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Abstract

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SYNTHESIS OF TETRAPEPTIDES WITHOUT ISOLATION OF INTERMEDIATE PEPTIDES

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Recently, disubstituted carbodiimides have found wide application for the synthesis of peptide and internucleotide bonds. The most widely used of these is dicyclohexylcarbodiimide. Along with this carbodiimide, which is insoluble in water, water-soluble carbodiimides have been synthesized ([1]). Solubility in water is ensured by the fact that one of the substituents contains a tertiary amino group or a quaternary ammonium group. One such compound, in particular, used in the present work proved to be cyclohexyl-β-[N-(N-methylmorpholinium)]ethylcarbodiimide *p*-toluenesulfonate:

$$\left[\mathrm{O} \left\langle \begin{matrix} \mathrm{CH_2-CH_2} \\ \mathrm{CH_2-CH_2} \end{matrix} \right\rangle \overset{\oplus}{\mathrm{N}}(\mathrm{CH_3}) - \mathrm{CH_2} - \mathrm{CH_2} - \mathrm{N} = \mathrm{C} = \mathrm{N} - \mathrm{C_6H_{11}} \right] [\mathrm{CH_3} - \langle\!\langle - \mathrm{SO_3^{\ominus}}]$$

Hereinafter, for brevity, this compound will be called CME-carbodiimide. With the aid of water-soluble carbodiimides, the synthesis of several peptides was carried out; the solubility of the carbodiimides in water was used to separate the disubstituted ureas formed from them from the water-insoluble peptides.

In our opinion, the synthesis of peptides in aqueous solutions with the aid of water-soluble carbodiimides has several advantages over existing methods of peptide synthesis. First, because in peptide synthesis one component has a protected amino group and the other a protected carboxyl group, the peptide obtained is the only electroneutral compound. Unreacted starting substances and by-products of the reaction are ions or readily form ions in the corresponding pH range. Therefore the peptide obtained can easily be freed from them with the aid of ion-exchange resins. Let us consider, as an example, the interaction of a formylamino acid with an amino-acid ester in the presence of CME-carbodiimide. In such a system, essentially three processes will occur:

$$\overset{\oplus}{\mathrm{R}} - \mathrm{N} = \mathrm{C} = \mathrm{N} - \mathrm{R'} + \mathrm{HCONHCHXCOOH} \rightarrow \overset{\oplus}{\mathrm{R}} - \mathrm{N} - \mathrm{CO} - \mathrm{NH} - \mathrm{R'}$$

$$\begin{array}{c} | \\ \mathrm{CO-CHXNHCHO} \end{array}$$

(formylaminoacyl-CME-urea),

$$\overset{\oplus}{\mathrm{R}}-\mathrm{N}=\mathrm{C}=\mathrm{N}-\mathrm{R}'+\mathrm{HCONHCHXCOOH}+\mathrm{NH_2CHX_1COOC_2H_5}\rightarrow$$

$$\rightarrow \mathrm{HCONHCHXCONHCHX_1COOC_2H_5}+\overset{\oplus}{\mathrm{R}}-\mathrm{NH}-\mathrm{CO}-\mathrm{NH}-\mathrm{R}'$$

$$\overset{\oplus}{\mathrm{R}}-\mathrm{N}=\mathrm{C}=\mathrm{N}-\mathrm{R}'+\mathrm{H_2O}\rightarrow\overset{\oplus}{\mathrm{R}}-\mathrm{NH}-\mathrm{CO}-\mathrm{NH}-\mathrm{R}'$$

where

$$\overset{\oplus}{\mathrm{R}}=\mathrm{O}\left\langle\begin{array}{l}\mathrm{CH_2-CH_2}\\ \mathrm{CH_2-CH_2}\end{array}\right\rangle\overset{\oplus}{\mathrm{N}}(\mathrm{CH_3})-\mathrm{CH_2}-\mathrm{CH_2}-,\qquad \mathrm{R}'=\text{cyclohexyl}$$

It is not difficult to see that at pH values close to neutral, all substances except the reaction product—the ester of formyldipeptide—formylamino acid (pK of the order of 3.5), amino acid ester (pK of the order of 8), CME-carbodiimide, CME-urea, and formylaminoacyl-CME-urea (the last three have a quaternary ammonium group in the radical $R^{\oplus}$) are either anions or cations. The situation will not change if, instead of formylamino acids, one starts from a formylpeptide, and instead of an amino acid ester—from a peptide ester.

Secondly, the reaction product is obtained in aqueous solution, where the ester group can be removed by alkaline hydrolysis and thus, without changing the solvent, a peptide ready for the addition of the next unit can be obtained. Consequently, the synthesis of long peptides can be carried out without isolating intermediate peptides.

In the present work, using the indicated features of water-soluble carbodiimides, we carried out the synthesis of two ethyl esters of tetrapeptides—formylglycylglycylglycylalanine and formylglycylglycylalanylleucine—without isolating intermediate di- and tripeptides. The work was carried out with CME-carbodiimide, since it is the most readily available water-soluble carbodiimide, and with formyl protection of amino groups, since the formyl group, unlike other protective groups used, hardly reduces the solubility of amino acids and peptides in water.

Experimental Part

CME-carbodiimide was obtained by the method of Shikhan and Hlavka from N-β-aminoethylmorpholine and cyclohexyl isothiocyanate through the corresponding thiourea ([1]).

Formylglycine was obtained by Fischer' s method ([2]).

Preliminary studies of the reaction of formylglycine and ethyl glycinate with CME-carbodiimide showed that the reaction gives a noticeable yield of the formyldipeptide ester only in the narrow pH range from 4 to 5.5. At the same time, the change in pH during the reaction showed that continuous alkalinization of the system occurs up to values of the order of 6.5, which makes further progress of the process impossible. Therefore, we began to carry out peptide syntheses in a pH-meter cell, continuously titrating the reaction mixture with $8\,N$ H_2SO_4 from a micropipette to the optimum pH value of 4.9. It proved that, when the reaction was carried out at room temperature and at initial concentrations of formylglycine and ethyl glycinate of 0.1 g-mol/l, the yield of the ethyl ester of formyldipeptide with a stoichiometric amount of CME-carbodiimide was 5.3% without titration and 18.2% with titration. In addition, by carrying out the reaction at constant pH, after the first portion of CME-carbodiimide had been consumed we could add a new portion and thus double the yield based on amino acid.

Synthesis of ethyl formylglycylglycylglycylalaninate. 2 mmoles of formylglycine (0.206 g) were dissolved in 20 ml of $0.1\,N$ NaOH. To the solution were added 2 mmoles (0.279 g) of ethyl glycinate hydrochloride, and the pH of the solution was adjusted to 4.9. CDI was added in 2 portions of 2 mmoles each (a total of 1.69 g) at an interval of 20 min. The pH value was kept constant by titration with $8\,N$ H_2SO_4. The resulting solution was filtered and passed through a column containing 12 g of Dowex 50×2 in the H^+-form; the column was washed with 20 ml of water, and the eluate was neutralized with 12 g of Dowex 1×2 in the HCO_3^--form. The solution was lyophilically evaporated to a volume of 4 ml. Then 9 ml of $0.1\,N$ NaOH were added to it, and the excess alkali was titrated after 2 hours with $0.1\,N$ HCl. The titration required 0.5 ml of $0.1\,N$ HCl. Consequently, 8.5 ml of $0.1\,N$ NaOH were required for hydrolysis of the ester bond, i.e., the solution contained 0.85 mmole of formyldipeptide. To the solution (13.5 ml) were added 0.85 mmole of ethyl glycinate hydrochloride (122.6 mg). Then CME-carbodi-

imide in two portions of 0.85 mmole each (0.718 g total). The reaction mixture was subjected to the ion-exchange resin purification described above. The solution was lyophilically dried to a volume of 6 ml. To the solution was added 0.1 N NaOH to remove the ester group, calculated for a 50% yield of the resulting formyltripeptide ester (4.25 ml). Excess alkali was back-titrated with 0.1 N HCl. The titration required 0.75 ml. To the formyltripeptide solution (11 ml) was added 0.34 mmole of alanine ethyl ester hydrochloride (0.053 g). Then CMC-carbodiimide was added in two portions of 0.34 mmole each (0.294 g total). The

ion-exchange resin purification procedure described above and lyophilic drying of the eluate were repeated.

Yield 35 mg (33% based on the starting tripeptide).

Found, %: C 45.25; H 6.38; N 17.58
$C_{12}H_{20}N_4O_6$. Calculated, %: C 45.57; H 6.32; N 17.72

The substance has not been described in the literature.

Ethyl ester of formylglycylglycylalanylleucine was obtained analogously to the synthesis described above of ethyl formylglycylglycylglycylalaninate.

Yield: 30 mg (16% based on the starting tripeptide).

Found, %: C 51.68; H 7.12; N 14.91
$C_{16}H_{28}N_4O_6$. Calculated, %: C 51.61; H 7.52; N 15.05

The product was also subjected to hydrolysis at 100° with 6 N HCl for 16 hr. The hydrolysate was purified by sorption on Dowex 50×2 in the H^+ form, followed by elution with a strong ammonia solution. The eluate was chromatographed in the system n-butanol—acetic acid—water $(4 : 1 : 1)$. On the chromatogram, distinct spots of glycine, alanine, and leucine were detected.

As far as we know, the synthesis of tetrapeptides without isolation of intermediate di- and tripeptides has not previously been carried out. A method has been described in the literature for the synthesis of a tripeptide without isolation of a dipeptide ([3]). The method consists in the condensation of a carbobenzoxyamino acid and the p-nitrophenyl ester of an amino acid with the aid of dicyclohexylcarbodiimide, and the direct use of the resulting p-nitrophenyl ester of the carbobenzoxydipeptide for elongating the next unit. At this point, however, the procedure ends.

The method proposed by us can, in principle, be used for constructing peptides of any desired length. The yields obtained in the present work are considerably lower than in the classical methods for obtaining peptides, such as the mixed anhydride method, the azide method, and syntheses with the aid of dicyclohexylcarbodiimide. This, however, is not of fundamental significance, since the method we propose is applicable to any carbodiimides containing a quaternary ammonium group. Water-soluble carbodiimides have been described in the literature that give considerably better yields than CMC-carbodiimide (for example, cyclohexyl p-toluenesulfonate, cyclohexyl p-methyldiethylammonium carbodiimide). The method has been tested only on the simplest amino acids. In passing to other amino acids, difficulties are encountered that are associated with the insolubility in water of the corresponding derivatives.

For some amino acids, protective groups have been described that permit work in aqueous solutions (for example, dihydropyran protection for cysteine, formyl protection of the ε-amino group of lysine). But for other amino acids (tyrosine, tryptophan), special protective groups will have to be developed. The simplicity

of the proposed synthetic method, in our opinion, fully justifies searches in this direction.

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Note: Figure translations are in progress. See original paper for figures.