BULLETIN OF THE CHEMICAL SOCIETY OF JAPAN VOL. 40 656—661 (1967)

A Proposal for Using Mild Bases in the Preparation of Optically Pure Peptides*1

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(Received September 12, 1966)

Base catalyzed racemization of *N*-carbobenzoxy-*S*-benzyl-L-cysteine *p*-nitrophenyl ester was studied with various tertiary amines; *N, N*-dialkylglycine esters and *N*-ethylmorpholine were found to induce less racemization than triethylamine. Furthermore, these mild bases were strong enough in many cases to liberate free amino acid or peptide esters from their salts. It was also confirmed that a mixture of an amino acid or peptide ester salt and such a mild base could be used in the place of a previously prepared free ester for peptide synthesis, and practically no racemization was observed during the coupling reaction with an acylamino acid active ester. Anderson's racemization test was also carried out by the mixed anhydride procedure with these mild bases instead of triethylamine; the mild bases were found to give better results in optical purity.

Although carbobenzoxyamino acid *p*-nitrophenyl esters are useful intermediates for peptide synthesis, they are apt to be racemized in the usual organic solvent containing triethylamine.[1,2] This base-catalyzed racemization might occur when one kind of the active ester is subjected to reaction with an amino acid or peptide ester in a simplified procedure where the peptide or amino acid ester is added as an equimolar mixture of the strong acid salt and triethylamine.[3] Therefore, the accepted procedure for obtaining optically pure peptide is to add the amino acid or peptide ester as a free ester into the reaction; that is, the acid moiety of the ester salt should be removed in a suitable way before adding the ester in the peptide-forming reaction.[3] However, since removal of the acid moiety from ester salts is not always successful, especially in the case of a longer peptide salt, the simplified method is still favored by many workers at the risk of partial racemization. Bodanszky *et al.*[4] first showed that pyridine can be used as a mild base for coupling carbobenzoxy-L-citrulline *p*-nitrophenyl ester with glycine ethyl ester hydrochloride. However, the pyridine procedure was found to be unsuccessful in general cases because of its weak basicity.

TABLE 1. APPARENT pK_a VALUES OF TERTIARY AMINES, L-AMINO ACID ESTERS AND A PEPTIDE ESTER

Compound	pK_a'[a]	pK_a''[b]
HCl·H-Pro-OBZL	8.35	8.00
HCl·H-Ala-OMe	7.90	7.65
HCl·H-Leu-NH_2	7.90	7.65
HCl·H-Gly-OEt	7.80	7.55
TosOH·H-Ile-OBZL	7.70	7.30
TosOH·H-Val-OBZL	7.55	7.15
TosOH·Glu(OBZL)$_2$	—	6.70
HBr·H-Glu(γ-OMe)-α-ONB	6.95	6.60
HBr·H-Asp(β-OMe)-α-ONB	6.30	6.15
TosOH·H-Try-OBZL	—	6.95
HCl·H-Phe-OMe	7.15	6.80
TosOH·H-Phe-OBZL	6.95	6.75
H-Pro-Leu-Gly-NH_2	8.40	8.05
N-Ethylmorpholine	7.90[c]	7.55
N, N-Diethylglycine *n*-propyl ester	7.85	7.50
N, N-Di-*n*-butylglycine ethyl ester	—	7.40
N, N-Diethylglycine ethyl ester	7.90	7.40
Triethylamine	10.65[d]	

a) Apparent pK_a value in water. See Experimental part.
b) Apparent pK_a value in a mixture of water and methanol. See Experimental part.
c) Cited: pK_a 7.70; H. K. Hall, Jr., *J. Phys. Chem.*, **60**, 63 (1956).
d) J. Hanson, *Svensk Kem. Tidskrift*, **67**. 256 (1955).
OBZL: benzyl ester. ONB: *p*-Nitrobenzyl ester. TosOH: *p*-toluenesulfonic acid.

*1 This study was presented at the 4th Symposium on Peptide Chemistry, Institute for Protein Research, Osaka University, Dec. 3, 1965; "The 4th Symposium on Peptide Chemistry," p. 31 (1965).

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1) M. Bodanszky and C. A. Birkhimer, *Chimia*, **14**, 365 (1960).
2) M. Goodman and W. J. McGahren, *J. Am. Chem. Soc.*, **87**, 3028 (1965).
3) M. A. Smart, G. T. Young and M. W. Williams, *J. Chem. Soc.*, **1960**, 3902; M. W. Williams and G. T. Young, *ibid.*, **1963**, 881; "Peptide Symposium," Oxford (1962), p. 119, Pergamon Press, New York (1963).
4) M. Bodanszky and C. A. Birkhimer, *J. Am. Chem. Soc.*, **84**, 4943 (1962).

Table 2. Optical rotation change of *N*-carbobenzoxy-*S*-benzyl-L-cysteine *p*-nitrophenyl ester in dimethylformamide containing various tertiary amines[a]

Amine and its concentration in DMF	hr	$[\alpha]_D^t$	hr	$[\alpha]_D^t$
No base	1/4	-42.8^{26}	69.5	-42.7^{24}
Triethylamine, 1%	1/2	-34.7^{25}	24	-0.2^{26}
Triethylamine hydrochloride, satd.	1/4	-42.0^{25}	48	$-40.1^{25.5}$
N,*N*-Diethylglycine ethyl ester, 2%	1/2	$-43.0^{24.5}$	46.5	-39.1^{25}
N,*N*-Di-*n*-butylglycine ethyl ester, 2%	1/6	-42.5^{25}	49.5	$-40.0^{24.5}$
N,*N*-Diethylglycine *n*-propyl ester, 2%	1/6	$-41.3^{25.5}$	55	-39.0^{26}
N-Ethylmorpholine, 2%	1/3	-40.1^{23}	27	$-33.3^{23.5}$

a) Concentration of the *p*-nitrophenyl ester in dimethylformamide was 0.2 g per 10 ml. The solution was kept at room temperature (23—26°C) without temperature control.

Table 3. Base effect on the synthesis of *N*-carbobenzoxy-*S*-benzyl-*L*-cysteinylglycine ethyl ester under various conditions by the *p*-nitrophenyl ester method[a]

Base	Eq.	Solvent	Product					
			M. p. °C	Yield %	$[\alpha]_D^t$ (*C* 6, AcOH)[b]	Anal.[c] % C	H	N
Triethylamine	1[e]	DMF	96—98	100	-27.4^{23}	61.21	5.94	6.48
Triethylamine	3[d]	$CHCl_3$-DMF	96—98	95.3	-27.1^{26}	61.21	6.18	6.22
Triethylamine	3[e]	DMF	91—94	82.7	-26.3^{20}	61.62	6.05	6.55
Triethylamine	3[e]	DMF[f]	88—91	80.4	-20.5^{20}	61.76	6.23	6.47
N,*N*-Diethylglycine ethyl ester	1[e]	DMF	95—97	97.7	-28.6^{27}	61.48	6.30	6.28
N,*N*-Diethylglycine ethyl ester	3[d]	$CHCl_3$-DMF	96.5—98	93.4	-28.3^{26}	61.20	6.28	6.24
N,*N*-Diethylglycine ethyl ester	3[e]	DMF	97.5—99	96.3	-28.0^{24}	61.71	6.12	6.20

a) Each reaction mixture was allowed to react for 24 hr at room temperature.
b) Highest value appeared in the literature is -28.7^{20} [J. A. Maclaren, W. E. Savige and J. M. Swan, *Aust. J. Chem.*, **11**, 345 (1958)].
c) Calcd for $C_{22}H_{26}O_5N_2S$; C, 61.37; H, 6.09; N, 6.51%.
d) Three eq. of glycine ethyl ester hydrochloride was used.
e) One eq. of glycine ethyl ester hydrochloride was used.
f) The reaction mixture was allowed to react for 72 hr at room temperature.

Table 4. Base effect on Anderson's racemization test by the mixed anhydride procedure with isobutyl chloroformate (Preparation of ethyl carbobenzoxyglycyl-L-phenylalanylglycinate)

Base	Solvent	Activation condition	Total yield %	Yield of racemate %
Triethylamine[a]	Chloroform	−5°C, 25 min	38.4	69.4
N,*N*-Diethylglycine ethyl ester[a]	Chloroform	−5°C, 25 min	16.6	0.22
N,*N*-Diethylglycine ethyl ester[b]	Chloroform	−5°C, 5 min	8.9	0.20
N-Ethylmorpholine[b]	Chloroform	−5°C, 25 min	51.8	2.07
N,*N*-Diethylglycine ethyl ester[b]	Tetrahydrofuran	−10°C, 10 min	31.1	0
N-Ethylmorpholine[b]	Tetrahydrofuran	−10°C, 10 min	61.0	0

a) Glycine ethyl ester was added as the distilled free ester.
b) Glycine ethyl ester was added as a mixture of the hydrochloride and respective base, which was used for the mixed anhydride formation, in dimethylformamide.

TABLE 5. SUMMARIZED DATA OF VARIOUS PEPTIDE SYNTHESES WITH MILD BASES

Starting materials		Mild base	Product	Yield %	Mp °C	$[\alpha]_D^t$
Carboxyl component	Amine component					
Z-L-Val-ONSu	HCl·H-L-Tyr-OMe	A	Z-L-Val-L-Tyr-OMe	86.3	152 —153 155.5—156[a]	+11.7[26] (*c* 4.8, Pyridine) +10.2[25] (*c* 4.8, Pyridine)[a]
Z-Gly-ONP	HCl·H-L-Phe-OEt	A	Z-Gly-L-Phe-OH[c]	70.0	129.5—131 125 —126[b]	+38.6[25] (*c* 5, EtOH) +38.8±0.4[25] (*c* 5, EtOH)[b]
Z-L-Phe-ONP	HCl·H-L-Pro-OBZL	A	Z-L-Phe-L-Pro-OH[c]	65.6	105 —106.5	−64.3[21.5] (*c* 2.6, Pyridine)
Z-L-Phe-ONP	HCl·H-L-Pro-OBZL	B	Z-L-Phe-L-Pro-OH[c]	74.0	105 —106.5	−65.4[20] (*c* 2.6, Pydridine)
Z-L-CyS(BZL)-ONP	HCl·H-L-Pro-L-Leu-Gly-NH_2	A	Z-L-CyS(BZL)-L-Pro-L-Leu-Gly-NH_2	77.8	168 —169.5 170 —171.5[d]	−60.2[20.5] (*c* 2, DMF) −60.0[21] (*c* 2, DMF)[d]
Z-L-Leu-OH[g]	HCl·H-Gly-OEt	B	Z-L-Leu-Gly-OEt	83.1	102.5—103.5 104 —105.5[e]	−27.5[20] (*c* 5, EtOH) −27.1[22] (*c* 5, EtOH)[e]
Z-L-Arg(NO_2)-OH[g]	HCl·H-Gly-OEt	A	Z-L-Arg(NO_2)-Gly-OEt	44.5	115 —117 118 —120[f]	−13.9[20.5] (*c* 2, MeOH) −13.4[27] (*c* 2, MeOH)[f]
Z-L-Arg(NO_2)-OH[g]	HCl·H-Gly-OEt	B	Z-L-Arg(NO_2)-Gly-OEt	66.4	115.5—116.5	−12.9[22] (*c* 2, MeOH)

a) H. Schwarz, F. M. Bumpus and I. H. Page, *J. Am. Chem. Soc.*, **79**, 5697 (1957).
b) K. Hofmann and M. Bergmann, *J. Biol. Chem.*, **134**, 225 (1940), J. R. Vaughan, Jr., *J. Am. Chem. Soc.*, **74**, 6137 (1952).
c) The obtained ester was directly hydrolyzed to free acid in methanol with N-sodium hydroxide.
d) M. Bodanszky and V. du Vigneaud, *J. Am. Chem. Soc.*, **81**, 2506 (1959).
e) M. Bodanszky and V. du Vigneaud, *ibid.*, **81**, 5688 (1959).
f) H. Gibian and E. Schroeder, *Ann.*, **642**, 145 (1961).
g) Mixed anhydride method.
A: *N, N*-Diethylglycine ethyl ester, B: *N*-Ethylmorpholine, *Z*: Carbobenzoxy,
ONP: *p*-Nitrophenyl ester, ONSu: *N*-Hydroxysuccinimide ester.

Previously, the exclusive use of *N, N*-diethylglycine ethyl ester has been reported for the preparation of *t*-amyloxycarbonylamino acid esters.[5] *N, N*-Dialkylglycine esters and *N*-ethylmorpholine are similar in basicity to the usual amino acid esters as shown in Table 1, and it was supposed that such mild bases may minimize the base-catalyzed racemization of acylamino acid active esters. This was tested using *N*-carbobenzoxy-*S*-benzyl-L-cysteine *p*-nitrophenyl ester (I) which is known to be one of the most readily racemizable compounds among many *p*-nitrophenyl esters[1] (Table 2). Optical rotation of the active ester I fell to almost zero after 24 hr in the presence of 1% triethylamine in dimethylformamide, as Bodanszky[1] mentioned previously, whereas it was unaffected by dimethylformamide itself even after 70 hr at room temperature. On the other hand, decrease in the optical rotation of I was within 10% during 50 hr in the following mixtures: Dimethylformamide saturated with triethylamine hydrochloride; 2% *N, N*-diethylglycine ethyl ester in dimethylformamide; 2% *N, N*-diethylglycine propyl ester in dimethylformamide; 2% *N, N*-dibutylglycine ethyl ester in dimethylformamide. Although *N*-ethylmorpholine showed the same apparent pK_a value as *N, N*-diethylglycine ethyl ester, it was found to be somewhat less effective for retaining the optical purity of compound I in comparison with *N, N*-dialkylglycine esters, but much more effective than triethylamine. As Young *et al.*[3] pointed out, triethylamine hydrochloride induces the racemization of benzoyl-L-leucine *p*-nitrophenyl ester in hot chloroform. In the present experimental conditions, however, the triethylamine hydrochloride brought only slight racemization of compound I, and this fact may be due to the low solubility of the hydrochloride in dimethylformamide at room temperature.

Next, racemization of *S*-benzyl-L-cysteinyl residues during the actual reaction was tested by checking each reaction product of compound I with a mixture of glycine ethyl ester hydrochloride and triethylamine or *N, N*-diethylglycine ethyl ester in various reaction conditions; the data are presented in Table 3. Excess triethylamine greatly reduced the optical purity of the product as was expected, but excellent optical rotation values of the product were obtained when *N, N*-diethylglycine ethyl ester was used in the reaction, even when it was used at three times the

5) S. Sakakibara and M. Itoh, This Bulletin, **40**, 646 (1967).

theoretical amount. During the experiments, further racemization was observed when the reaction product was kept with triethylamine at room temperature for two days after completion of the reaction; this fact indicated that the triethylamine-catalyzed racemization should continue even after completion of the peptide-forming reaction.

In connection with the racemization reaction by the active-ester method, the effects of various bases in the mixed anhydride procedure were compared using Anderson's racemization test.[6] The tertiary amine salts of carbobenzoxyglycyl-L-phenylalanylglycine were caused to react with isobutyl chloroformate at −5°C for 25 min in chloroform and then with ethyl glycinate to give ethyl carbobenzoxyglycyl-L-phenylalanylglycinate; according to Vaughan *et al.*,[7] complete racemization should occur under this reaction condition when triethylamine is used as the required base. Formed racemic tripeptide was separated by fractional recrystallization. Reaction conditions and the results of the presents study are listed in Table 4. Although maximum racemization was observed when triethylamine was used as the base, as expected, one equivalent *N, N*-diethylglycine ethyl ester or *N*-ethylmorpholine brought only slight racemization, whereas yields of the desired product were not satisfactory. As will be shown later, however, *N*-ethylmorpholine was generally a better reagent than *N, N*-diethylglycine ester in the normal mixed anhydride procedure, and no racemate was detectable in Anderson's test when tetrahydrofuran was used as the solvent.

During the preparation of this paper, an interesting result has been announced independently by Anderson *et al.*,[8] in which they recommended the use of *N*-methylmorpholine, instead of the *N*-ethyl derivative, to obtain satisfactory yields without racemization by the mixed anhydride procedure. The presence of an *N*-methyl group in the tertiary base may be necessary to get a better result in the mixed anhydride procedure.

Finally, several peptides were prepared using these mild bases to see how they can actually be used in the reactions. The data are listed in Table 5. Yields and optical purities of these products were quite satisfactory in comparison with those which appeared in the literature. Thus, such mild bases can be recommended as useful tertiary bases in peptide synthesis.*3

Experimental

Materials. *N*-Ethylmorpholine was obtained from Nakarai Chemicals, Ltd., Kyoto. *N, N*-Diethylglycine ethylester was prepared as described in preceding paper.[5]

***N, N*-Di-*n*-butylglycine Ethyl Ester.** Ethyl monochloroacetate (122.6 g, 1 mol) was added dropwise into a solution of di-*n*-butylamine (258.5 g, 2 mol) in ether (300 m*l*) at 25—30°C. After the reaction mixture was allowed to stand overnight in a refrigerator, formed amine hydrochloride was removed by filtration. The filtrate was washed twice with water, and then dried over anhydrous magnesium sulfate. After removal of the solvent there remained some oily materials, which were distilled to yield the product (120 g, 56%); bp 136—137°C/35 mmHg

Found: C, 66.86; H, 11.84%. Calcd for $C_{12}H_{25}O_2N$: C, 66.93; H, 11.70%.

***N, N*-Diethylglycine *n*-Propyl Ester.** The material was prepared from diethylamine and ethyl monochloroacetate as described before. Yield 62%, bp 96—98°C/28 mmHg,

Found: C, 61.84; H, 11.11%. Calcd for $C_9H_{19}O_2N$: C, 62.38; H, 11.05%.

Determination of Apparent pK_a Values. Each sample (0.00015 mol) was dissolved in 0.1 M sodium chloride solution (20.0 m*l*), in which *N*-hydrochloric acid (0.20 m*l*) was added, the mixture was titrated with N sodium hydroxide with a Radiometer auto-titrator. The pK_a' value was calculated from the titration curve by the method of Parke and Davis.[9] Since some samples were hard to dissolve in the above solution, methanol (10.0 m*l*) was added to the mixture to get a clear solution, and titration was carried out in the same manner. In this case, the observed value was reported as pK_a''. (Table 1).

***N*-Carbobenzoxy-*S*-benzyl-L-cysteinylglycine Ethyl Ester.** *A: Examples with e) as a suffix in Table 3.* Triethylamine or *N, N*-diethylglycine ethyl ester (0.005 or 0.015 mol) was added to a suspension of glycine ethyl ester hydrochloride (0.70 g, 0.005 mol) in dimethyl formamide (10 m*l*). To the clear solution, *N*-carbobenzoxy-*S*-benzyl-L-cysteine *p*-nitrophenyl ester (2.32 g, 0.005 mol) was added, and the mixture was allowed to react for 24 hr at room temperature. The product was extracted with ethyl acetate from the reaction mixture, to which water had been added, and the extract was washed successively with N ammonia, water, N hydrochloric acid and water, and dried over anhydrous magnesium sulfate. The dried solution was concentrated to obtain the product as crystals.

B: Examples with d) as a Suffix in Table 3. Triethylamine or *N, N*-diethylglycine ethyl ester (0.015 mol) was added to a suspension of glycine ethyl ester hydrochloride (2.10 g, 0.015 mol) in a mixture of chloroform (20 m*l*) and dimethylformamide (5 m*l*). The mixture was allowed to react with *N*-carbobenzoxy-*S*-benzyl-L-cysteine *p*-nitrophenyl ester (2.32 g, 0.005

6) G. W. Anderson and F. M. Callahan, *J. Am. Chem. Soc.*, **80**, 2902 (1958); G. W. Anderson and R. Paul, *ibid.*, **82**, 4596 (1960).

7) J. R. Vaughan, Jr., *ibid.*, **74**, 6137 (1952).

8) G. W. Anderson, J. E. Zimmerman and F. M. Callahan, *ibid.*, **88**, 1338 (1966).

*3 Recently, a few examples of peptide-forming reactions by the active-ester method were found to proceed very slowly with these mild bases; for instance, a reaction between *t*-amyloxycarbonyl-*S*-benzyl-L-cysteine *p*-nitrophenyl ester and L-serine benzyl ester tosylate proceeded not with *N, N*-diethylglycine ethyl ester, but with triethylamine, which indicates a limitation of the procedure.

9) T. V. Parke and W. W. Davis, *Anal. Chem.*, **26**, 642 (1954).

mol) for 24 hr at room temperature. The reaction product was treated as described in procedure A.

Carbobenzoxyglycyl-L-phenylalanylglycine Ethyl Ester. (Anderson's Test, Table 4) *A:* Triethylamine, *N*, *N*-diethylglycine ethyl ester or *N*-ethylmorpholine (0.005 mol) was added to a solution of carbobenzoxyglycyl-L-phenylalanine (1.78 g, 0.005 mol) in chloroform (40 ml) at −5°C, and then a solution of isobutyl chloroformate (0.69 g, 0.005 mol) in chloroform (10 ml) was added slowly at the same temperature. The reaction mixture was allowed to react with stirring for 25 min at −5°C. Then, a solution of freshly distilled glycine ethyl ester (0.52 g, 0.005 ml) in chloroform (10 ml) was added to the reaction mixture; the whole mixture was allowed to react for 30 min at −5°C and then for 20 hr at room temperature. After distilling out the solvent, the reaction product was extracted with ethyl acetate; the extract was washed successively with 5% sodium bicarbonate and water, and dried over magnesium sulfate. The dried solution was concentrated to a syrup, and the residue was crystallized by triturating with petroleum ether. The crude product was recrystallized from absolute ethanol as described by Anderson *et al.*[6]

B: *N*, *N*-Diethylglycine ethyl ester or *N*-ethylmorpholine (0.01 mol) was added to a solution of carbobenzoxyglycyl-L-phenylalanine (3.56 g, 0.01 mol) in tetrahydrofuran (50 ml), and isobutyl chloroformate (1.36 g, 0.01 mol) was added to the mixture with stirring at −10°C. After 10 min at −10°C, a solution of glycine ethyl ester hydrochloride (1.40 g, 0.01 mol) and *N*, *N*-diethylglycine ethyl ester or *N*-ethylmorpholine (0.01 mol) in dimethylformamide (30 ml) was added to the reaction mixture. The reaction was allowed to proceed with stirring at −10°C for 30 min and then at room temperature for 20 hr. Then, the reaction mixture was treated as described in procedure A.

Carbobenzoxy-L-valyl-L-tyrosine Methyl Ester. Into a suspension of L-tyrosine methyl ester hydrochloride (11.6 g, 0.05 mol) in a mixture of chloroform (80 ml) and dimethylformamide (40 ml), there was added *N*, *N*-diethylglycine ethyl ester (8.7 ml, 0.05 mol). Then, carbobenzoxy-L-valine *N*-hydroxysuccinimide ester[10] (17.0 g, 0.049 mol) was added to the mixture, and the whole mixture was allowed to react for 3 days at room temperature. After the reaction was over, chloroform was removed by distillation under reduced pressure and the product was extracted with ethyl acetate. The ethyl acetate extract was washed with 10% sodium bicarbonate, water, N hydrochloric acid and water, successively, and dried over magnesium sulfate. Concentration of the dried solution yielded crude crystals (21.0 g), which were recrystallized from ethyl acetate - petroleum ether to obtain needles. The data are presented in Table 5.

Found: C, 64.38; H, 6.57; N, 6.45%. Calcd for $C_{23}H_{28}O_6N_2$: C, 64.47; H, 6.59; N, 6.54%.

Carbobenzoxyglycyl - L - phenylalanine. Carbobenzoxyglycine *p*-nitrophenyl ester[11] (33.0 g, 0.1 mol) was added to a clear solution of L-phenylalanine ethyl ester hydrochloride (23.0 g, 0.1 mol) and *N*, *N*-diethylglycine ethyl ester (17.3 ml, 0.01 mol) in chloroform (200 ml), and the mixture was allowed to react for 3 days at room temperature. After that period, the solvent was removed and the residue was redissolved in ethyl acetate. The ethyl acetate solution was washed successively with water, N ammonia, N hydrochloric acid and water, and dried over magnesium sulfate. The dried solution was concentrated to a syrup which was dissolved in methanol (70 ml) and hydrolysis of the ester group was carried out with N sodium hydroxide (100 ml) at 15°C for 2 hr. The reaction mixture was acidified with N hydrochloric acid, the methanol was removed under reduced pressure, and the product was extracted with ethyl acetate. The ethyl acetate extract was washed with water, and then the main product was taken up into sodium bicarbonate solution. The sodium bicarbonate extract was washed once with fresh ethyl acetate, and then acidified with hydrochloric acid. The oil formed was extracted with ethyl acetate, the extract was washed with water, and dried over magnesium sulfate. Removal of the solvent gave crystals which recrystallized from ethyl acetate - petroleum ether; wt 26.0 g, mp 125—126.5°C. Further recrystallization was carried out with the same solvent system. The final data are listed in Table 5.

Carbobenzoxy-L-phenylalanyl-L-proline. *N*, *N*-Diethylglycine ethyl ester or *N*-ethylmorpholine (0.01 mol) was added to a solution of L-proline benzyl ester hydrochloride (2.42 g, 0.01 mol) in dimethylformamide (25 ml), and into the mixture, there was added carbobenzoxy-L-phenylalanine *p*-nitrophenyl ester[12] (4.20 g, 0.01 mol) and acetic acid (0.3 ml). The whole mixture was allowed to react for 4 days at room temperature, and the reaction product was obtained as an oil after treatment as described in the preceding section. Hydrolysis of the *C*-terminal benzyl ester group was carried out in a mixture of acetone (20 ml) and methanol (50 ml) using N sodium hydroxide (15 ml) at room temperature for 1.5 hr. The reaction mixture was treated as described before, and the final product was recrystallized from ethyl acetate-petroleum ether. The data are shown in Table 5.

Found: C, 66.28; H, 6.15; N, 6.90%. Calcd for $C_{22}H_{24}O_5N_2$: C, 66.65; H, 6.10; N, 7.07%.

Carbobenzoxy - *S* - benzyl-L-cysteinyl-L-prolyl-L-leucylglycine amide. Carbobenzoxy - *S* - benzyl - L-cysteine *p*-nitrophenyl ester[13] (2.32 g, 0.005 mol) was added to a solution of L-prolyl-L-leucylglycine amide hydrochloride (1.6 g, 0.005 mol) and *N*, *N*-diethylglycine ethyl ester (0.87 ml, 0.005 mol) in dimethylformamide (20 ml) and the reaction mixture was allowed to react for 3 days at room temperature. Water was added to the mixture, the product was extracted with ethyl acetate, the extract was wahed with 5% sodium bicarbonate, water, N hydrochloric acid and water, and dried over magnesium sulfate. The dried solution was concentrated to about 20 ml, and the product was obtained as crystals after keeping the concentrated solution in a refrigerator; wt 2.63 g. Recrystallization was carried out from methanol (30 ml) and water (20 ml). The data are shown in Table 5.

Carbobenzoxy-L-leucylglycine Ethyl Ester. Carbobenzoxy-L-leucine used was purified by following

10) G. W. Anderson, J. E. Zimmerman and F. M. Callahan, *J. Am. Chem. Soc.*, **86**, 1839 (1964).
11) "Biochemical Preparations," Vol. 9, John Wiley & Sons, Inc., New York (1962), p. 110.
12) M. Bodanszky and V. du Vigneaud, *J. Am. Chem. Soc.*, **81**, 6072 (1959).
13) M. Bodanszky and V. du Vigneaud, *ibid.*, **81**, 2504 (1959).

the description of Fujino.[14] Carbobenzoxy-L-leucine was crystallized as piperazine salt, and the salt was purified by successive recrystallization from methanol; mp 155.5—156.5°C, $[\alpha]_D^{21}$ −14.1 (*c* 1.4, methanol). The piperazine carbobenzoxy-L-leucinate (3.08 g, 0.005 mol) was shaken in a mixture of ethyl acetate (100 m*l*), ethanol (10 m*l*) and N hydrochloric acid (20 m*l*), and liberated carbobenzoxy-L-leucine was extracted with ethyl acetate. The extract was washed with water, and dried over magnesium sulfate. The dried solution was concentrated to a syrup which was flushed twice with toluene to remove volatile materials. Purified carbobenzoxy-L-leucine thus obtained was dissolved in dry tetrahydrofuran (50 m*l*), and *N*-ethylmorpholine (1.29 m*l*, 0.01 mol) was added to the solution. Then, isobutyl chloroformate (1.36 g, 0.01 mol) was added slowly to the mixture at −10°C with stirring. After 10 min, a solution of glycine ethyl ester hydrochloride (1.40 g, 0.01 mol) and *N*-ethylmorpholine (1.29 m*l*, 0.01 mol) in dimethylformamide (30 m*l*) was added to the reaction mixture at −10°C. Stirring was continued at the same temperature for 30 min and then at room temperature for 20 hr, and the reaction mixture was treated in the normal way; the yield of crude product was 3.2 g (91%). Recrystallization was carried out from ethyl acetate (40 m*l*) and petroleum ether (80 m*l*). The data are shown in Table 5.

N^{α}-Carbobenzoxy-N^{g}-nitro-L-arginylglycine Ethyl Ester. A mixed anhydride was prepared from N^{α}-carbobenzoxy-N^{g}-nitro-L-arginine (3.53 g, 0.01 mol) and isobutyl chloroformate (1.36 g, 0.01 mol) at −10°C in dry tetrahydrofuran (50 m*l*) using *N*-ethylmorpholine or *N*, *N*-diethylglycine ethyl ester (0.01 mol). After 10 min activation at −10°C, glycine ethyl ester hydrochloride (1.40 g, 0.01 mol) was added to the reaction mixture as a dimethylformamide solution (30 m*l*) together with *N*-ehtylmorpholine or *N*, *N*-diethylglycine ethyl ester (0.01 mol). The reaction mixture was allowed to react at −10°C for 30 min and then at room temperature for 20 hr. Then, the reaction mixture was treated in the usual way; the data are presented in Table 5.

14) M. Fujino, "The 3rd. Symposium on Peptide Chemistry," Institute for Protein Research, Osaka University (1964), p. 91.