The Synthesis and Bacitracin-Catalysed Hydrolysis of Aryl Esters of *N*-Acylamino Acids

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1. A new method for synthesizing aryl esters of *N*-acylamino acids is described. The unsymmetrical anhydride resulting from the interaction of an *N*-acylamino acid and diphenylketen is allowed to react with a phenol. Cleavage of the anhydride by the phenol usually occurs in the desired direction. 2. Bacitracin has been examined as an enzyme model by determining its catalytic activity towards the hydrolysis of aryl esters. In general, it is less effective than imidazole. The variation of the catalytic constant with pH, together with other evidence, suggests that the histidine residue in bacitracin is the effective catalytic centre. 3. The stereoisomers of *N*-methoxycarbonylalanine *p*-nitrophenyl ester were hydrolysed at the same rate, but bacitracin was stereoselective towards the stereoisomers of the corresponding phenylalanine derivative.

There is considerable evidence that histidine forms part of the active centre of a number of enzymes that catalyse hydrolytic and group-transfer reactions (Barnard & Stein, 1958). The imidazole ring of histidine may function as either a general-base catalyst or a nucleophilic catalyst. Model experiments have revealed that imidazole and its simple derivatives have some catalytic activity in reactions involving the hydrolysis of aryl esters but not alkyl esters (Bender & Turnquest, 1957*a,b*; Bruice & Schmir, 1957, 1958). In the hope that a more complex model might exhibit other properties of enzymes such as stereospecificity, we have examined the catalytic properties of bacitracin towards the hydrolysis of esters, especially those of *N*-methoxycarbonylamino acids (Elmore & Smyth, 1963*a*). Possible structures of bacitracin A (Swallow & Abraham, 1959, and references cited therein), the major component of commercial bacitracin (Newton & Abraham, 1953*a*), are given in Fig. 1: as well as the imidazole ring of the histidine residue, there are various groups that could participate in a catalytic process.

During the present work a new procedure was developed for the preparation of aryl esters of *N*-acylamino acids that may find application in the field of peptide synthesis (Elmore & Smyth, 1963*b*).

EXPERIMENTAL

Materials

Bacitracin (50 units/mg.) was purchased from Nutritional Biochemicals Corp. (Cleveland, Ohio, U.S.A.). 2-Methyl-Δ²-thiazoline (L. Light and Co. Ltd., Colnbrook,

Bucks.) was purified by fractional distillation under reduced pressure. *p*-Nitrophenyl acetate (m.p. 77–78°) was prepared by the method of Chattaway (1931). *N*-Benzoyl-D-alanine and -L-alanine were prepared by the method of Pacsu & Mullen (1940), and had $[\alpha]_D^{19} -36.7^\circ$ (c 6.433 in 0.3 N-NaOH) and $[\alpha]_D^{19} +37.0^\circ$ (c 6.433 in 0.3 N-NaOH) respectively. Diphenylketen was prepared by the method of Smith & Hoen (1940). 2-Methylpropan-2-ol was fractionally distilled through a Widmer column.

N-Methoxycarbonylamino acids. These derivatives were obtained by treating aqueous solutions of amino acids (0.01 mole) with a solution of methyl chloroformate (0.011 mole) in ether (20 ml.) during 1 hr. at 5°. The pH was controlled at 7.5 by means of a pH-stat. When the reaction was complete (negative ninhydrin test), the solution was extracted with ether, acidified to pH 2 with 4 N-HCl and either concentrated to small bulk or evaporated to dryness under reduced pressure. The concentrate or residue was extracted with ethyl acetate and the extract was washed with a small volume of water and then dried (over MgSO₄). Removal of solvent under reduced pressure gave an oil that, in some cases, could be solidified by trituration with light petroleum (b.p. 60–80°). Yields usually averaged 80–85%. *N*-Methoxycarbonylglycine (Leuchs, 1906) had m.p. 95° after recrystallization from methylene chloride–light petroleum (b.p. 60–80°). *N*-Methoxycarbonyl-L-alanine and -D-alanine (Go & Tanni, 1939) and *N*-methoxycarbonyl-L-phenylalanine and -D-phenylalanine were obtained as oils and used directly. *N*-Methoxycarbonyl-DL-alanine had m.p. 80° after recrystallization from methylene chloride–light petroleum (b.p. 60–80°) (Found: C, 41.0; H, 5.9; N, 9.9. C₅H₉NO₄ requires C, 40.8; H, 6.2; N, 9.5%). *N*-Methoxycarbonyl-L-asparagine was isolated by acidification of the reaction mixture followed by continuous extraction with ethyl acetate. The product (65%), after crystallization from ethyl acetate–light petroleum (b.p. 40–60°) and then from ethanol–ether, had m.p. 143°, $[\alpha]_D^{20} -12.2^\circ$ (c 5 in

Fig. 1. Alternative possible structures of bacitracin. The broken line indicates a linkage whose precise nature has not yet been established.

NN-dimethylformamide) (Found: C, 37.8; H, 5.2; N, 14.4. $\text{C}_6\text{H}_{10}\text{N}_2\text{O}_5$ requires C, 37.9; H, 5.3; N, 14.7%). The *D*-isomer had m.p. 143°, $[\alpha]_D^{20} + 12.1^\circ$ (c 5 in *NN*-dimethylformamide) (Found: C, 38.1; H, 5.5; N, 14.4%). 1-Methoxycarbonylamino cyclohexane-1-carboxylic acid was obtained as crystals by acidification of the reaction mixture from methyl chloroformate and 1-aminocyclohexane-1-carboxylic acid. It was washed with water, dried and recrystallized from ethyl acetate-light petroleum (b.p. 60–80°), when it had m.p. 189° (Found: C, 53.6; H, 7.2; N, 7.0. $\text{C}_9\text{H}_{15}\text{NO}_4$ requires C, 53.7; H, 7.5; N, 7.0%).

p-Nitrophenyl esters of *N*-methoxycarbonylamino acids. Two methods were used to synthesize aryl esters. Comparison of the products obtained by the two routes served as a check of their optical purity.

(a) A solution of the *N*-acylamino acid (0.01 mole) in dry tetrahydrofuran (10 ml.) at -15° was treated successively with triethylamine (2 ml. of a 1M solution in tetrahydrofuran) and diphenylketen (0.01 mole) in tetrahydrofuran (10 ml.). After 1 min., the phenol (0.01 mole) in tetrahydrofuran (10 ml.) was added and the reaction mixture was allowed to warm to room temperature and left overnight. Solvent was removed under reduced pressure and the residue was dissolved in cold ethyl acetate. The solution was washed successively with ice-cold saturated NaHCO_3 and ice-cold water, dried (over MgSO_4) and evaporated under reduced pressure. The product (60–65%) was recrystallized from methylene chloride-light petroleum (b.p. 60–80°).

(b) To a chilled solution of *N*-acylamino acid (0.01 mole) in dry tetrahydrofuran (10 ml.) was added successively a solution of the phenol (0.01 mole) in dry tetrahydrofuran (10 ml.) followed by a solution of *NN'*-dicyclohexylcarbodi-imide (0.011 mole) in tetrahydrofuran (10 ml.), and the mixture was left overnight at room temperature. *NN'*-Dicyclohexylurea was collected and the solvent was removed under reduced pressure. The residue was dissolved in ethyl acetate and the product (80–85%) was worked up as before.

The following esters were synthesized by the methods (a) or (b) as indicated in parentheses: *N*-methoxycarbonylglycine *p*-nitrophenyl ester (b), having m.p. 86° (Found: C, 47.1; H, 4.1; N, 11.2. $\text{C}_{10}\text{H}_{10}\text{N}_2\text{O}_6$ requires C, 47.3; H, 4.0; N, 11.0%); *N*-methoxycarbonyl-L-alanine *p*-nitrophenyl ester (a and b), having m.p. 99°, $[\alpha]_D^{20} - 69.2^\circ$ (c 5 in tetrahydrofuran) (Found: C, 48.9; H, 4.2; N, 10.7. $\text{C}_{11}\text{H}_{12}\text{N}_2\text{O}_6$ requires C, 49.3; H, 4.5; N, 10.4%); *N*-methoxycarbonyl-D-alanine (a and b), having m.p. 99°, $[\alpha]_D^{20} + 69.1^\circ$ (c 5 in tetrahydrofuran) (Found: C, 49.5; H, 4.5; N, 10.6%); *N*-methoxycarbonyl-DL-alanine *p*-nitrophenyl ester (a and b), having m.p. 130° (Found: C, 49.2; H, 4.4; N, 10.4%); *N*-methoxycarbonyl-L-phenylalanine *p*-nitrophenyl ester (a and b), having m.p. 103°, $[\alpha]_D^{20} - 9.6^\circ$ (c 5 in tetrahydrofuran) (Found: C, 59.3; H, 4.7; N, 7.9. $\text{C}_{17}\text{H}_{16}\text{N}_2\text{O}_6$ requires C, 59.3; H, 4.7; N, 8.1%); *N*-methoxycarbonyl-D-phenylalanine *p*-nitrophenyl ester (b), having m.p. 104°, $[\alpha]_D^{20} + 9.8^\circ$ (c 5 in tetrahydrofuran) (Found: C, 59.1; H, 4.5; N, 8.1%); *p*-nitrophenyl 1-methoxycarbonylamino cyclohexane-1-carboxylate (b), having m.p. 55° (Found: C, 55.7; H, 5.6; N, 8.8. $\text{C}_{15}\text{H}_{18}\text{N}_2\text{O}_6$ requires C, 55.9; H, 5.6; N, 8.7%) [method (a) gave only *p*-nitrophenyl diphenylacetate (80%), having m.p. 89°]. *N*-Methoxycarbonyl-L-asparagine *p*-nitrophenyl ester was synthesized by method (b) except that *NN*-dimethylformamide was used as solvent. After removal of

NN'-dicyclohexylurea, the product (80%) was precipitated by water, collected, dissolved in hot ethyl acetate, dried (over MgSO_4) and crystallized by the addition of light petroleum (b.p. 60–80°). After recrystallization, it had m.p. 163°, $[\alpha]_D^{20} -40.7^\circ$ (c 5 in *NN*-dimethylformamide) (Found: C, 46.5; H, 4.2; N, 13.7. $\text{C}_{12}\text{H}_{13}\text{N}_3\text{O}_7$ requires C, 46.3; H, 4.2; N, 13.5%). The *D*-isomer had m.p. 163°, $[\alpha]_D^{20} +40.7^\circ$ (c 5 in *NN*-dimethylformamide) (Found: C, 46.5; H, 4.5; N, 13.3%). *N*-Benzoyl-DL-alanine *p*-nitrophenyl ester, synthesized from *N*-benzoyl-DL-alanine by methods (a) and (b), had m.p. 142° (Found: C, 61.2; H, 4.4; N, 8.9. $\text{C}_{16}\text{H}_{14}\text{N}_2\text{O}_5$ requires C, 61.1; H, 4.5; N, 8.9%). In similar attempts to synthesize the *D*- and *L*-stereoisomers, both methods gave only the racemic ester.

Kinetic technique

The liberation of *p*-nitrophenoxide ion resulting from the hydrolysis of the aryl esters was followed spectrophotometrically at 400 $m\mu$ in either a Unicam SP.500 or an Optica CF4 spectrophotometer; each instrument was fitted with a thermostatically-controlled cell holder. All reactions were carried out at 25°. Thrice-distilled water was used throughout. The reaction mixture consisted of 0.067 *M*-sodium phosphate buffer (2 ml.) of various pH values, 0.8 *M*-NaCl (0.5 ml.), water or an aqueous solution of catalyst (0.5 ml.), and a solution of substrate in 2-methylpropan-2-ol-water (2:5, v/v) (1 ml.). The last component was made by dilution with water of a solution of substrate in 2-methylpropan-2-ol; it was maintained at 25° and added to the cell when the remainder of the reaction mixture had attained constant temperature. The extinction at zero time, E_0 , was measured as soon as the solutions were mixed, and subsequent readings, E_t , were obtained at intervals of 3–12 min. The extinction, E_∞ , was again measured after 3 days, when hydrolysis was complete. The first-order rate constant, k_{obs} , was derived by computing the regression of $\ln(E_\infty - E_0)/(E_\infty - E_t)$ on t by the method of least squares. Initial velocities were computed by the method of Elmore, Kingston & Shields (1963).

RESULTS AND DISCUSSION

For the present study, it was desirable that the compounds selected as substrates should satisfy the following criteria: (1) they should contain *N*-acyl and ester groups such that the substrates would hydrolyse at a convenient rate at 25°; (2) they should have minimal hydrophobic character so that the kinetic studies could be carried out in solutions containing a high proportion of water; (3) both stereoisomeric forms, when they exist, should be easily accessible; (4) hydrolysis of the esters should be accompanied by spectral changes sufficiently large to permit accurate spectrophotometric studies. Eventually, it became clear that *p*-nitrophenyl esters of *N*-methoxycarbonylamino acids were most satisfactory and these, together with *p*-nitrophenyl acetate, were used in the present investigation.

The observed first-order rate constants, k_{obs} , for the hydrolysis of *p*-nitrophenyl acetate and the

Fig. 2. Effect of catalyst concentration, $[C]$, on k_{obs} for *p*-nitrophenyl acetate. $\circ$, Imidazole, pH 8.00; $\times$, bacitracin, pH 8.00; $\square$, bacitracin, pH 7.72; $\bullet$, bacitracin, pH 7.38; $\blacktriangle$, bacitracin, pH 7.11.

Fig. 3. Effect of catalyst concentration, $[C]$, on k_{obs} for *N*-methoxycarbonylglycine *p*-nitrophenyl ester. $\circ$, Imidazole, pH 8.00; $\times$, bacitracin, pH 8.04; $\blacktriangle$, bacitracin, pH 7.17.

p-nitrophenyl esters of the *N*-methoxycarbonyl derivatives of glycine, alanine and phenylalanine varied linearly with concentration of catalyst

Fig. 4. Effect of catalyst concentration, $[C]$, on k_{obs} , for *N*-methoxycarbonylalanine *p*-nitrophenyl ester. $\circ$, Imidazole, pH 8.04; $\square$, bacitracin and D-ester, pH 8.04; $\times$, bacitracin and L-ester, pH 8.04; $\triangle$, bacitracin and L-ester, pH 7.73; $\bullet$, bacitracin and L-ester, pH 7.38; $\diamond$, bacitracin and L-ester, pH 7.17; $+$, bacitracin and D-ester, pH 7.17.

Fig. 5. Effect of catalyst concentration, $[C]$, on k_{obs} , for *N*-methoxycarbonylphenylalanine *p*-nitrophenyl ester. $\circ$, Imidazole, pH 8.04; $\times$, bacitracin and L-ester, pH 8.04; $\square$, bacitracin and D-ester, pH 8.04.

(Figs. 2, 3, 4 and 5). For a reaction that is first-order with respect to catalyst, the catalytic constant, k_c , and k_{obs} , are related by the equation:

$$k_{\text{obs}} = k_w + k_c[C]$$

where k_w is the rate constant for the hydrolysis of the substrate in the absence of catalyst and $[C]$ is the concentration of catalyst. Computation of the regression of k_{obs} on $[C]$ by the method of least squares gave k_c (Table 1): k_w is lowest for *p*-nitrophenyl acetate and highest for *N*-methoxycarbonylglycine *p*-nitrophenyl ester. These results may be explained by the inductive effect of the *N*-methoxycarbonyl group and the inductive and steric effects of the side chains of alanine and phenylalanine.

The reactions were shown to be first-order with respect to substrate by using the integrated first-order rate equation:

$$\ln(E_{\infty} - E_0)/(E_{\infty} - E_t) = k_{\text{obs}} t$$

and by the linear relationship between the initial velocity, v_0 , and substrate concentration, $[S]_0$ (Fig. 6). For the phenylalanine derivative, the substrate was too insoluble to study over a range of concentrations in 2-methylpropan-2-ol-water (1:9, v/v), but the reaction was shown to be first-order with respect to substrate for a limited range of substrate concentrations in 2-methylpropan-2-ol-water (1:3, v/v) (Fig. 6).

At first sight there are a number of groups in bacitracin that might participate in the catalytic mechanism of hydrolysis of aryl esters. There is good evidence that the thiol group in bacitracin is masked in the form of a Δ^2 -thiazoline ring (Newton & Abraham, 1963b; Craig, Hausmann & Weisiger, 1954). The model compound, 2-methyl- Δ^2 -thiazoline, however, did not catalyse the hydrolysis of *p*-nitrophenyl acetate at pH 7.38 and 7.72. It is therefore considered unlikely that the Δ^2 -thiazoline ring in bacitracin contributes to its catalytic activity. The inactivity of this ring system is not surprising in view of its low pK_a (Martin & Parcell, 1961), although basicity is only one of the factors that determine the nucleophilicity of a reagent. The α -amino group of the *N*-terminal amino acid, isoleucine, is rather unreactive to 1-fluoro-2,4-dinitrobenzene (Craig *et al.* 1954), and is therefore considered unlikely to contribute appreciably to the catalytic activity of bacitracin.

Further evidence on the nature of the catalytic group in bacitracin stemmed from a study of the pH-dependence of k_c . If the catalytic activity resides in a base whose conjugate acid has a dissociation constant, K_a , the catalytic constant, k'_c , at an H^+ concentration, $[H^+]$, is related to the maximum catalytic constant, k_c , when the base is completely unprotonated, by the equation:

$$1/k'_c = [H^+]/k_c K_a + 1/k_c$$

In practice, the regression of $1/k'_c$ on $[H^+]$ was found to be linear (Fig. 7), and K_a was computed from the quotient, ordinal intercept/slope, by the

Table 1. Comparison of k_c for the hydrolysis of *p*-nitrophenyl esters in presence of imidazole and bacitracin at pH 8.04 and 25° in 2-methylpropan-2-ol-water (1:9, v/v)

Values for k_c are given $\pm$ S.D.

Substrate (R·CO·O·C ₆ H ₄ ·NO ₂ - <i>p</i>)	k_c (M ⁻¹ min. ⁻¹)		Catalytic index (II/I)
	Imidazole (I)	Bacitracin (II)	
CH ₃ -	12.44 $\pm$ 0.22	9.60 $\pm$ 0.12	0.77
CH ₃ ·O·CO·NH·CH ₂ -	49.52 $\pm$ 2.24	38.76 $\pm$ 0.73	0.78
L-CH ₃ ·O·CO·NH·CH(CH ₃)-	45.99 $\pm$ 1.41	22.87 $\pm$ 1.00	0.50
D-CH ₃ ·O·CO·NH·CH(CH ₃)-		25.12 $\pm$ 1.07	0.55
L-CH ₃ ·O·CO·NH·CH(CH ₂ ·C ₆ H ₅)-	46.50 $\pm$ 1.64	48.10 $\pm$ 0.95	1.03
D-CH ₃ ·O·CO·NH·CH(CH ₂ ·C ₆ H ₅)-		21.40 $\pm$ 1.22	0.46

Fig. 6. Relationship between initial substrate concentration, $[S]_0$, and initial velocity of hydrolysis of substrate, v_0 , in the presence of bacitracin (0.267 mM). $\circ$, *p*-Nitrophenyl acetate at pH 7.38 in 2-methylpropan-2-ol-water (1:9, v/v); $\triangle$, *N*-methoxycarbonylglycine *p*-nitrophenyl ester at pH 7.17 in 2-methylpropan-2-ol-water (1:9, v/v); $\square$, *N*-methoxycarbonyl-D-alanine *p*-nitrophenyl ester at pH 7.17 in 2-methylpropan-2-ol-water (1:9, v/v); $\bullet$, *N*-methoxycarbonyl-L-phenylalanine *p*-nitrophenyl ester at pH 8.04 in 2-methylpropan-2-ol-water (1:3, v/v). The initial velocity, v_0 , is given as the rate of increase of extinction at 400 m μ ($\Delta E_{400m\mu}/\text{min.}$).

method of weighted least squares. For the hydrolyses of *p*-nitrophenyl acetate and *N*-methoxycarbonyl-L-alanine *p*-nitrophenyl ester, values for pK_a were 7.01 ± 0.08 and 7.18 ± 0.17 respectively. These are compatible with a mechanism involving catalysis by the unprotonated imidazole group of histidine but not for the participation of the unprotonated δ -amino group of ornithine, which

Fig. 7. Relationship between $[H^+]$ and $1/k_c$ for: $\circ$, *p*-nitrophenyl acetate; $\triangle$, *N*-methoxycarbonyl-L-alanine *p*-nitrophenyl ester.

would be expected to have pK_a greater than 8.5. Moreover, the catalytic behaviour of bacitracin strongly resembles that of imidazole, especially for the hydrolysis of the smaller substrates. It is not clear how far the parallel between the catalytic behaviour of bacitracin and imidazole can be pursued mechanistically. Enhancement of the rate of hydrolysis of aryl acetates by imidazole involves both nucleophilic and general-base catalysis (Bruice & Benkovic, 1964). Nucleophilic catalysis by imidazole has been recognized by the spectrophotometric identification of an *N*-acyl-imidazole as an intermediate in the reaction. This criterion could not be applied in the present case, since bacitracin absorbed too strongly in the ultra-violet region. The precise nature of the mechanism of catalysis by bacitracin must therefore remain open.

In general, the catalytic efficiency of bacitracin is less than that of imidazole. Table 1 shows that the catalytic ratio, k_c for bacitracin/ k_c for imidazole, with one exception discussed below, tends to fall

with increasing size of the substrate. This probably reflects the steric inaccessibility of the histidine residue in bacitracin to the approach of the substrate. Perhaps the most interesting observation in the present work is the stereoselectivity of bacitracin towards the *p*-nitrophenyl esters of *N*-methoxycarbonyl-L-phenylalanine and -D-phenylalanine. No significant difference in k_e was detectable for the *p*-nitrophenyl esters of *N*-methoxycarbonyl-L-alanine and -D-alanine, but the preference for the derivative of L-phenylalanine was sufficiently pronounced for the catalytic ratio to rise to approximately unity, a value higher than for any other substrate examined. Clearly, some additional factor operates to overcome the steric retardation that might have been expected to result from the presence of the bulky side chain. It is possible that some kind of hydrophobic bond is formed between the aromatic rings in the substrate and the phenylalanyl residue that is adjacent to histidine in bacitracin. Such a bond could be present in a ground-state bacitracin-substrate complex or a transition-state complex or both. A reaction that involved the formation of a ground-state complex would follow Michaelis-Menten-type kinetics, but attempts to examine this possibility for the *p*-nitrophenyl ester of *N*-methoxycarbonyl-L-phenylalanine were frustrated by the insolubility of the substrate, mentioned above.

Two other esters that we synthesized proved unsuitable as substrates for bacitracin. The hydrolysis of *p*-nitrophenyl 1-methoxycarbonylamino-cyclohexane-1-carboxylate was extremely slow, doubtless as a result of steric hindrance. On the other hand, *N*-methoxycarbonylasparagine *p*-nitrophenyl ester was hydrolysed extremely rapidly, probably owing to intramolecular catalysis by the amide group and the formation of a cyclic imide. Such a mechanism involving participation by an amide group has been established for the alkaline solvolysis of alkyl esters of *N*-benzyloxycarbonylasparagine (Sondheimer & Holley, 1954; Bernhard *et al.* 1962).

The *p*-nitrophenyl esters required for the present work were synthesized by using *NN'*-dicyclohexylcarbodi-imide and by a new method described briefly by Elmore & Smyth (1963b). The latter route, which was suggested by the work of Losse & Demuth (1961), involved the formation of an unsymmetrical anhydride from an *N*-acylamino acid and diphenylketene in the presence of a catalytic amount of tertiary base in cold tetrahydrofuran. We have found that these anhydrides are attacked by phenols such as *p*-nitrophenol and 3-hydroxypyridine to give optically pure aryl

esters of *N*-acylamino acids. Steric hindrance by the two phenyl groups usually directs fission of the anhydride in the desired direction. Although we have so far had satisfactory results with *N*-methoxycarbonyl- and *N*-benzyloxycarbonyl-amino acids by this method, like other procedures for the synthesis of aryl esters of *N*-acylamino acids, it should be used with caution. For example, *p*-nitrophenyl 1-methoxycarbonylamino-cyclohexane-1-carboxylate could not be made by this procedure, probably as a result of steric hindrance to attack by the phenol on the anhydride. Further work is needed before this method can be generally applied to peptide synthesis.

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