163. *Amino-acids and Peptides. Part XVI.*[1] *Further Studies of Racemisation during Peptide Synthesis.*

By M. W. Williams and G. T. Young.

The racemisation occurring when benzoyl-L-leucine is condensed with glycine ethyl ester by various methods has been studied. In each case so far examined, this model reaction has yielded crude product of a high degree of chemical purity, and the degree of racemisation can therefore be estimated directly from the optical rotation. No racemate could be found in the product of the azide route, in that from the cyanomethyl ester, or in that from the *p*-nitrophenyl ester in the absence of chloride ion. The use of dicyclohexylcarbodi-imide or tetraethyl pyrophosphite, and the carbonic mixed anhydride and phosphorazo-methods, gave varying degrees of racemisation. The use of 2-ethyl-5-*m*-sulphonatophenylisoxazolium in acetonitrile gave no detectable racemate with free glycine ester, but a little was formed from the ester hydrochloride; when the solvent was nitromethane, a considerable amount of racemate was formed, whether or not chloride was present.

Part XV [1] of this series reported an evaluation of the racemisation that occurs during coupling of acetyl-L-leucine with glycine ethyl ester by various procedures, and earlier work on the racemisation problem was reviewed there and in a previous paper.[2] It was pointed out that methods which yield fully active peptides from benzyloxycarbonylamino-acids may cause racemisation when benzyloxycarbonyldipeptides are coupled—a fact which we correlate with the inability of the former compounds to yield azlactones under normal conditions. Thus the retention of activity when benzyloxycarbonylamino-acids are coupled cannot be used as evidence that racemisation will not occur in other cases. It is necessary to reiterate this point, since Goldschmidt and Rosculet [3] have contrasted their preparation of optically pure L-leucylglycine by coupling benzyloxycarbonyl-L-leucine with glycine ethyl ester by the "phosphorazo-method," [4] with our experience [1] of nearly complete racemisation when acetyl-L-leucine is coupled analogously. No racemisation would be expected in the former case, whatever method of coupling were adopted, but as Grassmann, Wünsch, and Riedel [5] have shown, racemisation can ocur when benzyloxycarbonylpeptides are further coupled by the phosphorazo-method. A further example is reported below.

The model reaction with acetyl-L-leucine showed that all the methods examined, except that through the acid azide, could result in the formation of some racemate, but in many cases quantitative estimation of the degree of racemisation was made difficult by the fact that a considerable proportion of the product was syrupy. This paper describes a model system—the coupling of benzoyl-L-leucine with glycine ethyl ester—in which the crude product, in each case so far encountered, is crystalline and, in nearly every case, analytically pure. The degree of racemisation may then be measured directly by the optical rotation. Confirmation of racemisation by the isolation of racemic (or largely racemic) material is in some cases more difficult than with the acetyl analogue, since fractional crystallisation yields racemate only when it is present in high proportion. However, benzoyl-DL-leucylglycine crystallises much more readily than does its L-isomer, and with synthetic mixtures it was possible to detect the presence of 2% or more of benzoyl-DL-leucylglycine ethyl ester, admixed with the L-ester, by saponification of the mixture and careful crystallisation of the acid so obtained. In some experiments, the first crystals

[1] Part XV, Smart, Young, and M. W. Williams, *J.*, 1960, 3902.
[2] G. T. Young, Proc. Symposium on Methods of Peptide Synthesis, Prague, 1958 (*Coll. Czech. Chem. Comm.*, 1959, **24**, Special Issue, p. 39).
[3] Goldschmidt and Rosculet, *Chem. Ber.*, 1960, **93**, 2387.
[4] Goldschmidt and Lautenschlager, *Annalen*, 1953, **580**, 68.
[5] Grassmann, Wünsch, and Riedel, *Chem. Ber.*, 1958, **91**, 455.

TABLE.

	Conditions [a]	Solvent	Benzoyl-L-leucine (g.)	Crude product: Wt. (g.)	Yield (%)	M. p.	$[\alpha]_D$ [b]	L-Isomer [c] (%)	Found (%) [d]: C	H	N
	1. *Acid azide method*										
(i)	A	Et_2O	—[e]	2·77	87	153—155°	−34·0°	100	63·7	7·6	8·9
(ii)	A	,,	—[f]	1·18	74	152—155	−29·8	88	63·7	7·7	8·7
(iii)	C	,,	—[f]	1·07	67	148—151	−30·9	91	63·5	7·6	9·1
(iv)	C	,,	—[f]	1·21	76	153—154	−31·0	91	63·4	7·6	9·0
	2. *Cyanomethyl ester method*										
(i)	A; D	EtOAc	2·35	2·87	90	127—134	−30·1	89	63·5	7·5	9·0
(ii)	A; D	,,	0·59	0·65	81	135—139	−31·9	94	63·8	7·3	8·7
(iii)	A; E	,,	1·18	1·12	70	149—152	−33·6	99	64·1	7·2	9·1
(iv)	A; E	,,	1·18	1·19	74	153—155	−32·3	95	63·4	7·6	9·1
	3. *p-Nitrophenyl ester method*										
(i)	A	EtOAc	—[g]	1·01	95	156—157	−34·3	100	63·1	7·3	9·1
(ii)	B(NMP)	,,	—[g]	0·95	89	148—153	−26·8	80	63·5	7·3	8·8
(iii)	A	$CHCl_3$	—[g]	0·69	86	148—153	−32·6	96	63·7	7·7	8·5
(iv)	B(NMP)	,,	—[g]	0·89	84	150—155	−25·6	75	63·9	7·4	9·5
	4. *Dicyclohexylcarbodi-imide method*										
(i)	A	CH_2Cl_2	1·18	1·34	84	131—134	−18·1	53	63·8	7·1	8·9
(ii)	A	,,	1·18	1·18	74	130—134	−18·3	54	64·1	7·7	8·4
(iii)	B(NEt_3)	,,	1·18	1·12	70	130—135	−5·5	16	64·5	7·3	8·5
(iv)	A; 0°	,,	1·18	1·24	77	138—141	−19·2	55	64·0	7·6	8·6
(v)	A; 0°	,,	1·18	1·25	78	139—143	−17·4	51	63·7	7·5	8·3
	5. *Tetraethyl pyrophosphite method* [h]										
(i)	A	Et_2PHO_3	0·59	0·59	74	142—148	−18·1	53	63·0	7·5	8·9
(ii)	A	,,	0·30	0·30	75	145—149	−16·2	48	63·2	7·6	8·9
(iii)	B(NEP)	,,	0·30	0·29	75	142—149	−8·4	25	63·4	7·5	8·7
(iv)	A	$CHCl_3$	2·35	2·61	82	146—150	−29·2	86	63·3	7·6	8·7
(v)	A	,,	2·35	2·68	84	148—152	−30·6	90	63·2	7·6	9·0
(vi)	B(NEP)	,,	0·59	0·64	80	142—147	−14·9	44	63·4	7·6	9·0
(vii)	B(NEP)	,,	0·59	0·68	85	141—145	−14·7	43	63·3	7·6	8·7
(viii)	B(NEt_3)	,,	2·35	2·65	83	139—143	−17·5	52	63·5	7·6	8·8
(ix)	B(NEt_3)	,,	2·35	2·56	80	141—144	−16·7	49	63·7	7·6	8·7
(x)	A [h]	,,	1·18	1·55	97	138—141	−16·6	48	63·3	7·6	8·8
(xi)	B(NEt_3) [h]	,,	1·18	1·25	79	141—144	−9·3	27	63·2	7·5	8·8
	6. *Carbonic mixed anhydride method*										
(i)	A	THF [i]	1·18	1·40	88	126—130	−6·1	18	63·8	7·3	9·0
(ii)	A	,,	1·18	1·36	85	126—131	−7·6	22	63·1	7·4	8·9
	7. *Phosphorazo-method*										
(i)	—	Pyridine	0·58	0·49	61	137—142	−0·6	2	63·6	7·4	9·0
(ii)	—	,,	0·58	0·46	58	134—140	−0·4	1	63·2	7·6	8·8
	8. *Phenylisoxazolium method*										
(i)	A	Me·CN	1·012	0·98	77	155—157	−32·8	96	63·8	7·7	8·6
(ii)	A	,,	1·012	0·96	75	156—157	−32·5	96	63·6	7·6	8·8
(iii)	B(NEt_3)	,,	1·012	1·04	81	145—148	−29·8	88	63·9	7·6	8·4
(iv)	B(NEt_3)	,,	1·012	0·99	75	150—153	−30·6	90	63·7	7·4	8·9
(v)	A	$MeNO_2$	0·71	0·75	78	140—143	−22·5	66	63·9	7·3	8·6
(vi)	A	,,	0·71	0·85	89	142—144	−21·6	63	64·1	7·5	8·5
(vii)	A	,,	0·24	0·27	84	143—146	−22·2	65	63·7	7·55	8·4
(viii)	B(NEt_3)	,,	0·71	0·77	80	141—146	−24·9	73	63·8	7·2	8·4
(ix)	B(NEt_3)	,,	0·71	0·79	82	141—144	−23·7	70	63·8	7·8	8·7
(x)	B(NEt_3)	,,	0·24	0·26	82	141—145	−23·1	68	63·5	7·7	8·4

TABLE. (*Continued.*)

Racemate isolated in above experiments.

Compound	M. p.	$[\alpha]_D$	Expts.	Compound	M. p.	$[\alpha]_D$	Expts.
None	—	—	1 (ii, iii), 2 (ii, iii), 3 (i, iii), 8 (i, ii)	DL-Ester	144—145°	−2·2°	5 (x)
				,,	145·5—146	−3·5	5 (xi)
DL-Acid	162·5—164°	—	3 (ii)	,,	144·5—145·5	−2·7	6 (i)
,,	,,	−4·5°	3 (iv)	,,	144—144·5	0	7 (i)
DL-Ester	143—144	−4·0	4 (i)	,,	144·5—145·5	0	7(ii), 8 (vi)
,,	145·0—145·5	−1·0	4 (iii)	DL-Acid	159—161	−4·5	8 (iii)
,,	144—145	—	5 (iii)	,,	162—163	−4·1	8 (iv)
DL-Acid	154—156	−5·8	5 (iv)	DL-Ester	144·5—146	−1·6	8 (viii)
DL-Ester	145·0—145·5	0	5 (ix)				

[a] Conditions: A, Distilled glycine ethyl ester used. B, Ester hydrochloride with an equivalent of the named tertiary amine (NMP = 1-methylpiperidine; NEP = 1-ethylpiperidine). C, 1 Equivalent of 1-methylpiperidine added to the azide. D, Cyanomethyl ester prepared at b. p. E, Cyanomethyl ester prepared at 0°. [b] Optical rotations were measured at 18—23° (*c* 2—4 in EtOH) in a 1 dm. tube. [c] Excluding L-isomer present as racemate. [d] Calc. for benzoyl-leucylglycine ethyl ester, $C_{17}H_{24}N_2O_4$: C, 63·7; H, 7·55; N, 8·75%. [e] 2·49 g. of benzoyl-L-leucylhydrazide. [f] 1·25 g. of hydrazide. [g] 1·19 g. of benzoyl-L-leucine *p*-nitrophenyl ester. [h] In expt. 5 (i—ix) the "standard procedure" was used, *i.e.*, the phosphite reagent was added to the solution of acid- and amino-components; in expt. 5 (x, xi) the "amide procedure" was used, *i.e.*, the phosphite reagent was caused to react with the amino-compound before addition of the acid-component. [i] THF = tetrahydrofuran.

contained some benzoyl-DL-leucine (readily identified by melting point and mixed melting point), but in every case this contaminant was removed by recrystallisation. The procedure used in this model reaction is, therefore, to establish the chemical purity of the crude product by elementary analysis, to determine the proportion of L-isomer in this material from its optical rotation, and to confirm this conclusion by fractional crystallisation of the ester or the acid.

There has been much divergence in the constants reported for benzoyl-L-leucine.[6–8] We benzoylated L-leucine (shown by paper chromatography [9] to be free from isoleucine) under controlled conditions, and purified the product as the cyclohexylammonium salt. From this was obtained benzoyl-L-leucine with specific rotation in excellent agreement with that found by Fischer.[6] We found, as did Fischer, that the optical rotation of the leucine formed by acid hydrolysis was low and varied with the conditions used for hydrolysis, but we believe our product to be optically pure because (*a*) diazomethane formed a methyl ester with the same rotation as that obtained by the benzoylation of L-leucine methyl ester, and (*b*) coupling with glycine ethyl ester by several procedures (including the acid azide route) gave peptide with the same rotation as that obtained by the benzoylation of L-leucylglycine ethyl ester. The differential solubility test,[10] with tetrachloroethane as solvent, proved insufficiently sensitive to be helpful in this case, owing to the small effect of solute on the refractive index of the solvent. It is likely that the easy racemisation during benzoylation of leucine is due to the formation of a mixed benzoic anhydride, and thence azlactone, and this is best avoided by keeping the solution strongly alkaline. Authentic benzoyl-L-leucylglycine ethyl ester was prepared by benzoylation of L-leucylglycine ethyl ester, and benzoyl-L-leucylglycine was prepared analogously.

The Table gives the results of an examination of several important coupling methods in this model reaction. First, only experiments with free glycine ester (Condition A) will be considered. Once again, no racemate could be isolated from the product of the acid azide route,[11] even when the coupling was carried out in the presence of a tertiary

[6] Fischer, *Ber.*, 1900, **33**, 2370.
[7] Karrer and Kehl, *Helv. Chim. Acta*, 1930, **13**, 50.
[8] Squibb Institute for Medical Research, referred to by Cornforth, "Chemistry of Penicillin," Princeton Univ. Press, 1949, p. 802.
[9] Work, *Biochim. Biophys. Acta*, 1949, **3**, 400.
[10] E. W. Williams and Young, *J.*, 1951, 1745.
[11] Curtius, *Ber.*, 1902, **35**, 3226.

amine. It had not proved possible to evaluate the cyanomethyl ester method [12] satisfactorily by coupling acetyl-L-leucine with glycine ester, since the bulk of the product was syrupy,[2] but with the benzoyl analogue the crude product crystallised and had a high rotation; no racemate could be isolated.

Recent work has shown that racemisation may occur during the preparation of *p*-nitrophenyl esters from benzyloxycarbonyldipeptides by the action of di-*p*-nitrophenyl sulphite [13] or tri-*p*-nitrophenyl phosphite,[14] and by the use of dicyclohexylcarbodi-imide with *p*-nitrophenol.[13] There have also been reports [15,16] of the loss of optical activity of such *p*-nitrophenyl esters in basic conditions. However, Goodman and Stueben [17] found that condensation of benzyloxycarbonylglycine with L-phenylalanine *p*-nitrophenyl ester (added as the hydrobromide with triethylamine) by means of dicyclohexylcarbodi-imide, followed by condensation of the product with glycine ethyl ester (added as the hydrochloride with triethylamine), gave no racemate. We prepared benzoyl-L-leucine *p*-nitrophenyl ester by benzoylation of L-leucine *p*-nitrophenyl ester; as shown in the Table, reaction with glycine ethyl ester in ethyl acetate or in chloroform gave peptide of high specific rotation, from which no racemate could be isolated, even after hydrolysis to the acid. Coupling by means of dicyclohexylcarbodi-imide [18] in dichloromethane caused markedly greater racemisation than with the acetyl analogue,[1] and reducing the coupling temperature to 0° [19] had little effect on the optical rotation of the product. The carbonic mixed anhydride method [20] also gave rise to greater racemisation in this case than with the acetyl derivative.[1] The use of tetraethyl pyrophosphite [21] in the " standard procedure " with the diethyl phosphite as solvent led to considerable racemisation, which was greatly reduced when chloroform was the solvent; we have found this solvent to be very satisfactory in these conditions, although the " amide procedure " gave much racemate.

The increased racemisation noted in the previous paper [1] to occur whenever glycine ester hydrochloride and an equivalent of tertiary amine were used in place of free glycine ester (*i.e.*, Condition B) is again apparent. With the carbodi-imide or the pyrophosphite method, there was a substantial fall in the optical activity of the product and even with the *p*-nitrophenyl ester some racemisation occurred. The experiments with pyrophosphite in chloroform are particularly instructive here, since the tertiary amine reacts with the glycine ethyl ester hydrochloride before addition of the pyrophosphite, and therefore racemisation cannot be caused by a temporary excess of tertiary amine, as may happen in other cases.[1] We have investigated this effect further, and we shall show, in a later paper, that halide anions can cause racemisation in such conditions. The " phosphorazo "-method,[4] with glycine ethyl ester hydrochloride in pyridine, gave nearly inactive product.

We were glad to be given the opportunity to investigate the use of 2-ethyl-5-*m*-sulphonatophenylisoxazolium. In the original report [22] on the use of this reagent, it was stated that coupling of benzyloxycarbonylglycyl-L-phenylalanine with glycine ethyl ester in acetonitrile gave 90% of L-peptide but also 2·2% of DL-peptide (the " Anderson test " [21]). Dr. Woodward informs us that this racemic product arose from a small amount of racemate in the benzyloxycarbonylglycylphenylalanine; when the experiment was repeated with optically pure dipeptide derivative, no racemisation was observed. In our model reaction, in which glycine ester hydrochloride was used with triethylamine, the optical rotation of

[12] Schwyzer, Iselin, and Feurer, *Helv. Chim. Acta*, 1955, **38**, 69.
[13] Iselin and Schwyzer, *Helv. Chim. Acta*, 1960, **43**, 1760.
[14] St. Guttmann, *Chimia*, 1960, **14**, 368.
[15] Bodanszky and Birkhimer, *Chimia*, 1960, **14**, 368.
[16] Stueben, *Diss. Abs.*, 1960, **20**, 4532.
[17] Goodman and Stueben, *J. Amer. Chem. Soc.*, 1959, **81**, 3980.
[18] Sheehan and Hess, *J. Amer. Chem. Soc.*, 1955, **77**, 1067.
[19] Anderson and Callahan, *J. Amer. Chem. Soc.*, 1958, **80**, 2902.
[20] Boissonnas, *Helv. Chim. Acta*, 1951, **34**, 874; Vaughan, *J. Amer. Chem. Soc.*, 1951, **73**, 3547: Wieland and Bernhard, *Annalen*, 1951, **572**, 190.
[21] Anderson, Blodinger, and Welcher, *J. Amer. Chem. Soc.*, 1952, **74**, 5309.
[22] Woodward, Olofson, and Mayer, *J. Amer. Chem. Soc.*, 1961, **83**, 1010.

the crude product allowed the presence of a small amount of racemate, and some nearly-racemic acid was isolated by fractional crystallisation. However, with free glycine ester, the optical rotation of the crude product was high, and no racemate could be isolated after hydrolysis to the acid. There was a marked difference when nitromethane was the solvent; considerable racemisation then occurred, even with free glycine ester.

As would be expected, the coupling of benzoyl-L-leucine appears to be more susceptible to racemisation than is that of the acetyl analogue, and it must again be emphasised that racemisation in these model reactions does not imply that it will necessarily occur in more normal cases. It is, however, to be noted that even with benzoyl-leucine no racemate could be found in the product from the azide method, in that from the active esters, or when the phenylisoxazolium reagent in acetonitrile was used provided that chloride ion was absent. The simple model reaction described here should therefore prove valuable in selecting methods of coupling which give no racemisation even with unfavourable acid components such as benzoyl-leucine.

Experimental

M. p.s were taken on a Kofler block. Infrared spectra were recorded on a Perkin-Elmer model 21 spectrophotometer. Solutions in organic solvents were dried over $MgSO_4$; evaporation was on the water-pump unless otherwise stated; light petroleum was of b. p. 60—80°; tertiary amines were dried over sodium and redistilled.

Benzoyl-L-leucine.—L-Leucine (13·1 g.; shown by paper chromatography with t-pentyl alcohol saturated with water to be free from isoleucine [9]) in 2N-sodium hydroxide (50 ml.) was benzoylated at 0—5° by benzoyl chloride (11·6 ml.) and 2N-sodium hydroxide (60 ml.) with stirring, the solution being kept always strongly alkaline. 15 Min. after the last addition, the solution was extracted with ether, the aqueous layer was made acid to Congo Red, and the oily product was extracted in ether. Cyclohexylamine (10 ml.) was added to the combined and dried ether extracts, giving *benzoyl-L-leucine cyclohexylammonium salt* (26 g., 78%). Recrystallisation from methanol–ether gave needles, m. p. 145—146°, $[\alpha]_D^{19}$ +14·4° (*c* 4·25 in EtOH) (Found: C, 68·2; H, 8·9; N, 8·4. $C_{19}H_{30}N_2O_3$ requires C, 68·3; H, 9·0; N, 8·4%). The salt (12·3 g.) was suspended in ethyl acetate (100 ml.) and shaken with 2N-hydrochloric aid (100 ml.). After separation, the aqueous layer was again extracted with ethyl acetate, the combined extracts were dried and then concentrated, whereafter addition of light petroleum gave crystalline benzoyl-L-leucine (7·6 g., 88%). Recrystallisation from ethyl acetate and light petroleum, or from chloroform and light petroleum, gave material of m. p. 106°. The specific rotation varies markedly with concentration: in N-KOH, $[\alpha]_D^{19}$ +6·1° (*c* 3·5), +5·7° (*c* 5·8), +4·8° (*c* 10·4), +3·9° (*c* 13·4); in EtOH, $[\alpha]_D^{23}$ −6·9° (*c* 2·6), −6·2° (*c* 4·35), −5·6° (*c* 7·7), −4·9° (*c* 10·3). For comparison with Fischer's product, a solution of 0·2110 g. in 1·2 ml. of N-KOH was made up to 2·4316 g. with water, giving $[\alpha]_D^{23}$ +6·5°; Fischer [6] gives $[\alpha]_D^{23}$ +6·59°, and m. p. 105—107°. Karrer and Kehl [7] obtained an oil of $[\alpha]_D^{20}$ −10·82° (in EtOH), $[\alpha]_D^{20}$ +6·36° (in N-KOH).

Benzoyl-L-leucine Methyl Ester.—(*a*) Benzoyl-L-leucine (1·0 g.) in methanol (5 ml.) was treated with ethereal diazomethane until the yellow colour persisted. After a further 30 min. the solvent was removed, leaving ester of m. p. 103—104°, $[\alpha]_D^{23}$ −22° (*c* 3·6 in EtOH) {lit.[7], m. p. 102°, $[\alpha]_D^{20}$ −21·14° (in EtOH)}.

(*b*) A solution of L-leucine methyl ester hydrochloride (18·2 g.) in water (10 ml.) was covered with ether (70 ml.), and the mixture was stirred. Sodium hydrogen carbonate (9·2 g.) was added, and then benzoyl chloride (14·4 ml.) in ethyl acetate (50 ml.) was added gradually and simultaneously with 2N-sodium hydrogen carbonate (160 ml.). Stirring was continued for a further hour, then pyridine was added and the solution was washed with water, hydrochloric acid, and aqueous potassium hydrogen carbonate, and dried. Removal of the solvent gave crystalline ester (24·2 g., 97%). Recrystallisation from ethyl acetate–light petroleum gave needles, m. p. 104°, $[\alpha]_D^{23}$ −22° (*c* 2·5 in EtOH).

Benzoyl-L-leucylglycine Ethyl Ester.—L-Leucylglycine ethyl ester acetate (syrup; obtained by catalytic hydrogenation of 7·0 g. of benzyloxycarbonyl-L-leucylglycine ethyl ester [23] in the presence of acetic acid [1]) was dissolved in water (20 ml.); ether (50 ml.) and sodium hydrogen

[23] Vaughan and Osato, *J. Amer. Chem. Soc.*, 1952, **74**, 676.

carbonate (3·7 g.) were added, followed by portions of benzoyl chloride (2·8 ml.) in ethyl acetate (40 ml.) and 2N-sodium hydrogen carbonate (20 ml.). Stirring was continued for 15 min. after the last addition, and the ester (4·68 g., 73%) was isolated as described above for benzoyl-L-leucine methyl ester. Recrystallisation from ethyl acetate–light petroleum gave *product* of m. p. 156·5—157°, $[\alpha]_D^{20}$ −34·0° (*c* 3·1 in EtOH) (Found: C, 63·7; H, 7·5; N, 8·8. $C_{17}H_{24}N_2O_4$ requires C, 63·7; H, 7·6; N, 8·8%).

Benzoyl-L-leucylglycine.—(*a*) L-Leucylglycine hydrobromide [24] (1·08 g.) was dissolved in water (10 ml.) containing sodium carbonate (2·12 g.), and benzoyl chloride (0·46 ml.) was added. The mixture was shaken at room temperature for 30 min., during which the sodium salt of the product separated; acidification to Congo Red was followed by extraction into ethyl acetate, which was then dried and evaporated. The resulting syrup (0·92 g., 79%) crystallised on seeding, and recrystallisation from ethyl acetate gave *benzoyl-L-leucylglycine* of m. p. 134—135°, $[\alpha]_D^{20}$ −26·4° (*c* 4·1 in ethanol) (Found: C, 61·9; H, 7·1; N, 9·5. $C_{15}H_{20}N_2O_4$ requires C, 61·6; H, 6·9; N, 9·6%).

(*b*) Benzoyl-L-leucylglycine ethyl ester (1·60 g.) was shaken with N-sodium hydroxide (5 ml.) in aqueous dioxan (3 : 1) at room temperature for 1 hr.; complete dissolution had then occurred. The solution was acidified to Congo Red, and the oil was extracted into ether (2 × 15 ml.), which was then dried. Evaporation left the *acid* as a glass, $[\alpha]_D^{23}$ −24·8° (*c* 4·1 in EtOH) (Found: C, 61·2; H, 7·3; N, 9·3%). The glass was dissolved in ether, and a solution of dicyclohexylamine in ether was added, to give the *dicyclohexylammonium salt*, which after recrystallisation from methanol–ether had m. p. 184—185°, $[\alpha]_D^{23}$ −5·0° (*c* 1·7 in EtOH) (Found: C, 68·6; H, 9·1; N, 8·9. $C_{27}H_{43}N_3O_4$ requires C, 68·5; H, 9·2; N, 8·9%). The purified salt was shaken with ethyl acetate and dilute sulphuric acid, and the organic layer was dried. Evaporation gave an uncrystallisable syrup. In an attempt to crystallise the oil which appeared on acidification at the end of the hydrolysis, it was dissolved by warming it with the addition of water, and the solution was put aside at room temperature. After several weeks, crystals (40 mg.; m. p. 137—139°) appeared and were shown to be benzoyl-DL-leucine by mixed m. p.

Benzoyl-DL-leucylglycine Ethyl Ester.—Benzoyl-DL-leucine [6] (4·7 g.), glycine ethyl ester (2·0 ml.), and tetraethyl pyrophosphite in dry chloroform (25 ml.) were heated under reflux for 30 min. After cooling and dilution with chloroform, the solution was washed with dilute hydrochloric acid, aqueous sodium hydrogen carbonate, and water, and dried. Evaporation at 15 mm. and then at 100°/0·2 mm. gave crystals (6·3 g., 98%). Recrystallisation from ethyl acetate–light petroleum gave *ester* of m. p. 146° (Found: C, 63·5; H, 7·6; N, 8·4%).

Benzoyl-DL-leucylglycine.—The above ester was saponified as in the case of the L-isomer. Acidification after the hydrolysis gave an oil, which was redissolved by warming and dilution with water. Crystals were deposited on cooling, and recrystallisation from water gave *acid* of m. p. 165° (Found: C, 61·4; H, 6·9; N, 9·8%).

Procedure for Confirming the Partial Racemisation of Benzoyl-leucylglycine Ethyl Ester: Control Experiments with Synthetic Mixtures.—Mixtures containing 0—20% of racemate were saponified by shaking 1·0 g. with N-sodium hydroxide (5 ml.) in aqueous dioxan (3 : 1) at room temperature for 1 hr. The solution was made just acid to Congo Red with dilute hydrochloric acid, and just sufficient water was added to dissolve the oil at 100°. If no crystals appeared on cooling, the solution was seeded with benzoyl-DL-leucylglycine and left at room temperature for 1—2 weeks. The crystals which appeared were recrystallised from water to remove benzoyl-DL-leucine (m. p. 141°). No crystals were deposited from the solution obtained from the pure ester, or from the mixture containing 1% of racemate. The amount of recrystallised material obtained from the 2% mixture was insufficient for reliable measurement of optical rotation, but its identity was confirmed by a mixed m. p. determination. Details are tabulated.

DL-Isomer in mixture (%)	Crystals deposited		Recryst. material		
	Wt. (g.)	M. p.	Wt. (g.)	M. p.	$[\alpha]_D^{23}$*
2	0·13	138—139°	0·009	162—163·5°	—
5	0·047	137—139	0·019	162·5—163·5	−3·4°
10	0·07	146—150	0·014	163—164	−2·2
20	0·08	159—161	0·04	163·5—164·5	0·0

* *c* 1—2 in EtOH.

[24] Farrington, Hextall, Kenner, and Turner, *J.*, 1957, 1407.

Investigation of Racemisation during Coupling: General.—The normal procedure after each coupling reaction was to wash the solution of the product in the organic solvent with small volumes of 2N-hydrochloric acid, saturated aqueous sodium hydrogen carbonate, and water; the solution was dried and evaporated. The solid residue was weighed and analysed, and the specific rotation was determined. From this, the percentage of L-peptide (excluding that present as racemate) was calculated. When the specific rotation was low, recrystallisation of the whole or part of the crude product from acetone–ether usually gave largely racemic ester (characterised by m. p. and low specific rotation); otherwise, the crude product was saponified as described above for the experiment with synthetic mixtures, and attempts were made to detect racemic acid. The constants of the racemic ester or acid, obtained in this way, are given in the Table. Figures not given in the descriptions which follow will be found in the Table.

(1) *Acid Azide Method.*[11]—(*a*) *Benzoyl-L-leucylhydrazide.* Benzoyl-L-leucine methyl ester (2·92 g.) was dissolved in ethanol (20 ml.) at room temperature and 100% hydrazine hydrate (1·1 ml.) was added. Next day the solvent was removed, leaving a solid which was recrystallised from ethyl acetate–light petroleum, giving the *hydrazide* (2·55 g., 87%), m. p. 155°, $[\alpha]_D^{20}$ −5·4° (*c* 3·0 in N-HCl) (Found: C, 62·7; H, 7·4; N, 16·7. $C_{13}H_{19}N_3O_2$ requires C, 62·6; H, 7·7; N, 16·9%).

(*b*) *Coupling through the acid azide.* (i) Benzoyl-L-leucylhydrazide (2·49 g.) was dissolved in a mixture of water (10 ml.), concentrated hydrochloric acid (2 ml.), and glacial acetic acid (2 ml.). The solution was cooled to 0° and sodium nitrite (1·4 g.) in the minimum amount of water was added dropwise, with stirring. A colourless solid separated, which dissolved during extraction with ether (4 × 15 ml.). The ethereal solution of the azide was dried at 0° for 10 min., filtered, and cooled to 0°, then glycine ethyl ester (2·0 ml.) was added dropwise with stirring. A pale brown oil separated, which partially solidified after the solution had been stirred overnight at room temperature. Ethyl acetate (40 ml.) and chloroform (25 ml.) were added, and the product was then isolated as usual. In Expt. (ii) the scale was halved. In Expt. (iii) and (iv) 1-methylpiperidine (1·2 ml., 2 mol.) was added to the ethereal solution of azide before the addition of glycine ester.

(2) *Cyanomethyl Ester Method.*[12]—(*a*) *Ester formed at the b. p.* (i) Benzoyl-L-leucine (2·35 g.), chloroacetonitrile (1·27 ml.), and triethylamine (2·07 ml.) were dissolved in ethyl acetate (10 ml.) and boiled under reflux for 3 hr. The solution was then diluted with ethyl acetate, filtered, washed with water, dilute hydrochloric acid, and again water, and dried. Evaporation gave a syrup which solidified to a white solid (2·70 g., 100%). This cyanomethyl ester was dissolved in ethyl acetate (10 ml.), and glycine ethyl ester (1·1 ml.) was added. Next day, ethyl acetate (30 ml.) was added to dissolve crystals which had separated, and the product was then isolated as usual. Expt. (ii) was similar.

(*b*) *Ester formed at* 0°. (iii) Benzoyl-L-leucine (1·18 g.) was added portionwise to a stirred mixture of triethylamine (1·05 ml.) and chloroacetonitrile (0·64 ml.) at 0° during 15 min. Stirring at 0° was continued for a further 30 min., and then at room temperature overnight. Ethyl acetate (20 ml.) was added, and thereafter the procedure followed that in (*a*) above, except that 0·5 ml. of glycine ester was used. Expt. (iv) was similar.

(3) p-*Nitrophenyl Ester Method.*[25]—(*a*) *Benzoyl-L-leucine* p-*nitrophenyl ester.* L-Leucine *p*-nitrophenyl ester hydrobromide [17] (3·33 g.) was suspended in ethyl acetate (25 ml.) containing benzoyl chloride (1·16 ml.); the mixture was stirred vigorously while a solution of sodium carbonate (2·65 g.) in water (15 ml.) was added. Stirring was continued for a further 30 min., and the organic layer separated, dried, and evaporated, to give a syrup which crystallised. Recrystallisation from ethyl acetate–light petroleum gave the *ester* (3·0 g.), m. p. 96—97°, $[\alpha]_D^{23}$ −43·2° (*c* 3·4 in EtOH) (Found: C, 63·9; H, 5·5; N, 7·9. $C_{19}H_{20}N_2O_5$ requires C, 64·0; H, 5·6; N, 7·9%).

(*b*) *Coupling of the* p-*nitrophenyl ester.* (i) To a solution of benzoyl-L-leucine *p*-nitrophenyl ester (1·19 g.) in ethyl acetate (3 ml.) was added glycine ethyl ester (0·34 ml.). Next day the product was isolated in the usual way. In Expt. (ii) the glycine ester was replaced by glycine ethyl ester hydrochloride (0·47 g.) and 1-methylpiperidine (0·40 ml.); Expt. (iii) and (iv) were analogous, but with chloroform as solvent.

(4) *Carbodi-imide Method.*[18]—(i) Benzoyl-L-leucine (1·18 g.) and glycine ethyl ester (0·5 ml.) were dissolved in dry dichloromethane (15 ml.), and a solution of dicyclohexylcarbodi-imide

[25] Bodanszky, *Acta Chim. Acad. Sci. Hung.*, 1957, **10**, 335.

(1·03 g.) in dichloromethane (10 ml.) was added slowly with stirring, which was continued for 23 hr. A few drops of glacial acetic acid were added, and after 30 min. the dicyclohexylurea was filtered off and the solvent was evaporated. The residue was taken up in ethyl acetate (50 ml.), and the product was isolated as usual. Expt. (ii) was similar; in Expt. (iii), glycine ethyl ester hydrochloride (0·70 g.) and triethylamine (0·69 ml.) replaced the glycine ester. Expt. (iv) and (v) were analogous to (i) and (ii) except that the reaction was carried out at 0°.

(5) *Tetraethyl Pyrophosphite Method.*[21]—The tetraethyl pyrophosphite was prepared by Maclaren's method [26] and had b. p. 60—75°/0·3 mm., n_D^{25} 1·4301; it was free from chloride. The quantities of reagents used in these experiments are given in the annexed Table.

Expt.	(i)	(ii)	(iii)	(iv)	(v)	(vi)	(vii)	(viii)	(ix)	(x)	(xi)
Benzoyl-L-leucine, g.	0·59	0·30	0·30	2·35	2·35	0·59	0·59	2·35	2·35	1·18	1·18
Glycine ester, ml. ...	0·25	0·13	—	1·10	1·10	—	—	—	—	0·50	—
Glycine ester HCl, g.	—	—	0·17	—	—	0·35	0·35	1·40	1·40	—	0·70
Tert. amine, ml. ...	—	—	0·17 (NEP)	—	—	0·35 (NEP)	0·35 (NEP)	1·38 (NEt_3)	1·38 (NEt_3)	—	0·70 (NEt_3)
Et_2 phosphite, ml.[4]	4	2	2	—	—	—	—	—	—	—	—
Chloroform, ml.	—	—	—	10	10	2·5	2·5	10	10	5	5
Et_4 pyrophosphite, ml.	0·8	0·4	0·4	3·0	3·0	0·75	0·75	3·0	3·0	1·5	1·5

NEP = 1-ethylpiperidine.

(*a*) "*Standard procedure*" [Expt. (i)—(ix)]. The benzoyl-leucine and glycine ethyl ester were dissolved in the stated solvent and tetraethyl pyrophosphite was added. When diethyl phosphite was the solvent, the solution was heated at 100° for 30 min., and then evaporated to dryness at 100°/20 mm. When chloroform was the solvent, the solution was refluxed for 30 min., then evaporated to dryness on the water-pump. In each case the residue was taken up in ethyl acetate, and the product was isolated in the usual way.

(*b*) "*Amide procedure*" [Expt. (x) and (xi)]. The glycine ethyl ester was dissolved in chloroform, tetraethyl pyrophosphite was added, and the solution was refluxed for 2 min. Benzoyl-leucine was added, and boiling was continued for another 30 min. A further 15 ml. of chloroform were added, and the product was isolated as usual.

(6) *Carbonic Mixed Anhydride Method.*[20]—(i) Benzoyl-L-leucine (1·18 g.) was dissolved in dry tetrahydrofuran (12 ml.) and cooled to −5°. Triethylamine (0·70 ml.) was added, followed by isobutyl chloroformate (b. p. 128—130°; 0·66 ml.) dropwise and with stirring. After 3 min., glycine ethyl ester (0·5 ml.) in tetrahydrofuran (6 ml.) was added slowly. Stirring was continued at room temperature overnight. The solution was then filtered, the residue was washed with ethyl acetate, and the combined solutions were evaporated to dryness. The crystalline residue was taken up in ethyl acetate (20 ml.) and chloroform (5 ml.), and the product was isolated as usual. Expt. (ii) was a duplicate.

(7) *The "Phosphorazo-method."* [4]—Glycine ethyl ester hydrochloride (0·35 g.) was added to pyridine (4 ml.), and the suspension was cooled to 0°. Phosphorus trichloride (0·11 ml.) in pyridine (1 ml.) was added, and the yellow solution was left at room temperature for 30 min. Benzoyl-L-leucine (0·58 g.) and pyridine (1 ml.) were then added, and the solution was heated to 100° for 3 hr. After cooling, water (10 ml.) and ethyl acetate (30 ml.) were added; the peptide was isolated as usual, and then triturated with light petroleum.

(8) *Phenylisoxazolium Method.*[22]—(i) 2-Ethyl-5-*m*-sulphonatophenylisoxazolium (1·012 g.) was stirred in acetonitrile (10 ml.) at 0°. Benzoyl-L-leucine (0·94 g.) and 0·5M-triethylamine in acetonitrile (8 ml.; 1 equiv. of amine) were added and stirring at 0° was continued for 1 hr.; then glycine ethyl ester (0·4 ml.) was added. Stirring was continued at room temperature for 22 hr.; dissolution was almost complete. The solvent was removed by evaporation, the residue taken up in ethyl acetate (80 ml.), and the product isolated as usual. Expt. (ii) was a duplicate. Expt. (iii) and (iv) were similar except that glycine ethyl ester hydrochloride (0·56 g.) and triethylamine (0·55 ml.) replaced the glycine ester. The experiments with nitromethane as solvent were analogous except that the appropriate amount of the phenylisoxazolium reagent was stirred with the benzoyl-L-leucine for 7 min. at room temperature, before addition of the ester.

[26] Maclaren, Proc. Internat. Wool Textile Res. Conf., Australia, 1955, Vol. C, p. 168.

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THE DYSON PERRINS LABORATORY, OXFORD UNIVERSITY. [*Received, June 24th,* 1962.]