with DCM several times. Lactam bridge formation between the γ-carboxyl group of glutamic acid and the ε-amino group of the C-terminal lysine residue was carried out by the aid of 6 eq PyBOP in presence of 12 eq DIEA in DMF. The reaction was proceeded for 6h at RT. The cyclization process was monitored by ninhydrin assay. The monocyclic peptide was cleaved from the resin with anhydrous HF at 0°C for 90 min. After evaporation of HF the crude product was precipitated with dry ether, washed several times with ether, then dissolved in diluted acetic acid. The resin was filtered out and the product was isolated by liophylization. The crude preparation was purified by HPLC using semipreparative column. $C_{45}H_{76}N_{12}O_{14}$; Yield: 34 mg (12.5%); FAB-MS *m/z* 1009.3$[M+1]^+$; calculated 1009.6; Rt (HPLC): 15.1 min; amino acid analysis; found(calculated): Asp 1.04 (1), Ser 0.95 (1), Glu 1.07 (1), Pro 1.11 (1), Gly 0.96 (1), Ala 1.04 (1), Val 0.96 (1), Leu 1.93 (2), Lys 0.99 (1).

15 mg (14.9 µmol) monocyclic product was dissolved in 150 ml DMF and 6 eq PyBOP and 12 eq DIEA were added to the solution. The cyclization reaction continued at RT overnight. The solution was concentrated *in vacuo* and the remaining oil was diluted to 2 ml with eluent A and B (1:1, v/v) and purified on semipreparative HPLC column. $C_{45}H_{74}N_{12}O_{13}$; Yield: 3.8 mg (25.7%); FAB-MS *m/z* 991.4 $[M+1]^+$; calculated 991.5; Rt (HPLC): 16.3 min; amino acid analysis; found(calculated): Asp 1.03 (1), Ser 0.95 (1), Glu 1.06 (1), Pro 1.07 (1), Gly 0.97 (1), Ala 1.00 (1), Val 0.96 (1), Leu 1.93 (2), Lys 0.98 (1).

Synthesis of Choc-Ala-Val-Lys(Choc)-Asp(OcHex)-Glu(OcHex)-Leu-OH. The protected peptide was built up on Boc-Leu-Merrifield resin (500 mg, 0.71 mmol/g) by Boc strategy. Side chain carboxyl groups of Asp and Glu were protected as cyclohexyl esters while the amino groups (ε-amino group of Lys and α-amino group of Ala) were blocked by cyclohexyloxycarbonyl group. The Boc groups were removed with 35% TFA/DCM (1+20 min) followed by washing with DCM (5x0.5 min) and neutralization with 10% DIEA/DCM (3x1 min) of the resin. Three equivalent of protected amino acid derivatives, DCC and HOBt calculated to the capacity of solid support were applied for the coupling. In all cases a single coupling (1h) was effective according to the ninhydrin assay. From the dried resin (730 mg) the protected peptide was cleaved by 1M TMSOTf-thioanisole/TFA (1.94:1.20:6.89, v/v/v) mixture at 0°C for 1h. The resin was filtered out, washed with 5 ml cold TFA and twice with DCM. The collected cold filtrate was evaporated *in vacuo* under 10°C. 50 ml d.i. water was added to the remaining oil and the solution was adjusted to pH 6 with ammonia. The precipitated material was collected on a glass filter, washed several times with d.i. water and ether—*n*-hexan (1:2, v/v) mixture. The crude product was recrystallized from ethyl acetate—*n*-hexan. Yield: 280 mg (87.5%); M.p. 179-181°C; tlc.: 0.72 (ethyl acetate—pyridine—acetic acid—water = 333:20:6:11(v/v/v/v) on Merck precoated plate, DC-Alufolien Kieselgel 5553); FAB-MS *m/z* 1090.4 $[M+1]^+$, 1107.5 $[M+H_2O]$, 1112.5$[M+Na]^+$, 1128.5 $[M+K]^+$; $C_{55}H_{91}N_7O_{15}$ requires M 1089.7; elemental analysis(calculated for 3% inorganic salt content) found(calc.): C: 58.68%(58.77%), H: 8.19%(8.16%), N: 8.78%(8.72%).

REFERENCES AND NOTES

1. Abbreviations used in this paper for amino acids and for the designations of peptides follow the rules of the IUPAC-IUB Comission of Biochemical Nomenclature in *European J. Biochem.*, **1984**, *138*, 9-37 and *J. Biol. Chem.*, **1989**, *264*, 633-673. The following additional abbreviations are used: Boc, *tert*-butyl-oxycarbonyl; BHA, benzhydrylamine resin; Bzl, benzyl; cHex, cyclohexyl; Choc, cyclohexyloxycarbonyl; ClZ, 2-chloro-benzyloxycarbonyl; DCC, *N,N'*-dicyclohexyl-carbodiimide; DCM, dichloromethane; DIEA, *N,N'*-diisopropylethylamine; DMF, *N,N*-dimethylformamide; FAB-MS, fast atom bombardment mass spectrometry; Fm, 9-fluorenylmethyl; Fmoc, 9-fluorenylmethoxycarbonyl; HF, hydrogen-fluoride; HOBt,

1-hydroxy-benzotriazole; HSV, Herpes simplex virus; ODS, octadecyl silane; PAM, 4-oxymethylphenyl-acetamidomethyl; PyBOP, (benzotriazolyl)-*N*-oxypyrrolidinephosphonium hexafluorophosphate; RP-HPLC, reverse phase high-performance liquid chromatography; TFA, trifluoracetic acid; tlc., thin-layer chromatography; TMSOTf, trimethylsilyl trifluoromethanesulphonate; Z, benzyloxycarbonyl. Amino acid symbols denote L-configuration unless indicated otherwise.

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