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The Preparation of Peptides Using Mixed Carbonic–Carboxylic Acid Anhydrides

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Mixed anhydrides of N-substituted aminoacids or peptides with alkylcarbonic acids are formed at low temperature under anhydrous conditions and allowed to react with aminoacid or peptide esters or with aminoacid salts to give good yields of the corresponding peptides or higher peptides. Choice of the alkylcarbonic acid used is not critical and the reaction is not complicated by the formation of unfavorable by-products. The optimum conditions for the reaction have been determined and applied in the preparation of a series of peptides esters. No racemization of optically-active materials has been observed by this method.

In a continuation of our investigations on the use of mixed anhydrides as acylating agents for the synthesis of peptides,[1] an examination was made of the behavior of mixed anhydrides of N-substituted aminoacids and alkylcarbonic acids. These anhydrides, or acylalkylcarbonates, were of particular interest since theoretically they offered the advantage that, in any predominantly amide-forming reaction, the only by-products formed would be carbon dioxide and an alcohol derived from the alkylcarbonic acid used; *i.e.*

$$X\text{—NHCHRCOOCOOR}' + H_2NR'' \longrightarrow X\text{—NHCHRCONHR}'' + CO_2 + R'OH$$

Amides or peptides prepared by this method, therefore, should be obtained initially in a high state of purity.

In preliminary experiments, it was found that anhydrides of the desired type were unstable under ordinary conditions but could be prepared readily provided a sufficiently low temperature was used. In this manner, a mixed anhydride of N-carbobenzoxyglycine and ethylcarbonic acid was formed by allowing the triethylamine salt of the N-substituted aminoacid to react with ethyl chlorocarbonate in toluene solution at −5°. On addition of an equivalent of aniline to this solution, carbon dioxide was rapidly evolved and carbobenzoxyglycinanilide was formed in good yield and in a high state of purity.[2]

Since the reaction occurred readily and in the manner desired, a set of standardized conditions was selected, and the use of other alkylchlorocarbonates in the preparation of carbobenzoxyglycinanilide was investigated. In view of our previous observations on the influence of both steric and inductive effects on the acylation ratios obtained using mixed carboxylic acid anhydrides,[1] the comparison of a series of chlorocarbonates containing progressively branched alkyl groups was of particular interest. The results of this comparison are summarized in Table I.

Only slight differences in yields were observed among the various alkylchlorocarbonates tested and steric effects of the particular alkyl group used, therefore, appear to be of minor importance. The *s*- and isobutylchlorocarbonates, however, seemed to offer some slight advantage among the simpler members of the series and were selected for further study.

(1) J. R. Vaughan, Jr. and R. L. Osato, THIS JOURNAL, **73**, 5553 (1951).

(2) Since the completion of the work reported here, publications have appeared simultaneously by R. A. Boissonnas, *Helv. Chim. Acta*, **34**, 874 (1951), and by T. Wieland and H. Bernhard, *Ann.*, **572**, 190 (1951), in which essentially the same general reaction and conditions are described.

TABLE I

PREPARATION OF N-CARBOBENZOXYGLYCINANILIDE

Alkylchlorocarbonate	Yield, %[a]	M.p., °C. (cor.)[b]
Ethyl	58[c]	146–147
Isopropyl	49	146–147
Isobutyl	68	146–147
s-Butyl	70	146–147
2-Ethylbutyl	55	144–145
1,3-Dimethylbutyl	64	144–146
1-Ethylamyl	67	141–145
1-(2-Methylpropyl)-3-methylbutyl	65	144–145
Cyclohexyl	61	145–146
3,3,5-Trimethylcyclohexyl	61	145–146
Benzyl	44	146–147

[a] Initially isolated material before recrystallization. [b] The literature m.p. is 144° (ref. 5). [c] Recrystallized from methanol. The initial yield was 72% melting at 135–142°.

To determine optimum reaction conditions for the preparation of N-substituted peptide esters using these reagents, variations in reaction time, temperature and concentration were made in the preparation of a single peptide, namely, ethyl carbobenzoxyglycyl-DL-phenylalaninate, using *s*-butylchlorocarbonate. The relative amount of solvent used or slight excesses of either aminoacid reactant or of triethylamine were without effect. An excess (50%) of the chlorocarbonate, however, led to an impure product, although the yield after crystallization was not seriously affected. Moisture in the toluene or chloroform used as solvents caused a 10–15% lowering of yield. Temperature was the most critical condition and the reaction was found to be essentially inoperative at greater than +15° and yields were found to drop off rapidly at less than −20°. The anhydride-forming reaction appeared to be complete in 25 minutes at −5° and the amide-forming reaction in varying times, depending upon the time required for the reaction mixture to warm to room temperature after the addition of the aminoacid ester.

From the above observations, a reaction time of 25−30 minutes at −5° was selected for the anhydride-forming step and overnight standing at room temperature, after addition of the aminoacid or peptide ester to the cold mixture, for the amide-forming step. These general conditions were then applied to the preparation of the N-substituted peptide esters listed in Table II. The quantities of chloroform or toluene or mixtures of these used as solvents in the preparation of the peptides listed varied with the individual solubilities of the reagents but, in general, were between 25–50 cc. per 0.01 mole of reactant. In all cases,

TABLE II

PREPARATION OF N-SUBSTITUTED PEPTIDE ESTERS

We are indebted to Dr. J. A. Kuck and his staff of these laboratories for the microanalyses. The values reported are the average of two values differing by not more than 0.30.

Product	Anhydride-forming reagent: s-Butylchlorocarbonate Yield, %	M.p., °C. (cor.)	$[\alpha]^{24}D$ (ethanol)	Isobutylchlorocarbonate Yield, %	M.p., °C. (cor.)	$[\alpha]^{24}D$ (ethanol)
Ethyl carbobenzoxyglycyl-DL-phenylalaninate[a]	71	91–92		64	91–92	
Ethyl carbobenzoxyglycyl-L-tyrosinate[b]	51	124–125	+19.8° ± 0.2(c 5)	68	125–126.5	+19.3° ± 0.1(c 10)
Ethyl carbobenzoxy-DL-alanyl-DL-phenylaninate[c]	41	110–112				
Ethyl carbobenzoxy-DL-valyl-DL-alanininate[d]	65	111–113				
Ethyl carbobenzoxy-DL-valyl-L-leucinate[e]	68	103–104				
Ethyl carbobenzoxy-DL-valyl-DL-phenylalaninate[f]	47	138–140				
Ethyl carbobenzoxy-L-leucylglycinate[g]	61	104–105	−25.6 ± 0.2(c 5)	53	104–105	−25.2 ± 0.4(c 2.5)
Methyl carbobenzoxy-L-leucyl-L-leucinate[h]	64	93–95	−35.8 ± 0.2(c 2.5)	71	94–96	−35.6 ± 0.2(c 2.5)
Ethyl carbobenzoxy-L-leucyl-L-tyrosinate[i]	63	116–118	−14.9 ± 0.1(c 10)			
Ethyl dicarbobenzoxy-L-lysylglycinate[j]	64	92–93	−12.0 ± 0.3(c 4)	64	92–93	−11.6 ± 0.2(c 5)
Ethyl carbobenzoxy-L-phenylalanyl-L-tyrosinate[k]	46	159–160	−9.4 ± 0.1(c 10)			
Ethyl carbobenzoxyglycyl-DL-phenylalanylglycinate[l]	76	131–132		83	132–134	
Ethyl carbobenzoxyglycyl-DL-phenylalanylglyclglycinate[m]	65	172–174				
Ethyl carbobenzoxyglycyl-DL-phenylalanyl-DL-phenylalanyl-glycylglycinate[n]	59	180–184				
Ethyl carbobenzoxy-DL-alanyl-DL-phenylalanyl-DL-valyl-L-leucinate[o]	36	180–190				
Ethyl phthaloylglycyl-L-leucinate[p]	67	143–145	−28.5 ± 0.5 (c 2)	57	144–145	−29.0 ± 0.5(c 2)
Ethyl phthaloylglycyl-L-tyrosinate[q]	58	164–165	+46.0 ± 0.5(c 2)	58	164–165	+44.5 ± 0.5(c 2)
Ethyl phthaloyl-DL-leucyl-DL-phenylalaninate[r]	55	107–110				
Ethyl phthaloyl-DL-phenylalanylglycylglycinate[s]	67	162–163				

[a] H. Neurath, *et al.*, *J. Biol. Chem.*, **170**, 221 (1947), give m.p. 90–91° (cor.). [b] M. Bergmann and J. S. Fruton, *ibid.*, **118**, 405 (1937), give m.p. 118°; ref. 1 gives m.p. 125–127° and $[\alpha]^{24}D$ +18.4° (*c* 5, ethanol). [c] Ref. 1 gives m.p. 114–116° (cor.). [d] Calcd. for $C_{18}H_{26}N_2O_5$: C, 61.70; H, 7.47; N, 8.00. Found: C, 61.47; H, 7.59; N, 8.23. [e] Calcd. for $C_{21}H_{32}N_2O_5$: C, 64.26; H, 8.22; N, 7.14. Found: C, 64.39; H, 8.39; N, 7.18. [f] Calcd. for $C_{24}H_{30}N_2O_5$: C, 67.58; H, 7.09; N, 6.57. Found: C, 67.74; H, 7.27; N, 6.54. [g] M. Bergmann, *et al.*, *J. Biol. Chem.*, 111, 225 (1935), give m.p. 103–104°; M. A. Nyman and R. M. Herbst, *J. Org. Chem.*, **15**, 108 (1950), give m.p. 99° and $[\alpha]^{25}D$ −26.8° (*c* 2.6, ethanol). [h] M. A. Nyman and R. M. Herbst, ref. *g*, give m.p. 97–98°. Ref. 1 gives $[\alpha]^{24}D$ −35.3° (*c* 10, ethanol). [i] Ref. 1 gives m.p. 115–117° and $[\alpha]^{24}D$ −15.2° (*c* 5, ethanol). [j] M. Bergmann, *et al.*, *Z. phyisol. Chem.*, **224**, 26 (1934), give m.p. 90°; ref. 1 gives m.p. 89–91° and $[\alpha]^{24}D$ −12.0° (*c* 5, ethanol). [k] J. S. Fruton and M. Bergmann, *J. Biol. Chem.*, **145**, 262 (1942), give m.p. 162°. [l] Prepared from carbobenzoxyglycyl-DL-phenylalanine and ethyl glycinate. Ref. 1 gives m.p. 132–133°. [m] Prepared from carbobenzoxyglycyl-DL-phenylalanine and ethyl glycylglycinate. Calcd. for $C_{25}H_{30}N_4O_7$: C, 60.23; H, 6.07; N, 11.24. Found: C, 60.15; H, 6.15; N, 11.40. [n] Prepared from carbobenzoglycyl-DL-phenylalanine and ethyl DL-phenylalanylglycylglycinate. Calcd. for $C_{34}H_{39}N_5O_8$: C, 63.24; H, 6.09; N, 10.85. Found: C, 63.24; H, 6.33; N, 11.08. [o] Prepared from carbobenzoxy-DL-alanyl-DL-phenylalanine and ethyl DL-valyl-L-leucinate. Calcd. for $C_{33}H_{46}N_4O_7$: C, 64.90; H, 7.59; N, 9.17. Found: C, 64.88; H, 7.60; N, 9.15. [p] Ref. 1 gives m.p. 139–140° and $[\alpha]^{24}D$ −24.5° (*c* 2, ethanol). [q] Ref. 1 gives m.p. 163–164° and $[\alpha]^{24}D$ +43.0 (*c* 2, ethanol). [r] Calcd. for $C_{25}H_{28}N_2O_5$: C, 68.79; H, 6.47; N, 6.42. Found: C, 68.80; H, 6.57; N, 6.54. [s] Calcd. for $C_{23}H_{23}N_3O_6$: C, 63.15; H, 5.30; N, 9.61. Found: C, 63.36; H, 5.36; N, 9.82.

purification was effected by recrystallization from ethyl acetate–petroleum ether or alcohol–water mixtures.

As originally anticipated, the reaction leads to products of high initial purity. Other advantages have been found to be the rapidity with which the method may be carried through, the ease of purification of the products and the apparent complete lack of by-product formation. In some of the preparations of the racemic peptide esters, small amounts of secondary products have been isolated, but these have been shown by analysis to be simply a lower melting mixture of racemates and chemically the same as the main product obtained. Racemization apparently does not occur under these conditions, since the optically-active peptides prepared by this and by other methods have the same rotations.

It has also been possible to apply this reaction to the preparation of the free acids of N-substituted peptides, but the yields have been inferior to those obtained with the esters, probably due to the necessity of having to use an aqueous medium to obtain solution of the aminoacid salt. In this modification, the intermediate mixed anhydride is formed as described above and a solution of one equivalent of an aminoacid in one equivalent of 1 or 2 *N* sodium hydroxide is then added. After stirring the heterogeneous reaction mixture vigorously for several hours, during which time it is allowed to warm to room temperature, the aqueous layer is separated, extracted with ether and acidified to precipitate the formed peptide acid as an oil which slowly crystallizes. Several examples of this reaction are given in the Experimental section.

Experimental[2]

Preparation of Alkylchlorocarbonates.—The general procedure used consisted of first condensing a 50% molar excess of phosgene in a reaction vessel cooled in a Dry Ice–acetone-bath. The vessel was then transferred to an ice-bath and the alcohol added dropwise with stirring over a 20-minute period. No solvent was used, except with 3,3,5-trimethylcyclohexanol in which case the solid alcohol was dissolved in benzene to facilitate its addition. After overnight standing at room temperature, the reaction mixture was placed under vacuum for 1–2 hours to remove excess phosgene and the product was then obtained by distillation. The previously unreported chlorocarbonates used are listed below along with their properties and the melting point and analysis of their carbamate derivatives. With the exception of *s*-butanol, the alcohols were obtained from Union Carbide and Carbon Corporation.

Carbobenzoxyglycinanilide.—A solution of 5.23 g. (0.025 mole) of carbobenzoxyglycine and 2.55 g. (0.025 mole) of triethylamine in 50 cc. of toluene was cooled to −5° and

(2) All melting points were taken on a Fischer–Johns block and are corrected.

TABLE III

Chlorocarbonate	Yield, %	B.p., °C.	Mm.	n^tD	t	Carbamate m.p., °C.	Formula	Analyses, % Calculated C	H	N	Found C	H	N
s-Butyl-	50	115–117		1.4037	24	85.5–87.5	$C_8H_{16}NO_2$	51.26	9.47	11.95	51.27	9.67	12.00
2-Ethylbutyl-	56	67	13	1.4219	27	82–83.5	$C_7H_{16}NO_2$	57.90	10.41	9.65	57.83	10.60	9.52
1,3-Dimethylbutyl-	71	47–50	11	1.4142	25	80.5–81.5	$C_7H_{16}NO_2$	57.90	10.41	9.65	57.78	10.50	9.54
1-Ethylamyl-	33	68	11	1.4215	25	45–46	$C_8H_{17}NO_2$	60.34	10.76	8.80	60.49	10.69	8.97
1-(2-Methylpropyl)-3-methylbutyl-	31	81	14	1.4231	25	113–113.5	$C_{10}H_{21}NO_2$	64.13	11.30	7.48	64.11	11.21	7.27
3,3,5-Trimethylcyclohexyl-	81	97	14	1.4508	25	86–87.5	$C_{10}H_{19}NO_2$	64.83	10.34	7.56	64.80	10.16	7.52

3.42 g. (0.025 mole) of *s*-butylchlorocarbonate added. After 25 minutes at this temperature, during which time triethylamine hydrochloride separated, 2.34 g. (0.025 mole) of aniline was added. Rapid carbon dioxide evolution began immediately and was essentially complete after several minutes. The reaction mixture was then allowed to warm to room temperature and stand overnight. The carbobenzoxyglycinanilide crystallized from the reaction mixture and was filtered off together with triethylamine hydrochloride, washed with water, dilute sodium hydroxide solution and dilute hydrochloric acid and dried; wt. 4.97 g. (70%); m.p. 146–147°. The same reaction conditions were followed exactly in comparing the yields obtained with the different chlorocarbonates listed in Table I. An additional 2–4% of product could be obtained from the toluene filtrates in most cases, but this was usually low melting and was ignored in the above comparison.

Preparation of N-Substituted Peptide Esters and Acids.—In general, the peptide esters were prepared as described above for carbobenzoxyglycinanilide. Their isolation and purification, however, varied considerably and several examples are given to illustrate these differences. The preparation of three peptide acids is described separately. All solvents and reagents were dried and purified and precautions were taken to exclude moisture from the reaction mixtures, except when water was used in the preparation of the peptide acids.

Ethyl Carbobenzoxy-DL-valyl-DL-alaninate.—A solution of 25.1 g. (0.1 mole) of carbobenzoxy-DL-valine and 10.2 g. (0.1 mole) of triethylamine in 200 cc. of toluene was cooled to −5° and 13.7 g. (0.1 mole) of *s*-butylchlorocarbonate added. The reaction mixture set to a white solid mass. After 25 minutes a precooled solution of 11.7 g. (0.1 mole) of ethyl DL-alaninate in 100 cc. of toluene was added. Extremely rapid carbon dioxide evolution occurred at once and cooling, therefore, was continued for 2 hours to allow this initial reaction to subside. The reaction mixture was then allowed to stand at room temperature overnight, and the product which crystallized out (36%) was filtered off, along with triethylamine hydrochloride, and washed with water. The organic phase was separated from the filtrate, washed with 3% sodium bicarbonate solution and dried over sodium sulfate. On diluting this solution until cloudy with petroleum ether and cooling, a second crop (42%) of colorless product crystallized. The two crops were combined and recrystallized from 1 liter of ethyl acetate–petroleum ether (1:9) to give 22.7 g. (65%) of pure material, m.p. 111–113°.

Carbobenzoxy-DL-valyl-L-leucinate.—A solution of the valyl mixed anhydride was prepared as described above and a second precooled solution of 19.6 g. (0.1 mole) of ethyl L-leucinate hydrochloride and 10.2 g. (0.1 mole) of triethylamine in 200 cc. of chloroform added. Carbon dioxide evolution was very rapid. After 2 hours, the reaction mixture was removed from the cooling bath and allowed to stand overnight at room temperature. The reaction mixture, which contained some insoluble triethylamine hydrochloride, was washed with water and with 3% sodium bicarbonate solution and dried over sodium sulfate. The solution was then filtered and concentrated in an air stream on a steam-bath until practically all of the solvent was removed. The residue was diluted with 1 liter of petroleum ether to give a clear solution from which the product rapidly crystallized as colorless needles on cooling; wt. 28.3 g. (72%); m.p. 100–102°. Recrystallization from a mixture of 50 cc. of ethyl acetate and 800 cc. of petroleum ether gave 26.8 g. (68%) of pure material; m.p. 103–104°.

Ethyl Carbobenzoxyglycyl-DL-phenylalanylglycylglycinate.—A solution of 7.13 g. (0.02 mole) of carbobenzoxyglycyl-DL-phenylalanine and 2.04 g. (0.02 mole) of triethylamine in a mixture of 50 cc. of toluene and 25 cc. of chloroform was cooled to −5° and 2.73 g. (0.02 mole) of *s*-butylchlorocarbonate added. After 25 minutes a second, precooled solution of 3.93 g. (0.02 mole) of ethyl glycylglycinate hydrochloride and 2.04 g. (0.02 mole) of triethylamine in 35 cc. of chloroform was added with good mixing. The reaction mixture was then allowed to stand overnight at room temperature. The product, which had crystallized, was filtered off along with triethylamine hydrochloride and washed well with water; wt. 8.80 g. (88%); m.p. 169–171°. Recrystallization of this from 1 liter of 50% alcohol gave 6.45 g. (65%) of pure product melting at 172–174°. No additional product was found in the organic phase of the original reaction mixture.

Carbobenzoxyglycyl-DL-phenylalanine.—A solution of 4.18 g. (0.02 mole) of carbobenzoxyglycine and 2.04 g. (0.02 mole) of triethylamine in 50 cc. of toluene was cooled to −5° and 2.73 g. (0.02 mole) of isobutylchlorocarbonate added. After 25 minutes a solution of 3.28 g. (0.02 mole) of DL-phenylalanine in 20 cc. of 1 *N* sodium hydroxide was added and the mixture was stirred vigorously for 2–3 hours while allowing it to warm to room temperature. Carbon dioxide was given off during this time. The aqueous phase was isolated, extracted with ether and acidified with hydrochloric acid to precipitate the product as a colorless oil which crystallized rapidly on cooling. After two recrystallizations from ethanol–water, 4.50 g. (63%) of pure product of m.p. 160–162° was obtained. The literature m.p. is 159.5–160.5°.[4]

Carbobenzoxyglycyl-DL-valine.—A solution of the intermediate mixed anhydride was prepared as just described but using *s*-butyl instead of isobutylchlorocarbonate. After standing for 25 minutes at −5°, a second solution of 2.34 g. (0.02 mole) of DL-valine in 10 cc. of 2 *N* sodium hydroxide was added and the mixture was stirred vigorously and allowed to warm to room temperature during 1.5 hours. The aqueous phase was then separated, extracted with ether and acidified with hydrochloric acid to precipitate the product as a colorless oil which slowly set to a waxy solid. This was redissolved in dilute alkali, treated with decolorizing carbon (Darco) and reprecipitated with acid to give a crystalline material. After an additional crystallization from ethanol–water, there was obtained 3.0 g. (49%) of product which melts at 127–128° and then resolidifies and remelts at 146–147°. The literature m.p. is 146°.[5]

Carbobenzoxy-DL-alanyl-DL-phenylalanine.—A solution of 11.16 g. (0.05 mole) of carbobenzoxy-DL-alanine and 5.1 g. (0.05 mole) of triethylamine in 150 cc. of toluene was cooled to −5° and 6.83 g. (0.05 mole) of *s*-butylchlorocarbonate added. After 25 minutes a solution of 8.21 g. (0.05 mole) of DL-phenylalanine in 25 cc. of 2 *N* sodium hydroxide was added and the mixture was stirred vigorously for 30 minutes at 0° and then for 1.5 hours at room temperature. A large amount (18%) of the sodium salt of the formed peptide separated directly from the reaction mixture and was filtered off, redissolved in 300 cc. of water and precipitated as the crystalline acid by acidifying the solution with hydrochloric acid. The original aqueous layer from the reaction mixture was also separated and acidified to precipitate an additional amount (57%) of product as a colorless oil which slowly crystallized. The two crops were combined, redissolved in 200 cc. of dilute sodium hydroxide, treated with Darco and reprecipitated as a colorless solid. After an additional crystallization from ethanol–water, there was obtained 9.25 g. (50%) of pure material; m.p. 145–146°. The literature m.p. is 139°.[5]

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(4) H. Neurath, *et al.*, *J. Biol. Chem.*, **170**, 221 (1947).

(5) T. Wieland and R. Sehring, *Ann.*, **519**, 122 (1950).