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IMPROVED ISOXAZOLIUM SALT REAGENTS IN PEPTIDE SYNTHESIS—I

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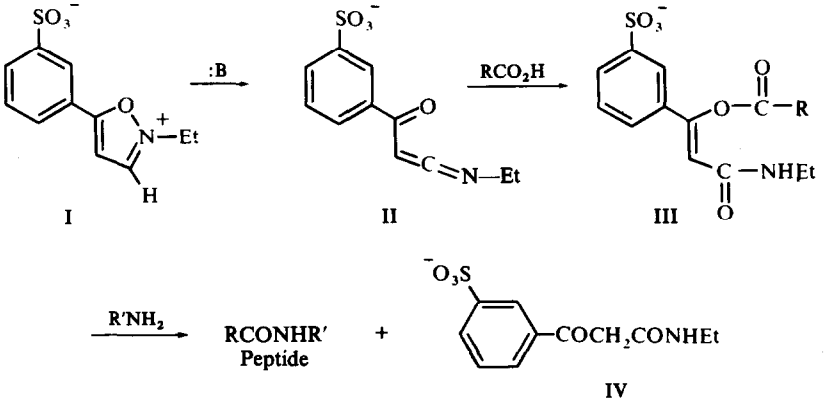
Abstract—Model studies directed toward the design and development of a better and more practical isoxazolium salt reagent for the synthesis of peptides are described.

SINCE the introduction of the amide bond forming reagent, N-ethyl-5-phenyl-isoxazolium-3′-sulfonate (I), in 1961[1–3] this compound has found wide application in the construction of peptide linkages[4] and has in fact even been utilized in certain key steps of the monumental total syntheses of insulin and ACTH.[4]



Its very success has inspired a number of efforts to design an even more useful reagent but so far this elusive goal has not been attained. In this paper a number of successful model studies directed toward this end are described; experiments which it is hoped will ultimately culminate in the synthesis of a better and more practical isoxazolium salt reagent.

LOGIC OF APPROACH

Any attempt to design a more useful synthetic method must necessarily be founded on a knowledge of the mechanism of action, limitations, scope and disadvantages of the reagent to be improved upon. In the present case the mechanism is exceedingly complex but it has been painstakingly elucidated in careful detail by Woodward, Olofson, Kemp, and Woodman.[1–3, 5–11] The presently accepted pathway for activation of a carboxylic acid in peptide synthesis (generation of the enol ester III) by the isoxazolium sulfonate (I) is depicted in Scheme I.

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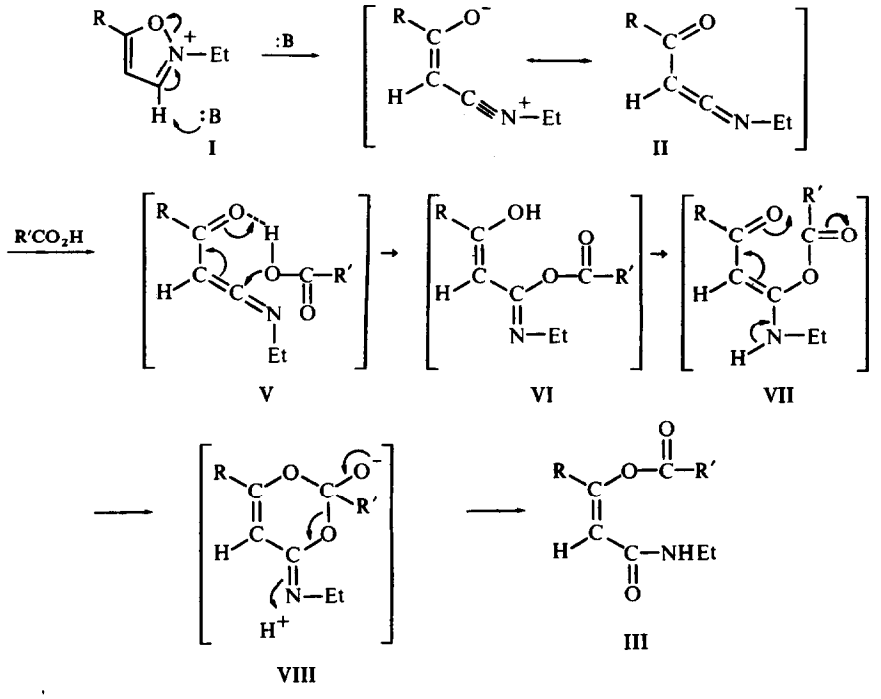
† Adapted from the M.S. Thesis of Y.L.M., The Pennsylvania State University, March 1968.

SCHEME I (R = m-sulfonatophenyl)

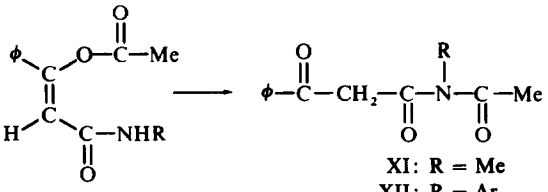


Evidence has been specifically adduced for: (1) the base-induced abstraction of the proton in I;[1, 3] (2) the concertedness of this process with ring cleavage;[12] (3) the counterclockwise direction of electron flow in the ring scission, I → II;[1, 3] (4) the intermediacy of the ketoketenimine (II);[1, 3] (5) the reaction of this species with free carboxylic acid rather than carboxylate anion;[1, 3, 9] (6) the geometry of the transition state (V) and the electrocyclic, Diels-Alder like, nature of the process, II → VI (note that this reaction is initiated by a hydrogen bonding interaction between the carboxylic acid proton and the ketoketenimine oxygen);[1, 3] and (7) the structure of the enol ester (III).[1, 3] The activated ester is then treated with an amine and the product peptide is formed *via* the usual aminolysis mechanism.

Besides the very high product yields even in difficult systems, the isoxazolium sulfonate has two other advantages in peptide synthesis. First: the by-products are all water soluble and therefore easily removed from the product peptide derivative. Second: the coupling process occurs with very little or no racemization under optimum conditions. The disadvantages of this method are: (1) the high cost of the isoxazolium salt reagent, (2) a side rearrangement which can be mitigated but still keeps the peptide yields from being quantitative, and (3) the formation of some racemic peptide in certain useful reaction environments.

The side reaction was first studied by Woodward and Olofson[1, 3] who demonstrated that the enol ester rearranges to the inert imide under the reaction conditions. In the example, β-acetoxy-N-methylcinnamamide (IX), the O → N acyl migration to the imide (XI) occurs with a half-life of about twenty minutes in acetonitrile at 30°.

Racemization in peptide synthesis using isoxazolium salts is known to take place

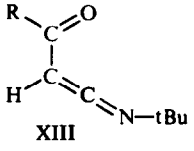


IX: R = Me
X: R = Ar

XI: R = Me
XII: R = Ar

primarily by the well known azlactone mechanism involving decomposition in this system of the iminoanhydride like intermediate (VI- with $R'CO_2H$ being at least a dipeptide acid) to the azlactone.[1, 3, 8, 9] The enol ester itself (III) ordinarily yields only optically pure peptides.*

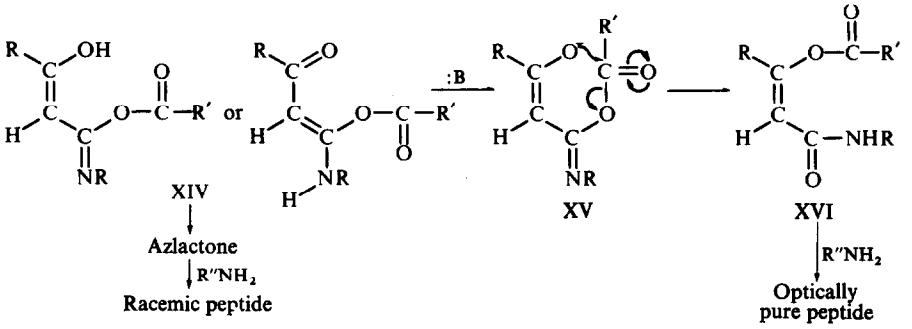
Much effort has been expended on attempts to design reagents in which the O → N acyl shift side reaction is eliminated. Woodward and Olofson showed that the rearrangement rate is decreased when the N-methyl group is replaced by the bulkier N-ethyl moiety.[1–3] This is the main reason that the N-ethyl isoxazolium sulfonate is the reagent of choice rather than the N-methyl salt; in simple cases peptide yields are increased 5–10%.[1, 3] From this it was apparent that one solution to the rearrangement problem might require the preparation of isoxazolium cations with even bulkier substituents on nitrogen. The hindered N-arylisoxazolium salts were the first compounds to be tested in response to this hypothesis.[9, 11] The imide rearrangement, X → XII, however occurred much more rapidly because the process is base catalyzed[3] and it is easier to deprotonate an anilide than an N-alkyl amide. With N-t-butylisoxazolium salts[9–11] O → N acyl migration was finally eliminated[9, 10] and stable enol esters were formed. Unfortunately the isolable t-butyl ketenimine (XIII)[9, 10] derived from this cation was so unreactive that it did not easily yield enol esters with peptide acids at the desired low concentrations; the activation step was so slow that side reactions became significant.



XIII

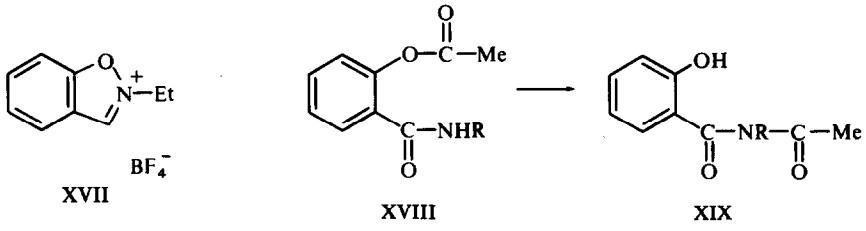
Woodward and Woodman also attempted to minimize the racemization problem.[9] They reasoned that since racemization occurred *via* azlactone formation from the imino anhydride (XIV) the solution to the problem was to increase the rate of rearrangement of this species to the enol ester (XVI). This transformation should be catalyzed by base which would generate the activated intermediate (XV). They discovered this to be true; the ratio of enol ester to azlactone did increase on addition of triethylamine. Unfortunately, in the presence of triethylamine the enol ester itself

* The main evidence supporting this statement is the fact that the enol ester from hippuric acid is not converted to azlactone under the reaction conditions though it can be induced to do so by the addition of excess triethylamine.[9] This test is described in more detail later in the discussion.



slowly decomposed to azlactone.* They needed a base which would be strong enough to induce ionization of the active proton in XIV but weak enough so it would not catalyze azlactone formation from XVI. By using 2-picoline as the base and solvent, they were finally able to minimize azlactone formation but this method did not in general prove to be very satisfactory.

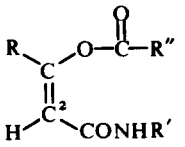
When we began our own experiments on the synthesis of isoxazolium salt reagents we were not only acquainted with the work described above but also with the elegant studies of Kemp and Woodward on peptide synthesis utilizing the benzisoxazolium cation (XVII),[5–7] and this latter research provided an important clue on which we based our own approach. In the course of his studies Kemp[7] discovered that the enol ester (XVIII, R = Et) does not rearrange to the imide although the primary amide (XVIII, R = H) does undergo the O → N acyl migration.[14]



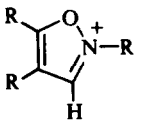
Kemp rationalized these results by suggesting that the imide derived from XVIII (R = Et) might be destabilized relative to XIX (R = H) because the bulky N-ethyl moiety might interfere with the imide group achieving planarity with the benzene ring and therefore the full benefit of π-electron delocalization. A number of additional experiments were in agreement with this hypothesis.

After a study of Kemp's work we postulated that other steric interactions might destabilize an imide relative to an enol ester by restricting the imide's ability to achieve its preferred planar conformation. One modification of our basic enol ester system (XX) which we felt should have the desired conformationally restrictive effect would involve substitution of the hydrogen at C-2 with a larger group.

* More recently it has been carefully established that azlactone formation is base catalyzed.[8, 13] The present experiment worked because of the probable greater acidity of the active proton in XIV than an ordinary amide proton.



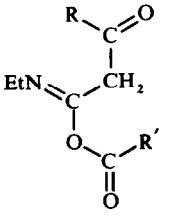
XX



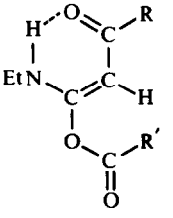
XXI

We therefore concluded that an improved reagent could best be described by the general structure (XXI).

We next considered the racemization problem and specifically tried to answer the question: is there any way to increase the rate of rearrangement of the racemization prone iminoanhydride intermediate to the more racemization resistant enol ester without creating the special problems involved in tampering with the acidity of the reaction medium? One factor influencing the rate of that rearrangement is the number of possible tautomers and conformational isomers of the iminoanhydride (VI), many of which could be in facile equilibrium under the reaction conditions. Some of these species, for example XXII and XXIII, would decrease the acyl migration rate in proportion (1) to their concentration and (2) to the ease of their conversion to other possible intermediates from which the rearrangement is geometrically possible.

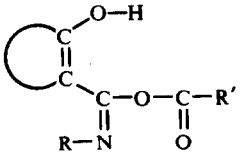


XXII

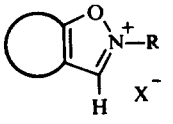


XXIII

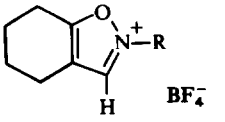
We therefore postulated that any structural restraint we could put on V which would decrease the number of inactive tautomers, geometrical and conformational isomers, would also increase the rate of its rearrangement to enol ester and thus minimize racemization. The most useful constraint on VI for this purpose is embodied in the iminoanhydride structure (XXIV).



XXIV



XXV



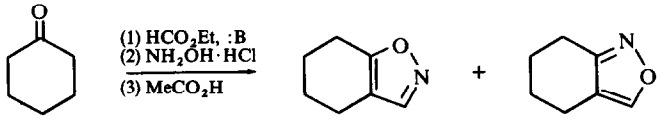
XXVI (R = Me)
XXVII (R = Et)

With this structure many unreactive isomers are eliminated, specifically some conformations involving extended systems such as XXII or some geometrical isomers such as XXIII in which the functions which are destined to react with one another are trans-substituted on a double bond. If this argument has any validity an improved isoxazolium salt reagent should best be described by the general structure (XXV). Note that this species also encompasses our earlier goal, structure (XXI).

In this work our initial experimental objective then was the synthesis of isoxazolium salts of the general structure (XXV). In order for these compounds to be improved reagents in peptide chemistry four additional requirements must be met by XXV: (1) the R group must not be too bulky; (2) its synthesis must be simple and economical; (3) the keto amide derived from it in peptide forming reactions must be easily separable from the product peptide; and (4) it must be easily purified and therefore nicely crystalline and nonhygroscopic. Since some of these latter requirements are only capable of empirical solution we concluded it would be valuable to first test our initial hypotheses on a model substrate and we chose for this purpose the N-methyl and N-ethyl-4,5-tetramethyleneisoxazolium fluoroborates (XXVI and XXVII).

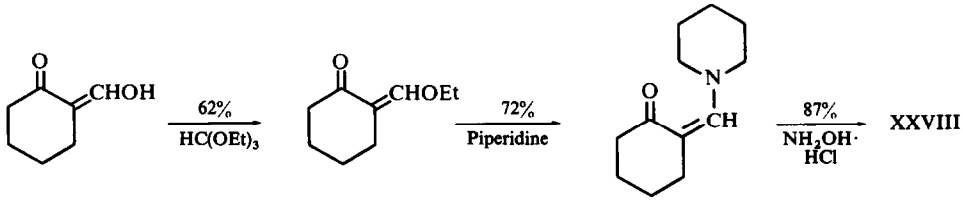
DISCUSSION OF RESULTS

Cyclohexanone has been converted by von Auwers[15] and later by Johnson[16] into a mixture of the two isomeric tetramethyleneisoxazoles (XXVIII and XXIX) by standard procedures but the mixture has never been separated and XXVIII has never been isolated in pure form.



XXVIII XXIX

In our hands under various reaction conditions the product mixture contained between 75 and 80% of XXVIII by NMR analysis.[17] The only contaminant was the isomer which could not be separated from XXVIII by vpc, thin layer chromatography on alumina or silica, or fractional distillation on a spinning band column. We were, however, able to prepare pure XXVIII by a scheme which was adapted with numerous modifications from a synthesis of 5-methylisoxazole by Wilson and Burness.[18]



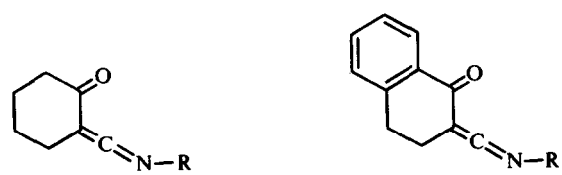
The isoxazole (XXVIII) could be methylated (92%) with trimethyloxonium fluoroborate*[19] to yield the desired N-methylisoxazolium fluoroborate (XXVI) as a slightly hygroscopic solid. The N-ethylisoxazolium fluoroborate (XXVII), a colorless oil, was similarly prepared using triethyloxonium fluoroborate[20] as the alkylating agent. Finally the isoxazolium salts (XXXI and XXXII) from α-tetralone were also synthesized; these compounds proved especially useful in resolving a key point in later experiments (*vide infra*). In this system there was no isomer problem in obtaining the precursor, 4,5-dihydronapht[1,2-*c*]isoxazole (XXX)[16] by the classical route.

* We thank Mr. Richard B. Silverman for furnishing us with a generous sample of this material (R. B. Silverman and R. A. Olofson, Chem. Comm., 1313 (1968)).

XXX

XXXI: R = Me
XXXII: R = Et

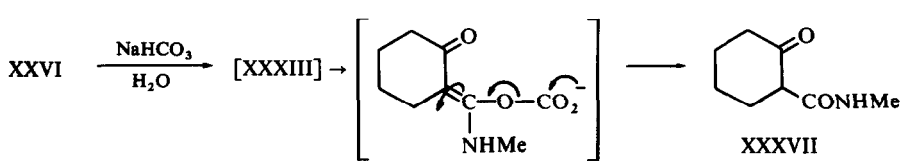
As a structural check the new isoxazolium salts were first characterized by treatment with triethylamine in methylene chloride. In each case the isoxazolium cation was converted to the corresponding ketoketenime (XXXIII–XXXVI) which was detected by its infrared band at 4·85 μ.



XXXIII: R = Me
XXXIV: R = Et

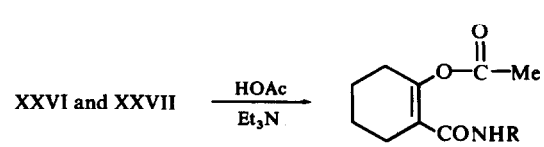
XXXV: R = Me
XXXVI: R = Et

The ketoketenimines (XXXIII and XXXIV) from the tetramethyleneisoxazolium cations were stable in solution for about two hours (about the same stability as the ketoketenime derived from N-ethyl-5-phenyl isoxazolium fluoroborate[1, 3]). The ketoketenime (XXXVI) was however stable in solution for over a week and could in fact be isolated as a yellow viscous oil and identified by IR and NMR spectroscopy. The isoxazolium salt (XXVI) was further characterized by hydrolysis to the expected keto amide (XXXVII) on treatment with sodium bicarbonate in water.



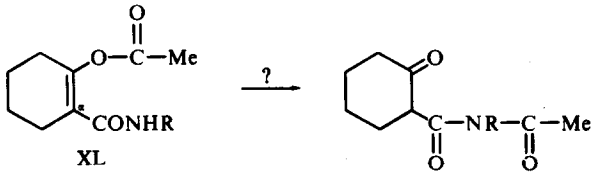
XXXVII

The isoxazolium salts (XXVI and XXVII) also yielded the anticipated enol acetates (XXXVIII and XXXIX) on treatment with acetic acid and triethylamine in acetonitrile.



XXXVIII: R = Me
XXXIX: R = Et

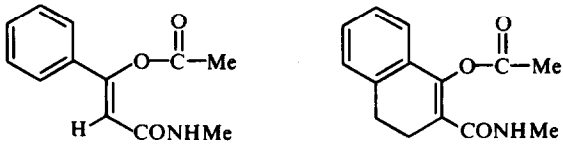
We next set out to discover if the crucial enol ester to imide rearrangement (XL → XLI) would in fact take place more slowly than in α-unsubstituted systems as we had induced from all the previous work in this area.



XL XLI

We were encouraged by the fact that XXXVIII and XXXIX could be recrystallized from warm cyclohexane without rearrangement and we were delighted when these enol esters were stable when refluxed overnight in dioxane or acetonitrile. The only noticeable change in the residue was the presence of small amounts of the β-keto amides expected from a hydrolytic process (detected by IR spectroscopy). In one molar triethylamine in acetonitrile the enol ester decomposed within a few hours at reflux temperature and was about 20 per cent decomposed after thirty hours at room temperature. From spectral analysis it seemed that base induced hydrolysis and carbonyl condensation reactions were the primary decomposition processes. Though rearrangement to the imide might have taken place it was not a major decomposition pathway. These final reaction conditions are orders of magnitude more drastic than those employed in peptide synthesis so our goal of preventing imide rearrangement has been achieved.

It is still possible that the O → N acyl migration has been suppressed, not because of the steric factors described above, but because the activating phenyl substituent in β-acetoxy-N-methyl cinnamamide (XLII) (rearrangement rate: $t_{\frac{1}{2}} \sim 20$ min. in acetonitrile at 30°[1, 3]) was not present in this model.

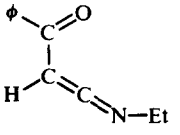


XLII XLIII

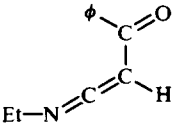
As a test of this hypothesis the enol ester (XLIII) was prepared from the isoxazolium salt (XXXI) in the usual manner and subjected to the rearrangement conditions. This compound was also stable in hot acetonitrile or dioxane though it decomposed more rapidly than the enol ester (XXXVIII) in one molar triethylamine in acetonitrile, again mainly by a hydrolytic pathway. An imide product could not be isolated. Though XLIII is more sensitive to hydrolysis than XXXVIII it is still very much less susceptible to O → N acyl migration than XLII in accord with our major postulate.

In order to show that acetic acid rather than the acetate ion is the species attacking these ketenimines two parallel experiments were performed. A solution of the keto-ketenimine (XXXVI) was dissolved in methylene chloride and treated with a weighed

amount of acetic acid. The IR spectrum was recorded immediately but the ketenimine peak at 4·85μ had already disappeared. The complete reaction therefore had to occur in less than one minute. A new band attributable to the enol ester appeared in the carbonyl region at 5·66μ. This experiment was repeated using triethylammonium acetate rather than acetic acid (same concentrations). Under these conditions the ketenimine peak decreased more slowly and was completely gone after ten minutes. This is convincing evidence that these fused ring ketoketenimines react with carboxylic acid by the same pathway as the corresponding intermediates from the 5-substituted isoxazolium salts (*vide supra*). The reaction of XXXVI with acetic acid is much faster than the similar reaction of the previously studied ketenimine (XLIV).[1, 3]



XLIV

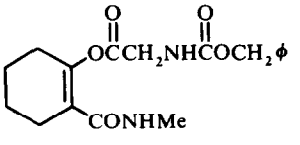


XLV

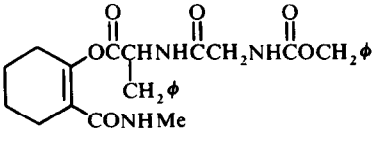
This suggests, if one accepts the evidence that the reaction occurs by the electrocyclic process described in the previous section, that the ketenimines from 5-substituted isoxazolium salts exist in solution as a mixture of s-*cis* and s-*trans* isomers, i.e., XLIV and XLV.

We next initiated a preliminary study of the utility of these systems in peptide synthesis to determine: (1) whether the enol esters of peptides would be easily formed; (2) whether these active esters would react readily with amines to yield peptides; and (3) whether racemization problems would be eliminated.

Reaction of the isoxazolium salt (XXVI) with carbobenzoxyglycine and triethylamine in acetonitrile yielded the enol ester (XLVI) and a similar experiment using carbobenzoxyglycyl-L-phenylalanine afforded the analogous ester (XLVII).



XLVI

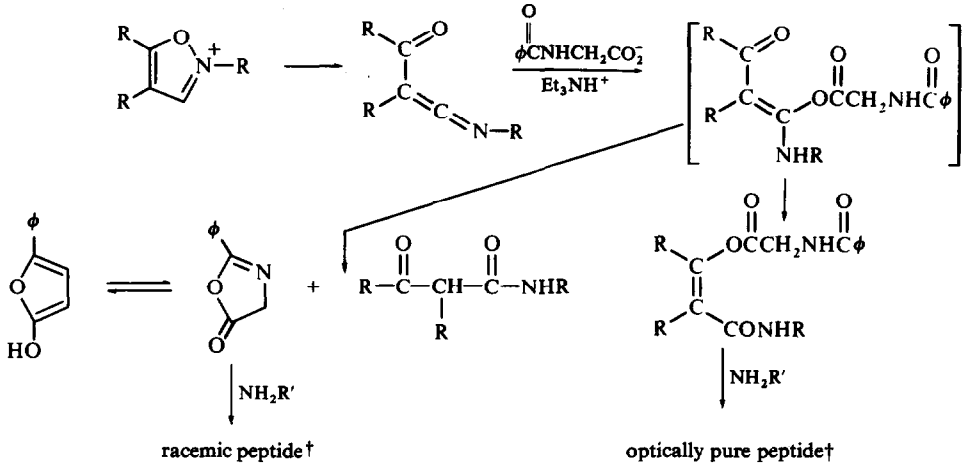


XLVII

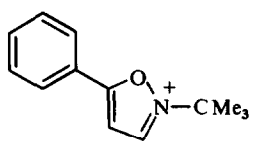
Treatment of XLVI with benzylamine yielded carbobenzoxyglycine benzylamide (compared with an authentic sample) and reaction of XLVII with glycine ethyl ester hydrochloride plus triethylamine in dimethylformamide afforded carbobenzoxyglycyl-L-phenylalanyl-glycine ethyl ester. The formation of this tripeptide ester was also carried out under Anderson test[1, 21] conditions and in this very accurate and sensitive test for racemization no D,L-isomer was isolated.

Recently an exceedingly stringent test for racemization in peptide synthesis using isoxazolium salt reagents has been designed by Woodward *et al.*[5, 9] In this test an isoxazolium salt is treated with triethylammonium hippurate in acetonitrile. The IR spectrum of the reaction mixture is recorded repeatedly until the ketenimine band at 4·85μ has completely disappeared and the ratio of the azlactone of hippuric acid (IR at 5·45μ) to hippuric acid enol ester (IR at 5·65μ) is then determined from the

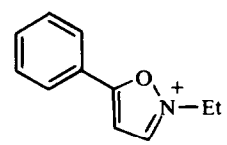
relative intensities of the CO stretching peaks for the two species.* Since azlactone is the main active intermediate in the formation of racemic peptides whereas the enol ester is the precursor to most of the unracemized peptide the ratio of these two compounds in the reaction mixture is a useful test for racemization. Also since this test generally gives higher results than other racemization tests using more classical peptide-like acylamino acids, it is especially valuable in that it exaggerates any future racemization problem.



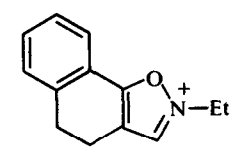
In Fig. 1 the IR spectra of the reaction mixtures utilizing the following isoxazolium salt substrates are recorded.



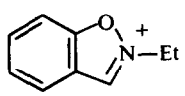
Salt A



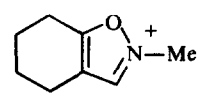
Salt B



Salt C (XXXII)



Salt D (XVII)



Salt E (XXVI)

Note that the isoxazolium salt C(XXXII) reacts very much faster in this system than N-ethyl-5-phenylisoxazolium fluoroborate (Salt B) in accord with our previously described and rationalized control experiment. Concerning the racemization question, the spectra reproduced in Fig. 1 speak for themselves; they are definitive and

* The use of CO absorption as an assay for azlactones has been described.[22]

† In systems where the azlactone and enol esters would be formed from an optically active acylamino acid.

entirely in accord with our hypothesis and our greatest expectations. Future papers will be dedicated to the translation of these model study results into a practical peptide bond forming reagent.

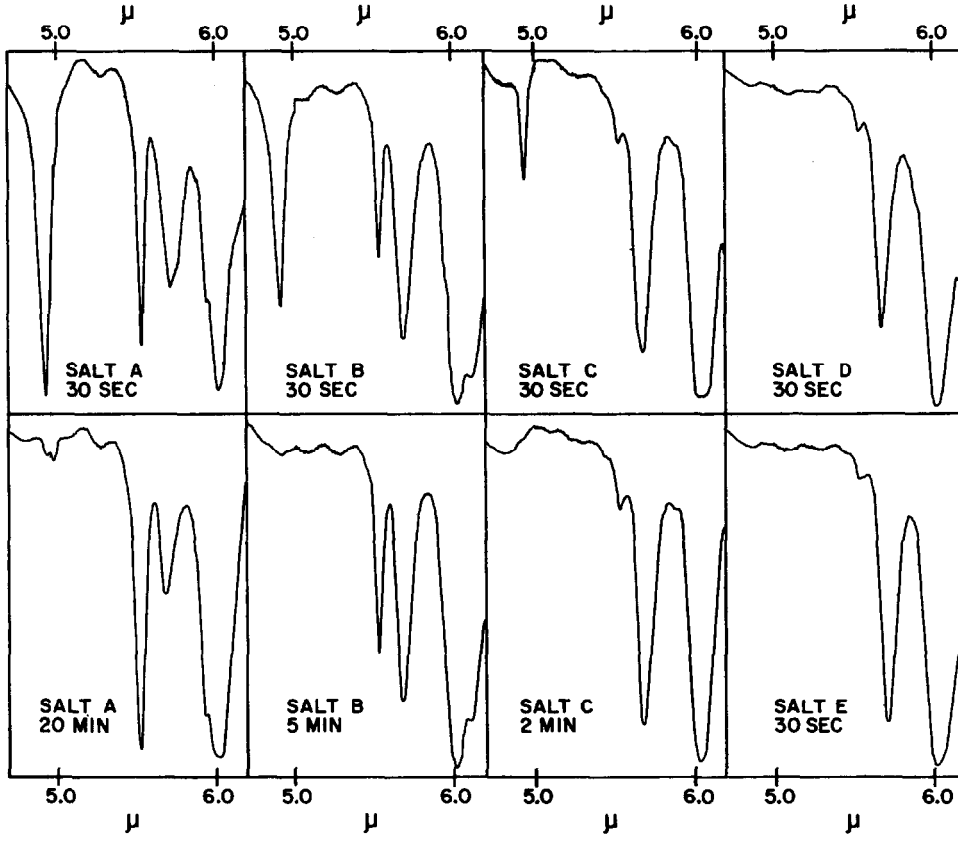


FIG. 1 IR spectra of reaction solutions from the treatment of one equivalent of isoxazolium salt (A–E) with one equivalent of triethylammonium hippurate in acetonitrile, all reactants at 0·005M concentration. The IR peak at ~ 4·85 μ is attributed to ketenimine, that at ~ 5·45 μ to hippuric acid azlactone, and that at ~ 5·65 μ to hippuric acid enol ester. The reactions were all performed at room temperature and the IR spectra were recorded on a Perkin–Elmer Model 257 Grating Infrared Spectrophotometer.

EXPERIMENTAL

M.ps were taken in Kimax, soft glass capillary tubes using a Thomas–Hoover "Uni-Melt", Model 6406K, Capillary M.P. apparatus with a calibrated thermometer. IR spectra were measured on a Perkin–Elmer, Model 137, NaCl Spectrophotometer (calibrated against the 6·238μ band of polystyrene). A Varian Associates, Model A-60 Analytical NMR Spectrophotometer was used to record NMR spectra. Mass spectral data was obtained with a Nuclide Low Resolution Mass Spectrometer. The solvents and reactants were of the best commercial grade available and were used without further purification. Optimum experimental conditions were sometimes not worked out since our main interest was only in the use of these compounds in model experiments.

Tetramethyleneisoxazole mixture (XXVII *and* XXIX). The experimental procedure used by Johnson[16] for cyclizations in the tetralone-l series starting with 2-hydroxy-methylene-cyclohexanone[23] was followed. This was later modified and the reaction mixture was stirred for 1 hr at 140° instead of the usual 8 hr at 70–80°. Identical yields (85–90%) were obtained using either procedure but the isomer ratio changed from 75 ± 3% to 80 ± 3% of the desired isomer (NMR analysis).

The mixture of isomeric isoxazoles could not be separated by VPC using the following columns: ⅜″ × 12′ Polypropylene glycol on Chrom. W., U.C. oil LB 550-X; F. S-1265(QF-1), fluorinated silicone oil and silicone gum rubber on Chromosorb P(8A). Separation attempts using TLC on silica or alumina and using fractional distillation on a Nester/Faust annular teflon spinning band distillation column also failed.[17]

2-N-*Piperidinomethylenecyclohexanone.* 2-Ethoxymethylenecyclohexanone[24] (48 g, 0·31M), piperidine (83 g, 0·63 M) and *p*-toluenesulfonic acid (1·5 g) were dissolved in 375 ml benzene and placed in a 1 l. round bottom flask equipped with a magnetic stirring device, a Dean–Stark trap and a reflux condenser fitted with a $CaCl_2$ drying tube. The stirred mixture was heated to reflux in an oil bath at 90–95° for 24 hr. The oil bath temp was raised to 105° and 2/3 of the solvent removed by distillation.

The reaction flask was then cooled and the volatile materials distilled at room temp *in vacuo*. The residue, a mixture of oil and solids, was filtered to remove much of the oil and the remainder was washed from the filter cake with ether. (This solid, probably a toluenesulfonate salt, was not further investigated.) The total filtrate was then taken to dryness at reduced press yielding powdery light yellow hygroscopic crystals; 43 g or 72%; m.p. 49–51°. This material was used directly in the next step. NMR(τ) CCl_4: 2·76(s), 6·38–6·76(m), 7·28–7.63(m), 7.72–8.00(m), 8.05–8.55(m); ratio 1:4:2:2:10; Mol. Wt. 193(MS).

4,5-*Tetramethyleneisoxazole* (XXVIII). 2-N-piperidinomethylenecyclohexanone (52 g, 0·27M) dissolved in the minimum amount of water was placed in a 200 ml round-bottomed flask immersed in a cold bath. A concentrated aqueous soln of NH_2OH·HCl (18.8 g, 0·27M) was added with stirring. The reaction flask was fitted with a condenser and then heated on a steam bath for 2 hr. A light brown oil separated. The soln was neutralized to pH 7·2–7·4, extracted with ether and the ether extracts dried over Na_2SO_4. Concentration of the solution at reduced press followed by vacuum distillation afforded 28·9 g or 87% of the pure colorless isoxazole; b.p. 52–53° at 0·9 mm. The lit.[15] b.p. for a mixture of tetramethyleneisoxazoles is 90–95° at 14 mm (NMR(τ) neat: 2·01(s), 7·17–7.83(m), 7·93–8.58(m); ratio 1:4:4.

N-*Methyl*-4,5-*tetramethyleneisoxazolium fluoroborate** (XXVI). Trimethyloxonium fluoroborate[19] (4·58 g, 0·031 M), XXVIII (3·76 g, 0·031 M) and CH_3NO_2 (25 ml) were placed in a 50 ml round-bottomed flask equipped with a glass stopper. The soln was allowed to stand at room temp for 3 hr and the solvent then stripped off. The crude white crystalline salt was recrystallized 3 times from acetone–ether and then dried *in vacuo*; m.p. 49–51° (slightly hyg.); 6·45 g or 92%; NMR (τ) $CDCl_3$: 1·04(s), 5·66(s), 6·87–7.50(m), 7·73–8.33(m); ratio 1:3:4:4. (Found: C, 42·49; H, 5·42; N, 5·94. $C_8H_{12}ONBF_4$ requires: C, 42·70; H, 5·38; N, 6·23%).

N-*Ethyl*-4,5-*tetramethyleneisoxazolium fluoroborate* (XXVII). A soln of XXVIII (4·92 g, 0·04M) dissolved in CH_2Cl_2 (20 ml) was added to a soln of triethyloxonium fluoroborate[20] (7.60 g, 0·04M) in an equal volume of solvent in a 125 ml erlenmeyer. The reaction mixture was then left overnight at room temp protected with a $CaCl_2$ drying tube. A colorless oil which could not be crystallized was obtained after solvent removal; 8·2 g or 86%; NMR (τ) $CDCl_3$: 1·02(s), 5·10–5·57(q), 6·92–7·52(m), 7·78–8·18(m), 8·18–8·53(t); ratio 1:2:4:3.

4,5-*Dihydronaphth*-[1,2-c]-*isoxazole* (XXX). 2-Hydroxymethylenetetralone-l, NH_2OH·HCl and HOAc were combined according to the procedure published by Johnson.[16] Purification by vacuum distillation, (b.p. 124° at 0·7 mm) yielded 43 g or 62% or pure isoxazole. NMR showed the presence of only one isomer though Johnson had reported the isolation of a 79:9% mixture of isoxazole isomers; NMR (τ) CCl_4: 1·92(s), 2·35–2·64(m), 2·68–3·03(m), 6·94–7·64(m); ratio 1:1:3:4.

N-*Methyl*-4,5-*dihydronaphth*-[1,2-c]-*isoxazolium fluoroborate* (XXXI). XXX (2·0 g, 0·01 M) and trimethyloxonium fluoroborate (1·48 g, 0·01M) were combined according to the procedure described for the synthesis of XXVI; m.p. 139–142°; 2·4 g or 89%; NMR (τ) CH_3NO_2: 0·96(s), 2·08–2·33(m), 2·35–2·63(m), 5·55(s), 6·80–7·08(m); ratio: 1:1:3:3:4. (Found: C, 52·91; H, 4·61. $C_{12}H_{12}ONBF_4$ requires: C, 52·78; H, 4·43%).

N-*Ethyl*-4,5-*dihydronaphth*-[1,2-c]-*isoxazolium fluoroborate* (XXXII). Triethyloxonium fluoroborate (7·60 g, 0·04M) and XXX (6·84 g, 0·04M) were combined following the procedure for the synthesis of XXVII; 91%; m.p. 140–143°; NMR (τ) CH_3NO_2: 0·91(s), 2·03–2·27(m), 2·30–2·60(m), 5·17(q), 6·75–7·06(m), 8·26(t); ratio: 1:1:3:2:4:3. (Found: C, 54·38; H, 5·32. $C_{13}H_{14}ONBF_4$ requires: C, 54·39; H, 4·92%).

* See Ref. [*a*]

Generation and stability of the ketoketenimine (XXXIII). XXVI (0·11 g, 5·0 × 10^{-4}M) was dissolved in CH_2Cl_2 (5 ml) and placed in a 10 ml volumetric. An Et_3N soln (0·050 g, 5·0 × 10^{-4}M) in the same solvent was then added and the flask rapidly filled to the mark with CH_2Cl_2. The IR spectrum was immediately recorded and then retaken at intervals until the ketenimine band at 4·85μ had almost disappeared (2 hr).

The other ketenimines (XXXIV-XXXVI) were generated by the same procedure. In the case of XXXVI the 4·85μ band was still present in sols kept over a week suggesting that this compound should be isolable.

Isolation of the ketoketenimine (XXXVI). A soln of Et_3N (0·34 g, 3·4 × 10^{-3}M) in 10 ml CH_2Cl_2 was placed in a 50 ml erlenmeyer equipped with a Claisen adapter, long capillary tube and a magnetic stirrer. The reaction mixture was kept under N_2 and immersed in a Dry-Ice–acetone bath. While stirring, a solution of XXXII (1·00 g, 3·4 × 10^{-3}M) in the same solvent was added dropwise. The yellow reaction mixture was then poured into a flask containing light petroleum (50 ml) and the supernatant decanted from the precipitated amine salt after the system reached room temp. The solvent was removed *in vacuo* and a viscous yellow oil was obtained. This non-distillable oil could not be recrystallized and slowly darkened on standing; it is best kept under N_2 in a deep freeze; IR (μ) CH_2Cl_2: 4·85, 5·93, 6·11, 6·21, 6·55; NMR (τ) CCl_4: 1·80–2·13(m), 2·53–3·00(m), 6·27(q), 6·95–7·50(m), 8·64(t); ratio: 1:3:2:4:3.

2-[*N-Methylformamido*]-*cyclohexanone* (XXXVII). Concentrated aqueous solns of XXVI (1·50 g, 0·007M) and $NaHCO_3$ (1·18 g, 0·014M) were combined at room temp in a 50 ml erlenmeyer. After 25 min the reaction mixture was placed in the refrigerator and left there overnight. The soln was extracted twice with ether, the ether extracts washed twice with water and then dried over Na_2SO_4. Evaporation of the ether at reduced press afforded a white powdery solid which was washed with cold n-hexane and cyclohexane, and then dried *in vacuo*, m.p. 82–84°; 0·45 g or 41%; IR (μ) $CHCl_3$: 2·91, 3·00(sh), 5·85, 6·10, 6·20(sh), 6·55. The intensities of the carbonyl absorptions varied with time suggesting a change in tautomer equilibrium position; NMR (τ) $CDCl_3$: 2·66–3·12, 3·89–4·38, 6·57–6·97, 7·14 (close d), 7·23 (close d), 7·41–8·53; ratio: 1:1:1:1½:1½:8. After exchange with D_2O the 3 low field peaks were no longer present and the 2 doublets became singlets; MS: Mol. Wt. 155. (Found: C, 61·69; H, 8·65; N, 8·89. $C_8H_{13}O_2N$ requires: C, 61·91; H, 8·44; N, 9·03%).

N-Methyl-3,4,5,6-*tetrahydro*-2-*acetoxybenzamide* (XXXVIII). XXVI (0·75 g, 0·003M) and 5 ml of CH_3CN were placed in a 25 ml round-bottomed flask equipped with a ground glass stopper. HOAc (0·18 g, 0·003M) in 5 ml CH_3CN was then added, followed by a solution of Et_3N (0·30 g, 0·003M) in CH_3CN (5 ml). The pale yellow soln was left overnight at room temp, the solvent then removed under vacuum, and the residue dissolved in an EtOAc–water mixture. The organic layer was separated, washed, 1 × 15 ml water, dried over Na_2SO_4 and the solvent stripped off under vacuum. The oily residue was dissolved in the minimum amount of ether and the soln was then cooled in a Dry-Ice–acetone bath. Upon the addition of n-hexane to this cold soln, a white powdery solid precipitated; this was filtered and recrystallized; m.p. 97–99°; 0·48 g or 81%; IR (μ) $CHCl_3$: 2·90, 5·69, 6·02, 6·55; MS:Mol. wt. 197; NMR (τ) $CDCl_3$: 3·71–4·17, 7·00–7·20 (d), 7·40–7·92 (m) (CH_3 at 7·82), 8·05–8·48 (m); ratio: 1:3:7:4. (Found: C, 61·00; H, 7·88; N, 7·28. $C_{10}H_{15}O_3N$ requires: C, 60·89; H, 7·67; N, 7·10%).

N-Ethyl-3,4,5,6-*tetrahydro*-2-*acetoxybenzamide* (XXXIX). XXVII (2·38 g, 0·009M), HOAc (0·54 g, 0·009M), Et_3N (0·91 g, 0·009M) and MeCN (20 ml) were combined following the procedure described for XXXVIII. A white solid was obtained directly after the EtOAc was stripped off under vacuum. This was filtered off, washed with cold n-hexane and purified by precipitation from a concentrated EtOAc soln with n-hexane: m.p. 65–67°; 1·46 g or 77%; IR (μ) $CHCl_3$: 2·92, 5·71, 6·05, 6·65; MS:Mol. Wt. 211; NMR (τ) $CDCl_3$: 3·61–4·25 (broad singlet), 6·50–7·00 (quartet split into doublets), 7·43–8·00 (m) (CH_3 at 7·87); 8·11–8·50 (m), 8·72–9·08 (t); ratio: 1:2:7:4:3. (Found: C, 62·67; H, 7·97; N, 6·87. $C_{11}H_{17}O_3N$ requires: C, 62·53; H, 8·11; N, 6·63%).

The enol acetate (XLIII). XXXI (0·273 g, 0·001M), HOAc (0·60 g, 0·001 mole), Et_3N (0·100 g, 0·001M) and MeCN (5 ml) were combined following the procedure described for the synthesis of XXXVIII. The solid obtained after the work up procedure was recrystallized from acetone–hexane; m.p. 110–111°; 0·217 g or 89%; UV λ_{max} at 274 mμ in CH_2Cl_2 (UV λ_{max} of β-acetoxy-N-methyl-cinnamamide (XLII) is at 267 mμ in CH_2Cl_2[3]); IR (μ) CH_2Cl_2: 5·65, 6·03; NMR (τ) $CDCl_3$: 1·9–3·0 (m), 6·7–8·1 (m), 7·17–7·28 (d), 8·01 (s). (Found: C, 68·85; H, 6·28. $C_{14}H_{15}O_3N$ requires: C, 68·55; H, 6·16%).

Thermal decomposition of enol acetates. XXXVIII and XXXIX were refluxed in MeCN for 1 hr. The IR spectra of the residues after solvent removal were unchanged. When the esters were refluxed overnight in dioxane-acetonitrile the reisolated materials were shown by IR to be contaminated by small amounts of the respective hydrolysis products, the β-ketoamides (also identified by TLC). This was attributed to the presence of traces of water in the solvents. When dissolved in a solution of Et_3N (1 Molar) in MeCN the

enol esters decomposed within a few hr at reflux temp and were ~20% decomposed after 30 hr at room temp.

The enol ester (XLIII) also decomposed in 1 molar Et_3N in MeCN again mainly by a hydrolytic pathway (IR peaks at 6·0μ); about half decomposition overnight at room temp.

Reaction of the ketoketenimine (XXXVI) *with* HOAc:*spectral study.* Crude freshly prepared XXXVI (0·60 g, 0·003M) dissolved in CH_2Cl_2 (5 ml) was placed in a 10 ml volumetric. HOAc (0·14 g, 0·0023M) in the same solvent was then added and the flask filled to the mark with CH_2Cl_2. The IR spectrum was recorded immediately but no band at 4·85 μ was observed. Complete disappearance of the ketenimine peak must occur in less than one min. The presence of new absorption in the CO region at 5·66 μ showed that the corresponding enol ester had in fact formed.

Crude XXXVI (0·60 g, 0·003M) dissolved in a small amount of CH_2Cl_2 was treated with a soln of HOAc (0·14 g, 0·0023M) and Et_3N (0·23 g, 0·0023M) in the same solvent, total volume of 10 ml. The ketenimine band at 4·85 μ decreased in intensity with time and was completely gone after 10 min. During this period the CO stretch band attributed to the enol ester increased in intensity.

2-*Carbobenzoxyglycyloxy*-N-*methyl*-3,4,5,6-*tetrahydrobenzamide* (XLVI). Carbobenzoxyglycine (0·92 g, 0·004M), XXVI (1·0 g, 0·004M), and MeCN (20 ml) were placed in a 50 ml tightly stoppered round-bottomed flask. A soln of Et_3N (0·44 g, 0·004M) in MeCN (5 ml) was added and the reaction mixture was allowed to stand at room temp for 40 min. The isolation procedure used for XXXVIII was followed: m.p. 106–107°; (IR (μ) $CHCl_3$: 2·92, 3·42, 5·65, 5·80, 6·05, 6·66; NMR (τ) $CDCl_3$: 2·70(s), 3·75–4·32, 4·90(s), 5·97–6·07(d), 7·20–7·28(d), 7·50–8·05(m), 8·16–8·55(m); ratio: 5:2:2:2:3:4:4. (Found: C, 62·44; H, 6·63; N, 8·20. $C_{18}H_{22}N_2O_5$ requires: C, 62·41; H, 6·40; N, 8·09%).

The enol ester was further characterized by conversion to carbobenzoxyglycine benzylamide (identical with authentic sample[1]) on treatment with benzylamine in CH_3NO_2.

2-*Carbobenzoxyglycyl*-L-*phenylalanyloxy*-N-*methyl*-3,4,5,6-*tetrahydrobenzamide* (XLVII). A soln of XXVI (1·00 g, 0·0044M) in MeCN (5 ml) was placed in a 50 ml Erlenmeyer equipped with a glass stopper and a magnetic stirring device. The reaction flask was then immersed in an ice bath and, while stirring, a soln of carbobenzoxyglycyl L-phenylalanine (1·59 g, 0·0044M) and Et_3N (0·44 g, 0·0044M) in the same solvent (15 ml) was added. The reaction mixture quickly thickened with the formation of a white ppt so more MeCN was added and the mixture stirred for 30 min at room temp. The ppt was filtered off, washed with n-hexane, water and ether and then dried *in vacuo*, m.p. 134–136°; 1·6 g or 74%. The enol ester was further purified for analysis by precipitation from an acetone soln with n-hexane, m.p. 139–140·5°; NMR (τ) $CDCl_3$: 2·64(s), 2·74(s), 2·90–3·27 (broad singlet), 3·77–4·14 (broad singlet), 4·22–4·52 (broad singlet), 4·39(s), 5·18 (sextet), 6·15(d), 6·91(d), 7·25(d), 7·47–8·52(m); ratio: 5:5:1:1:1:2:1:2:2:3:8. (Found: C, 65·45; H, 6·49; N, 8·69. $C_{27}H_{31}N_3O_6$ requires: C, 65·70; H, 6·23; N, 8·52%).

Anderson test studies. The active ester (XLVII, 1·380 g, 0·003M) dissolved in the minimum amount of DMF was placed in a 25 ml Erlenmeyer equipped with a magnetic stirrer. The reaction flask was stirred effectively and glycine ethyl ester hydrochloride (0·838 g, 0·006M) and a soln of Et_3N (0·606 g, 0·006M) in DMF was added (total volume 10 ml). The reaction mixture was stirred for another hr and then left standing overnight. The soln including the precipitated amine salt was dissolved in EtOAc (50 ml) and ether (50 ml) and extracted 1 × 20 ml water. The organic layer was separated and washed with 5 × 10 ml 10% Na_2CO_3, 2 × 10 ml 1NHCl, 1 × 10 ml H_2O, dried over Na_2SO_4 and the solvent evaporated at reduced press. After this residue was dried *in vacuo* a solid was obtained. Recrystallization from acetone–n-hexane gave 0·94 g or 71% carbobenzoxyglycyl-L-phenylalanylglycine ethyl ester, m.p. 112–116° (Lit.[1] m.p. 117–118°). The residue (0·24 g, 18%) contained about 80% product by IR.

In a parallel experiment the Anderson[21] test procedure was followed but no racemic product was isolated from the 2% EtOH soln seeded with authentic carbobenzoxyglycyl-D,L-phenylalanylglycine ethyl ester.[1]

Reaction of isoxazolium salts with triethylammonium hippurate—spectral comparison of azlactone formation. A soln of hippuric acid (0·896 g, 0·005M) and Et_3N (0·506 g, 0·005M) in MeCN was prepared in a 100 ml volumetric. An aliquot of this soln (10 ml) was stirred vigorously at room temp in an Erlenmeyer and 0·0005M of the isoxazolium salt substrate was quickly added. The IR spectrum was recorded repeatedly until the ketenimine band had disappeared completely and no further change in the spectrum was observed. The following salts were tested:

A. N-t-butyl-5-phenylisoxazolium perchlorate (0·1511 g).
B. N-ethyl-5-phenylisoxazolium fluoroborate (0·1308 g).
C. N-ethyl-4,5-dihydronaphth-[1,2, -c] isoxazolium fluoroborate (0·1433 g).

D. N-ethyl-benzisoxazolium fluoroborate (0·1175 g).
E. N-methyl-4,5-tetramethyleneisoxazolium fluoroborate (0·1124 g).
The IR spectra are reproduced in Fig. 1.

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