A Reinvestigation of the Mixed Carbonic Anhydride Method of Peptide Synthesis[1]

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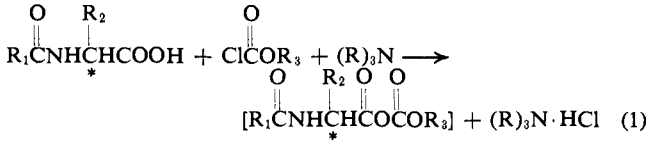
Abstract: The factors which affect yield and racemization in peptide synthesis by the mixed carbonic anhydride method have been investigated. Good results in test cases were obtained when the tertiary amine necessary for formation of the mixed anhydride contained at least one N-methyl group, and it was not used in excess, indicating the importance of steric factors. Reduction of "basicity" of the amine was found to be favorable; complete racemization was found with an excess of trimethylamine as the tertiary base, and none with an excess of N-methylmorpholine in the test Z-Gly-Phe-Gly-OEt synthesis in ethyl acetate or tetrahydrofuran. The more sensitive Bz-Leu-Gly-OEt synthesis gave no racemate with 1 equiv of N-methylmorpholine but some racemate with 2 equiv. The effects on racemization of change of solvent, temperature, time allowed for mixed anhydride formation, and several anions are reported. Evidence for racemization *via* oxazolones is given. Best yields were obtained with isobutyl chloroformate as the reagent. The principles disclosed here should be useful in improving other acylations.

The use of alkyl chloroformates as peptide-forming reagents (eq 1 and 2) was independently proposed



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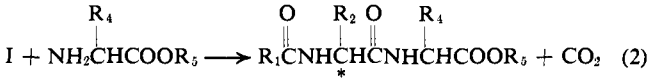


(2)

in 1951 by three groups.[2] The method became popular because of advantages in speed, yield, and relative purity of products. The scope and limitations of the method as then known were competently discussed in a 1962 review article by Albertson[3] and will not be reported in detail here. The most serious limitation, which is common to other methods, was the danger of racemization when acylpeptides were coupling components. Experience has shown that acylamino acids can be used without racemization when common protecting groups such as benzyloxycarbonyl and *t*-butyloxycarbonyl are used, and the result has been that the most popular procedure today for building a peptide chain is the stepwise addition of an acylamino acid followed by removal of the acyl group and repetition of the process. Coupling of acylpeptides is safe provided that the carboxy terminal amino acid fragment is glycyl or prolyl. With other amino acids, the α-carbon atom (starred in eq 1) has a labile hydrogen attached to an asymmetric carbon, and exchange of this results in racemization if, as is the case with naturally occurring peptides, the carbon is present as L or D. Coupling of acylpeptides without racemization is possible with the azide method, but this has disadvantages, mainly low yields and contamination of the product with by-products.[4] Methods of coupling without racemization are thus needed, particularly for the synthesis of long chain peptides. With this as a goal, we began a comprehensive reinvestigation of the mixed anhydride method several years ago;[5] successful results have been briefly reported.[1] We have used our racemization test[6] based on coupling Z-Gly-Phe-OH with H-Gly-OEt as the basic tool, and have also applied the super-sensitive Young test[7] involving the synthesis of Bz-Leu-Gly-OEt. The investigation began with an attempt to confirm the report of Determann and Wieland[8] that racemization of Z-Gly-Phe-OH (L) does not begin in the first 10 min after formation of the mixed anhydride from ethyl chloroformate in tetrahydrofuran at $-15°$ with triethylamine as hydrogen chloride acceptor; we found instead that racemization begins immediately and the initial rate follows first-order kinetics.[5]

Tertiary Amine Effect. With the possibility that racemization in the Z-Gly-Phe-Gly-OEt synthesis is caused by the presence of small amounts of unreacted tertiary amine, a comparison of the effect of 1 equiv to 2 equiv of tertiary amine was made, using ethyl chloroformate in the procedure of example 1 in the Experimental Section where 12 min at $-15°$ was allowed for anhydride formation. When 1 equiv of triethylamine was used, an average of 13% DL and 46% L was obtained; with 2 equiv, 17% DL and 62% L resulted. With tri-*n*-propylamine, the results were 4% DL, 31% L with 1 equiv and 6% DL, 72% L with 2 equiv. Thus the effect of 100% excess of tertiary amine was mainly one of increase in over-all yields. A comparison of several other amines using 2 equiv was then made. Results were: ethyl diisopropylamine, 0.2% DL, 3% L; tri-*n*-octylamine, 0.1% DL, 81% L; pyridine, 4% DL, 37% L; N,N-dimethylaniline, 0% DL, 1% L; trimethylamine, 65% DL, trace of L; and quinuclidine, 69% DL, 0% L. The results with trioctylamine were encouraging, but in subsequent experiments DL was

(1) Preliminary communication: G. W. Anderson, J. E. Zimmerman, and F. M. Callahan, *J. Am. Chem. Soc.*, **88**, 1338 (1966).
(2) T. Wieland and H. Bernhard, *Ann*, **572**, 190 (1951); R. A. Boissonnas, *Helv. Chim. Acta*, **34**, 874 (1951); J. R. Vaughan, Jr., and R. L. Osato, *J. Am. Chem. Soc.*, **73**, 3547 (1951).
(3) N. F. Albertson, *Org. Reactions*, **12**, 157 (1962).
(4) E. Schnabel, *Ann.*, **659**, 168 (1962).
(5) G. W. Anderson, F. M. Callahan, and J. E. Zimmerman, *Acta Chim. Acad. Sci. Hung.*, **44**, 51 (1965).
(6) G. W. Anderson and F. M. Callahan, *J. Am. Chem. Soc.*, **80**, 2902 (1958).
(7) M. W. Williams and G. T. Young, *J. Chem. Soc.*, 881 (1963).
(8) H. Determann and T. Wieland, *Ann.*, **670**, 136 (1963).

always found in small amounts, and the insolubility of trioctylamine hydrochloride in water made purification cumbersome. The trimethylamine result suggested a steric effect in the racemization, and the quinuclidine experiment was done to check this at the suggestion of Dr. R. Paul. Ethyldiisopropylamine was tried because it is known that it does not complex with acid chlorides because of steric inhibition; the very low yields suggested that the chloroformate must react with the tertiary amine as a first step toward anhydride formation. It now seemed possible that complete reaction of the chloroformate with a sterically uninhibited tertiary base of sufficient strength would remove all tertiary base and thus the possibility that any would be present to racemize the mixed anhydride being made. A reaction with an exact equivalent of trimethylamine confirmed this; no racemate was obtained with yields of 88 and 91% pure L tripeptide in two experiments. Isobutyl chloroformate was used in these experiments because yields are reported to be better than with ethyl chloroformate[9] (our experiments described below subsequently confirmed this).

A series of experiments comparing different amines, using isobutyl chloroformate, in tetrahydrofuran (THF) with a 12-min period for anhydride formation (activation time) was now carried out. Selected examples are given in Table I. With 1 equiv of amine, a

Table I. Z-Gly-Phe-Gly-OEt Synthesis in THF at −15° Using Isobutyl Chloroformate and a 12-min Activation Time with Various Amines

Amine	Amine equiv	% yield Crude	DL	L
Triethyl	1	97	8	82
Triethyl	2	84	16	59
Methyldiethyl	1	98	...	94
Methyldiethyl	2	90	18	68
Dimethylethyl	1	99	...	98
Dimethylethyl	2	87	62	15
Trimethyl	1	100	...	90
Trimethyl	2	76	68	Trace
Propyldimethyl	1	97	...	91
Propyldimethyl	2	96	49	33
Diisopropylmethyl	1	98	...	94
Diisopropylmethyl	2	98	3	85
N-Methylmorpholine	1	96	...	92
N-Methylmorpholine	2	98	...	93
N,N′-Dimethylpiperazine	2	98	...	95
β-Dimethylaminopropionitrile	2	95	...	91
Bis(dimethylaminomethyl) acetylene	1	99	...	93
Bis(dimethylaminomethyl) acetylene	2	95	...	82

5% excess of chloroformate was used to ensure that no excess amine was present. In addition to the amines listed in the table, good yields and no racemate were obtained when 1 equiv of cyclohexyldimethylamine, benzyldimethylamine, N-methylpyrrolidine, or N-methylpiperidine was used; varying amounts of racemate were found with 2 equiv. No peptide was obtained with N-methylethylenimine, quinoline, imidazole, N-methylimidazole, diethylhydroxylamine, or potassium *t*-butylate. One experiment with N-ethylmorpholine (2 equiv) gave no racemate but only 78% L. These results amply show that any tertiary amine containing at least one N-methyl group will give no racemate provided it is sufficiently basic and is not used in excess, and such amines with low but sufficient basicity may be used in excess. We have chosen N-methylmorpholine as the best, considering water solubility, availability, etc.

Activation Time. In our first report of this study[5] we showed that racemization is a function of activation time when triethylamine is the tertiary base, and concluded that 2 min gave satisfactory results in avoiding racemization. Further study has shown that excess amine lowers the activation time necessary for good yields with triethylamine. Thus, with methyl chloroformate as the reagent in THF at −15°, 1 equiv of triethylamine gave a 23% of L and no DL tripeptide, and 2 equiv gave 80% L and no DL when the activation time was 1 min; 1 equiv of amine and 12 min yielded 56% L and 17% DL. Trioctylamine (2 equiv) was more sluggish, giving only 8% L and no DL with ethyl chloroformate and 2 min; at 12 min, a trace of DL and 80% L were obtained. In contrast, 1 equiv of methylmorpholine in THF with isobutyl chloroformate at −15° and a 30-sec activation time yielded 93% L and no DL tripeptide. Neglecting any small effect from changes in the chloroformate, one can conclude that complexing of the chloroformate with a methylamine and subsequent reaction with Z-Gly-Phe-OH are very rapid; an immediate precipitate on addition of the chloroformate is also observed. In experiments with 1 equiv of trimethylamine, complete and rapid reaction is indicated also by lack of racemization, since this base is a very strong racemizer.

Basicity of Amines and Racemization. The relative basicity of different amines in water does not carry over to nonaqueous solvents.[10] Pearson and Vogelsong[10] have described a procedure for basicity comparisons in nonaqueous solvents which involves the spectrophotometric measurement of equilibrium constants using 2,4-dinitrophenol as the reference acid. Applying this procedure to several amines (Table II), we found that there is no correlation between equilibrium constant and racemizing ability in our test synthesis. Thus, trimethylamine, the best racemizer, gives a lower constant than triethylamine, and tri-*n*-propylamine, which is a poorer racemizer than triethylamine, has a lower constant than trimethylamine.

Table II. Equilibrium Constants for 2,4-Dinitrophenol and Amines in Tetrahydrofuran[a]

Amine	Constant ± 200
Trimethylamine[b]	5,600
Triethylamine[b]	11,600
Tri-*n*-propylamine	2,040
N,N-Dimethylethylamine[b]	6,800
N,N-Diethylmethylamine[b]	8,200
N-Methylpyrrolidine	14,450
N-Ethylpyrrolidine	15,600
N-Methylpiperidine	5,500
N-Ethylpiperidine	7,180

[a] Determined by S. Venetianer by the method of Pearson and Vogelsong.[10] [b] Aliquots of titrated (0.1 *N* HCl) solutions in THF instead of weighed samples were used.

Correlation between Racemization in the Z-Gly-Phe-Gly-OEt Synthesis and Loss of Optical Rotations of

(9) J. R. Vaughan, Jr., and R. L. Osato, *J. Am. Chem. Soc.*, 74, 676 (1952).

(10) R. G. Pearson and D. C. Vogelsong, *ibid.*, 80, 1038 (1958).

Phth-Ala-ONP with Time in the Presence of Tertiary Amines. Molar equivalents of various tertiary amines were added to a 2% solution of phthaloyl-L-alanine *p*-nitrophenyl ester in tetrahydrofuran, and the optical rotation was measured at fixed time intervals for 24 hr. A good correlation of the rate of loss of rotation with racemizing ability in the Z-Gly-Phe-Gly-OEt test was obtained; thus, quinuclidine > trimethylamine > dimethylethylamine > triethylamine > trioctylamine. Amines which caused no loss in optical rotation included N-methylmorpholine, pyridine, and quinoline. This test is therefore a simple one for screening amines which might be useful in peptide synthesis.

Complexing of Amines and Chloroformate as the First Step. As shown in example 2 in the Experimental Section, the addition of the chloroformate to a solution of triethylamine or trimethylamine in tetrahydrofuran followed in 1 or 12 min by the Z-Gly-Phe-OH, then the H-Gly-OEt, gave good yields and no racemate. An experiment with a 100% excess of trimethylamine gave complete racemization (70% yield) as expected. Yields were a little lower than in the standard procedure of example 1.

Racemization by Anions. On the assumption that the anion of Z-Gly-Phe-OH, formed by the usual addition of triethylamine to a solution of the dipeptide in nonaqueous solvent before the addition of the chloroformate, racemizes the mixed anhydride during its formation, Applewhite and Nelson[11] proposed that "inverse addition" and an excess of chloroformate would diminish racemization. Therefore, they formed the mixed anhydride by slow addition of the triethylammonium salt of the dipeptide to a stirred, cooled solution of ethyl chloroformate in tetrahydrofuran, and 5–10 min later added ethyl glycinate to complete the reaction. No racemate was found in the product when excesses of chloroformate and ethyl glycinate were used. On attempts to repeat their experiment (Experimental Section, example 3), we always found racemate (5%) when triethylamine was the base. However, trimethylamine under the same conditions gave no racemate, and quinuclidine only a trace. Since these two bases are excellent racemizers if not neutralized, these results can be explained by complete and rapid reaction to form the mixed anhydride when they are present, but a slower and incomplete reaction with triethylamine. In the latter case, racemization could be caused by triethylamine or by Z-Gly-Phe-OH anion. Several experiments by the normal procedure (example 1) with isobutyl chloroformate were done. With a 5-min activation time at −5°, a 10% excess of triethylamine resulted in 8% DL and 79% L tripeptide; 10% excess of triethylamine and Z-Gly-Phe-OH gave 18% DL and 67% L. This indicates that the anion is the better racemizer. Similar experiments with trimethylamine and a 2-min activation time resulted in 19% DL, 50% L when only the amine was in excess, and 11% DL, 55% L when both were in excess; here, the anion is the poorer racemizer. Thus trimethylamine > anion > triethylamine as racemizers.

A set of experiments with N-methylmorpholine gave valuable results. Since it has been shown that no racemate is formed in the presence of a 100% excess of N-methylmorpholine in the tripeptide synthesis at −15° with a 12-min activation time, the same experiment with an added 100% excess of Z-Gly-Phe-OH was done. The result was yields of 15% DL and 77% L tripeptide, clearly showing that Z-Gly-Phe-O⁻ is the racemizer. Furthermore, the excess Z-Gly-Phe-OH was recovered and used to make the tripeptide in a normal synthesis with equivalents of N-methylmorpholine and isobutyl chloroformate; an 80% yield of L and no DL tripeptide was obtained. This eliminates the possibility of racemate in the first case by formation of the symmetrical anhydride of Z-Gly-Phe-OH; since racemate was found in the first, it would have to be present in the second because the two parts of the symmetrical anhydride would be equally racemized. To complete the possibilities, an experiment with a 100% excess of Z-Gly-Phe-OH and no excess of N-methylmorpholine gave no racemate. It should be noted that the collective evidence indicates that chloride anion is not a racemizer in our experiments. Although the tertiary amine hydrochloride is almost completely insoluble in most cases, it is soluble when long chain alkylamines such as trioctylamine are used; as discussed, only a trace of racemate was found with the latter.

Comparison of Chloroformates. In early work, Vaughan[9] found that isobutyl and *sec*-butyl chloroformates were superior to a number of other alkyl chloroformates in giving the best yields of products. We have confirmed this in the Z-Gly-Phe-Gly-OEt synthesis at −15° with 1 equiv of N-methylmorpholine as the tertiary base and a 30-sec activation time. Recrystallized yields of the L form (no DL was found) were: methyl, 91; ethyl, 84; isopropyl, 90; *sec*-butyl, 91; isobutyl, 93; and cyclopentyl, 91%. Isobutyl chloroformate is stable on storage, in contrast to chloroformates derived from secondary alcohols, and it is readily available. Thus it appears to be the best reagent over-all.

Solvents. The Z-Gly-Phe-Gly-OEt synthesis with isobutyl chloroformate and 1 equiv of N-methylmorpholine and a 12-min activation time was used to compare a number of solvents. Ethyl acetate and tetrahydrofuran gave the best yields and no racemate. Also good (better than 90% yields) were dimethoxymethane, dimethoxyethane, 1,3-dioxolane, and acetonitrile. Lower yields but no racemate were found in methyl cellosolve acetate, triethyl phosphate, and N,N-dimethylacetamide. Racemate in the 2 to 8% range was isolated from methylene chloride, N-methylpyrrolidone, and dimethylformamide. Lower yields and more racemate occurred in hexamethylphosphoramide. The same conditions with trimethylamine as the tertiary base and a 5% excess of chloroformate yielded no racemate from tetrahydrofuran or methyl cellosolve acetate (only 65% yield of L in the latter case) and almost complete racemization in N-methylpyrrolidone, N,N-dimethylacetamide, or acetonitrile. In dimethylformamide, 45% DL and 18% L were found; when a 50% excess of chloroformate was used, the yields were 26% DL and 70% L.

With 2 equiv of N-methylmorpholine present in the reaction, good yields and no racemate were found only in ethyl acetate, tetrahydrofuran, and dioxolane. Triethyl phosphate yielded6 % DL and 81% L; acetonitrile

(11) T. H. Applewhite and J. S. Nelson, *Tetrahedron Letters*, 819 (1964).

32% DL and 64% L, and dimethoxymethane 0.2% DL and 57% L.

It seemed likely that solvent participation can occur with amide-type solvents, particularly dimethylformamide. Thus, ethyldiisopropylamine, which does not complex with chloroformates, gives no reaction when used as the tertiary base in dimethoxyethane or dimethoxymethane, yet yields of 26% DL, 52% L were obtained from a reaction in dimethylformamide and 11% DL, 73% L in N-methylpyrrolidone; 14% DL, 50% L in dimethylacetamide; 69% DL, no L in hexamethylphosphoramide; and 4% DL, no L in acetonitrile. As reported above, 0.2% DL and 3% L were obtained with 2 equiv of ethyldiisopropylamine in tetrahydrofuran.

Since dimethylformamide is a common solvent for peptide synthesis, several tertiary amines other than N-methylmorpholine were tried in it. Those which gave good yields also gave some racemate, and even the weak base N,N-dimethylaniline yielded 0.6% DL and 19% L tripeptide. The safest amide solvent is N,N-dimethylacetamide; other than N-methylmorpholine as tertiary base, N,N'-dimethylpiperazine gave no racemate and a good yield (81%) of L tripeptide.

Temperature and Activation Time. Our original choice of $-15°$ for mixed anhydride formation was made for direct comparison with literature procedures.[5] Since experience has shown that mixed anhydride formation in ethyl acetate or tetrahydrofuran is complete in 30 sec or less when a methylamine is the tertiary base, this is a practical working temperature. Obviously lower temperatures can be used, and good results are obtained at $-5°$. Confirming the results of Vaughan and Osato,[9] results were poorer at room temperature, probably because of instability of the mixed anhydride. Although an activation time of 12 min was used in most of our experiments to exaggerate racemization when it occurred, we recommend the 30-sec period as a practical working time. Quite likely experience will show that some peptide reactions will have other optimum activation times.

Use of Ethyl Glycinate Hydrochloride. It is normally convenient to add the amine reactant in peptide synthesis as a salt, with sufficient tertiary base present to liberate the amine. In older mixed anhydride procedures, this was frequently done with the extra equivalent of base present during the formation of the mixed anhydride. Since we now know that the bases then used are racemizers, it was of interest to test the system with N-methylmorpholine. First, the mixed anhydride from 5 mmoles of Z-Gly-Phe-OH was prepared at $-15°$ in 25 ml of tetrahydrofuran (THF) with 1 equiv of N-methylmorpholine. A mixture of ethyl glycinate hydrochloride and 1 equiv of N-methylmorpholine in 10 ml of THF (solution not obtained) was added, and the regular procedure was followed thereafter. Result: an oily product in 80% yield from which yields of 13% DL and 16% L tripeptide were isolated. This was at first interpreted to mean insufficient basicity of N-methylmorpholine to liberate ethyl glycinate, which is a strong base. A later experiment with a 30-sec activation time and using triethylamine to neutralize the ethyl glycinate hydrochloride gave a 57% yield of tripeptide which was separated into yields of 10% DL and 39% L. Since triethylamine is a strong base, and addition to ethyl glycinate hydrochloride suspended in THF did not cause solution, it was suspected that reaction was incomplete, and the unreacted triethylamine was the racemizer. An experiment (Experimental Section, example 4) in which a solution was obtained in dimethylformamide (DMF), and this was added to the mixed anhydride formed in THF, was successful in giving a 91% yield and no racemate. This also demonstrated that, although DMF as the solvent for mixed anhydride formation induces racemization, it is safe for the reaction of a preformed mixed anhydride. An experiment similar to example 4 (Experimental Section) with N-methylmorpholine as the base gave a 97% yield and no racemate. Thus N-methylmorpholine is a strong enough base to liberate ethyl glycinate, and the earlier result in THF must have been a result of insolubility of the hydrochloride; the mixed anhydride would thus not have reacted completely and oxazolone formed on warming which reacted slowly with ethyl glycinate as it was liberated from the base. We can conclude that complete neutralization of ethyl glycinate hydrochloride by the tertiary base is required before adding to the mixed anhydride; this is best achieved by complete solution. Successful experiments similar to example 4, by solution of H-Gly-OEt·HCl and 1 equiv of tri-*n*-propylamine in DMF (93% yield of L tripeptide, no DL) and with solution of H-Gly-OEt·HCl with N-methylmorpholine in hexamethylphosphoramide (89% L, no DL) confirm the conclusion.

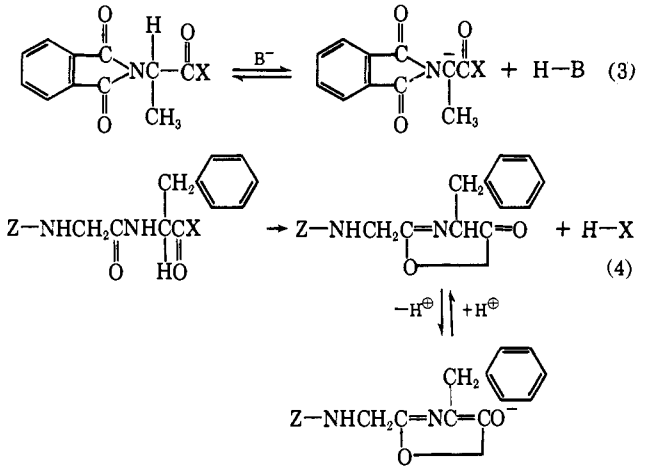
The use of a variety of powerful solvents for the amine component (as free amine or amine hydrochloride plus tertiary base) along with mixed anhydride formation of the acid component in THF or ethyl acetate should simplify peptide formation with otherwise insoluble reactants.

Studies With Ethyl Benzoylleucyl Glycinate (Bz-Leu-Gly-OEt). The Young test synthesis[7] is recognized to be supersensitive, hence any procedure which gives no racemization with it should be safe for general use in peptide synthesis. In our brief communication on the present work,[1] we reported a modification of Young's procedure designed to disclose racemate in amounts less than 2%, which is the limit of his detection. Our procedure, involving extraction of the Bz-Leu-Gly-OEt with ether and fractionation from alcohol–water, can detect <1% racemate as reported. The use of a 4-min activation time at $-15°$ followed by reaction of the mixed anhydride with H-Gly-OEt is described in detail in example 5, Experimental Section. With 1 equiv of N-methylmorpholine, a 93% yield of optically pure Bz-Leu-Gly-OEt was obtained. With a 12-min activation time, no racemate was found; the yield was 84%, but some product was accidentally lost in the work-up. A similar experiment with 2 equiv of N-methylmorpholine and a 12-min activation time (erroneously reported[1] in our communication as 4 min) yielded 50% L and 16% DL dipeptide. We have found that mixed anhydride formation is complete in less than 30 sec in this system; thus it is likely that the 16% DL can be essentially eliminated by a short activation time, although we have not done the experiment. The synthesis with 1 equiv of trimethylamine, a 10% excess of isobutyl chloroformate, and a 60-sec activation time yielded 93% L, no DL at $-14°$ and 88% L, 1% DL at $-5°$.

Isolation of Mixed Anhydrides. In work reported separately,[12] active esters were made by reaction of mixed anhydrides with appropriate hydroxy compounds. A synthesis of the N-hydroxysuccinimide ester of phthaloyl-L-alanine (Phth-Ala-OSu) gave a by-product which proved to be the mixed anhydride with isobutylcarbonic acid (Phth-Ala-$OCOOCH_2CH(CH_3)_2$). This was subsequently made in the absence of N-hydroxysuccinimide (HOSu) as a crystalline solid, mp 68°, in 84% yield (example 6, Experimental Section). Although reaction with an amine was not studied, this compound reacted with HOSu to give the ester in good yield. Tarbell and Leister[13] have isolated crystalline mixed anhydrides of other esters, but this is the first such amino acid derivative to our knowledge. It was found to be unstable to the extent that the cap was blown from a vial of the material after several days at room temperature. However, other preparations kept better. Attempts to isolate Z-Gly-Phe-$OCOOCH_2CH(CH_3)_2$ gave an oil which bubbled slowly at room temperature; this was partially soluble in isopropyl ether, but subsequent reaction of both portions with H-Gly-OEt gave partially racemized Z-Gly-Phe-Gly-OEt in both cases.

Discussion

Racemization Mechanisms. There is much evidence that racemization of activated acylamino acids or acylpeptides can occur by direct proton abstraction (eq 3) or by formation of oxazolone intermediates which are readily racemized (eq 4); a recent discussion by Bodanszky and Ondetti[14] summarizes present beliefs.



As already stated, we obtained a good correlation of the racemizing effect of various tertiary amines in the Z-Gly-Phe-Gly-OEt synthesis with the rate of loss of optical rotation of solutions of L-Phth-Ala-ONP caused by the same bases. The simplest explanation to account for the results which we and others have obtained is that, when oxazolone formation can readily take place, racemization proceeds by the mechanism illustrated by eq 4, and in other cases by direct proton abstraction as illustrated in eq 3. Both mechanisms could be operating on occasion.

Experimentally, the oxazolone formed in (4) can be measured directly. Also, when X is $-OC(=O)OCH_2-CH(CH_3)_2$ as in most of the work reported here, the H–X formed is a carbonic acid monoester which decomposes to CO_2 and isobutyl alcohol; these also can be measured. In an experiment (example 7 in Experimental Section) in which Z-Gly-Phe-$C(=O)OCH_2-CH(CH_3)_2$ was made in the presence of N-methylmorpholine, then treated with trimethylamine, isobutyl alcohol was isolated (75% of the theoretical amount). The formation of the oxazolone was shown by infrared. Similar experiments in which H-Gly-OEt was added yielded completely racemized Z-Gly-Phe-Gly-OEt. These results do not exclude the possibility of racemization of the mixed anhydride by proton abstraction before oxazolone formation.

(12) G. W. Anderson, F. M. Callahan, and J. E. Zimmerman, *J. Am. Chem. Soc.*, **89**, 178 (1967).
(13) D. S. Tarbell and N. A. Leister, *J. Org. Chem.*, **23**, 1149 (1958).
(14) M. Bodanszky and M. A. Ondetti, "Peptide Synthesis," Interscience Publishers, Inc., New York, N. Y., 1966, p 137.

Summary and Conclusions

The results show that the tertiary base used for mixed anhydride formation by reaction of an acylamino acid or acylpeptide with an alkyl chloroformate is not merely a hydrogen chloride acceptor. It first reacts with the chloroformate to form a quaternary compound which in turn reacts with the carboxylic acid. Methylamines give the fastest reactions, showing the importance of steric factors. Methylamines of suitable basicity are also the best racemizers, indicating that racemization is also sterically controlled. The discovery that weaker bases such as N-methylmorpholine give rapid anhydride formation without racemization in test cases shows that racemization is a separate process from anhydride formation. The fact that trimethylamine, a good racemizer when used in excess, may be used without racemization if not in excess indicates a rapid and complete formation of the mixed anhydride in its presence.

It can be concluded that the mixed anhydride method should be useful in coupling acylpeptides to amino acid or peptide derivatives without racemization when properly applied. This not only extends the application of the method, but provides a much-needed way to couple peptides safely. Flexibility in peptide synthesis is thus considerably increased.

The principles disclosed here should be applicable to nonpeptide forming reactions also. Reactions of acid halides in general, and also other reactions where tertiary amines are used as "acid acceptors," are the most obvious subjects for improvement.

Experimental Section

Example 1. Z-Gly-Phe-Gly-OEt Synthesis. A solution of 1.78 g (5 mmoles) of benzyloxycarbonylglycyl-L-phenylalanine (Z-Gly-Phe-OH) in 25 ml of tetrahydrofuran (THF) (distilled from calcium hydride and stored over calcium hydride) was stirred and chilled to −15° by a Dry Ice–acetone bath. Triethylamine (0.70 ml, 5 mmoles) was added, followed by ethyl chloroformate (0.47 ml, 5 mmoles) giving a precipitate. After 12 min, ethyl glycinate (H-Gly-OEt; 0.53 ml, 5 mmoles) was added, stirring was continued for a minute or so at −15°, then the bath was removed, and the mixture was allowed to warm to room temperature. The solvent was removed under vacuum; ethyl acetate (75 ml) and then 25 ml of 5% sodium bicarbonate solution were added. Following shaking and separation, the ethyl acetate layer was washed with 25 ml of water, 25 ml of 1 *N* hydrochloric acid, and finally 25 ml of water. After drying over anhydrous sodium sulfate, evaporation of the ethyl acetate solution left 1.42 g of crude Z-Gly-Phe-Gly-OEt (64% yield). This was fractionated from 2% solution in anhydrous ethanol,[6] giving a 12% yield of DL tripeptide and a 44% yield of the L form. Repetition of the experiment gave 15% DL and 48% L.

Example 2. Complexing of Triethylamine with Isobutylchloroformate Prior to Z-Gly-Phe-Gly-OEt Synthesis. A solution of 0.70

ml of triethylamine in 10 ml of THF was chilled to −15° and 0.47 ml of isobutyl chloroformate was added. After 1 min ("complexing time"), 1.78 g of Z-Gly-Phe-OH was added; 2 min later, 0.53 ml of H-Gly-OEt was added. The work-up was the same as in example 1. From a crude yield of 83%, fractionation from alcohol gave no DL and a 72% yield of L tripeptide. A 12-min complexing time gave similar results. With 1 equiv of trimethylamine, the crude yields were higher (86 and 83%) but no racemate was formed.

Example 3. Z-Gly-Phe-Gly-OEt by "Inverse Addition." (Compare Ref 11). A solution of 0.94 ml (10 mmoles) of ethyl chloroformate in 25 ml of tetrahydrofuran (THF) was stirred and cooled to −5°. To this was added dropwise during 3 min a previously cooled (−5°) solution of triethylamine (0.70 ml, 5 mmoles) and Z-Gly-Phe-OH(L) (1.78 g, 5 mmoles) in 12.5 ml of THF. The mixture was stirred at −5° for 8 min (activation time), then a solution of H-Gly-OEt (1.54 g, 15 mmoles) in 30 ml of THF was added. After warming to room temperature, the mixture was worked up as described in example 1. Fractionation from absolute alcohol yielded 5% DL and 75% L tripeptide. Activation times of 12 or 16 min gave the same results (3 to 6% DL, 76 to 80% L). Substituting trimethylamine for the triethylamine gave no DL and 89% L; quinuclidine yielded a trace of DL and 83% L.

Example 4. Use of HCl·H-Gly-OEt in the Z-Gly-Phe-Gly-OEt Synthesis. A solution of 5 mmoles of Z-Gly-Phe-OH and 5 mmoles of N-methylmorpholine in 25 ml of THF was chilled to −15° with stirring; then 0.67 ml (5 mmoles) of isobutyl chloroformate was added. After about 45 sec, a solution of HCl·H-Gly-OEt (5 mmoles) and triethylamine (0.70 ml, 5 mmoles) in 10 ml of dry DMF (made by dissolving the hydrochloride by warming, cooling in a water bath to room temperature, adding the triethylamine, and swirling) was added, using wash DMF. After 5 min, the bath was 5°. The hydrochlorides were filtered off; the filtrate was concentrated under vacuum and 25 ml of water added, giving immediate crystallization of the tripeptide. Following filtration and washing with water, dilute sodium bicarbonate solution, and water, the product was dried; weight 2.00 g (91% yield), mp 118–120°. Fractionation from absolute alcohol gave no racemate and 1.86 g (85% yield) of L tripeptide, mp 120–121°.

Example 5. Synthesis of Bz-Leu-Gly-OEt. A solution of 2.35 g (10 mmoles) of benzoyl-L-leucine in 50 ml of dry tetrahydrofuran was chilled to −15° with stirring; 1.10 ml (10 mmoles) of N-methylmorpholine was added, then 1.39 ml (10.5 mmoles) of isobutyl chloroformate. Four minutes later, 1.11 ml (10.5 mmoles) of ethyl glycinate was pipetted in. After a minute or so, the bath was removed and the solution allowed to stand for 0.5 hr. It was then concentrated under vacuum, the residue taken up in 15 ml of chloroform plus 85 ml of ethyl acetate plus 10 ml of water. After shaking, the water layer was removed and the organic was washed successively with 10 ml of saturated sodium bicarbonate solution, 10 ml of water, 10 ml of 1 *N* hydrochloric acid, and 10 ml of water. The solution was dried with sodium sulfate, then concentrated under vacuum. The crystalline residue was washed out of the flask with 20 ml of warm absolute ether, followed by an ether wash; dry weight 2.90 g, mp 157–158°. Concentration of the ether extracts to a small volume yielded 94 mg, mp 155–157°, and the addition of petroleum ether (bp 30–60°) to the filtrate gave 24 mg, mp 142–149°. Solution of the latter in a little warm alcohol and the addition of water yielded 12 mg, mp 156.5–157°. Similar recrystallization of the 94 mg gave 82 mg, mp 157–158°. The combined yield of L peptide was 2.99 g (93%). As previously reported,[1] our rotation values are consistently $[\alpha]^{25}D$ −32.5 ± 0.5° (*c* 3, EtOH) and a different thermal analysis gives a single endotherm at 159°. The value of −34° reported by Williams and Young[7] was never obtained.[15]

When DL peptide was present in other experiments it usually appeared in the last fraction from ether, and alcohol–water recrystallization gave material with mp 143–144°. Thin layer chromatography on silica gel using chloroform–methanol (2:1) gave spots at R_f 0.80 for both L and DL (chlorine–tolidine reagent).

Example 6. Phth-Ala-OCOOCH$_2$CH(CH$_3$)$_2$. A solution of 3.29 g (15 mmoles) of phthaloyl-L-alanine and 1.65 ml (15 mmoles) of N-methylmorpholine in 50 ml of dry ethyl acetate was chilled to −18° with stirring; then 2.01 ml (15 mmoles) of isobutylchloroformate was added, giving an immediate recipitate. After a minute or so, the bath was removed and 25 ml of water added. The ethyl acetate layer was separated in a chilled funnel and washed with 10 ml of water. After drying over two portions of anhydrous sodium sulfate, the colorless solution was separated by decanting, then concentrated on a rotary evaporator under vacuum with a bath to 37° for the first few minutes. The resulting oil crystallized. It was dissolved in a few milliliters of isopropyl alcohol by warming, then cooled to 20°. The resulting crystals were collected and dried in a vacuum desiccator: yield 4.00 g (84%), mp 68° sharp. A previous sample isolated from an attempted reaction with HOSu had mp 67.5–68.5° and $[\alpha]^{25}D$ −36.4 ± 1° (*c* 2.008, dioxane). *Anal.* Calcd for $C_{16}H_{17}NO_6$: C, 60.18; H, 5.37; N, 4.39. Found: C, 59.98; H, 5.70; N, 4.52.

Example 7. Oxazolone Formation from Z-Gly-Phe-OCOOCH$_2$-CH(CH$_3$)$_2$. Z-Gly-L-Phe-OH (1.78 g, 5 mmoles) and N-methylmorpholine (0.55 ml, 5 mmoles) were dissolved in 8 ml of dry THF and chilled to −15°, and 0.67 ml (5 mmoles) of isobutylchloroformate was added. The precipitated N-methylmorpholine hydrochloride was filtered off on a cold funnel and washed with 2.5 ml of cold (−80°) THF. The filtrate was held at −19°, and 2 ml of 1.3 *N* trimethylamine in THF was added. After 2 min, the solution was put in a −80° bath, and 80 ml of cold heptane was added, giving a precipitate. This was removed by decanting and filtering; it bubbled on warming. An infrared curve showed the characteristic absorption at 5.5 μ for an oxazolone.

Analysis of the filtrate by gas chromatography gave 0.34 ml of isobutyl alcohol (75% of the theoretical 0.46-ml by-product from oxazolone formation).

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(15) We have recently exchanged samples with Professor Young, and both laboratories have obtained a value of −33.5°. Our earlier results are best explained by volume errors on our part. We did not check these by weighing the solvent, a procedure recommended by Professor Young.