Curve c of Fig. 2 was drawn to examine such possible correspondence. We are examining this proposition more thoroughly through the use of binary mixtures of phosphatic monoesters of differing second dissociation constants, and by quantitatively evaluating the numerous factors which determine or influence the shape of the pH–rate profile.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, POLYTECHNIC INSTITUTE OF BROOKLYN, BROOKLYN, N. Y.]

Peptide Synthesis *via* Active Esters. IV. Racemization and Ring-Opening Reactions of Optically Active Oxazolones

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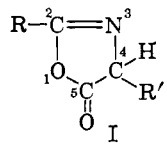
An optically active oxazolone, 2-phenyl-L-4-benzyloxazolin-5-one, was synthesized from benzoyl-L-phenylalanine by allowing benzoylamino acid to react in an acetic anhydride–dioxane solution. The reaction was followed polarimetrically. At the point of greatest negative rotation the solvent was removed and the oxazolone isolated and purified. The rates of racemization of 2-phenyl-L-4-benzyloxazolin-5-one, using various nucleophiles, were determined. These nucleophiles were, in order of decreasing racemization rates: *p*-nitrophenylate, phenylalanine methyl ester, and pyridine. Second-order rate constants were calculated from three pseudo-first-order rate constants for each nucleophile used. Ring-opening rates were measured spectrophotometrically. The concentration of nucleophile necessary to cause ring opening must be much greater than that necessary to bring about racemization in approximately the same time. No ring-opening reactions take place over the time scale necessary for racemization. It is found that rates of reaction for ring opening follow the order: *p*-nitrophenylate > phenylalanine methyl ester ≫ pH "8" buffer solution > water. The equilibrium reactions of oxazolone to yield benzoylphenylalanine *p*-nitrophenyl ester, and the reverse, benzoylphenylalanine *p*-nitrophenyl ester to give oxazolone, were measured using infrared spectrophotometry. From the calculated rates, the equilibrium constant was found to favor the formation of the *p*-nitrophenyl ester. The relevance of this equilibrium racemization during peptide synthesis is discussed.

Introduction

Partial or complete racemization is observed in syntheses involving optically active N-acylamino acids and in reactions of N-protected peptides, using various condensing reagents.[2] Solvent, temperature, and condensing agent[2-4] have been shown to be important in determining the extent of racemization.

The oxazolone I is the intermediate which most likely



I

undergoes racemization. These compounds were first isolated in reactions of optically active amino acids with acetic anhydride,[5-7] ketene,[8,9] and, more recently, with trifluoroacetic anhydride.[10] However, the oxazolones obtained were optically inactive, and the reactions of oxazolines which have been reported were carried out on racemized compounds.[11-13]

Racemization of optically active peptides, such as benzyloxycarbonylglycyl-L-phenylalanine *p*-nitrophenyl ester, is thought to involve the oxazolone intermediate[14] but, to date, no peptide oxazolone has been isolated.

Optically active oxazolones were isolated relatively recently,[15] although isolation of optically active thiohydantoins, derived from optically active oxazolones, was reported earlier.[16] Since no quantitative information is available on the reactions of optically active oxazolones, and because a study of these compounds would lead to a better understanding of racemization mechanisms, we prepared optically active 2-phenyl-L-4-benzyloxazolin-5-one, and studied its racemization and ring-opening reactions with various nucleophiles.

Results and Discussion

Benzoyl-L-phenylalanine was synthesized under Schotten–Baumann conditions. A 2% solution of this compound in 1:1 acetic anhydride–dioxane was prepared, and the change in rotation followed. The point of maximum negative rotation was reached in 60 min., $[\alpha]^{25}D$ −40°. At this point solvent was removed by distillation and the 2-phenyl-L-4-benzyloxazolin-5-one collected. The solvent used with the acetic anhydride and the concentration of the acylamino acid have a profound effect on the value of the observed

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TABLE I

RATE CONSTANTS FOR THE RACEMIZATION OF 2-PHENYL-L-4-BENZYLOXAZOLIN-5-ONE

Reagent causing racemization[a]	pK_a	Ratio reagent:oxazolone	$k_1 \times 10^2$, min.$^{-1}$	k_2, l./mole-min.	$t_{1/2}$,[b] min.
Pyridine	5.23[19]	134:1	2.87 ± 0.09	0.03	3814
		100:1	1.87 ± .05		
		82:1	1.01 ± .05		
Phenylalanine methyl ester	7.06[20]	2.4:1	7.46 ± .67	3.69	31
		1.8:1	3.84 ± .23		
		1:1	1.85 ± .06		
p-Nitrophenol and tri-*n*-butylamine	6.85[c]	0.25:1[d]	24.0 ± .7	115	0.99
		0.125:1[d]	13.1 ± .4		
		0.065:1[d]	6.09 ± .19		

[a] Solvent is dioxane in all cases. [b] Concentration of oxazolone is 8.74×10^{-3} *M* for all calculations. [c] $pK_a = 14$; pK_a *p*-nitrophenol = 14–7.15.[19] [d] Ratio amine to oxazolone only, phenol:oxazolone = 3:1.

TABLE II

BASE-CATALYZED RING-OPENING REACTIONS OF 2-PHENYL-L-4-BENZYLOXAZOLIN-5-ONE

Ring-opening reagent	Ratio reagents: oxazolone	$k_1 \times 10^2$, min.$^{-1}$	$t_{1/2}$, min.	Products[a]
p-Nitrophenol and tri-*n*-butylamine	10:10:1	3.55 ± 0.12	19.5	Benzoyl-phe *p*-nitrophenyl ester
Phenylalanine methyl ester	41:1	0.284 ± 0.11	244	Benzoyl-phe-phe methyl ester
Water	3.01×10^5:1	0.342 ± 0.20	203	Benzoyl-phe-OH
pH "8" buffer[b]	7560:1	2.89	24	Benzoyl-phe-OH
Pyridine	69:1	No reaction	...	*dl*-Oxazolone

[a] Phenylalanine is abbreviated pheOH. [b] Buffer solution in presence of dioxane.

maximum negative rotation.[15,17] The magnitude of the negative rotation was found to be inversely proportional to the acylamino acid concentration. Racemization of the oxazolone occurs least in dioxane under equivalent conditions. Other solvents tried were acetone and ethyl acetate. Acetic acid, produced in the reaction, may be complexed by the dioxane thus retarding racemization. Such a complex has recently been shown to exist.[18]

Kinetics

1. Racemization.—Rate constants are listed in Table I for the racemization of 2-phenyl-L-4-benzyloxazolin-5-one.

The results show that the strongest nucleophile gives the fastest rate of racemization. A very slow reaction takes place between *p*-nitrophenol and oxazolone (molar ratio 3:1), which results in a decrease in optical rotation at 546 mμ from −85 to −33° over a 20-hr. period. Instantaneous racemization of the oxazolone was observed in pure pyridine. This effect is due to the electron-withdrawing 2-phenyl group, since 2-methyl-L-4-benzyloxazolin-5-one gave a much slower racemization rate, $k_1 = 3.64 \times 10^{-2}$ min.$^{-1}$,[17] in the same solvent.

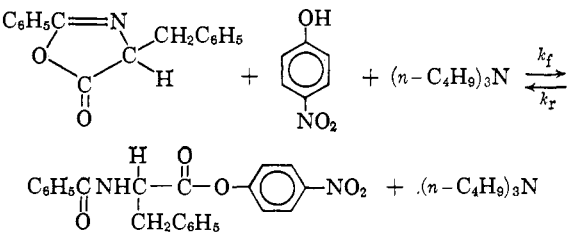
2. Ring-Opening Reactions.—The base-catalyzed ring-opening reactions of 2-phenyl-L-4-benzyloxazolin-5-one were measured either by infrared or ultraviolet spectroscopy. The results are listed in Table II.

It is interesting to observe that whereas racemization occurs in pyridine solutions, ring-opening cannot occur since pyridine does not contain a dissociable hydrogen. A high degree of nucleophilicity is also necessary for ring opening to occur. For example very weakly basic secondary amines have been shown not to open oxazolone rings.[21]

The first-order plot, Fig. 1, of the ring-opening reaction with pH "8" buffer (see Experimental section) gives a slower rate after 11 min. By this point, 3.56×10^{-5} *M* of benzoylphenylalanine is produced. A reaction between product acid and buffer takes place which changes the buffer capacity in this reaction medium. Goodman and Stueben[14] observed that in the reaction of benzyloxycarbonylglycyl-L-phenylalanine *p*-nitrophenyl ester with the same pH "8" buffer, it was necessary to add more base during the reaction to maintain the solution at constant pH.

The amount of water and pH "8" buffer necessary to effect ring opening compared to the other nucleophiles studied is very great. From this we conclude that the order of nucleophilicities in the ring-opening reactions is *p*-nitrophenylate > phenylalanine methyl ester >>> pH "8" buffer > water.

Equilibrium between Oxazolone and Benzoylphenylalanine *p*-Nitrophenyl Ester.—Young[22] reported the formation of oxazolone in the reaction of benzoylleucine *p*-nitrophenyl ester with triethylamine. By means of quantitative infrared spectroscopy we have determined the equilibrium constant for the reaction



$$K = \frac{k_f}{k_r} = \frac{3.55 \times 10^{-2}}{2.07 \times 10^{-3}} = 17.1$$

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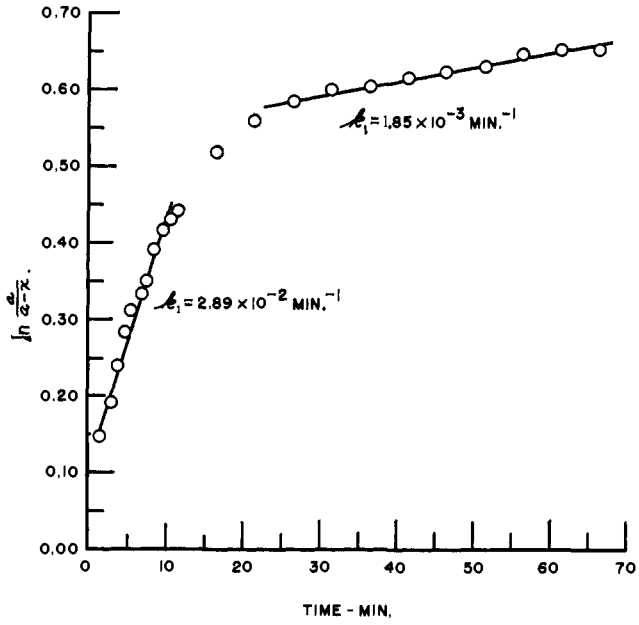


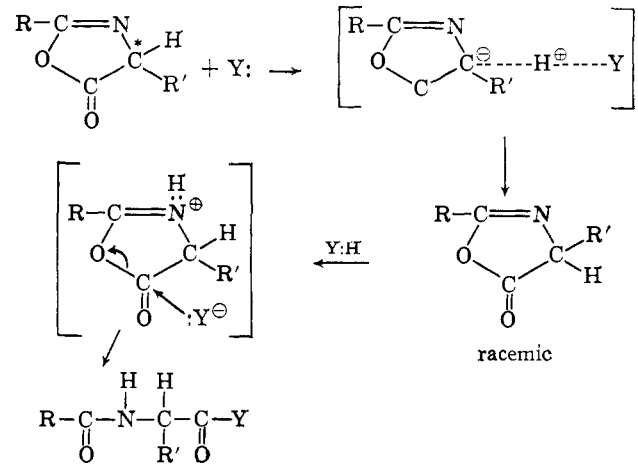
Fig. 1.—Ring-opening reaction of 2-phenyl-4-benzyloxazolin-5-one with pH "8" buffer.

The forward reaction was performed using 1 mole of oxazolone, 10 moles of *p*-nitrophenol, and 10 moles of tri-*n*-butylamine, while the reverse reaction was carried out using 1 mole of benzoylphenylalanine *p*-nitrophenyl ester, 9 moles of *p*-nitrophenol, and 10 moles of tri-*n*-butylamine. The results indicate that the equilibrium constant lies far to the right. A correction in optical density was made for the forward reaction to account for oxazolone which formed and did not react further. When the reaction was carried out using 3:3:1 *p*-nitrophenol–tri-*n*-butylamine–oxazolone, no reaction could be detected after 8 min. This equilibrium reaction has implications for peptide formation through active esters.[23-25] As a typical case for peptide synthesis, we studied a reaction between an oxazolone and phenylalanine methyl ester, where the product-forming step is irreversible. In this case, once again racemization occurs much more rapidly than ring opening.

The racemization and ring-opening reactions of optically active oxazolines are pictured as occurring in the following manner.

The base for ring-opening reactions must involve an available proton. In order to effect ring-opening in the same time that racemization takes place a much greater concentration of nucleophile is necessary. We conclude that under the conditions of our racemization experiments, very little, if any, ring opening of the oxazolone occurs.

In most peptide-forming reactions, the amount of oxazolone formed must be very small, and probably follows steady-state kinetics. Evidence for this comes from the fact that in most reactions some optically active product is formed. Either the optically active oxazolone ring opens to yield optically active material, which seems doubtful from our results, or the reaction proceeds *via* a different mechanism such as a pathway



involving a simple nucleophilic addition to the activated multiple bond.[4]

Experimental[26,27]

A. Materials.—Reagent grade dioxane which had been refluxed over sodium for 6 hr. was distilled into a flask containing calcium hydride. It was redistilled and stored over calcium hydride (b.p. 101.0–101.5°). A pipet which was flushed with dry nitrogen immediately before use was used to withdraw the dioxane as needed. Matheson, Coleman and Bell spectroscopic grade dioxane was distilled from lithium aluminum hydride and stored over calcium hydride. It was pipetted as above. Pyridine (b.p. 115.0–115.5°) and tri-*n*-butylamine (b.p. 89°, 12 mm.) were distilled from and stored over calcium hydride and pipetted as above. Absolute methanol was prepared by the method of Lund and Bjerrum.[28] Lastly, *p*-nitrophenol was recrystallized from 2 *N* hydrochloric acid (m.p. 114.0–114.8°).

B. Preparation of Compounds. 1. Benzoyl-L-phenylalanine was prepared by the method given by Greenstein and Winitz.[29] Recrystallization from water afforded 16 g. (59%) of product, m.p. 142–143° (lit.[30] m.p. 145–146° for benzoyl-D-phenylalanine), $[\alpha]^{25}D$ + 38.74°, $[\alpha]^{25}_{546}$ +45.73° (*c* 1.6, dioxane).

2. Changes in Optical Rotation of Benzoyl-L-phenylalanine.—A 2% solution of benzoyl-L-phenylalanine was made by dissolving 40 mg. in 1 ml. of dioxane and adding 1 ml. of acetic anhydride. Changes in rotation with time were followed in a 2-dm. tube. The most negative observed rotation was −1.587° at 66 min., λ 589 mμ.

3. 2-Phenyl-L-4-benzyloxazolin-5-one.—Benzoyl-L-phenylalanine (2 g., 0.0074 mole) was dissolved in 50 ml. of dioxane and 50 ml. of acetic anhydride was added to the solution. After 1 hr. the solvent was removed *in vacuo* (0.1 mm., 25°). A solid formed in the flask as the last traces of solvent were removed. The crude oxazolone contained acetic anhydride. To decompose it the residue was shaken for a few minutes with 25 ml. of an ice-cold saturated sodium bicarbonate solution. After decanting the bicarbonate solution, the residue was dissolved in 60 ml. of ether and extracted twice (25 and 10 ml.) with cold, saturated sodium bicarbonate solutions. The organic layer was dried over magnesium sulfate and evaporated. The residue still contained acetic anhydride. It was removed from the oxazolone by dissolving the residue in 25 ml. of dry xylene and removing the solvents *in vacuo* (0.1 mm., 25°).[31] The oxazolone was recrystallized from ether–hexane and allowed to crystallize in the refrigerator. Several crops of oxazolone were obtained by filtration. The filtrate was stored in the freezing compartment of the refrigerator until sufficient crystallization occurred. The flask was then stored overnight at normal refrigerator temperature. This operation was repeated until no solid remained after leaving the precipitate in the refrigerator overnight. The last crop was col-

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lected from a solution which had been stored in the freezing compartment. This method yielded 0.92 g. (49%) of oxazolone, m.p. 86.6–87.2°, $[\alpha]^{25}D$ −71.20°, $[\alpha]^{25}_{546}$ −82.76° (*c* 0.5, dioxane). Rotations reported here were for material having the highest optical purity. The ultraviolet spectrum gave a λ_{max} 245 mμ (ε 14,900) in dioxane; reported λ_{max} 244 mμ (ε 17,300) in ether.[15] The infrared spectrum, Nujol mull, showed strong absorption at 1830 and 1815 (C=O), 1825 (dioxane solution), 1660 (C=N), 921, 900, and 800 cm.$^{-1}$ (unassigned).[16]

Anal. Calcd. for $C_{16}H_{13}NO_2$: C, 76.47; H, 5.21; N, 5.57. Found: C, 76.72; H, 5.26; N, 5.83.

4. Benzoylphenylalanine *p*-Nitrophenyl Ester.—Benzoyl-L-phenylalanine (0.54 g., 0.002 mole) and *p*-nitrophenol (0.34 g., 0.002 mole + 20% excess) were dissolved in 5 ml. of ethyl acetate. N,N′-Dicyclohexylcarbodiimide (0.45 g., 0.002 mole) was added with swirling to the solution which was maintained at 0° for 0.5 hr. and at room temperature for 1 hr. The N,N′-dicyclohexylurea (0.430 g., theoretical) was filtered and the solution evaporated almost to dryness. Dry hexane was added to precipitate the ester which was recrystallized from ethyl acetate–hexane and afforded a total of 85 mg., m.p. first crop 149.4–149.6°, second crop, 155.6–156.2°. The optical rotation of the second crop was zero.

Anal. Calcd. for $C_{22}H_{16}N_2O_5$: C, 67.68; H, 4.65; N, 7.18. Found: C, 67.71; H, 4.67; N, 7.20.

From the Oxazolone.—The compound *p*-nitrophenol (0.070 g., 0.0005 mole) and tri-*n*-butylamine (0.01 ml., 0.000042 mole) were dissolved in 5 ml. of dry dioxane. After addition of 2-phenyl-L-4-benzyloxazolin-5-one (0.126 g., 0.0005 mole), the solution remained 1 hr. at room temperature. The ester was precipitated by adding water to the solution to give 0.161 g. (82.5%) of ester, m.p. 159.0–159.4°, [α] 0° (*c* 1.25, dioxane).

5. Benzoyl-L-phenylalanine Methyl Ester.—Diazomethane was generated by dissolving N-nitroso-N′-methylurea, 1.13 g., in an ice-cold solution of 5 ml. of 40% potassium hydroxide and 13 ml. of ether. The ethereal diazomethane solution (13 ml.) was decanted into a flask which contained benzoyl-L-phenylalanine (2 g., 0.0075 mole) in 5 ml. of methanol. No yellow color was produced. The solvent was removed by evaporation, the residue dissolved in ether, and extracted three times with saturated sodium bicarbonate solutions. After drying the organic layer over magnesium sulfate and removing the solvent, the residue was recrystallized from ether–hexane to give 1.09 g. (51%), m.p. 83.6–84.6°, $[\alpha]^{25}D$ +24.22°, $[\alpha]^{25}_{546}$ +28.79° (*c* 0.56, dioxane).

From the Oxazolone.—2-Phenyl-L-4-benzoyloxazolin-5-one (48 mg., 0.00019 mole) was dissolved in 2 ml. of methanol. This solution remained at room temperature for 2 weeks and then the methanol was removed by evaporation. The residue was dissolved in dioxane to a total volume of 2 ml. and its absorbance at 1750 cm.$^{-1}$ was determined. By use of a calibration curve the concentration of ester was found to be 0.0454 g. in 2 ml. (83%), $[\alpha]^{25}_{546}$ +3.54° (*c* 2.3, dioxane).

6. DL-Phenylalanine Methyl Ester.—DL-phenylalanine methyl ester hydrochloride (1.3 g., 0.006 mole) was dissolved in a minimum of water, and this solution was added to 25 ml. of a 40% potassium carbonate solution. The methyl ester was extracted with two 7-ml. portions of ether and dried over magnesium sulfate. After the ether was evaporated, the oil was distilled *in vacuo* (b.p. 75°, 0.05 mm.). There was obtained 0.567 g. (52%) of product, λ_{max} 258 mμ (ε 166), λ_{max} 253 mμ (ε 144). After 1 week crystals of diketopiperazine began to appear.

7. L-Phenylalanine methyl ester was prepared as above using L-phenylalanine methyl ester hydrochloride (1 g., 0.0046 mole), producing 0.525 g. of product (63.7%), $[\alpha]^{25}D$ +27.09°, $[\alpha]^{25}_{546}$ +32.01°, (*c* 2.8, dioxane).

8. Benzoylphenylalanylphenylalanine Methyl Ester.—To a solution of benzoyl-L-phenylalanine (0.808 g., 0.003 mole) in 25 ml. of methylene chloride maintained at 0° was added L-phenylalanine methyl ester hydrochloride (0.646 g., 0.003 mole) followed by triethylamine (0.42 ml., 0.003 mole). N,N′-Dicyclohexylcarbodiimide (0.619 g., 0.003 mole) was then added to the solution. The solution was allowed to come to room temperature overnight. Ten drops of acetic acid was added to the solution the next day and the N,N′-dicyclohexylurea was filtered. The methylene chloride was evaporated and replaced by ethyl acetate. The organic layer was extracted twice with aqueous sodium bicarbonate and twice with 2 *N* hydrochloric acid. The solution was dried over magnesium sulfate and the solvent removed under reduced pressure. Recrystallization from ethyl acetate–hexane afforded 0.661 g. (50%) of product, m.p. 164–165°, $[\alpha]^{25}D$ +19.71°, $[\alpha]^{25}_{546}$ +23.71° (*c* 1.4, dioxane). No tests for racemization were made.

From the Oxazolone.—Oxazolone (0.08760 g., 0.00035 mole) was added to L-phenylalanine methyl ester (0.46884 g., 0.0026 mole) dissolved in 5 ml. of dry dioxane. After standing 24 hr. the product was precipitated by adding water to the solution. Two crops were isolated weighing 0.127 g. (84.8%). The first crop (0.080 g.) had $[\alpha]^{25}D$ +12.68°, $[\alpha]^{25}_{546}$ +14.37° (*c* 0.9, dioxane), m.p. 164.8–165.2°.

Anal. Calcd. for $C_{26}H_{26}N_2O_4$: C, 72.54; H, 6.09; N, 6.51. Found: C, 72.77; H, 6.14; N, 6.30.

C. Buffer solutions were prepared according to the directions given by Goodman and Stueben.[14]

D. Instruments and Apparatus.—Polarimetric studies were carried out on a Model 80 Rudolph polarimeter equipped with a Model 200 A oscillating polarizer. Monochromatic light of different wave lengths was obtained by using a Bausch and Lomb grating monochromator equipped with a xenon–mercury light source (Hanovia 901B). Polarimeter tubes used were 2 or 4 dm. in length with a bore not less than 3 mm. in diameter. Center-fill tubes were employed. The temperature of the tube compartment was kept constant by a circulating pump connected to a constant temperature bath.

The voltage applied to the photoelectric cell was varied by means of a Keithley Model 240 voltage supply.

All ultraviolet spectrophotometric measurements were made using a Cary Model 14 recording spectrophotometer equipped with a constant temperature cell compartment. Matched, 1-cm. quartz cells were used.

Infrared spectra were recorded on a Perkin–Elmer Model 21 spectrophotometer using sodium chloride optics. Solution spectra were measured in matched 0.5-mm. cells except for the absorbances of benzoylphenylalanine methyl ester which were measured using 0.05-mm. matched cells.

E. Kinetic Procedure. 1. Polarimetry.—All kinetics were followed at 546 mμ on solutions in a center-fill, 4-dm. tube. Each reaction was started by the addition of one component to another component which was contained in a 5-ml. volumetric flask placed in a constant temperature bath maintained at 25 ± 0.1°. A stopwatch timed the initiation of reaction. After the mixing of reactants the volumetric flask was quickly taken from the bath and the contents poured into the polarimeter tube. After putting the tube into the polarimeter, the stopwatch was stopped and an electric timer started. The stopwatch time was added to the timer time in all experiments. The specific rotation at zero time was found by extrapolating calculated specific rotations back to zero time.

In some experiments oxazolone which did not have the highest optical rotation was used.

2. Ultraviolet Absorption Spectropnotometry.—Experiments were initiated as above using only the electric timer. Measurements were taken at 247.5 and at 225 mμ. In a 3:2 dioxane–water mixture, the extinction coefficients for oxazolone and for benzoylphenylalanine were found to be

	$\epsilon_{247.5}$ mμ	$\epsilon_{225.0}$ mμ
Oxazolone (c_1)	15,055	7,809
Benzoylphenylalanine (c_2)	7,609	13,826

The concentration of oxazolone for any time could be found by using the equations

$$A_{247.5} = 15{,}055c_1 + 7{,}609c_2$$

$$A_{225.0} = 7{,}809c_1 + 13{,}826c_2$$

$$1.82A_{247.5} - A_{225.0} = 19{,}591c_1$$

For the kinetic run using pH "8" buffer, a 3-ml. solution of oxazolone in dioxane was added to 2 ml. of water containing 0.050 ml. of pH "8" buffer. The pH "8" buffer solution was made from Coleman buffer tablets.

3. Infrared Absorption Spectrophotometry.—Either the change in absorbance of the oxazolone peak at 1823 cm.$^{-1}$ or the change in absorbance of the *p*-nitrophenyl ester peak at 1770 cm.$^{-1}$ was followed by withdrawing aliquots from the reaction flask. The absorbance at zero time was found by extrapolation.

4. Reaction between Oxazolone, *p*-Nitrophenol, and Tri-*n*-butylamine.—Oxazolone (0.04020 g., 0.00016 mole) was weighed

into a 10-ml. flask and dissolved in 1 ml. of dioxane. Another 10-ml. dioxane solution containing *p*-nitrophenol (0.24170 g., 0.00173 mole) and tri-*n*-butylamine (0.42 ml., 0.00173 mole) was prepared. To initiate the reaction, 9 ml. of the *p*-nitrophenol and tri-*n*-butylamine solution was pipetted into the oxazolone solution. The final ratio of *p*-nitrophenol:amine:oxazolone was 10:10:1.

5. Reaction between Oxazolone and Phenylalanine Methyl Ester.—Phenylalanine methyl ester (0.67126 g., 0.00375 mole) was weighed into a 5-ml. volumetric flask to which 4 ml. of dioxane was added. A 2-ml. dioxane solution of 2-phenyl-4-benzyloxazolin-5-one (0.04631 g., 0.000189 mole) was prepared. To initiate reaction, 1 ml. of the oxazolone solution was added to the 4 ml. solution of phenylalanine methyl ester. The ratio phenylalanine methyl ester:oxazolone was 41:1.

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Studies on the Esterase Action of Carboxypeptidase A. Kinetics of the Hydrolysis of Acetyl-L-mandelate[1,2]

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O-Acyl esters of mandelic acid have been found to be excellent substrates for kinetic measurements on the esterase activity of carboxypeptidase A. A detailed study of the carboxypeptidase-catalyzed hydrolysis of O-acetylmandelate has been carried out, using primarily the optically pure L-compound. The value of K_m for the hydrolysis of acetyl-L-mandelate is 0.070 ± 0.014 mole/liter at pH 7.5. At this pH competitive inhibition of the hydrolysis by one of the products, L-mandelate, is observed with a K_i of 1.76 ± 0.16 × 10^{-3} mole/liter. The pH–rate profile for the hydrolysis of acetyl-L-mandelate exhibits a maximum near pH 7.5, a value close to the optimal pH reported previously for peptidase activity. Other α-acyloxy acids have also been examined as potential substrates for carboxypeptidase A with a view toward determining the specificity and reactivity requirements of the enzyme as an esterase.

Introduction

This paper is the first of a projected series on the esterase action of carboxypeptidase A. A zinc-containing metalloenzyme with a molecular weight of about 34,300,[3-5] carboxypeptidase A is known to catalyze the hydrolysis of peptides in which a free carboxyl group is situated α to the hydrolytically labile amide linkage.[6] Its catalysis of the hydrolysis of some α-acyloxycarboxylic acids has also been reported.[6-8] However, although the specificity and the kinetics of the hydrolytic action of carboxypeptidase A on peptide substrates have been investigated in great detail, considerably less attention has been devoted to the examination of a number of the most basic features of its action on ester substrates. A survey of the literature reveals a lack of a thorough kinetic analysis of the carboxypeptidase A-catalyzed hydrolysis of an ester. The effect of pH upon esterase activity, the effects of inhibitors, and the relationships between the structure and stereochemistry of substrates and their hydrolytic reactivity all need clarification. In this paper the results of a kinetic study of the hydrolysis of a relatively uncomplicated ester, O-acetylmandelate, will be discussed, together with several experiments designed to delve into the specificity requirements of carboxypeptidase A.

Our choice of O-acetylmandelate as a suitable compound for study was motivated by the knowledge that mandelic acid can be readily resolved and that the preparation of its O-acyl esters in optically pure form can be accomplished without difficulty. Fortunately, since the hydrolysis of O-acetylmandelate catalyzed by carboxypeptidase A proceeds at a rate which is conveniently measurable using an automatic titrator, this substrate is particularly amenable to kinetic investigation.

Experimental

Materials. O-Acetyl-DL-mandelic Acid.—Racemic O-acetylmandelic acid was obtained from Aldrich Chemical Co. The commercial product was apparently partially hydrated. Recrystallization from hexane gave pure anhydrous O-acetyl-DL-mandelic acid, m.p. 80.5–81.5° (lit.[9] m.p. 79–80).

L-Mandelic Acid.—Mandelic acid was resolved through its strychnine salt. A mixture of 152 g. (1 mole) of racemic mandelic acid and 167 g. (0.5 mole) of strychnine in 1500 ml. of water was heated on a steam bath and enough concentrated ammonium hydroxide was added to give a clear yellow solution. The solution was seeded with a crystal of pure strychnine L-mandelate and was allowed to cool to room temperature. Recrystallization of the precipitated salt from 1 l. of water and air drying gave colorless needles of strychnine L-mandelate with indefinite m.p. in the range 107–116° (lit.[10] m.p. 115–116°). (This material was probably a hydrate since exhaustive desiccation raised the m.p. to 128–131°.) The salt was treated with hot water and excess ammonium hydroxide and, after cooling, the strychnine was collected. The filtrate was saturated with sodium chloride and was acidified with hydrochloric acid. Repeated extraction of the filtrate with ether and evaporation of the solvent from the dried extracts gave 30 g. (39%) of crude L-mandelic acid which was suitable for the preparation of acylated derivatives. The yield of recovered material could be increased substantially by using

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