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# SUPPRESSION OF RACEMIZATION DURING PEPTIDE SYNTHESES

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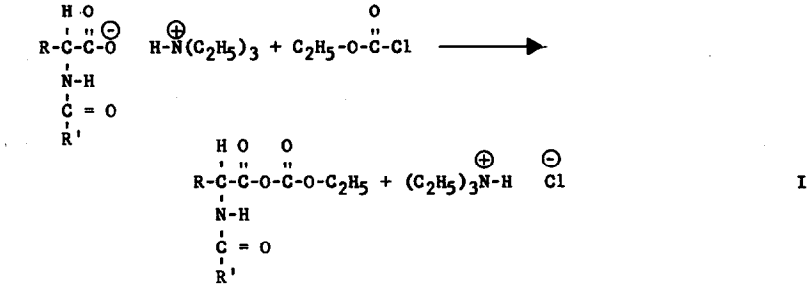
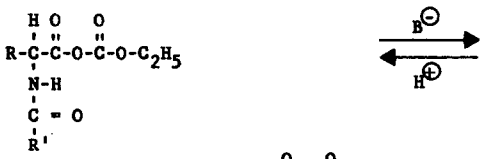
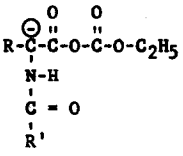
Current interest (1,2,3) in the base-catalyzed racemization of activated derivatives of N-protected amino acids and the continuing importance of this problem prompts us to report results of studies on the suppression of racemization during the use of the mixed carboxylic-carbonic anhydride method (4) in peptide syntheses.

Initially, we adopted Neuberger's suggestion (5) that the mixed anhydrides could directly undergo base-catalyzed racemization. Based on this assumption, the major reactions involved in systems using ethyl chloroformate and triethylamine are as follows:



I





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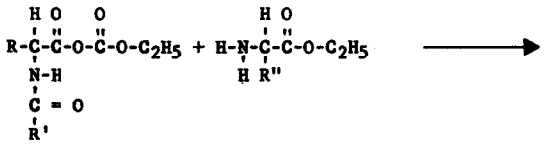
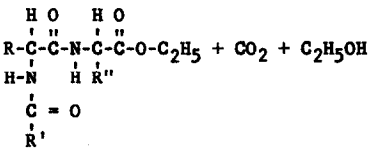




III

Equations I and III describe the accepted (4) course of the peptide-forming reaction. Equation II is suggested (1,2,3,5) as a potential route to racemized products.*

Consideration of the source of the base in equation II led us to speculate that the acylated amino acid or peptide anion functions as the base, $B^-$. The interaction of such ions as bases with anhydrides is well known, for example, in the Perkin reaction. Classically, mixed anhydrides are formed by addition of chloroformate ester to an excess of carboxylic acid anion (4). This results in ideal racemization conditions as indicated

*With our present data, we cannot completely reject other possible racemization mechanisms. Oxazolones historically have been suggested as potential racemization intermediates, although racemization of N-alkyl-N-acylated amino acid derivatives indicates that other mechanisms are likely (5). Liberek and coworkers (1,2,3) have presented evidence in support of situations similar to equation II, whereas Heard and Young (6) reported results that allow the possibility of oxazolone intermediates. We have chosen to base our studies on the less complex approach (1,2,3,5), recognizing that oxazolones, if they are formed, could conceivably yield equivalent results.

in equation II. Considering equations I and II as consecutive, competitive reactions, we developed preparative methods that accelerate reaction I and suppress reaction II.

To reduce the concentration of the acid anion we use inverse addition. In contrast to the classical method (4), a solution of the tertiary amine salt of the protected amino acid or peptide (0.2-0.4 M) is slowly added (1-2 ml./min.) to a vigorously stirred, cooled solution (-5° to 0°) of the chloroformate ester (typically ca. 0.4 M). As the chloroformate ester is depleted and the mixed anhydride concentration increases, however, there is greater probability of carboxylic acid anion reacting as in equation II. Use of excess chloroformate ester (1 to 5 equivalents) suppresses reaction II by accelerating reaction I. After 5-10 minutes enough glycine ethyl ester (in an 0.5 M solution) is added (1-2 ml./min.) to react with all of the chloroformate. The byproduct O-ethyl carbamate of ethyl glycinate can be readily extracted with water* before the usual (4) isolation procedures. Some typical results are shown in Table 1.

The syntheses of these particular peptides have been subjected to considerable earlier investigation (7,8,9,10,11,12,13,14) and were selected for study because they also offer reasonably severe tests of retention of activity. In the first two examples racemization was essentially suppressed using our modifications, while coupling N-benzoyl-L-leucine with ethyl glycinate yielded a product containing 84% of the L-isomer. Complete racemization was observed earlier (7) when formyl-L-phenylalanine was condensed with ethyl glycinate using ordinary mixed carboxylic-carbonic

*This obvious disadvantage can also be avoided by removal of the excess chloroformate before addition of the amine component. Such a procedure would undoubtedly be required with amino acid esters leading to less water soluble carbamates. Preliminary trials show this technique is practical (Phth-Gly-Gly·OEt was obtained in 91% yield after removal of a two-fold excess of ethyl chloroformate at 20° and 0.5 mm. Hg and addition of a 10% excess of ethyl glycinate) but the effect on racemization has not been evaluated by its application in optically active systems.

TABLE 1

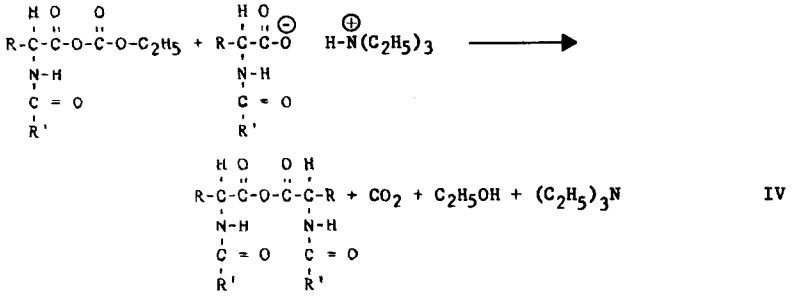
Peptides Prepared by Condensing "Inverse Addition" Mixed Anhydrides with Ethyl Glycinate in Tetrahydrofuran

| Peptide[a] | Quantity of Reactants millimoles | | | Crude Yield % | Analytical Sample | | |
|---|---|---|---|---|---|---|---|
| | Acid and triethylamine | Ethyl chloroformate | Ethyl glycinate | | m.p. °C | $[\alpha]_D^{25}$ (C) ethanol | L-isomer % |
| For-Phe-Gly·OEt | 10 | 10 | 10 | 86.5 | 127-8 | 2.11[b] (2.24) | 76 |
| | 10 | 10 | 10[c] | 80 | 127-7.5 | 1.77[b] (2.22) | 64 |
| | 5 | 10 | 15 | 69 | 128-9[d] | 2.63[b] (2.24) | 95 |
| Z-Gly-Phe-Gly·OEt | 2 | 2 | 2.2 | 84 | -- | --[e] | -- |
| | 2 | 4 | 6 | 90 | 116-7.5 | -12.5[f] (2) | 100 |
| | 2 | 4 | 6 | -- | 117-8 | -12.4[f] (2) | 100 |
| PhCO-Leu-Gly·OEt | 2 | 4 | 6 | 77 | 137-140 | -15.0 (3) | 44[g] |
| | 2 | 10 | 20 | 86 | 150-2 | -28.6 (3) | 84[g] |

[a] Symbols according to M. Goodman and G. W. Kenner, Ad. Protein Chem., 12, 465 (1957). All starting materials had acceptable physical constants. [b] ± 0.01° calculated as probable error of mean from replicate analyses. [c] Ethyl glycinate hydrochloride plus triethylamine in methylene chloride. [d] No depression of m.p. on admixture with authentic sample, m.p. 128-129°C, $[\alpha]_D^{25}$ + 2.78[b] (C 2.24, ethanol), prepared using N,N-dicyclohexylcarbodiimide (7). [e] DL-isomer (11%), m.p. 129.5-131°C, separated during fractional crystallization (8,9,10,11,12,13). L-isomer (58%), m.p. 114.5-116.5°C, $[\alpha]_D^{25}$ - 9.5 (C 2, ethanol) was recovered from mother liquors. Direct addition using Vaughan's "refluxed" procedure (10) in tetrahydrofuran gave 12.5% DL-isomer, m.p. 130-131.5° and 44% L-isomer, m.p. 117-118°, $[\alpha]_D^{25}$ - 10.4 (C 2, ethanol) on fractional crystallization (8,9,10,11,12,13); a modification using 100% excess ethyl glycinate and "room temperature" conditions (10) gave 15% DL-isomer, m.p. 129.5-131.5° and 54% L-isomer, m.p. 116-118°, $[\alpha]_D^{25}$ - 12.8 (C 2, ethanol). [f] No DL-isomer isolated on careful fractional crystallization (8,9,10,11,12,13). Properties of L-isomer are reported as: m.p. 116-118°C, $[\alpha]_D^{25}$ - 12.0 (C 2, ethanol) (8); m.p. 117-118°C, $[\alpha]_D^{24}$ - 12.3 (C 2, ethanol) (9); m.p. 118-119°C, $[\alpha]_D^{24}$ - 11.5 ± 0.5 (C 2, ethanol) (10); m.p. 117-118°C, $[\alpha]_D^{26}$ - 12.4 (ethanol) (11); m.p. 117-118°C, $[\alpha]_D^{25}$ - 12.0 (C 2, ethanol) (12); m.p.s 116.5-119.5°C and 120-120.5°C, $[\alpha]_D^{25}$ - 11.5 ± 1 and -13.2 ± 1 (C 2, ethanol) (13). [g] Based on $[\alpha]_D^{20}$ - 34.0 (C 3.1, ethanol), m.p. 156.5-157°C for pure compound (14).

anhydride techniques. Various levels of racemization were reported (10) in almost every instance during the direct addition coupling of N-carbobenzoyloxyglycyl-L-phenylalanine with ethyl glycinate in several solvent systems. We could not duplicate the direct addition results by explicitly following the published procedure (10) or modifications thereof using tetrahydrofuran as a solvent (cf. footnote e, Table 1). We succeeded only by inverse addition and use of excess chloroformate. Lastly, only 18-22% of the L-isomer was obtained (14) using the carboxylic-carbonic anhydride and direct addition in preparing ethyl N-benzoyl-L-leucylglycinate. This latter condensation appears to be an exceedingly stringent test for racemization.

The modified techniques proposed here also reduce the directly competing side reaction of symmetrical anhydride formation. Consideration of the classical method (4) suggests that formation of the mixed anhydride in the presence of excess carboxylate anion provides ideal conditions for the occurrence of this side reaction (equation IV).



Reactions of type IV have been cited (15,16) for the preparation of symmetrical anhydrides from mixed anhydrides. To our knowledge, however, little attention has been given to the possibility that this reaction rather than the commonly accepted disproportionation reaction (16) causes lower yields in mixed anhydride systems.

We investigated a striking example of this type in the mixed anhydride coupling of phthaloylglycine with ethyl glycinate (17,18). Direct addition in dioxane (17) or inverse addition in tetrahydrofuran employing equimolar quantities of reactants gave 69% yields. We noted particularly heavy precipitation during the anhydride-forming step. An 85% yield was reported using direct addition in chloroform (18). In methylene chloride a 79.5% yield was obtained from equimolar amounts of reactants in our direct addition studies while use of 100% excess chloroformate led to only an 88% yield. Employing inverse addition in the latter solvent we obtained 85% yield, and with excess ethyl chloroformate: a 10% excess led to a 95% yield; a 25% excess led to a 95% yield; and a 100% excess led to a 98% yield. In these experiments the time, temperature, and dilution parameters were similar to those mentioned above. All products had acceptable melting points. A likely interpretation of our results is that the competing reaction leading to symmetrical anhydride is reduced by the more rapid reaction of the carboxylic acid anion with the excess chloroformate ester. Also, solvents which prevent precipitation of the symmetrical anhydride reduce the extent of its formation. Disproportionation seems unlikely under our conditions since if this reaction is occurring in the manner suggested (16) one would anticipate little or no effect of addition order on the end result.

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