[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, MASSACHUSETTS INSTITUTE OF TECHNOLOGY]

The Use of Water-Soluble and Basic Carbodiimides in Peptide Synthesis

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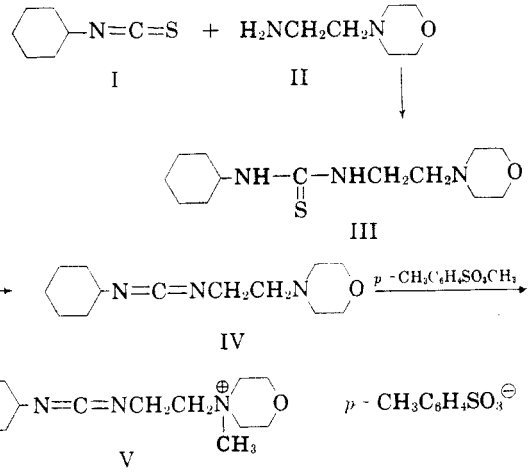
The synthesis of a new group of aliphatic carbodiimides bearing tertiary or quaternary amine substituents and the application of these reagents to peptide bond formation are described.

In a recent communication[2] from this laboratory there was described a convenient new method of forming peptide or other amide bonds by the use of 1,3-dicyclohexylcarbodiimide. In certain instances, as for example the synthesis of high molecular weight peptides, the co-product (1,3-dicyclohexylurea) and the peptide derivative may have similar solubility properties, thus complicating the isolation procedure. This communication reports the preparation of several new carbodiimides bearing tertiary or quaternary amine substituents. Since the corresponding urea derivatives are soluble in dilute acid or in pure water respectively, the separation from the peptide derivative is greatly facilitated. The yield of recrystallized peptide product is typically in the range 80–95%.

The tertiary amine substituted carbodiimides were prepared from the corresponding thioureas, which were obtained by interaction of an amine and an isothiocyanate. Quaternization was effected by treatment with methyl *p*-toluenesulfonate.



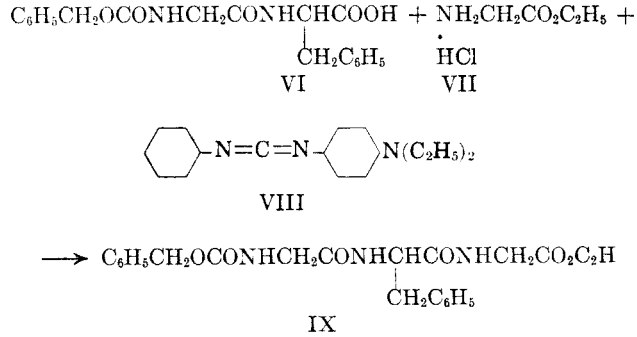
In a similar manner 1-cyclohexyl-3-(4-diethylaminocyclohexyl)carbodiimide (VIII), the corresponding metho *p*-toluenesulfonate, 1,3-di-(4-diethylaminocyclohexyl)carbodiimide and 1-cyclohexyl-3-(β-diethylaminoethyl) carbodiimide were prepared and shown to be effective in peptide synthesis.

The most promising of the carbodiimides we have investigated for general peptide bond synthesis are 1-cyclohexyl-3-(2-morpholinyl-(4)-ethyl)carbodiimide (IV) and the corresponding metho *p*-toluenesulfonate (V). They are readily prepared in good yield from commercially available intermediates, and the quaternized derivative V is crystalline and non-hygroscopic.

Of equal utility are 1-cyclohexyl-3-(4-diethylaminocyclohexyl) carbodiimide (VIII) and the corresponding metho *p*-toluenesulfonate; however, the amine intermediate for these compounds is not yet available commercially and the quaternary derivative has less favorable physical properties than V. Both quaternary salts and the urea coproducts are freely water-soluble.

In a separate experiment designed to test the stability of the carbodiimide in aqueous solution, the quaternary salt V was recovered substantially unchanged from a solution in water after a 7-hour storage period at 25°.

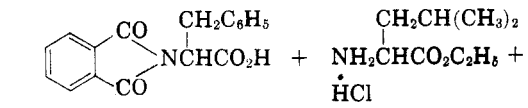
The synthesis of five typical peptide derivatives was studied using a variety of water-soluble and basic carbodiimides with different solvents. The preferred conditions are the use of a carbodiimide bearing a tertiary amine substituent (for example, VIII) directly with the amino acid ester hydrochloride in dioxane solution.



Alternatively, a carbodiimide carrying a quaternary ammonium salt function (for example, V) may be employed in acetonitrile solution with the amino acid ester hydrochloride and an equivalent of triethylamine. A longer reaction time is required under the latter conditions and the yield of peptide derivative tends to be lower.

(1) Aided by a contract from the Office of Naval Research and a Fellowship from the National Institutes of Health.

(2) Sheehan and Hess, *J. Am. Chem. Soc.*, 77, 1067 (1955).



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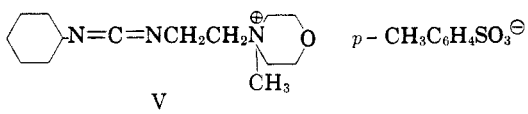
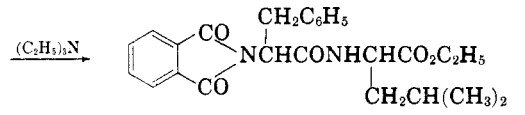


V



XII

These peptide syntheses may be conducted in the presence of water as a co-solvent with some diminution in yield. However, it is interesting to note that phthaloylglycylglycine ethyl ester was formed in pure water solution (no co-solvent present) in good yield, thus demonstrating that peptide bonds can be synthesized chemically in fully aqueous solutions directly from the free acid and amine components. Preliminary experiments indicate that these water-soluble carbodiimides are extremely promising agents for the chemical modification of proteins.

EXPERIMENTAL[3]

1-Cyclohexyl-3-(2-morpholinyl-(4)-ethyl)thiourea (III). A solution of 19.4 g. (0.138 mole) of cyclohexyl isothiocyanate[4] and 18.0 g. (0.138 mole) of N-(2-aminoethyl)morpholine (Carbide and Carbon Chemicals Co.) in 500 ml. of ether was heated under reflux for 10 minutes. After cooling, the crystalline thiourea separated, 36.0 g. (96%); m.p. 128–129°.

A portion was recrystallized from acetone-ligroin, m.p. 128–129°.

Anal. Calc'd for $C_{13}H_{25}N_3OS$: C, 57.56; H, 9.22; N, 15.50. Found: C, 57.51; H, 9.26; N, 15.68.

1-Cyclohexyl-3-(2-morpholinyl-(4)-ethyl)carbodiimide (IV). A mixture of 4.0 g. (0.0147 mole) of 1-cyclohexyl-3-(2-morpholinyl-(4)-ethyl)thiourea and 6.0 g. of mercuric oxide (Merck Yellow) in 50 ml. of acetone was heated under reflux for 6 hours. The mercuric sulfide formed was removed by filtration, a second 6.0-g. portion of mercuric oxide was added, and the suspension was heated at reflux for an additional 6 hours. The reaction mixture filtered again, and the filtrate was concentrated under reduced pressure and flushed twice with benzene. The oily residue was evaporatively distilled at 145°/0.2 mm.; yield, 2.4 g. (70%).

Anal. Calc'd for $C_{13}H_{23}N_3O$: C, 65.78; H, 9.77; N, 17.71. Found: C, 65.60; H, 9.59; N, 17.71.

1-Cyclohexyl-3-(2-morpholinyl-(4)-ethyl)carbodiimide metho-p-toluenesulfonate (V). A mixture of 0.5 g. (2.1 mmoles) of 1-cyclohexyl-3-(2-morpholinyl-(4)-ethyl)carbodiimide (IV) and 0.39 g. (2.1 mmoles) of methyl *p*-toluenesulfonate was heated on a steam-bath for 20 minutes. A solution in benzene deposited 0.6 g. (68%) of the crystalline quaternary salt, m.p. 113–115°.

Anal. Calc'd for $C_{21}H_{33}N_3O_4S$: C, 59.57; H, 7.80; N, 9.93. Found: C, 59.58; H, 8.05; N, 9.88.

In a second experiment the carbodiimide free base was not distilled but was used directly in the preparation of the quaternary salt with an over-all yield of 70%. The quaternary carbodiimide V was recovered unchanged after 7 hours in water solution at room temperature.

1-Cyclohexyl-3-(4-diethylaminocyclohexyl)thiourea. After heating under reflux for 15 minutes, a solution of 5.0 g. (0.029 mole) of N,N-diethyl-1,4-cyclohexyldiamine[5] and 4.2 g. (0.03 mole) of cyclohexyl isothiocyanate[4] in 10 ml. of ether was evaporated under reduced pressure. Crystallization of the residue from warm ligroin and recrystallization from an acetone-ligroin mixture afforded 7.2 g. (80%), m.p. 139–141°, of the pure thiourea.

Anal. Calc'd for $C_{17}H_{33}N_3S$: C, 65.59; H, 10.61; N, 13.50; S, 10.28. Found: C, 65.65; H, 10.66; N, 13.66; S, 10.36.

1-Cyclohexyl-3-(4-diethylaminocyclohexyl)carbodiimide (VIII). A suspension of 2.0 g. of mercuric oxide in 20 ml. of acetone containing 0.8 g. (0.003 mole) of 1-cyclohexyl-3-(4-diethylaminocyclohexyl)thiourea was refluxed for 20 hours. The mercuric sulfide formed was removed by filtration, the filtrate was concentrated under reduced pressure, and the residue was evaporatively distilled at 140°/0.03 mm., 0.6 g. (84%).

Anal. Calc'd for $C_{17}H_{31}N_3$: C, 73.59; H, 11.26; N, 15.15. Found: C, 73.19; H, 11.30; N, 15.29.

1-Cyclohexyl-3-(4-diethylaminocyclohexyl)carbodiimide metho p-toluenesulfonate. A solution of 3.0 g. (1.1 mmoles) of 1-cyclohexyl-3-(4-diethylaminocyclohexyl)carbodiimide (VIII) and 0.21 g. (1.1 mmoles) of methyl-*p*-toluenesulfonate in 6 ml. of benzene (sodium-dried) was heated under reflux on a steam-bath for 20 minutes. After storage overnight at room temperature, the solvent was evaporated to dryness under reduced pressure. The hygroscopic residue, amounting to 0.51 g. (100%) was completely water-soluble and was used without further purification.

Anal. Calc'd for $C_{25}H_{41}N_3O_3S$: C, 64.78; H, 8.85; N, 9.07. Found: C, 64.58; H, 8.71; N, 9.14.

1,3-Di-(4-diethylaminocyclohexyl)thiourea. A solution of 2.3 ml. (0.03 mole) of carbon disulfide in 4 ml. of methanol was added slowly to a cold solution of 2.0 g. (0.012 mole) of N,N-diethylcyclohexyldiamine[5] in 20 ml. of methanol. As the addition progressed a solid separated. This suspension was heated under reflux for 2 days during which time solution was effected. The reaction mixture was evaporated to dryness under reduced pressure and the residue was recrystallized twice from ligroin, yielding 0.8 g. (35%), m.p. 164–165°.

Anal. Calc'd for $C_{21}H_{42}N_4S$: C, 65.97; H, 11.00; N, 14.55; S, 8.36. Found: C, 65.80; H, 11.22; N, 14.74; S, 8.33.

1,3-Di-(4-diethylaminocyclohexyl)carbodiimide. A mixture of 2.4 g. of mercuric oxide and 0.95 g. (2.5 mmoles) of 1,3-di-(4-diethylaminocyclohexyl)thiourea in 30 ml. of acetone was heated with stirring under reflux for 1 hour. The filtered reaction mixture was concentrated under reduced pressure and the residue was evaporatively distilled at 180°/0.03 mm., 0.575 g. (66%).

Anal. Calc'd for $C_{21}H_{40}N_4$: C, 72.36; H, 11.57; N, 16.08. Found: C, 72.25; H, 11.50; N, 16.34.

1-Cyclohexyl-3-(β-diethylaminoethyl)carbodiimide. A solution of 7.0 g. (0.05 mole) of cyclohexylisothiocyanate[4] and 5.8 g. (0.05 mole) of N,N-diethylethylenediamine in ether was heated under reflux for 10 minutes. The ether was evaporated to dryness under reduced pressure leaving the thiourea as a yellow oil, weight 12.8 g.

A portion of the thiourea (5.0 g.) was dissolved in 75 ml. of acetone containing 7.0 g. of mercuric oxide. The reaction mixture was stirred and heated under reflux for 5 hours. The mercuric sulfide was removed by filtration and an additional 7.0 g. of mercuric oxide was added followed by an overnight reflux period. After evaporation of the solvent, the residual oil was purified by evaporative distillation at 100°/0.05 mm.; yield, 1.2 g.

(3) All melting points are corrected. We are indebted to Dr. S. M. Nagy and associates for the microanalytical data.

(4) Skita and Rolfes, *Ber.*, **53**, 1242 (1920).

(5) This material was supplied through the courtesy of Dr. J. E. Kirby, Chemical Department, E. I. du Pont de Nemours and Co., Inc., Wilmington, Delaware.

Anal. Calc'd for $C_{13}H_{25}N_3$: C, 69.90; H, 11.28; N, 18.81. Found: C, 69.91; H, 11.24; N, 18.75.

Phthaloyl-L-phenylalanyl-L-leucine ethyl ester (XII).[2] *A.* To a suspension of 0.27 g. (1.36 mmoles) of L-leucine ethyl ester hydrochloride (prepared by Fischer esterification) in 5 ml. of dioxane was added 0.39 g. (1.4 mmoles) of 1-cyclohexyl-3-(4-diethylaminocyclohexyl) carbodiimide (VIII) followed by 0.4 g. (1.36 mmoles) of phthaloyl-L-phenylalanine.[6] After stirring the mixture overnight, the dioxane was distilled under reduced pressure. A solution of the residue in ethyl acetate was washed with 6 ml. of 1 *N* hydrochloric acid followed by 6 ml. of 1 *N* potassium bicarbonate. After drying and removal of the solvent, the residue was recrystallized from ethyl acetate-ligroin yielding 0.50 g. (87%), m.p. 108–109°, of product; $[\alpha]_D^{26.0}$ −115° [ethanol]. The mixture m.p. with an authentic sample[2] did not show a depression.

B. This process was carried out as above with the variation that 2 ml. of water was added to the initial reaction mixture. The yield was 50%.

C. To 0.268 g. (1.36 mmoles) of L-leucine ethyl ester hydrochloride in 4 ml. of acetonitrile was added 0.19 ml. (1.36 mmoles) of triethylamine followed by 0.40 g. (1.36 mmoles) of phthaloyl-L-phenylalanine[6] and 0.58 g. (1.36 mmoles) of 1-cyclohexyl-3-(2-morpholinyl-(4)-ethyl)carbodiimide metho *p*-toluenesulfonate (V). The mixture was stirred two days at room temperature and the solvent was evaporated under reduced pressure. The residue was taken up in ether and extracted with acid, bicarbonate, and water. After drying, removal of the ether and recrystallization, the product amounted to 0.45 g. (80%). The m.p. and optical rotation of the products obtained in Runs *B* and *C* agree closely with the constants reported for Run *A*.

Carbobenzoxyglycyl-L-phenylalanylglycine ethyl ester (IX).[7] *A.* The experimental procedure outlined for Method *A* above was followed using 0.12 g. (0.85 mmole) of glycine ethyl ester hydrochloride, 3 ml. of dioxane, 0.24 g. (0.85 mmole) of 1-cyclohexyl-3-(4-diethylaminocyclohexyl)carbodiimide (VIII) and 0.3 g. (0.85 mmole) of carbobenzyloxyglycyl-L-phenylalanine. The peptide ester amounted to 0.30 g. (81%), m.p. 117–118°; $[\alpha]_D^{26.0}$ −12.4° [ethanol]. The reported[7] values are m.p. 116–118° and $[\alpha]_D^{25}$ −12.0°.

B. A solution of 0.195 g. (0.55 mmole) of carbobenzyloxyglycyl-L-phenylalanine, 0.057 g. (0.55 mmole) of glycine ethyl ester, and 0.24 g. (0.55 mmole) of 1-cyclohexyl-3-(2-morpholinyl-(4)-ethyl)carbodiimide metho *p*-toluenesulfonate (V) in 7 ml. of acetonitrile was stirred at room temperature for 2 days. The solvent was evaporated under reduced pressure and the residue was treated as described in Method *C* for phthaloyl-L-phenylalanyl-L-leucine ethyl ester. The product amounted to 0.15 g. (63%), m.p. 116–118°; $[\alpha]_D^{26.0}$ −12.0° [ethanol].

Phthaloyl-L-phenylalanyl-L-phenylalanine methyl ester. This peptide ester was prepared in the manner described previously using 0.216 g. (1.0 mmole) of L-phenylalanine methyl ester hydrochloride (prepared by Fischer esterification), dioxane as the solvent, 0.285 g. (1.1 mmoles) of 1-cyclohexyl-3-(4-diethylaminocyclohexyl) carbodiimide (VIII), and 0.295 g. (1.0 mmoles) of phthaloyl-L-phenylalanine.[6] The product was crystallized from an *n*-butyl ether-ether mixture, 0.40 g. (88%); m.p. 101–102°, $[\alpha]_D^{26.0}$ −128° [ethanol].

Anal. Calc'd for $C_{27}H_{24}N_2O_5$: C, 71.04; H, 5.30; N, 6.12. Found: 70.75; H, 5.54; N, 5.78.

Phthaloyl-L-phenylalanylglycine ethyl ester.[2] *A.* The procedure described for the preparation of phthaloyl-L-phenylalanyl-L-leucine ethyl ester was followed using 0.196 g. (1.4 mmoles) of glycine ethyl ester hydrochloride, 0.4 g. (1.7 mmoles) of 1-cyclohexyl-3-(2-morpholinyl-(4)-ethyl) carbodiimide (IV), and 0.415 g. (1.4 mmoles) of phthaloyl-L-phenylalanine.[6] Recrystallization of the product from ethanol yielded 0.43 g. (81%), m.p. 160–161°; $[\alpha]_D^{26.0}$ −146° [ethanol]. The recorded[2] values are m.p. 161–162° and $[\alpha]_D^{26.0}$ −146°.

B. To a solution of 0.196 g. (1.4 mmoles) of glycine ethyl ester hydrochloride and 0.41 g. (1.5 mmoles) of 1-cyclohexyl-3-(4-diethylaminocyclohexyl)carbodiimide (VIII) in 6 ml. of dioxane was added, with stirring, 0.415 g. (1.4 mmoles) of phthaloyl-L-phenylalanine. The resulting reaction mixture, processed as described previously, afforded 0.49 g. (90%), m.p. 160–161°; $[\alpha]_D^{26.0}$ −146° [ethanol].

C. To determine the effect of water on the course of the reaction, the reactant amounts for procedure *B* were used together with a mixture of 2 ml. of water and 6 ml. of dioxane as the solvent. The yield was lowered to 71%, m.p. 160–161°; $[\alpha]_D^{26.0}$ −146° [ethanol].

D. 1-Cyclohexyl-3-(β-diethylaminoethyl)carbodiimide (0.38 g., 1.7 mmoles) was used as the condensing agent in an experiment otherwise identical to Method *B*, yield 0.11 g. (20%).

Phthaloylglycylglycine ethyl ester.[8] To a solution of 0.18 g. (0.865 mmole) of phthaloylglycine[9] and 0.088 g. (0.865 mmole) of glycine ethyl ester in 6 ml. of water was added 0.4 g. (0.86 mmole) of 1-cyclohexyl-3-(4-diethylaminocyclohexyl) carbodiimide metho *p*-toluenesulfonate. A solid began to separate after 10 minutes of stirring and after two hours the solid was collected, weight 0.18 g. (75%); m.p. 194–195°.

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(6) Sheehan, Chapman, and Roth, *J. Am. Chem. Soc.*, **74**, 3822 (1952).

(7) Anderson and Young, *J. Am. Chem. Soc.*, **74**, 5307 (1952).

(8) Boissonnas, *Helv. Chim. Acta*, **34**, 874 (1951).

(9) Drechsel, *J. prakt. Chem.*, [II] **27**, 418 (1883).