Tetrahedron, Suppl. 8, Part I, pp. 321–346. Pergamon Press Ltd. Printed in Great Britain

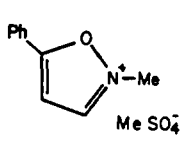
A USEFUL SYNTHESIS OF PEPTIDES[1]

R. B. WOODWARD, R. A. OLOFSON and H. MAYER
Converse Memorial Laboratory, Harvard University
Cambridge 02138, Massachusetts

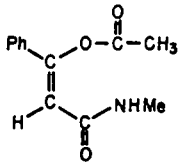
(*Received* 10 *June* 1966)

Abstract—Carboxylates react rapidly and smoothly with 3-unsubstituted isoxazolium salts under very mild conditions to yield enol esters. In this paper we report the application of this reaction as the carboxyl-activating step in a simple and practical synthesis of peptides. The utility of the specific peptide forming reagent, N-ethyl-5-phenylisoxazolium-3′-sulfonate, is described in some detail.

IN 1902 in a dissertation describing work carried out with Claisen at Kiel, Otto Mumm[2] reported the clean and facile precipitation of a white solid when N-methyl-5-phenylisoxazolium methosulfate (*aaa*) was treated with sodium acetate in water at room temperature. In the preceding paper we have described the evidence which led us to assign the enol ester structure *aab* to this compound.[3]

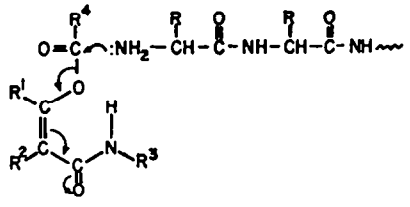


aaa



aab

Though we had disproved Mumm's extraordinary iminoanhydride formulation of the sodium acetate product, we were not disappointed with the results of our structure investigation, since enol esters of the type *aac* should be useful as acylating agents in peptide synthesis, the forming anion not only being stabilized by the amide carbonyl (cf. *aac* arrows) but also by a propitiously placed hydrogen bond (cf. *aac*).



aac

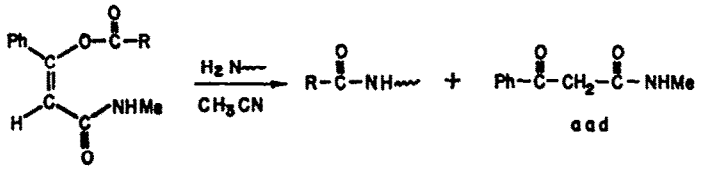
Further, we felt the synthetic scheme which produced *aab* could be employed to advantage in the construction of the wide variety of compounds encompassed by the generalized structure *aac*.

[1] For preliminary communications see: R. B. Woodward and R. A. Olofson, *J. Amer. Chem. Soc.* **83**, 1007 (1961); R. B. Woodward, R. A. Olofson, and H. Mayer, *Ibid.* **83**, 1010 (1961).

[2] O. Mumm, "Dissertation", Kiel (1902).

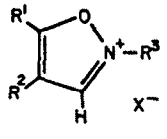
[3] R. B. Woodward and R. A. Olofson, *Tetrahedron, Suppl.* 7, *Stephen Memorial*, (1966).

In a preliminary test of the acylating ability of these enol esters, N-methyl-β-acetoxycinnamamide (*aab*), was treated with glycylglycine ethyl ester in ethyl acetate at room temperature, and we were gratified to discover that acetyldiglycine ethyl ester precipitated from this solution in high yield. Our initial studies on the activation of carboxyl groups with Mumm's isoxazolium salt (*aaa*) were not as successful. When the triethylamine salt of carbobenzoxyglycine was treated with *aaa* in aqueous solution, the precipitated enol ester was contaminated by large quantities of carbobenzoxyglycine, and when ethanol was used as the solvent, the major product was the iminoether tautomer derived from addition of ethanol to the intermediate ketenimine.[3] We were not, however, confined to hydroxylic solvents. By changing the anion of the isoxazolium salt, we were able to change its solubility properties to such an extent that we were able to carry out the reaction in almost any solvent. Use of the perchlorate salt of *aaa* in acetonitrile gave the most promising results; the carboxyl group of a number of nitrogen blocked amino acids could be activated almost quantitatively at room temperature in a few minutes in this way. Though acylation of the amine component by the activated ester proceeded smoothly, one major practical problem remained. The protected peptides formed in these reactions have in many cases solubility properties similar to those of the by-product, N-methyl-benzoylacetamide (*aad*), and though the peptide yields are high, the products are not easily purified.



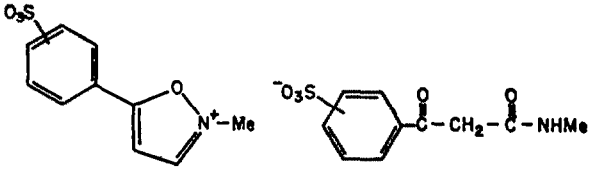
aad

The activating agent *aae* may be varied through specific choices for the groups R^1, R^2, and X^-, with a view to conferring a special degree of reactivity or particular physical properties on the reagent, the activated ester intermediate, or the by-product acylacetamide from the reaction of the enol ester with amines.



aae

Our first attempt as molecular architects resulted in the synthesis of the zwitterionic isoxazolium salt mixture *aaf*, *vide infra*.



aaf aag

Though this zwitterion is insoluble in all organic solvents, it is reactive enough to activate carboxyl groups in many of these same solvents. The by-product from peptide synthesis, the acylacetamide *aag*, a sulfonic acid salt, is exceedingly water soluble; in

fact, it is impossible to extract it from an aqueous solution into organic solvents. Purification of the product peptide is therefore very simple—all other products, by-products, side products, and remaining starting materials are either acids, bases, or salts!

In the synthesis of simple peptides, the zwitterion *aaf* gives yields of 80–90%. These are overall yields and involve essentially three reactions: (1) activation of the carboxyl component, (2) formation of the amino acid ester or peptide ester from the hydrochloride (the ester hydrochloride plus one equivalent of triethylamine is used directly in the reaction mixture; many other methods of peptide synthesis require the pure free amine ester), and (3) acylation of the amine to give the product peptide.

Using the synthesis of carbobenzoxyglycine benzyl amide as a model, we first carried out a number of experiments designed to discover the best conditions for the formation of the amide bond with this reagent. Carbobenzoxyglycine and *aaf* were suspended in solvent and triethylamine added. The mixture was stirred—usually until everything had dissolved, and the solution was clear (T_1 = time one). Then benzylamine was added and the solution stirred again for time T_2. Finally, the product was isolated. The effects of change in times (T_1 and T_2), temperature, solvent, reactant ratio, concentration, and a few other variables are presented in Tables 1 to 4.

Comments on Table 1. The slow change in yield with increasing T_2 (A-1, A-2, A-3) may be due to a slow reaction of the imide rearrangement product *aai* from the enol ester[3] with benzylamine to afford the correct acyl derivative. This hypothesis was confirmed in A-4 and A-5; increase in T_1 led to an increase in the amount of imide rearrangement product and therefore a larger difference in yield as T_2 was varied. The decreased yield indicates that the imide is not a particularly good acylating agent. Reaction of the ketoketenimine from *aaf* directly with benzylamine to form an amidine which could react further was probably the reason for the low yield in C-6. Since the peptide yield did not change significantly with concentration, two opposing factors may be cancelling each other. A higher concentration should increase the yield by increasing the reaction rate and therefore decreasing the time for intramolecular rearrangements. But a higher concentration should also increase the polarity of the solution and therefore decrease the peptide yield by increasing the rate of rearrangement of the enol ester to the imide.

TABLE 1. VARIATION OF T_1, T_2 AND CONCENTRATION[a]

Reaction no.	T_1[b] (min)	T_2 (hr)	Vol. Reaction Soln (ml)	Yield[c] mg (%)
A-1	25	1	5	370 (83)
A-2	25	4	5	372 (83)
A-3	25	15	5	385 (86)
A-4	120	1	5	318 (71)
A-5	120	15	5	358 (80)
A-6	0	15	5	219 (49)
A-7	25	15	2·5	378 (85)
A-8	25	15[d]	10	388 (87)

[a] The reactions were run in acetonitrile at room temp with equimolar quantities of the reactants (1·5 mM scale).

[b] In all cases except A-6 the soln was clear and colorless at the end of T_1.

[c] The product m.p. were all between 118·5–119·5° (analytical sample: m.p. 119–120°).

[d] Solid present in reaction soln at end of T_2.

TABLE 2. VARIATION OF THE RATIO OF REACTANTS[a]

Reaction no.	Ratio of Z-Gly/(*aaf*)/ Et_3N/benzylamine	T_1 (min)	T_2 (hr)	Yield mg (%)
A-3	1:1:1:1	25	15	385 (86)
A-9	1:1:1:0·9	25	15	345 (86)[b]
A-10	1:1:1:1·2	25	15[c]	387 (87)
A-11	1:1:1:2	25	15[d]	403 (90)
A-12	1:1:0·7:1	30[e]	15[e]	296 (66)
A-13	1:1:1·2:1	20[f]	15	319 (71)
A-14	1:1:2:1[g]	20	15	334 (75)
A-15	0·9:1:1:1	30[e]	15[e]	349 (87)[h]

[a] Reaction conditions identical with A-3 except for variations delineated in Table. Product m.p. were all between 118·5–119·5°.
[b] Yield based on benzylamine.
[c] Solid appeared after 20 min.
[d] Solid immediately ppd.
[e] Solid present throughout.
[f] Soln very yellow.
[g] 1 Eq. Et_3N added immediately; 1 eq. added at end of T_1.
[h] Yield based on Z-Gly.

TABLE 3. VARIATION OF SOLVENT[a]

Reaction no.	Solvent	T_1 (min)	T_2 (hr)	Yield mg (%)	M.p.
A-3	Acetonitrile[b]	25	15	385 (86)	118·5–119
A-16	Water[c]	5	18	113 (25)	89·5–94[d]
A-17	Abs. Ethanol[e]	90	18	141 (32)	107·5–116
A-18	Triethylamine[g]	30	18	89 (20)	119–119·5
A-19	Dimethylformamide[b]	25	1	330 (74)	119–119·5
A-20	Dimethylformamide[b]	25	18	343 (77)	118·5–119
A-21	Dimethylsulfoxide[h]	25	18	367 (82)	118–119
A-22	Nitromethane[i]	25	1	372 (83)	115–118
A-23	Nitromethane[j]	25	18	375 (84)	119–120
A-24	Nitromethane[j]	12	15	394 (88)	118·5–119
A-25	Dioxane[k]	60	18	183 (41)	119–120
A-26	Dioxane[k]	30	18	156 (35)	120–120·5
A-27	Tetrahydrofuran[k]	60	18	90 (20)	119–119·5
A-28	Ethyl acetate[l]	180	18	119 (27)	119–120
A-29	Acetonitrile/ethyl acetate[j, m]	25	15	382 (85)	118·5–119·5

[a] Reaction conditions identical with A-3 except for variations delineated in Table.
[b] Soln clear and colorless.
[c] Soln turned yellow-orange on addition of first drop of Et_3N; gum precipitated; this crystallized slowly.
[d] The IR spectrum indicated that only 20–30% of product was Z-Gly benzyl amide.
[e] Soln not clear after 90 min; light yellow after 18 h.
[f] The IR spectrum indicated that 80–90% of the product was Z-Gly benzyl amide.
[g] After addition of solvent a white gum formed which did not dissolve.
[h] Et_3N not very soluble in DMSO; soln yellow.
[i] Soln clear but yellow.
[j] Precipitate at end of T_2.
[k] *aaf* not dissolved; yellow oil after 18 hr.
[l] Solids present throughout reaction.
[m] Activation in 4 ml acetonitrile; benzylamine added in 3 ml AcOEt.

TABLE 4. A FEW DEVIATIONS FROM REACTION A-3

Reaction no.	Deviation	T_1 (min)	T_2 (hr)	Yield mg (%)	M.p.
A-3	Standard[a]	25	15	385 (86)	118·5–119
A-30	Used *aaf* monohydrate[a]	25	15	372 (83)	118·5–119
A-31	Reverse addn, benzylamine added at start T_1 and Et_3N at start T_2[b]	25	15	186 (42)	118–119
A-32	Equimolar soln of Et_3N and Z-Gly added to *aaf*[a]	25	6	387 (87)	118·5–119·5
A-33	Et_3N soln added over 20 mins to *aaf* and Z-Gly[a]	35[c]	15	378 (85)	118·5–119
A-34	Z-Gly treated with excess Et_3N soln, solvent removed at reduced press, residue dissolved in acetonitrile and added to *aaf*[d]	25	15	310 (69)	118–119
A-35	Reaction in cold room at 0°[e]	75	24	367 (82)	118·5–119·5
A-36	Used Z-Gly, Na salt instead of Z-Gly plus Et_3N[f]	30	15	99 (22)	118–119
A-37	Pyridine used instead of Et_3N[g]	30	15	209 (47)	117–118

[a] Soln clear and colorless.

[b] Did not all dissolve in 25 min, precipitated Z-Gly benzylamine salt at start T_1; this never dissolved.

[c] Included the 20 min.

[d] Not complete soln at end of T_1; soln clear at end of T_2.

[e] Same as *b*; substitute 75 min.

[f] Did not all dissolve during T_1; on addition of benzylamine gummy lump formed in bottom of flask; this never dissolved.

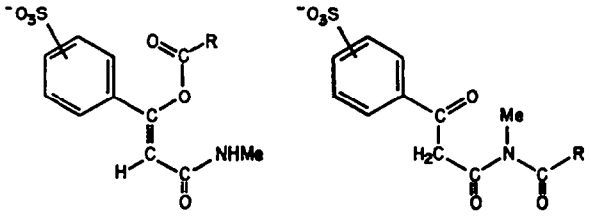
[g] Same as *b*; substitute 30 min.

Comments on Table 2. Most important, there was no major increase in yield by varying the ratio of the reactants from stoichiometric proportions. The use of excess triethylamine probably lowered the yield by catalyzing imide formation[3] (A-13, A-14).

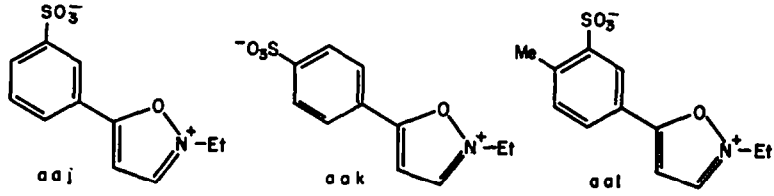
Comments on Table 3. Low yields in water (A-16) and ethanol (A-17) were due to reaction of the ketenimine from the isoxazolium salt (*aaf*) with the solvent; low yields in dioxane (A-25, A-26), tetrahydrofuran (A-27), and ethyl acetate (A-28) were due to incomplete formation of the enol ester in these solvents (not enough solvent power); and lowered yields in dimethylformamide (A-19, A-20) and dimethylsulfoxide (A-21) were probably due to an increase in the rate of the side reaction, imide formation, caused by the increased polarity of the solvent. Nitromethane, however, seemed to be at least as good a solvent if not an even better solvent than acetonitrile for formation of the amide bond.

Comments on Table 4. The Table itself includes most of the necessary comments, but the following points might be reiterated and the rationale of some of the experiments elucidated. Use of the hydrated zwitterion (A-30) did not lower the yield appreciably. Reaction A-31 was run in the hope that benzylamine would remain as unreactive benzylammonium ion until addition of the triethylamine. This would have made reaction with amino acid ester hydrochlorides more convenient in that all the solids could be weighed in first and only a solution of triethylamine need be added at the end of T_1. Pyridine did not seem to be a strong enough base to cause the reaction to proceed (A-37).

The preceding experiments revealed the presence of one major side reaction in the synthesis of peptides using the zwitterionic isoxazolium salt (*aaf*) to effect activation of the carboxyl group, namely rearrangement of the enol ester *aah* to an imide (*aai*).[4] We therefore set out to re-design our isoxazolium salt with a view to reducing the rate of



this rearrangement. The simplest way to do this is to increase the bulk of the N-alkyl substituent and thus decrease the accessibility of the nitrogen to electrophilic attack. Our attempts at altering molecular architecture resulted in the synthesis of the three N-ethyl zwitterionic isoxazolium salts (*aaj*, *aak* and *aal*) (*vide infra*). The physical properties of the three compounds are similar to those of the N-methyl-isoxazolium



salt (*aaf*); *aak* crystallized as a monohydrate (and was used as such), while *aaj* and *aal* crystallized as the anhydrous non-hygroscopic salts. Peptides could be synthesized in exceedingly high yields with these three isoxazolium salts using the procedures worked out with *aaf*. Most of our synthetic work was done with *aaj*, the *meta* substituted derivative, which gave the highest yields; *aak* was almost as valuable in peptide synthesis as *aaj*, while *aal*, though not quite as useful, gave significantly higher yields than the methyl zwitterion (*aaf*). We will first be concerned with some further experiments designed to determine the best conditions for peptide synthesis using the *meta* substituted zwitterion (*aaj*), using the synthesis of carbobenzoxytriglycine ethyl ester as a model. The isoxazolium salt was suspended in solvent, and a solution of carbobenzoxyglycine and triethylamine in the same solvent added. The mixture was stirred until the isoxazolium salt had dissolved and the solution was clear (T_1). Then the amine component either as the free ester or the ester hydrochloride plus triethylamine was added and the solution stirred for time T_2. The results are outlined in Tables 5 and 6.

Comments on Table 5. Of great practical importance was the finding that use of the amine hydrochloride plus an equivalent of triethylamine did not lead to significantly reduced yields in peptide synthesis. It was surprising that the yield in dimethylformamide (B-5) was as high as it was; this may be due to a decrease in the rate of rearrangement to the by-product imide versus the rate of amine acylation. That this rearrangement still proceeded, however, is seen in B-6; the peptide yield decreased as T_1 was

[4] Even when this side reaction is appreciable, the product imide does not interfere with peptide purification procedures, since it too is a water soluble sulfonic acid salt.

increased. The high peptide yield after a T_2 of only 1 hr (B-7) indicates that the enol ester is quite an active acylating agent. It is worth noting that when the activation step was performed at 0°, the peptide yield was increased slightly (B-9, B-10). The peptide syntheses with the methyl isoxazolium salt *aaf* (B-11, B-12, B-13) show its inferiority to the N-ethyl compounds.

TABLE 5. VARIATION OF T_1, T_2, SOLVENT, TEMPERATURE, AMINE COMPONENT, AND THE ZWITTERION USED[a]

Reaction no.	T_1 (min) T_2 (hr)	Temp	Amine comp[b]	Reagent solvent[c]		Yield mg (%)	M.p.
B-1	6 20	r.t.	B	*aaj*	Ni	470 (89)	167–8
B-2	6 20	r.t.	A	*aaj*	Ni	477 (91)	166–7
B-3	20 20	r.t.	B	*aaj*	Ac	463 (88)	167–8
B-4	20 20	r.t.	A	*aaj*	Ac	472 (90)	167–8
B-5	5 20	r.t.	B	*aaj*	DMF	436 (83)	165·5–7
B-6	60 20	r.t.	B	*aaj*	Ni	438 (83)	167–8
B-7	6 1	r.t.	B	*aaj*	Ni	460 (87)	166·5–7·5
B-8	60 20	0°	B	*aaj*	Ac	474 (90)	166·5–7·5
B-9	60 20	0°–r.t.	B	*aaj*	Ac	477 (91)	167–8
B-10	24 20	0°–r.t.[d]	B	*aaj*	Ni	473 (90)	167–8·5
B-11	10 20	r.t.	B	*aaf*	Ni	424 (81)	167–8
B-12	10 20	r.t.	A	*aaf*	Ni	443 (84)	167–7·5
B-13	25 20	r.t.	B	*aaf*	Ac	418 (79)	167·5–8·5
B-14	50 20	0°–r.t.[d]	B	*aal*	Ac	448 (85)	167–8
B-15	60 20	0°–r.t.[d]	B	*aak*[e]	Ac	469 (89)	167–8

[a] The experiments were run on a 1·5 mM scale using stoichiometric quantities of each reagent.
[b] A = free glycylglycine ethyl ester; B = glycylglycine ethyl ester hydrochloride plus 1 equiv. of Et_3N.
[c] Ni = nitromethane; Ac = acetonitrile.
[d] T_1 at 0°; T_2 at room temp.
[e] Used as the monohydrate.

TABLE 6. VARIATION OF THE AMINE COMPONENT AND STUDY OF POSSIBLE CATALYSIS[a]

Reaction no.	Amine component	Yield mg (%)	M.p.
B-1	1·5 mM GlyGlyOEt·HCl[b]	470 (89)	167–8
B-2	1·5 mM GlyGlyOEt	477 (91)	166–7
B-16	1·2 mM GlyGlyOEt·HCl[b]	378 (90)[c]	164–5
B-17	1·2 mM GlyGlyOEt	385 (91)[c]	164–5
B-18	2·0 mM GlyGlyOEt·HCl[b]	461 (88)[d]	167–8
B-19	2·0 mM GlyGlyOEt	475 (90)[d]	166·5–7·5
B-20	1·5 mM GlyGlyOEt[e]	434 (82)	167–8
B-21	1·5 mM GlyGlyOEt[f]	474 (90)	166·5–7

[a] The reaction conditions were identical with those of B-1 except for the variation in the quantity and type of amine component; 1·5 mM was stoichiometric.
[b] Plus an equal amount of Et_3N.
[c] Washed with 20 ml water and some bicarbonate instead of 10 ml water; the wash liquors were very yellow; yield based on the amine.
[d] Washed with 15 ml water instead of 10 ml; yield based on carbobenzoxyglycine.
[e] 0·3 mM of Et_3N added at start of T_2.
[f] 0·3 mM AcOH added at start of T_2.

Comments on Table 6. This Table indicates that any deviation from stoichiometry has little effect except to decrease the purity of the product. It is known that the peptide yield in some peptide syntheses proceeding through active esters is increased by the addition of triethylamine or acetic acid as catalysts.[5] This was not the case here. Triethylamine (B-20) decreased the yield by promoting the rearrangement to the imide; acetic acid (B-21) at least left well enough alone.

So far we have determined the conditions leading to the best yields in peptide synthesis using these zwitterionic isoxazolium salts. Prior to a study of the versatility of the method and the preparation of a number of peptides, it is necessary to determine the degree of racemization in peptide synthesis inherent in the method.

In the synthesis of peptides, the complete retention of optical activity is an important though rarely achieved goal.[6] While small quantities of racemized product can sometimes be removed by crystallization, it is often necessary to resort to more involved techniques in order to obtain the optically pure product. A very accurate test for racemization in peptide synthesis is the Anderson test,[7] the synthesis of carbobenzoxyglycyl ($_{DL}^{L}$) phenylalanylglycine ethyl ester using carbobenzoxyglycl-L-phenylalanine as the carboxyl component. The crude product from a particular method of synthesis was dissolved in ethanol, seeded with racemic tripeptide, and fractions collected until the optically pure material crystallized (determined by melting point). In our hands, the optically pure peptide did not begin to crystallize until the racemic material had been completely removed from solution. The test therefore gave very accurate and reproducible results (Table 7).

Comments on Table 7. (Our initial experiments on the extent of racemization in the Anderson test were run with commercial carbobenzoxyglycyl-L-phenylalanine (C-4 to C-9) and at least 2% of racemic tripeptide was obtained in every experiment. We suspected that this might be a result of the presence of some DL isomer in the dipeptide acid and this hypothesis was verified when we synthesized some optically pure carbobenzoxyglycyl-L-phenylalanine and repeated reactions C-8 (C-1) and C-6 (C-2).) The amount of racemization in the Anderson test using zwitterionic isoxazolium salts compared very favorably with results from the best of other methods of peptide synthesis. In fact there was no racemization with the N-ethyl zwitterionic isoxazolium salt (*aaj*) when the reaction was run in acetonitrile at 0° (C-1). That racemization was negligible even though glycine ethyl ester hydrochloride plus triethylamine was used as the amine component is of great practical importance. It should be noted that the use of excess triethylamine at the start of T_1 increased the extent of racemization (C-3).

We believe that if racemization occurs, it occurs via side reactions on an iminoanhydride intermediate;[3] the enol ester should be optically stable and therefore the rate of aminolysis of this species should have little connection with the extent of racemization.[8] In most other methods of peptide synthesis, the acylating species is also the

[5] R. Schwyzer, M. Feurer and B. Iselin, *Helv. Chim. Acta* **38**, 83 (1955).

[6] A goal which is in fact only reached by the azide method; this method, however, suffers the disadvantage of low yields due to extensive side reactions of the azide leading to side products of peptide-like solubility properties: (1) reduction to the amide; and (2) Curtius rearrangement to the isocyanate followed by addition of the amine component to afford the urea.

[7] For an example and references to the earlier literature, see G. W. Anderson and F. M. Callahan, *J. Amer. Chem. Soc.* **80**, 2902 (1958).

[8] Except under special conditions; see D. S. Kemp, Dissertation, Harvard University (1964).

one which is prone to racemization. Therefore in these cases, low solubility of the reacting components or any other factor which decreases the rate of peptide bond formation increases the amount of racemization.

TABLE 7. A STUDY OF THE EXTENT OF RACEMIZATION IN PEPTIDE SYNTHESIS: THE SYNTHESIS OF Z-GLY-($\frac{L}{DL}$)-PHE-GLYOET[a]

Reaction no.	Reagent	Solvent[b]	T_1 (min)	Temp	Crude yield %	Observed %DL	Observed %L	Actual[c] %DL	Actual[c] %L
C-1	*aaj*	Ac	55	0°–r.t.[e]	98	0·0	92	0·0	92
C-2	*aaj*	Ni	7	r.t.	98	3·9	87	3·9	87
C-3[d]	*aaj*	Ac	18	0°	93	2·5	82	2·5	82
C-4	*aaf*	Ac	20	r.t.	88	1·8	80	0·0	82
C-5	*aaj*	Ac	13	r.t.	94	3·6	86	1·4	88
C-6	*aaj*	Ni	7	r.t.	96	6·0	85	3·8	87
C-7	*aaj*	Ac	60	0°	96	2·3	89	0·0	91
C-8	*aaj*	Ac	60	0°–r.t.[e]	97	2·2	90	0·0	92

[a] The amine component was glycine ethyl ester hydrochloride plus an equiv of Et_3N. Our best m.p. for L tripeptide was 117·5–118·5° for 3 times recrystallized material. The yield of L tripeptide reported in this Table was for material melting between 116·5–118·5°. The reactions were run on a 2 mM scale; the reactant ratios were exactly stoichiometric.

[b] Ac = acetronitrile; Ni = nitromethane.

[c] Reactions C-4 to C-8 were run with commercial Z-Gly-L-PheOH which contained about 2·5% of the DL isomer. The yields are therefore corrected to show the true extent of racemization in the formation of the peptide.

[d] An additional equiv of Et_3N was added at the start of T_1.

[e] T_1 at 0°; T_2 at room temp.

So far we have prepared a number of peptides using zwitterionic isoxazolium salts as the carboxyl activating reagents (Table 8).

The general procedure is outlined as follows: *For activation*, an N-protected amino acid or peptide (exactly one mole), dissolved in acetonitrile or nitromethane containing triethylamine (exactly one mole), is added to a suspension of the zwitterionic isoxazolium salt (exactly one mole) in the same solvent and stirred at 0° or at room temperature until the reagent has dissolved. *For combination*, the amino acid ester hydrochloride or peptide ester hydrochloride (exactly one mole) and an equivalent of triethylamine are added and the reaction mixture is stirred overnight at room temperature. *For isolation*, the solvent is removed *in vacuo*, and further simple operations appropriate to the special characteristics of the product peptide are carried out. In many cases the residue may be simply triturated with warm water before crystallization from the same solvent (A). Alternatively, the residue may be distributed between water and ethyl acetate, the ethyl acetate layer extracted with bicarbonate and acid, and the peptide finally crystallized from a suitable solvent (B). When the peptide is water soluble, an aqueous solution of the residue may be extracted with ethyl acetate, the ethyl acetate removed *in vacuo*, and the product finally crystallized from a small amount of water (C). One crystallization (even under conditions of almost complete precipitation—in only one example in Table 8 is a second crop collected) usually suffices to yield pure material. In all cases removal of secondary products is rendered especially easy by their water solubility, and peptides are produced directly in an unusually high degree of purity.

TABLE 8. PEPTIDE SYNTHESIS WITH ZWITTERIONIC ISOXAZOLIUM SALTS[a]

Reaction no.[b]	Reagent	solvent[c]	T_1 (min)	Temp	Crude yield (%)	Recryst. yield	of M.p. (%)	Our best M.p.[d] (best lit. M.p.)[e]
				Z-Gly|benzyl amide				
A-38	*aaf*[f]	Ac	25	r.t.	—	86	119–119·5	119–120 (118–119)
A-39	*aaf*	Ni	10	r.t.	—	90	118·5–119·5	
A-40	*aaj*	Ni	6	r.t.	—	94	119–119·5	
				Z-Gly|Gly-GlyOEt				
B-22*	*aaj*	Ni	6	r.t.	—	90	167–168	167–168 (167–168)
B-23	*aaj*	Ni	6	r.t.	—	92	167–168	
B-24*	*aaj*	Ac	60	0°	—	91	167–168	
B-25*	*aal*	Ac	50	0°	—	85	167–168	
B-26*	*aak*[g]	Ac	60	0°	—	89	167–168	
B-27*	*aaf*	Ni	10	r.t.	—	82	167–168	
				Z-Gly-L-Phe|GlyOEt				
C-1*	*aaj*	Ac	55	0°	97	92	117–118·5	117–118
C-2*	*aaj*	Ni	7	r.t.	96	87	117–118	
C-4*	*aaf*	Ac	20	r.t.	88	82	117–117·5	
C-5*	*aaj*	Ac	13	r.t.	94	88	117–117·5	
				Z-Gly-DL-Phe|GlyOEt				
D-1*	*aaj*	Ni	8	r.t.	92	89	132–133	132·5–133 (132–134)
				diZ-L-Lys|GlyOEt				
E-1*	*aaj*	Ac	50	0°	98	95	90·5–92·5	91·5–93 (92–93)
				Z-L-Phe|GlyOEt				
F-1*	*aaj*	Ac	55	0°	98	93	109–110	109·5–110·5 (109–110)
F-2*	*aak*[g]	Ac	60	0°	96	92	109–110·5	
				Phth-Gly|GlyOEt				
G-1*	*aaj*	Ac	60	0°	—	88	193·5–194·5	193·5–194·5 (194–195)
				Z-L-Phe|L-LeuOMe				
H-1*	*aaj*	Ac	60	0°	94	90	106–107	109–109·5 (—)
				Z-L-Met|Gly-GlyOEt				
I-1*	*aaj*	Ac	45	0°	90	86	131·5–133	132·5–133·5 (131–133)
				Z-OH-L-Pro|Gly-GlyOEt				
J-1*	*aaj*	Ac	60	0°	—	80	145–146	145–146 (144–145)
				Z-Gly-Gly|L-TyrOMe				
K-1*	*aaj*	Ac	55	0°	88	84	159·5–161·5	159·5–161·5 (—)
				Z-L-Asp|GlyOEt NH_2				
L-1*	*aaj*	Ni	7	r.t.	—	80	185·5–187	186–187 (184–185)
L-2*	*aaj*	Ac	70	0°	—	79	186–187	
L-3*	*aaf*	Ac	25	r.t.	—	62	183–185	
L-4*	*aal*	Ni	6	r.t.	—	73	185–186	
				Z-L-Asp|L-LeuOMe NH_2				
M-1*	*aaj*	Ni	8	r.t.	—	76	176·5–178	177·5–178·5 (—)

TABLE 8.—*continued*

Reaction no.[b]	Reagent solvent[c]		T_1 (min) Temp		Crude yield (%)	Recryst. yield of M.p. (%)		Our best M.p.[d] (best lit. M.p.)[e]
					Z-L-Glu|L-ValOMe NH_2			
N-1*	*aaj*	Ni	10	r.t.	—	77	172·5–173	172·5–173·5 (173–175)
					Z-L-Glu|L-TyrOMe NH_2			
O-1*	*aaj*	Ni	9	r.t.	—	75	198–199	197·5–198·5 (198–201)
					Z-Gly|DL-PheOH			
P-1	*aaj*	$Ni-H_2O$	10	r.t.	29	19	161·5–162·5	162–162·5 (162)
					Z-Gly|L-PheOH			
Q-1*	*aak*[g]	Ac	50	0°	—	76[f]	129–130	129·5–130 (127)

[a] The reactions are described in further detail in the Experimental. The amide bond being formed is indicated by a vertical line.

[b] An asterisk indicates use of the hydrochloride plus Et_3N as the amine component.

[c] Ac = acetonitrile; Ni = nitromethane.

[d] After at least two further recrystallizations.

[e] References are given in the Experimental.

[f] Yields for many other peptides with *aaf* are not included, since the reactions were run before the best experimental conditions had been worked out. This reagent is, however, decidedly inferior to *aaj*.

[g] As the monohydrate.

[h] One author reports m.p's from 116–120° for pure material in different communications.

[i] A nitromethane soln of the activated ester was added to an aqueous soln of DL-phenylalanine and NaOH and the mixture stirred for 3 hr before isolation of the dipeptide acid.

[j] Overall yield for formation of the dipeptide ethyl ester (an oil) and hydrolysis to the dipeptide acid.

Comments on Tables 8 *and* 9. Table 8 lists the peptides we have prepared. Some comparisons of peptide yield *versus* the isoxazolium salt used are included, and it is seen that N-ethyl-5-phenylisoxazolium-3′-sulfonate (*aaj*) is the best activating agent we have yet devised for peptide synthesis. This fact should not, however, detract from the excellence of peptide yields using the other zwitterionic isoxazolium salts.

For convenience, Table 9 (abstracted from Table 8) has been prepared, and this table lists all the peptides we have synthesized with *aaj* as the carboxyl activating agent. The pure peptide yields in Table 9 are generally higher than the yields obtained in the best of the known methods of peptide synthesis. The greatest difference so far observed in peptide yields between the present method and other known methods of peptide synthesis is in the preparation of asparaginyl and glutaminyl peptides (L-O). Usually yields are very low due to a large number of side reactions involving the ω-amide group;[9] though these side reactions are probably still appreciable in reactions L to O, the side products do not interfere with the peptide isolation procedures. The ω-amide group is the only reactive amino acid side grouping which *cannot* ordinarily be blocked and thus deactivated in peptide synthesis.

From Table 9 it is also apparent that the hydroxyl of hydroxy-L-proline (J-1) and the phenolic hydroxyl of tyrosine (K-1, O-1) do not interfere in this method of peptide

[9] K. Medzchradszky, *Coll. Czech. Chem. Comm.* Special Issue, *Proceedings of the Symposium on Methods of Peptide Synthesis* p. 55. Prague, Czechoslovakia, September (1958).

synthesis. We are especially proud of the pure yield in the synthesis of carbobenzoxy-hydroxy-L-prolylglycylglycine ethyl ester, since this peptide is very water soluble. The isolation of this peptide is therefore a stringent test of the practicality of the method and illustrates the exceeding ease with which the peptide product can ordinarily be separated from the by-products.

TABLE 9. PEPTIDE SYNTHESES WITH THE ISOXAZOLIUM SALT (*aaj*[a])

Reaction no.	Peptide[b]	Solvent Temp of T_1	Crude yield (%)	Pure yield (%)
A-40	Z-Gly|benzyl amide	Nitromethane—r.t.	—	94
B-22	Z-Gly|Gly_2OEt	Nitromethane—r.t.	—	90
B-23	Z-Gly|Gly_2OEt	Nitromethane—r.t.	—	92
B-24	Z-Gly|Gly_2OEt	Acetonitrile—0°	—	91
C-5	Z-Gly-L-Phe|GlyOEt	Acetonitrile—0°	97	90
D-1	Z-Gly-DL-Phe|GlyOEt	Nitromethane—r.t.	92	89
E-1	diZ-L-Lys|GlyOEt	Acetonitrile—0°	98	95
F-1	Z-L-Phe|GlyOEt	Acetonitrile—0°	98	93
G-1	Phth-Gly|GlyOEt	Acetonitrile—0°	—	88
H-1	Z-L-Phe|L-LeuOMe	Acetonitrile—0°	94	90
I-1	Z-L-Met|Gly_2OEt	Acetonitrile—0°	90	86
J-1	Z-OH-L-Pro|Gly_2OEt	Acetonitrile—0°	—	80
K-1	Z-Gly_2|L-TyrOMe	Acetonitrile—0°	88	84
L-1	Z-L-Asp(NH$_2$)|GlyOEt	Nitromethane—r.t.	—	80
L-2	Z-L-Asp(NH$_2$)|GlyOEt	Acetonitrile—0°	—	79
M-1	Z-L-Asp(NH$_2$)|L-LeuOMe	Nitromethane—r.t.	—	76
N-1	Z-L-Glu(NH$_2$)|L-ValOMe	Nitromethane—r.t.	—	77
O-1	Z-L-Glu(NH$_2$)|L-TyrOMe	Nitromethane—r.t.	—	75

[a] Abstracted from Table 8.

[b] In all cases except A-40 and B-23, the ester hydrochloride plus Et_3N was used as the amine component. The ratio of all reactants was exactly stoichiometric. The amide bond being formed is indicated by a vertical line.

Since the publication of our preliminary communications on peptide syntheses with N-ethyl-5-phenylisoxazolium-3'-sulfonate[1] over thirty papers have appeared from other laboratories reporting the use of this reagent in the synthesis of amide bonds. From these papers we have selected a few examples of peptide syntheses which further confirm the utility of this reagent. These are listed in Table 10.

In conclusion, the following points might be reiterated. First: the peptide yields are very good even in the synthesis of asparaginyl, glutaminyl, arginyl, and seryl peptides which are ordinarily very difficult to prepare in reasonable yield. Second: in acetonitrile at 0° the coupling procedure is completely stereospecific as measured by the Anderson test for racemization. Third: the by-products are all water soluble and therefore easily removed from the product peptide derivative. One recrystallization, even under conditions of almost complete precipitation, usually suffices to yield pure

TABLE 10. EXAMPLES OF PEPTIDE SYNTHESES USING N-ETHYL-5-PHENYLISOXAZOLIUM-3'-SULFONATE (*aaj*)[a]

Ref.	Peptide	Yield (%)
b	Z-L-Arg|L-Arg-NH_2 (Tos, Tos)	73
c	Z-L-Arg|L-Phe-L-Pro-OH (!) (NO_2)	61
d	Tri-Gly-L-Trypt|Gly-L-Trypt-NH_2	—
e	Z-L-Phe-L-Ser|L-Phe-L-Arg-OMe (NO_2)	50
	t-BOC-Gly-OCH_2CO-Gly|Gly-OCH_2CO-Gly-OCH_2Ph-pNO_2	66
g	Tri-L-Lys-L-Lys|L-Arg-L-Arg-L-Pro-OMe (BOC, BOC, NO_2, NO_2)	81
h	Me-(BzO, NO_2)—CO-L-Ser|D-Val-L-Pro-Sar-L-MeVal-OBz	—
	For-Gly-L-Asp-L-Ser-Gly-Gly-L-Pro-L-Leu-L-Val-OMe (OBz, OBz)	—
	Z-L-Arg-Gly-L-Phe-L-Phe-L-Tyr-L-Thre-L-Pro-L-Lys-L-Ala-OMe (Tos, Tos)	87
k	L-Tyr-L-Ileu-L-Glu-L-Asp-L-Cys-L-Pro-L-Leu-Gly-NH_2 (NH_2, NH_2; → NH-CO-CH_2-CH_2-CH_2-S)	—
l	Z-L-Ala-L-Leu-L-Tyr-L-Val-L-Cys-Gly|L-Glu-L-Arg-Gly-L-Phe-L-Phe-L-Tyr-L-Thre-L-Pro-L-Lys-L-Ala-OMe (SBz; OBz, Tos; Tos)	38
m	Z-L-Ser-L-Tyr-L-Ser-L-Met-L-Glu-L-Hist-L-Phe-L-Arg-L-Trypt|Gly-L-Lys-L-Pro-L-Val-Gly-L-Lys-L-Lys-L-Arg-L-Arg-L-Pro-L-Val-L-Lys-L-Val-L-Tyr-L-Pro-L-Asp-Gly-O^{t}Bu (OBz; Tos; Tos, Tos; Tos, Tos, Tos; Tos, Tos; Tos; O^{t}Bu)	80

[a] The peptide bond was produced at the point indicated by a vertical stroke in the accompanying designations.

[b] J. Ramachandran, D. Chung, and C. H. Li, *J. Amer. Chem. Soc.* **87**, 2696 (1965).

[c] S. Lande, *J. Org. Chem.* **27**, 4558 (1962).

[d] D. M. Theodoropoulos and J. S. Fruton, *Biochem. J.* **1**, 933 (1962).

[e] M. Bodanszky, J. T. Sheehan, M. A. Ondetti, and S. Lande, *J. Amer. Chem. Soc.* **85**, 991 (1963).

[f] R. Schwyzer, J. P. Carrion, B. Gorup, H. Nolting, and A. Tun-Kyi, *Helv. Chim. Acta* **47**, 441 (1964).

[g] R. Schwyzer and H. Kappeler, *Ibid.* **46**, 1550 (1963).

[h] H. Brockman and H. Lackner, *Tet. Letters* 3517, 3523 (1964).

[i] All amide bonds prepared by this method: H. T. Cheung, T. S. Murthy, and E. R. Blout, *J. Amer. Chem. Soc.* **86**, 4200 (1964).

(*table footnotes continued at top of page 334*)

(table footnotes continued)

[j] P. G. Katsoyannis and K. Suzuki, *Ibid.* **85**, 2659 (1963)

[k] J. Rudinger and K. Jöst, *Exp.* **20**, 570 (1964).

[l] P. G. Katsoyannis and M. Tilak, *J. Amer. Chem. Soc.* **85**, 4028 (1963).

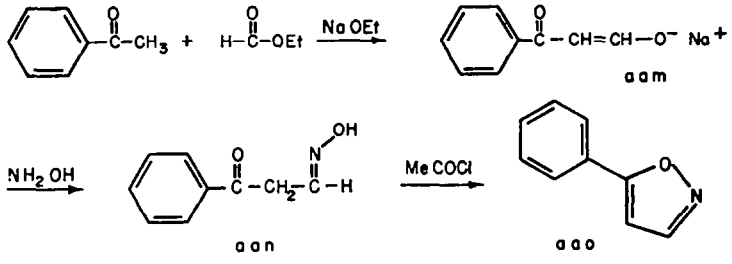
[m] J. Ramachandran and C. H. Li, *Ibid.* **87**, 2691 (1965); the reagent has been used by this group in the synthesis of a number of peptides related to ACTH (see this and previous papers).

material. A stringent test of this statement is the synthesis of carbobenzoxyhydroxy-L-prolylglycylglycine ethyl ester, a peptide which is itself very soluble in water.

There are two disadvantages to this method of peptide synthesis: (1) the high cost of the isoxazolium salt, and (2) the limitations on the choice of solvent.

SYNTHESIS OF THE ISOXAZOLIUM SALT REAGENTS

The isoxazole building blocks were prepared by standard methods;[10] 5-phenylisoxazole (*aao*) was synthesized by formylation of acetophenone with ethyl formate followed by oxime formation and dehydrative cyclization with acetyl chloride; 5-*p*-tolylisoxazole was prepared in the same way from *p*-methylacetophenone. We were,



however, forced to work out the experimental details ourselves, the procedures in the old literature[2, 11, 12] not being suited to large scale production. Some examples of this inadequacy are: (1) the oxime (*aan*) could only be prepared in batches starting with 8 to 50 g of the sodium salt of hydroxymethyleneacetophenone (*aam*) (the exact quantity seemed to depend only on the courage of the experimenter), and (2) isolation of the isoxazole was accomplished by an unnecessary neutralization, steam distillation and extraction prior to the final distillation. Surprisingly, use of acetic anhydride as the dehydrating agent yields not the isoxazole (*aao*) but benzoylacetonitrile.[12] In the synthesis of simple 5-arylisoxazoles, there is no contamination of the product by the 3-substituted derivative, a major by-product in the formation of some monoalkyl isoxazoles.[13]

[10] Comprehensive reviews of the methods of isoxazole synthesis are available; R. A. Barnes in *Heterocyclic Compounds* (Edited by R. C. Elderfield), Vol. 5; p. 452 *et seq.* Wiley, New York (1957); J. D. Loudon in *Chemistry of Carbon Compounds* (Edited by E. H. Rodd) Vol. 4; part A, p. 334 *et seq.* Elsevier, Amsterdam (1957); R. M. Acheson, *An Introduction to the Chemistry of Heterocyclic Compounds*, p. 270 *et seq.* Interscience, New York (1960).

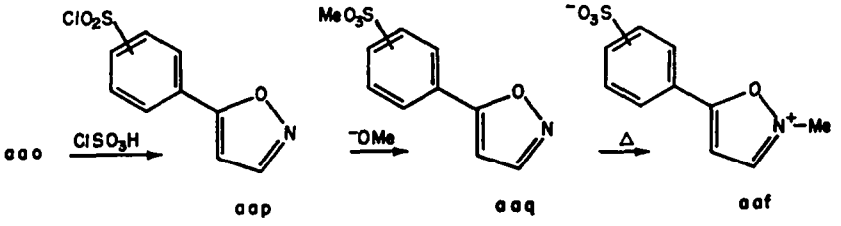
[11] H. Zöpfchen, Dissertation, Kiel (1899); G. Münchmeyer, Dissertation, Kiel (1910); A. Wirth, Dissertation, Kiel (1914); H. Hornhardt, Dissertation, Kiel (1937); O. Mumm and G. Münchmeyer, *Chem. Ber.* **43**, 3335 (1910); O. Mumm and H. Hornhardt, *Ibid.* **70**, 1930 (1937).

[12] R. Stock, Dissertation, Munich (1889); L. Claisen and R. Stock, *Chem. Ber.* **24**, 130 (1891).

[13] L. Claisen, *Chem. Ber.* **36**, 3664 (1903); **42**, 59 (1909); P. Thomaschewski, Dissertation, Kiel (1900).

5-Phenylisoxazole was chlorosulfonated with excess chlorosulfonic acid to yield what is probably a eutectic mixture of sulfonyl chlorides (*aap*) in which the benzene ring has been substituted in both the *meta* and *para* positions. The isomers could be partially separated by chromatography on silica; the ratio is about two parts of the *meta* isomer to one of the *para*. When the mixture of sulfonyl chlorides was treated with one equivalent of methoxide in methanol, an isolable but unstable sulfonic acid ester (*aaq*) was obtained which, when heated neat, yielded the zwitterionic isoxazolium salt (*aaf*) in a "bootstrap" reaction, the sulfonate ester acting both as the alkylating agent and the species alkylated.

Procedure I



The isoxazolium salt (*aaf*) is insoluble in organic solvents including even dimethylformamide, and is crystallized as the neutral zwitterion by precipitation from an aqueous hydrochloric acid solution with acetone. It crystallizes as a monohydrate, and though the mole of water can be removed *in vacuo* at room temperature, it is quickly restored on exposure to air.

The sulfonyl chloride mixture (*aap*) can also be alkylated with triethyloxonium fluoborate[14] to yield two easily separable isoxazolium fluoborates (*aar* and *aas*), the structures of which were proven by potassium hydroxide fusion of the zwitterionic salt derivatives (*aaj*) and (*aak*) to the respective hydroxybenzoic acids followed by comparison with authentic samples; the *meta* compound (*aar*) is isolated in 60% yield while the *para* compound (*aas*) is formed in about 30% yield. On hydrolysis in 2N HCl, the sulfonyl chlorides are converted in 80% yield to the corresponding zwitterionic isoxazolium sulfonates (*aaj*) and (*aak*).

In physical properties these two are quite similar to the mixture of methyl zwitterions (*aaf*); however, while the *para* compound (*aak*) crystallizes with one mole of water, the *meta* substituted derivative (*aaj*) crystallizes as the anhydrous species, is not hygroscopic, and is stable to light, time, and temperature; it decomposes above 200°.

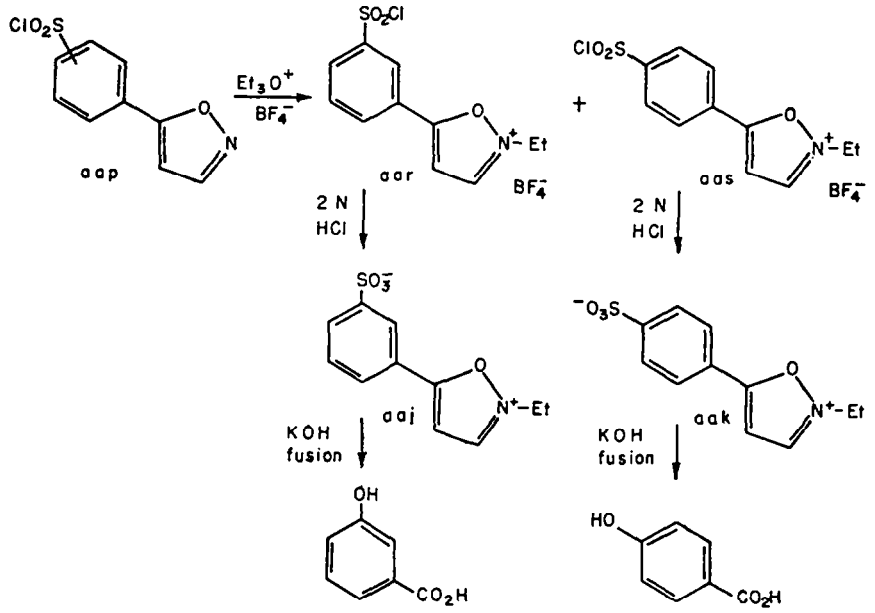
A mixture of *aaj* and *aak* has also been prepared by Procedure 1,[15] and we have used Procedure II to synthesize the methyl zwitterion (*aaf*), unfortunately without being able to separate the *meta* sulfonate from the *para* derivative.

We hoped that chlorosulfonation of an N-alkylisoxazolium salt would insure substitution in the *meta* position, since the positive charge might then be pushed completely into the isoxazole ring and further into the phenyl moiety. We therefore alkylated 5-phenylisoxazole with triethyloxonium fluoborate[14] to give the isoxazolium

[14] H. Meerwein, E. Battenberg, H. Gold, E. Pfeil, and G. Willfang. *J. Prakt. Chem.* **154**, 83 (1939).

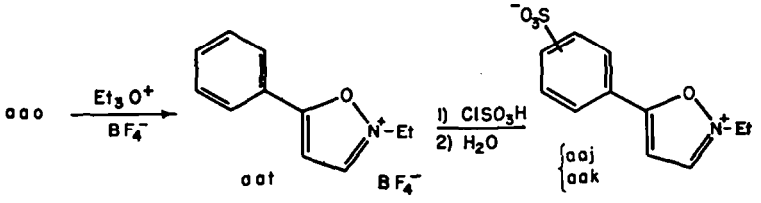
[15] This series of reactions was performed by Pilot Chemicals, Watertown 72, Massachusetts. We obtained the same mixture of *aaj* and *aak* by hydrolyzing the crude mixture of sulfonylchloride fluoborates (*aar* and *aas*) directly.

Procedure II



salt (*aat*) which was chlorosulfonated and hydrolyzed in one step (it is not practical to isolate the sulfonyl chloride) to yield the zwitterionic isoxazolium salt. However, once again the usual mixture of *meta* and *para* substituted sulfonates was obtained.

Procedure III



We were able to eliminate the annoyance of obtaining two products by preparing the zwitterionic isoxazolium salt from 5-*p*-tolylisoxazole. Both procedures II and III yielded the same product, N-ethyl-5-*p*-tolylisoxazolium-3′-sulfonate (*aal*). It is unfortunate that *aal* is not as valuable in peptide synthesis as the simple ethyl zwitterions (*aaj* and *aak*) because Procedure III is the most convenient and gives the highest overall yield. The product *aal* has the same solubility properties as the other zwitterionic salts, contains no water of crystallization and is not hygroscopic.

Acknowledgements.—We wish to express our appreciation of support by the National Science Foundation and the National Institutes of Health.

EXPERIMENTAL

All m.ps were taken in soft glass capillary tubes in a Hershberg m.p. apparatus using Anschütz thermometers. The IR spectra were run on a Perkin-Elmer Model 21 Double Beam Recording Spectrophotometer equipped with NaCl optics and the bands in the 5–7μ region were calibrated against the 5·88μ band of atmospheric water vapor; the UV spectra were run on a Cary Model 11 Recording Spectrophotometer. The best grade of commercial solvents was used without further purification.

Hydroxymethyleneacetophenone sodium salt (aam). A 53·5% dispersion of NaH in mineral oil (63 g, 33·6 g NaH or 1·40 M) was placed in a five-liter 3-neck flask equipped with a powerful blade stirrer, dropping funnel, and a reflux condenser outfitted with a gas outlet, and immersed in an ice bath. Absolute ether (2·5 l.) was added and stirring begun. Then abs EtOH (60 g, 1·3 M) was added over 30 min followed by a mixture of 170 g (1·41 M) high grade commercial acetophenone and 120 g (1·6 M) of ethyl formate (dried over $KHCO_3$). (As the soln thickened with pptd salts, more ether was added to a final volume of about 4 l.) This final addition took about 1 hr, and stirring was continued for an additional 4 hr at room temp. The reaction mixture was then filtered through a Buckner funnel of large surface area, pressed as dry as possible, washed with ether, and again pressed dry. Then the solid was dried overnight in the air, ground through a sieve, and dried for a short period under vacuum. This material was used directly in the next step. The yield was 89–99% of the theoretical 238 g.

On a pilot plant scale it may be safer to run the reaction in benzene; the procedure is the same, and the yields are then 73–77% (possibly a more economical procedure despite the lower yields).

Benzoylacetaldoxime (aan). The formylation product (*aam*; 170 g, 1·0 M) was placed in a 4-liter beaker immersed in an ice bath and equipped with a powerful stirrer. A mixture of water and ice (1300 ml) was added and stirring begun. When the salt had dissolved (10–20 min), 100 g (1·2 M) of a sat AcONa aq was added, quickly followed by 140 g (2 M) of a sat soln of hydroxylamine hydrochloride in water. If available a number of seed crystals of the product were then added. Vigorous stirring was continued for 30–40 min, small pieces of ice being continuously added to keep the soln as cold as possible. White crystals and yellow kernels precipitated from the soln, and the sides of the beaker were continually scratched to solidify the oily fraction. The cold soln was filtered, and any oil left on the sides of the beaker was washed with ice water and the washings also filtered. The ppt was washed with ice water and sucked dry for 20–30 min. The oil in the beaker was taken up in about 500 ml EtOH and the ppt added to the soln. Then the slurry was quickly taken to dryness at reduced press (30° or below) and the yellow-orange almost completely solid residue crystallized from benzene. The product was filtered, washed with a little cold benzene, and dried under vacuum at room temp. As many crops as possible were collected. This crude product was pure enough to use in the next step directly; yield: 90 g or 55%. The compd is unstable and should either be used directly or kept in the deep freeze.

The oxime could be purified; three recrystallizations from benzene yielded material of m.p. 87·5·88° (lit.[12] 86°).

5-Phenylisoxazole (aao). Benzoylacetaldoxime (*aan*); 120 g, 0·737 M) was placed in a 250 ml 3-neck flask outfitted with a dropping funnel, condenser, stirrer, and outlet tube, and immersed in an ice bath. Acetyl chloride (100 ml, 1·3 M) was added over a period of 30 min, slowly at first, faster toward the end of the addition. Stirring was continued for an additional ½ hr at room temp, and then the excess acetyl chloride was stripped off. The remainder was distilled through a short Vigreaux column at reduced press, the fraction boiling from 126–127° at 12 mm being collected; the yield of colorless product was 95 g or 89%: UV: $\lambda_{max}(\epsilon)$ 261 mμ (19,500) in 95% EtOH.

5-Phenylisoxazole (aao); *alternate procedure.* Without doubt the most difficult part of the original procedure was crystallization of the benzoylacetaldoxime, difficulties usually being due to incomplete removal of water from the product. However, an alternate procedure could be substituted, starting with the EtOH slurry of the crude material. The slurry was warmed to dissolve the solids completely, and then the water and EtOH were removed at 30° under vacuum as fast as possible to prevent excessive decomposition of the benzoylacetaldoxime. A vacuum of one mm or less was required to remove the last of the water. The remainder, a brown gum, was used directly in the synthesis of 5-phenylisoxazole, A magnetic stirring bar was put in the flask which was then equipped with a dropping funnel, condenser, and outlet tube, and placed in an ice bath. Acetyl chloride was added as before, the reaction mixture was distilled, and the fraction boiling between 100–130° at 12 mm was collected. This material was redistilled through a short Vigreaux column as above. The overall yield was only slightly below that of the other procedure.

Hydroxymethylene-p-methylacetophenone, sodium salt. Using the procedure already worked out for acetophenone, the yield averaged 90%. A slightly higher dilution of the reaction mixture proved advantageous.

p-Methylbenzoylacetaldoxime. Hornhardt's original procedure[11] was followed. The sodium salt of hydroxymethylene-p-methylacetophenone, (92 g) was placed in a 2-liter beaker equipped with an efficient stirrer and immersed in an ice bath, and 750 ml of an ice cold soln of 2 N NaOH was added along with enough ice to keep the mixture cold. Stirring was begun, and a cold conc aqueous soln of hydroxylamine hydrochloride (43 g) was added. After the mixture had been stirred for 1 hr, it was filtered to remove all undissolved material. While being kept cold, the filtrate was acidified to a pH of 5–6 by dropwise addition of 50% AcOH with vigorous stirring. The total addition took about 1 hr after the soln first turned milky. Then the reaction mixture was allowed to stand in the refrigerator for 6 hr, filtered, and the solid washed with cold water and dried overnight *in vacuo* at room temp. The product, 67 g (76%), was of sufficient purity to use directly in the next step.

5-p-Tolylisoxazole. The procedure devised for the synthesis of 5-phenylisoxazole was applicable to the preparation of the 5-*p*-tolyl analogue. After the AcCl had been stripped off, the product was distilled at aspirator press, precautions being taken to keep it from solidifying in the air condenser system (a heat gun directed at the positions were the product began to solidify proved most effective), and the product boiling within 5 degrees of the main fraction was collected. Finally the solid was crystallized once from aqueous MeOH to give material of m.p. 58–59° (lit.[11] 60° in 83% yield; UV: $\lambda_{max}(\epsilon)$ 266 mμ (18,500) in 95% EtOH.

N-Ethyl-5-p-tolylisoxazolium fluorobate. The procedure devised for the 5-phenyl analogue[3] (*aat*) was adopted. Two crystallizations from acetone–ether afforded 91% of a white solid of m.p. 137·5–138·5°. This compound also decomposes slowly on standing; UV: $\lambda_{max}(\epsilon)$ 309 mμ (25,100) in CH_2Cl_2. (Found: C, 52·52; H, 5·30; N, 4·66. $C_{12}H_{14}NOBF_4$ requires: C, 52·40; H, 5·13; N, 5·09%).

5-Phenylisoxazole-3′ (and 4′)-sulfonylchloride mixture (aap). A 500 ml 3-neck flask was equipped with a slipseal stirrer, 250 ml press equalizing dropping funnel, and an air condenser outfitted with a $CaCl_2$ drying tube and gas outlet, and immersed in an ice bath. 5-Phenylisoxazole (85 g, 0·586 M) was poured into the flask, and then 220 ml of redistilled chlorosulfonic acid (b.p. 149–151°; 3·3 M) was added with stirring over a period of 30 min, slowly at first and faster at the end. The dropping funnel and stirrer were removed and the joints stoppered with ground glass stoppers. Then the flask was placed in an oil bath, heated to 102°, and left at that temp for 30 hr. After the mixture had cooled, it was slowly poured onto 1600 g of ice with stirring, extracted 3 times with a total of 1200 ml of chf, dried over Na_2SO_4, and the chf stripped off at reduced press. The cream colored solid was crystallized from 300 ml of hot CCl_4, and dried in the air. Recrystallization yielded a nearly white solid melting between 90 and 92°, which was of sufficient purity to use in the next step; 85–90 g or 60–63%. The analytically pure material melted from 92·6–92·8°; UV: $\lambda_{max}(\epsilon)$ 269 mμ (16,000) in CH_2Cl_2. (Found: C, 44·36; H, 2·51; Cl, 14·68; N, 5·71. $C_9H_6ClNO_3S$ requires: C, 44·36; H, 2·48; Cl, 14·55; N, 5·75%).

Only starting material was obtained when the chlorosulfonation was run at room temp or at 60°; gas evolution, a useful measure of rate, did not take place until a temp of over 90° was reached. Some starting material could still be isolated if the chlorosulfonation was run for 6 hr at 105°. Addition of NaCl to the reaction mixture did not increase the rate or raise the product yield.

The sulfonylchloride mixture (300 mg) was placed on a silica gel column (18 g) and eluted with benzene. IR spectra of the various fractions indicated that two major components were present, though these components were only partly separated (no new bands were present in the spectra of the fractions). A strong band at 8·55μ probably belongs to the *meta* substituted compound while one at 8·40μ is attributable to the *para* substituted isomer.

5-Phenylisoxazole-3′(4′)-sulfonic acid methyl ester (aaq). The sulfonylchloride mixture (*aap*; 48·7 g, 0·2 M) was placed in a 1-liter round-bottomed flask, and 250 ml of abs MeOH was added. Then the flask was warmed slightly to dissolve the solid, and 0·2 M of a 0·5 molar soln of MeONa in MeOH was added with vigorous stirring, fast at first and then very slowly at the end of the addition. The soln was allowed to stand for 2 hr at room temp, and then the solvent was stripped off *in vacuo*. The residue was partitioned between water and chf, the chf layer separated, the water layer extracted twice with chf, the combined chf extracts dried over Na_2SO_4, and the solvent removed at reduced press. Though the crude solid could not be recrystallized easily, it was pure enough to use in the next step directly; yield: 25–30 g or 52–63%.

N-*Methyl-5-phenylisoxazolium-3'(4')-sulfonate mixture* (aaf). The methosulfonate (*aag*; 24 g, 0·1 M) was divided between 4 short wide mouth cork stoppered test tubes. These were placed in an oil bath, heated slowly to 67°, and left at that temp for an additional 20 min. (If the mixture was heated faster, the exothermic reaction which ensued on melting proceeded so vigorously that the test tubes exploded; 4 test tubes were used so critical mass was not exceeded.) On cooling the reaction mixture solidified to a brown glass. This was ground into a white milk with 100 ml water, and then 1500 ml acetone was added to precipitate the product completely. The ppt was filtered, washed with acetone, and dried for a few min. It was then dissolved in the minimum amount of 6N HCl and again pptd with acetone. This procedure was repeated: and the product was finally filtered, washed with a large amount of acetone, and dried at 0·1 mm of Hg or less for 12 hr at room temp to remove the water of hydration The product was kept in a tightly sealed brown bottle in the dark; yield: 18 g or 75%; decomposes while turning red from 202–205°. On exposure to air, the compound absorbed one mole of water within 30 min and could only be analyzed as the monohydrate. The water of hydration could be removed repeatedly without decomposing the product; UV: $\lambda_{max}(\epsilon)$ 284 mμ (22,000) in 0·1N HCl. (Found: C, 46·48; H, 4·45; N, 5·06; O, 31·00; S, 12·68; $C_{10}H_9NSO_4 \cdot H_2O$ requires: C, 46·68; H, 4·31; N, 5·45; O, 31·09; S, 12·47%).

N-*Ethyl-5-(3'-and 4'-chlorosulfonylphenyl-isoxazolium fluoborates* (aar *and* aas). Triethyloxonium fluoborate[14] (95 g, 0·5 M) was dissolved in 100 ml CH_2Cl_2, and a soln of 122 g (0·5 M) of the mixture of 5-phenyl-isoxazole sulfonylchlorides (*aap*) in the same solvent was added. The soln warmed up but otherwise remained clear and was kept overnight in a round-bottomed flask fitted with a drying tube. During this period some solid pptd. The solvent was stripped off at reduced press, and the mostly crystalline residue taken up in warm acetone and partially pptd with ether. Two recrystallizations afforded pure *meta* isomer of m.p. 161–162°; yield: about 100 g or 55%; UV: $\lambda_{max}(\epsilon)$ 281 mμ (21,700) in CH_2Cl_2. (Found: C, 36·97; H, 3·22; N, 3·79; S, 9·02. $C_{11}H_{11}ClNO_3SBF_4$ requires: C, 36·75; H, 3·08; N, 3·89; S, 8·92%).

The filtrates were concentrated and another 10 g of the *meta* isomer was removed by partial precipitation. The remaining oil, crude N-ethyl-5-(4'-chlorosulfonylphenyl) isoxazolium fluoborate (*aas*), usually was not purified further prior to hydrolysis. It could, however, be crystallized, though not easily. The crude yield varied from 25–30%; UV: λ_{max} 286 mμ (no ϵ crude) in CH_2Cl_2.

These two compounds are not stable and should be hydrolyzed within a day or two of preparation; the original white crystals are a light tan within 2 weeks. No advantage resulted from the use of excess triethyloxonium fluoborate, and the yield was lower when the alkylation was run in nitromethane as solvent.

N-*Ethyl-5-phenylisoxazolium-3'-sulfonate* (aaj).[16] N-Ethyl-5-(3'-chlorosulfonylphenyl) isoxazolium fluoborate (*aar*; 90 g, 0·25 M), was placed in a 2 liter round-bottomed flask, and 600 ml of 2 N HCl plus 250 ml EtOH were added while stirring to dissolve the isoxazolium salt. The soln was left overnight at room temp and then concentrated to about 150 ml *in vacuo* on the water bath at 35°. When acetone was slowly added to the concentrate, the product crystallized though almost 4 l. of acetone were required to effect complete pptn. After a few min the ppt was filtered, washed with acetone and dried for a short period. The product was dissolved in 1N HCl, precipitated with acetone, filtered, washed with acetone, and dried. Repetition of this process yielded 47–50 g or 75–80% of pure material which turned red while decomposing at 206–208°. Finally the product was dried *in vacuo* at room temp to remove the last traces of solvent; it is not hygroscopic; UV: λ_{max} (ϵ) 283 mμ (22,500) in 0·1 N HCl. (Found: C, 52·48; H, 4·20; N, 5·48; O, 25·18; S, 12·61. $C_{11}H_{11}NSO_4$ requires: C, 52·16; H, 4·38; N, 5·53; O, 25·27; S, 12·66%).

(It proved advantageous to precipitate the zwitterionic salt from a slight excess of dil HCl so the precipitated mass did not get too thick, and a pulpy crystal without hard seeds in the middle could be obtained. The product so obtained gives slightly improved yields in peptide synthesis.)

Fusion of aaj *with potassium hydroxide.* Potassium hydroxide (2·50 g) was ground together with 0·50 g of the *meta* substituted zwitterion (*aaj*); then the mixture was transferred to a nickel crucible and 0·5 ml water added. The reaction mixture was heated to 220° in a Wood's metal bath, kept at that temp for 45 min, cooled, dissolved in water, and extracted 5 times with ether. Then the aqueous soln was acidified with conc HCl, extracted 5 times with ether, the ether extract dried over Na_2SO_4, and the solvent evaporated. Light tan prisms (0·252 g or 92%) of m.p. 185–186° were obtained. The IR

[16] This compound is now commercially available from Pilot Chemicals, Watertown 72, Mass. and from the Aldrich Chemical Company, Inc. Milwaukee, Wisconsin.

spectrum in KBr was identical with that of commercial material. However, after recrystallization from water, m.p. 203–204°, the IR spectrum changed. Commercial *m*-hydroxybenzoic acid was recrystallized from water, m.p. 203–204°, and its spectrum was identical with that of the fusion product; it seems that two crystalline forms are isolable; mixed m.p. 203–204°.

N-*Ethyl-5-phenylisoxazolium-4'-sulfonate* (aak). The procedure devised for the *meta* sulfonyl chloride (*aar*) was adapted to the hydrolysis of the crude oil (*aas*) to yield a yellow soln which was worked up in the usual manner. The product was recrystallized by being dissolved in 6N HCl and pptd with acetone. After 3 such recrystallizations, the pure material was dried *in vacuo* at room temp; dec p. 200°; yield: 75%. The product was analyzed and used as the monohydrate; it proved difficult to remove much of the water under vacuum; UV: λ_{max} (ϵ) 288 mμ (28,000) in 0·1 N HCl. (Found: C, 49·01; H, 4·60; N, 5·21; O, 29·39; S, 11·65. $C_{11}H_{11}NO_4S \cdot H_2O$ requires: C, 48·70; H, 4·83; N, 5·16; O, 29·49; S, 11·82%).

In another experiment the mixture of phenylisoxazole sulfonylchlorides (*aap*) was treated with triethyloxonium fluoborate and the crude product mixture of isoxazolium fluoborates hydrolyzed directly in 1 N HCl to yield 82% of a mixture of *meta* and *para* isomers; dec. p. 185–186°. The ratio of *meta* to *para* as estimated from the IR spectra was about 2:1; the IR spectrum of this mixture was nearly identical with that obtained for the product from Procedure I (sample provided by Pilot Chemicals Inc.). Attempts at separation of the two isomers by fractional crystallization were not very successful.

Fusion of aak *with potassium hydroxide.* Potassium hydroxide fusion using the procedure devised for the *meta* zwitterion (*aaj*) resulted in 0·235 g (86%) of an almost white crystalline product of m.p. 213–215°. Recrystallization from water raised the m.p. to 216–217°; the KBr spectrum was identical with that of commercial *p*-hydroxybenzoic acid; m.p. 216–217°.

N-*Methyl-5-(3'-and 4'-chlorosulfonylphenyl) isoxazolium fluoborate mixture.* Trimethyloxonium fluoborate[14] (4·52 g) and an equimolar quantity of the 5-phenyl-isoxazole sulfonylchloride mixture (*aap*; 7·45 g) were dissolved in nitromethane, and the mixture was allowed to stand at room temp overnight. The solvent was stripped off and the residue crystallized twice from acetone–ether; m.p. 134·5–135°; yield: 9·05 g or 89% crude and 7·30 g or 71% pure. Since the product is very unstable, it should be hydrolyzed within a day of preparation; UV: λ_{max} (ϵ) 285 mμ (21,200) in CH_2Cl_2. (Found: C. 34·82; H, 2·86; N, 3·69; S, 9·50. $C_{10}H_9ClNO_3SBF_4$ requires: C, 34·76; H, 2·62; N, 4·05; S, 9·28%).

The isoxazolium fluoborate mixture was hydrolyzed with 2N HCl to the zwitterionic salt (*aaf*); dec p. 217–221°. Though its IR spectrum was identical with that of *aaf* from Procedure I, the decomposition point was much higher, indicating a different amount of fractionation during purification in the two procedures. However, contamination must only be by isomers since the yield in peptide synthesis with both products was the same.

N-*Ethyl-5-phenylisoxazolium-3'(4')-sulfonate mixture; Procedure* III. N-Ethyl-5-phenylisoxazolium fluoborate (*aat*[3]; 0·522 g, 2mM) was placed in a ground glass jointed 10 ml round-bottomed flask, and 1·16 g (0·01 M) of redistilled chlorosulfonic acid was slowly added. The addition was endothermic, and copious amounts of HF and BF_3 were evolved. Then the reaction mixture was heated (condenser and drying tube attached) in an oil bath for 2 hr at 90° and 12 hr at 110° and finally cooled. The light yellow reaction mixture was pipetted onto 5 g of ice and the nearly colorless solution kept at room temp overnight. Acetone was slowly added with cooling and scratching, and a white crystalline solid pptd after some min. After the ppt had been kept in the refrigerator for 1 hr, it was filtered, washed with acetone and dried; yield: 0·293 g or 58%; dec. p. 185–187°.

The IR spectrum was identical with that obtained from the product of hydrolysis of the crude mixture of sulfonylchloride fluoborates (*aar*) and (*aas*), again about 2:1 *meta* to *para*. Recrystallization from 1 N HCl–acetone did not change the isomer ratio.

5-p-*Tolylisoxazole-3'-sulfonylchloride.* This final series of reactions on the synthesis of the *p*-tolyl zwitterionic isoxazolium salt has only been run once in an exploratory fashion, and the yields are not necessarily the best possible; the earlier procedures were adapted without modification. Our interest in this series was greatly diminished when we learned that the isoxazolium salt (*aal*) was not as valuable in peptide synthesis as the simple ethyl zwitterions.

5-*p*-Tolylisoxazole was chlorosulfonated using the procedure devised for 5-phenylisoxazole to give the sulfonylchloride in 58% yield; m.p. 137·5–138·5°. (Found: C, 46·26; H, 3·15; Cl, 14·18; N, 5·19; $C_{10}H_8ClNO_3S$ requires: C, 46·61; H, 3·13; Cl, 13·76; N, 5·44%).

N-*Ethyl-5-p-(3'-chlorosulfonyltolyl) isoxazolium fluoborate.* 5-*p*-Tolyl-3'-sulfonylchloride was alkylated in the usual manner with triethyloxonium fluoborate to give the isoxazolium salt in 82% yield;

m.p. 154–155°. This compound decomposes on standing and should be used soon after prepn. (Found. C, 38·65; H, 3·87; N, 3·56; S, 9·25. $C_{12}H_{13}ClNO_3SBF_4$ requires: C, 38·58; H, 3·51; N, 3·75; S, 8·58%).

N-*Ethyl*-5-p-*tolylisoxazolium*-3′*sulfonate* (aal)

Procedure II. Hydrolysis of N-ethyl-5-*p*-tolylisoxazole-3′-sulfonylchloride to the zwitterionic salt was accomplished in 80% yield; decomposed while turning red at 208–211°. Though the zwitterion is not hygroscopic, it is slightly light sensitive; UV: λ_{max} (ϵ) 296 mμ (25,100) in 0·1 N HCl. (Found: C, 53·63; H, 4·85; O, 24·24; S, 12·06. $C_{12}H_{13}NO_4S$ requires: C, 53·92; H, 4·90; O, 23·94; S, 12·00%).

Procedure III. N-Ethyl-5-*p*-tolylisoxazolium fluoborate was chlorosulfonated and hydrolyzed on dilution with water using the procedure devised for the simple ethyl zwitterion mixture to give pure product in an overall yield of 61%; dec. p. 208·5–211°. This procedure is very convenient and gives the highest yield of all those tested.

Acetyldiglycine ethyl ester. N-methyl-β-acetoxycinnamamide (*aab*; 220 mg) was dissolved in 7 ml AcOEt, and 160 mg of glycylglycine ethyl ester (prepared from the hydrochloride by the method of Fischer and Fourneau;[17] m.p. 86–87° (lit.[18] 85–86°)) was added. After 2 hr, the pptd peptide was filtered, washed with AcOEt, and dried; yield: 178 mg or 88% of m.p. 151·5–152·5° (lit.[19] 152°). Recrystallization did not change the m.p.

Treatment of carbobenzoxyglycine sodium salt with N-*methyl*-5-*phenylisoxazolium methosulfate* (aaa). When the reaction was run in aq soln, the pptd enol ester was contaminated by an almost equal quantity of carbobenzoxyglycine. Use of a phosphate buffer at pH ~ 6·02 did not change the product distribution nor did use of the bisulfate salt with an additional equivalent of hydroxide ion. When the reaction was run in EtOH almost 80% of the product was the iminoether tautomer resulting from addition of EtOH to the intermediate ketenimine.[3] The remainder was a mixture of the enol ester and the imide. The product composition was determined with the aid of IR spectra.

Preparation of carbobenzoxyglycine benzyl amide using N-*methyl*-5-*phenylisoxazolium perchlorate.* Carbobenzoxyglycine potassium salt (494 mg) and the isoxazolium perchlorate (520 mg) were dissolved in 10 ml acetonitrile, and the mixture was allowed to stand at room temp for 10 min. Then 215 mg of benzylamine was added and the mixture allowed to stand for 4 hr while the peptide product pptd. The reaction mixture was filtered and a second crop of peptide obtained from the filtrate: first crop, 480 mg of m.p. 115·5–117°; second crop, 63 mg of m.p. 107–113° for an overall crude yield of 91%. The product was contaminated with N-methylbenzoylacetamide; the m.p. of pure Z-Gly benzyl amide is 119–120° (*vide infra*). Other simple peptides were prepared in the same way in varying degrees of purity. Carboxyl activation in acetonitrile followed by aminolysis in AcOEt did not result in an improved yield.

A study of reaction conditions

Carbobenzoxyglycine benzyl amide. The results are contained in Tables 1–4. A sample synthetic procedure, Reaction A-3, is described below.

Carbobenzoxyglycine[20] (314 mg, 1·5 mM), the N-methylisoxazolium sulfonate (*aaf*; 359 mg, 1·5 mM), and 2 ml of acetonitrile were placed in a 10 ml Erlenmeyer flask equipped with a ground-glass stopper, and the flask was suspended above a magnetic stirrer and stirring begun. Two ml of a triethylamine soln in acetonitrile (1·5 mM Et_3N) was added over a 1 min period, and stirring was continued until the isoxazolium salt had reacted and was completely in soln, 25 min (T_1). Then 1·0 ml of a soln of benzylamine and Et_3N in acetonitrile (1·5 mM benzylamine and 0·1 mM Et_3N) was added, and the soln was stirred for an additional 15 hr (T_2).

The product isolation procedure was exactly the same in all the reactions in the A series and was designed to give a very pure product without the invonvenience of recrystallization. This series of experiments was carried out to determine not the absolute yield but the change in yield as conditions were varied. All precautions were taken to insure that a constant quantity was lost in the isolation procedure.

The reaction mixture was transferred to a 25 ml round-bottomed flask, the final traces being washed in with excess solvent. The solvent was then stripped off at reduced press. Then 20 ml water was added to the solid residue and the mixture triturated and heated to about 50° on the steam bath in order to leach the solid further. The product was cooled for 3 hr or longer in the refrigerator and

[17] E. Fischer and E. Fourneau, *Chem. Ber.* **34**, 2868 (1901).
[18] H. N. Rydon and P. W. G. Smith, *J. Chem. Soc.* 2542 (1955).
[19] E. Fischer, *Chem. Ber.* **35**, 1095 (1902).
[20] *Organic Syntheses* Coll. Vol. III; p. 168. Wiley, New York (1955).

filtered, the last traces of solid being washed in with the filtrate. Then the product was washed carefully with 5 ml water, dried in the air to constant wt at room temp, and weighed (vacuum drying did not decrease the wt).

A study of reaction conditions

Carbobenzoxytriglycine ethyl ester. The results are outlined in Tables 5 and 6. A sample synthetic procedure, Reaction B-1, is described below.

The N-ethyl zwitterionic isoxazolium salt (*aaj*; 380 mg, 1·5 mM) and 5 ml nitromethane were placed in a 10 ml Erlenmeyer flask equipped with a ground-glass stopper and magnetic stirring bar. Stirring was begun, and 2 ml of a soln of carbobenzoxyglycine (314 mg, 1·5 mM) and Et_3N (152 mg, 1·5 mM) in nitromethane was added. Stirring was continued until the isoluble zwitterion had all reacted, and the soln was clear, six min (T_1). Then 295 mg (1·5 mM) of glycylglycine ethyl ester hydrochloride (twice recrystallized material from Nutritional Biochemicals Corp.; m.p. 181–182°) (lit.[21] 181–182°) was added along with 1·5 mM of Et_3N in 1·5 ml of nitromethane. The soln was stirred for 20 hr (T_2), and during this period a solid pptd.

The same isolation procedure was used in all the reactions of the B series. The soln was transferred to a 25 ml round-bottomed flask and the solvent removed *in vacuo*. Then 15 ml of water was added to the residue and the mixture triturated and nearly dissolved on the steam bath. The mixture was cooled in the refrigerator for 3 hr, and the ppt was finally filtered, washed with 10 ml of water, dried in the air at 40°, and weighed.

A study of racemization

The Anderson test. The results are summarized in Table 7. The experimental procedure for reaction C-8 is reproduced below.

The N-ethyl zwitterionic isoxazolium salt (*aaj*; 506 mg, 2 mM) was placed in a 25 ml Erlenmeyer flask along with 5 ml acetonitrile in a cold room at 0° and stirring begun, using a magnetic stirring apparatus. A soln of carbobenzoxyglycyl-L-phenylalanine[22] (twice recrystallized material from Mann Research Laboratories, Inc., m.p. 127·5–128·5°, $[\alpha]_D^{23}$ + 37·6° (c = 2, EtOH), (lit[23] 127°)) (713 mg 2 mM) and Et_3N (2 mM) in acetonitrile was added. Stirring was continued until the zwitterion had almost completely dissolved, and the soln was a very light yellow (1 hr). Then glycine ethyl ester hydro chloride (279 mg, 2 mM) and a soln of Et_3N (2 mM) in acetonitrile were added and the reaction mixture stirred overnight at room temp. The solvent was stripped off under vacuum, the residue partitioned between 40 ml of AcOEt and 10 ml water and separated, the organic layer washed with 2 × 10 ml 5% $NaHCO_3$, 1 × 10 ml water, 1 × 10 ml 1 N HCl, 1 × 5 ml water, dried over Na_2SO_4, and the AcOEt stripped off at reduced press. The white crystalline residue weighed 856 mg. (97%). Using the Anderson test procedure[7] for determining the degree of racemization, the following results were obtained:

Z-Gly-DL-Phe-GlyOEt	19 mg	2·2%
Z-Gly-L-Phe-GlyOEt	793 mg	90% m.p. 116·5–117·5°, $[\alpha]_D^{23}$ = − 12·6°, c = 2, EtOH
Residue	21 mg	2·4%
Loss on purification	23 mg	2·6%

Recrystallization of the optically pure material raised the m.p. to 117–118°, $[\alpha]_D^{23}$ = −12·7°, c = 2 (EtOH).[24]

Carbobenzoxyglycine benzyl amide

A-38. The above reaction procedure for carboxyl activation and aminolysis is the standard one. Henceforth only quantities, solvents, reaction times, and temps will be given.

Carbobenzoxyglycine (837 mg, 4 mM), zwitterion (*aaf*; 957 mg, 4 mM), Et_3N (405 mg) for a T_1 of 25 min in acetonitrile at room temp. Benzylamine (429 mg) overnight at room temp. The total volume of the reaction soln was 12 ml. The solvent was stripped off and the product triturated with hot water. The mixture was cooled in the refrigerator, and the ppt filtered, washed with water and dried; yield: 1·024 g or 86%; m.p. 119–119·5°.

[21] H. F. Schott, J. B. Larkin, L. B. Rockland and M. S. Dunn, *J. Org. Chem.* **12**, 490 (1947).

[22] This material, which contained about 2·5% racemic acid, was used in reactions C-4 to C-8. Optically pure dipeptide acid (cf. reaction Q-1) was used in reactions C-1 to C-3.

[23] G. W. Kenner and R. J. Stedman, *J. Chem. Soc.* 2069 (1952).

[24] See ref. 7; the same laboratory reports for pure material in various places m.ps from 116–120·5, $[\alpha]_D$ −11 to −14°.

A-39. Same as A-38 except in nitromethane for a T_1 of 10 min; yield: 1·069 g or 90%; m.p. 118·5–119·5°.

A-40. Same as A-38 except N-ethyl zwitterion (*aaj*; 1·012 g) for a T_1 of 6 min in nitromethane at room temp; yield: 1·121 g or 94%; m.p. 119–119·5°.

The product from reaction A-40 was recrystallized from acetone–water for analysis; m.p. 119–120° (lit.[5, 25] 113·4–114°, 118–119°). (Found: C, 68·41; H, 5·86; N, 9·26. Calc. for $C_{17}H_{18}N_2O_3$: C, 68·44; H, 6·08; N, 9·39%).

Carbobenzoxytriglycine ethyl ester

B-22. Carbobenzoxyglycine (836 mg, 4 mM), zwitterion (*aaj*; 1·012 g), Et_3N (405 mg) for a T_1 of 6 min in nitromethane at room temp. Glycylglycine ethyl ester hydrochloride (786 mg) and Et_3N (405 mg) overnight at room temp. The total volume of the reaction soln was 18 ml. The solvent was stripped off, the residue dissolved in hot water, the soln cooled, and the ppt filtered, washed with water and dried; yield: 1·265 g or 90%; m.p. 167–168°.

The product was recrystallized from EtOH–water for analysis; m.p. 167·5–168° (lit.[26] 167–168°). (Found: C, 54·54; H, 6·02; N, 11·55. Calc. for $C_{16}H_{21}N_3O_6$: C, 54·69; H, 6·02; N, 11·96%).

B-23. Glycylglycine ethyl ester (640 mg) was used as the amine component instead of the hydrochloride plus Et_3N; yield: 1·286 g or 92%; m.p. 167–168°.

B-24. Same as B-22 except T_1 was in acetonitrile at 0° for 60 min; yield: 1·280 g or 91%; m.p. 167–168°.

B-25. Same as B-22 except the *p*-tolyl zwitterion (*aal*; 1·069 g) was reacted for a T_1 of 60 min at 0° in acetonitrile; yield: 1·194 g or 85%; m.p. 167–168°.

B-26. Same as B-22 except the *para* substituted zwitterion monohydrate (*aak*; 1·085 g) was reacted for a T_1 of 60 min at 0° in acetonitrile; yield: 1·251 g or 89%; m.p. 167–168°.

B-27. Same as B-22 except the N-methyl zwitterion (*aaf*; 956 mg) was reacted for a T_1 of 10 min in nitromethane at room temp; yield: 1·154 g or 82%; m.p. 167–168°.

*Carbobenzoxyglycyl-*DL*-phenylalanylglycine ethyl ester*

D-1 Carbobenzoxyglycyl-DL-phenylalanine (twice recrystallized material from Mann Research Laboratories Inc ; m.p. 162–162·5° (lit [23] 162°) (1·070 g, 3 mM), zwitterion (*aaj*; 760 mg), Et_3N (304 mg) for a T_1 of 8 min in nitromethane at room temp. Glycine ethyl ester hydrochloride (420 mg) and Et_3N (304 mg) overnight at room temp. The total volume of the reaction solution was 20 ml. The isolation procedure adopted for the L-stereoisomer was followed; crude yield: 1·212 g or 92%. Recrystallization from acetone–water gave the pure peptide; yield: 1·176 g or 89%; m.p. 132–133°. Further recrystallization yielded material of m.p. 132·5–133° (lit.[27] 132–134°).

*Dicarbobenzoxy-*L*-lysylcglyine ethyl ester*

E-1. Dicarbobenzoxy-L-lysine (prepared from L-lysine[28] (*aaj*); m.p. 77–79° (lit.[28] 55–65°) (1·036 g. 2·5 mM) zwitterion (*aaj*; 633 mg) Et_3N (253 mg) for a T_1 of 50 min in acetonitrile at 0°. Glycine ethyl ester hydrochloride (349 mg) and Et_3N (253 mg) overnight at room temp (soln volume, 20 ml). Procedure B, the isolation procedure for carbobenzoxyglycyl-L-phenylalanylglycine ethyl ester, was followed; crude yield: 1·228 g or 98%. The peptide was recrystallized from AcOEt–pet. ether; yield: 1·181 g or 95%; m.p. 90·5–92·5°. Further recrystallization yielded peptide of m.p. 91·5–93° (lit.[27] 92–93°).

*Carbobenzoxy-*L*-phenylalanylglycine ethyl ester*

F-1. Carbobenzoxy-L-phenylalanine (prepared from L-phenylalanine[29]; m.p. 87–88° (lit.[29] 88–89°) (898 mg, 3 mM), zwitterion (*aaj*; 760 mg), Et_3N (304 mg) for a T_1 of 55 min at 0° in acetonitrile. Glycine ethyl ester hydrochloride (419 mg) and Et_3N (304 mg) overnight at room temp (soln volume, 18 ml).

[25] D. Ben-Ishai, *J. Amer. Chem. Soc.* **79**, 5736 (1957).
[26] G. W. Anderson, J. Blodinger and A. D. Welcher, *J. Amer. Chem. Soc.* **74**, 5309 (1952).
[27] J. R. Vaughan, Jr. and R. L. Osato, *J. Amer. Chem. Soc.* **74**, 676 (1952).
[28] E. Katchalski in *Methods in Enzymology* (Edited by S. P. Colowich and N. O. Kaplan), Vol. 3; p. 542. Academic Press, New York (1957).
[29] W. Grassman and E. Wünsch, *Chem. Ber.* **91**, 462 (1958).

Isolation of the peptide by Procedure B afforded 1·131 g or 98% of crude material. The peptide was recrystallized from AcOEt–pet. ether; yield: 1·071 g or 93%; m.p. 109–110°. Further recrystallization afforded peptide of m.p. 109·5–110° (lit[30] 109–110°).

F-2. Same as F-1 except the *para* substituted zwitterion monohydrate (*aak*; 814 mg) was reacted for T_1 of 60 min in acetonitrile at 0° to give 1·108 g or 96% of crude peptide; recrystallized yield: 1·063 g or 92%; m.p. 109–110·5°.

Phthaloyldiglycine ethyl ester

G-1. Phthaloylglycine (616 mg, 3 mM from phthalic anhydride and glycine; m.p. 192–193° (lit.[31] 191–192°), zwitterion (*aaj*) (760 mg), Et_3N (304 mg) for a T_1 of 60 min in acetonitrile. Glycine ethyl ester hydrochloride (419 mg) and Et_3N (304 mg) overnight at room temp (soln volume, 18 ml). The residue after the solvent was removed *in vacuo* was triturated in hot water, the soln cooled, and the ppt filtered, washed with water, and dried; yield: 726 mg or 88%; m.p. 193·5–194·5°. Recrystallization from EtOH–water did not change the m.p. (lit.[32] 194–195°).

Carbobenzoxy-L-phenylalanyl-L-leucine methyl ester

H-1. Carbobenzoxy-L-phenylalanine (2·991 g, 10 mM), zwitterion (*aaj*; 2·533 g), Et_3N (1·012 g) for a T_1 of 60 min in acetonitrile at 0°. L-Leucine methyl ester hydrochloride (1·817 g) and Et_3N (1·012 g) overnight at room temp (soln volume, 75 ml). Isolation Procedure B yielded 4·013 g or 94% of crude peptide. The product was recrystallized from AcOEt–pet. ether; yield: 3·831 g or 90%; m.p. 106–107°. A second recrystallization gave 3·641 g or 85% of peptide of m.p. 107·5–108·5°. The residue had the same IR spectrum as the product, indicating that the carbobenzoxy-L-phenylalanine may not have been optically pure. The analytical sample melted at 109–109·5°. (Found: C, 67·73; H, 6·92; N, 6·72. $C_{24}H_{30}O_5N_2$ requires: C, 67·59; H, 7·09; N, 6·56%).

The peptide ester was hydrolyzed to carbobenzoxy-L-phenylalanyl-L-leucine in methanolic NaOH aq. After recrystallization from AcOEt–pet. ether the peptide acid melted from 139·5–140·5°. (Found: C, 67·09, H, 6·80; N, 7·01. $C_{23}H_{28}N_2O_5$ requires: C, 66·97; H, 6·84; N, 6·80%).

Carbobenzoxy-L-methionylglycylglycine ethyl ester

I-1. Carbobenzoxy-L-methionine[33] (850 mg, 3 mM), zwitterion (*aaj*; 760 mg) and Et_3N (304 mg) for a T_1 of 45 min at 0° in acetonitrile. Glycylglycine ethyl ester hydrochloride (590 mg) and Et_3N (304 mg) overnight at room temp (soln volume, 20 ml). The residue after evaporation of the solvent was triturated in warm water and the ppt (after cooling) filtered, washed with water, and dried to yield 1·151 g or 90% of crude peptide of m.p. 128–131°. The peptide was recrystallized from acetone–water; yield: 1·095 g or 86%; m.p. 131·5–133°. Further recrystallization raised the m.p. to 132·5–133·5° (lit.[34] 131–133°).

Carbobenzoxyhydroxy-L-prolylglycylglycine ethyl ester

J-1. Carbobenzoxyhydroxy-L-proline (796 mg, 3 mM, twice recrystallized material from Mann Research Laboratories, Inc.; m.p. 106–106·5° (lit.[29] 106°), zwitterion (*aaj*; 760 mg) and Et_3N (304 mg) for a T_1 of 65 min at 0° acetonitrile. Glycylglycine ethyl ester hydrochloride (590 mg) and Et_3N (304 mg) overnight at room temp (soln volume, 16 ml). The solvent was stripped off at reduced press, and the residue was taken up in 10 ml 1% $NaHCO_3$ aq and 50 ml of AcOEt and the 2 phases separated. The aq soln was extracted with 3 × 10 ml AcOEt, the combined organic extracts dried over Na_2SO_4, and the solvent removed *in vacuo*. The residue was triturated with 20 ml water and the mixture cooled* in the refrigerator. Pure peptide was obtained in the first two crops; yield: 974 mg or 80%; m.p. 145–146°. The remaining residue (6%) was also mostly peptide. Further crystallization did not change the m.p. (lit.[35] 144–145°).

[30] G. W. Anderson and R. W. Young, *J. Amer. Chem. Soc.* **74**, 5307 (1952).

[31] E. Drechsel, *J. prakt. Chem.* (2), **27**, 418 (1883).

[32] J. C. Sheehan and J. J. Hlavka, *J. Org. Chem.* **21**, 439 (1956).

[33] Commercial carbobenzoxy-L-methionine obtained from Cyclo Chemical Corp. was actually a 1:1 mixture of the free acid and its sodium salt; m.p. 68–69°. Extraction of an AcOEt soln of the mixture with dil HCl acid yielded the free acid, m.p. 67–68° after two crystallizations from AcOEt–pet. ether. Lit. m.p. 68–69°; M. Brenner and R. W. Pfister, *Helv. Chim. Acta.* **34**, 2085 (1951).

[34] C. A. Dekker, S. P. Taylor, Jr. and J. S. Fruton, *J. Biol. Chem.* **180**, 155 (1949).

[35] N. C. Davis and E. L. Smith, *J. Biol. Chem.* **200**, 373 (1953).

Carbobenzoxyglycylglycyl-L-tyrosine methyl ester

K-1. Carbobenzoxyglycylglycine (799 mg, 3 mM, twice recrystallized material from Aldrich Chemical Co., Inc., m.p. 176–177·5° (lit[36] 178°), zwitterion (*aaj*; 760 mg) and Et_3N (304 mg) for a T_1 of 55 min at 0° in acetronitrile. L-Tyrosine methyl ester hydrochloride (695 mg, twice recrystallized material from Mann Research Laboratories, Inc., m.p. 189–190·5° (lit.[37] 190°) and Et_3N (304 mg) overnight at room temp (soln volume, 22 ml). After evapn of the solvent, the residue was triturated with hot water, the mixture cooled, and the ppt filtered off, washed with water, and dried to yield 1·175 g or 88% of crude peptide of m.p. 157–160°. The product was purified by recrystallization from EtOH–water; yield: 1,095 g or 84%; m.p. 159·5–161·5°. Further recrystallization did not change the m.p. (Found: C, 59·48; H, 5·78; N, 9·51. $C_{22}H_{25}N_3O_7$ requires: C, 59·58; H, 5·68; N, 9·48%).

Carbobenzoxy-L-asparaginylglycine ethyl ester

L-1. Carbobenzoxy-L-asparagine (798 mg, 3 mM, twice recrystallized material from Aldrich Chemical Co., Inc., m.p. 162–163° (lit.[37] 163°), *zwitterion* (*aaj*; 760 mg), and Et_3N (304 mg) for a T_1 of 7 min in nitromethane at room temp. Glycine ethyl ester hydrochloride (419 mg) and Et_3N (304 mg) overnight at room temp (soln vol, 19 ml). After the solvent had been evaporated, the residue was triturated with 40 ml of hot 0·5% $NaHCO_3$ aq, the mixture cooled, and the ppt filtered from a blue soln, washed with 10 ml water and dried; yield: 846 mg or 80%; m.p. 185·5–187°. Further crystallization from acetone–water raised the m.p. to 186–187° (lit.[38] 184–185°).

L-2. Same as L-1 except T_1 in acetonitrile for 70 min at 0°. At the end of T_1 the reaction mixture still contained a white ppt—probably the enol ester; yield: 835 mg or 79%; m.p. 186–187°.

L-3. Same as L-1 except that zwitterion (*aaf*; 717 mg) was reacted for a T_1 of 25 min in acetonitrile at room temp; yield: 651 mg or 62%; m.p. 183–185°.

L-4. Same as L-1 except that zwitterion (*aak*; 802 mg) was reacted for a T_1 of 6 min at room temp in nitromethane; yield: 771 mg or 73%; m.p. 185–186°.

Carbobenzoxy-L-asparaginyl-L-leucine methyl ester

M-1 Carbobenzoxy-L-asparagine (1·064 g, 4 mM), zwitterion (*aaj*; 1·012 g), and Et_3N (405 mg) for a T_1 of 8 min in nitromethane at room temp. Leucine methyl ester hydrochloride (726 mg) and Et_3N (405 mg) overnight at room temp (soln vol, 22 ml). The product was worked up in the same way as the asparagine peptide L-1 (Procedure A); yield: 1·198 g or 76%; m.p. 176·5–178°. The analytical sample obtained by recrystallization from acetone–water melted from 177·5–178·5°. (Found: C, 57·83; H, 6·84; N, 10·78. $C_{19}H_{27}N_3O_6$ requires: C, 58·00; H, 6·92; N, 10·68%).

Carbobenzoxy-L-glutaminyl-L-valine methyl ester

N-1. Carbobenzoxy-L-glutamine (841 mg, 3 mM, twice recrystallized material from Cyclo Chemical Corp., m.p. 133·5–135° (lit.[37] 135°), zwitterion (*aaj*; 760 mg), and Et_3N (304 mg) for a T_1 of 10 min in nitromethane at room temp. L-Valine methyl ester hydrochloride (502 mg, twice recrystallized material from Cyclo Chemical Corp., m.p. 167·5–168·5° (lit.[39] 170°) and Et_3N (304 mg) overnight at room temp (soln vol, 20 ml). The product was isolated according to Procedure A; yield: 904 mg or 77%; m.p. 172·5–173°. Further crystallization changed the m.p. to 172·5–173·5° (lit.[40] 173–175°).

Carbobenzoxy-L-glutaminyl-L-tyrosine methyl ester

O-1. Carbobenzoxy-L-glutamine (841 mg, 3 mM), zwitterion (*aaj*; 760 mg), and Et_3N (304 mg) for a T_1 of 9 min in nitromethane at room temp (soln vol, 20 ml). The product was isolated according to Procedure A; yield: 1·034 g or 75%; m.p. 198–199°. Recrystallization changed the m.p. to 197·5–198·5 (lit.[40] 198–201°).

Carbobenzoxyglycyl-DL-phenylalanine

P-1. Carbobenzoxyglycine (837 mg, 4 mM), zwitterion (*aaj*; 1·012 g) and Et_3N (405 mg) for a T_1 of 10 min in 12 ml of nitromethane at room temp. The clear reaction soln was then added to a soln of

[36] S. Goldschmidt and M. Wick, *Liebigs Ann.* **575**, 217 (1952).

[37] R. A. Boissonnas, St. Guttmann, P. A. Jaquenoud and J. P. Waller, *Helv. Chim. Acta* **38**, 1491 (1955).

[38] S. S. Leach and H. Lindley, *Austr. J. Chem.* **7**, 173 (1954).

[39] J. I. Harris and T. S. Work, *Biochem. J.* **46**, 582 (1950).

[40] E. Sondheimer and F. W. Holley, *J. Amer. Chem. Soc.* **76**, 2816 (1954).

661 mg of DL-phenylalanine in 4·5 ml of 1N NaOH aq and the mixture was stirred for 3 hr. The solvent was evaporated at reduced press and the residue taken up in water to which a little bicarbonate had been added. The aq soln was extracted with AcOEt, then acidified to pH 2 and again extracted with AcOEt. The AcOEt extract of the acidified soln was dried over Na_2SO_4 and the solvent stripped off *in vacuo*. Then the residue was crystallized from acetone–water to yield 419 mg or 29% of crude product. Two recrystallizations from acetone-water yielded 277 mg or 19% of peptide acid of m.p. 161·5–162·5° (lit.[23] 162°).

Carbobenzoxyglycyl-L-phenylalanine

Q-1. Carbobenzoxyglycine (8·36 g, 0·04 M), zwitterion (*aak*) monohydrate (10·84 g) and Et_3N (4·05 g) for a T_1 of 50 min in acetonitrile at 0°. L-Phenylalanine ethyl ester hydrochloride (9·18 g, prepared from L-phenylalanine (California Corporation for Biochemical Research) by Fischer esterification; m.p. 157–159° (lit.[23] 148–150°)) and Et_3N (4·05 g) overnight at room temp (soln vol, 200 ml). The product was isolated as an oil according to Procedure B. This was hydrolyzed directly in 40 ml MeOH and 40 ml 1·00N NaOH aq at room temp for 3 hr. The solvent was removed *in vacuo*, and the residue was dissolved in water to which a little bicarbonate had been added. The aqueous soln was extracted with AcOEt, then acidified to Congo Red and again extracted with AcOEt. This second AcOEt extract was dried and the solvent removed at reduced press. The residue was recrystallized from acetone–pet. ether; overall yield: 10·76 g or 76%; m.p. 129–130°. Recrystallization raised the m.p. to 129·5–130° (lit.[23] 127°).