Tetrahedron. Vol. 31, pp. 2659 to 2662. Pergamon Press 1975. Printed in Great Britain

THE BEHAVIOR OF AMIDES OF N-ACYLAMINO ACIDS TOWARD ANILINE AND SUBSTITUTED ANILINES UNDER PAPAIN CATALYSIS†

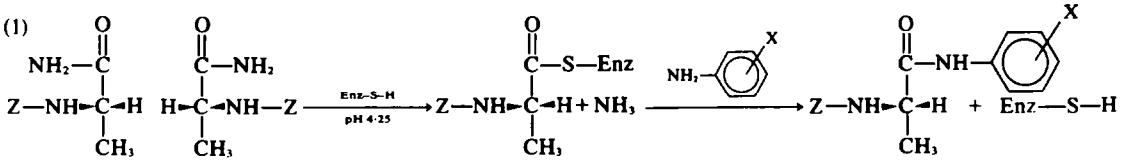
J. L. ABERNETHY,* F. G. HOWELL, A. LEDESMA, D. DOOSE and R. EVERETT
Department of Chemistry, California State Polytechnic University, Pomona, CA 91768, U.S.A.

(*Received in U.S.A.* 15 *April* 1975; *Received in UK* 16 *June* 1975)

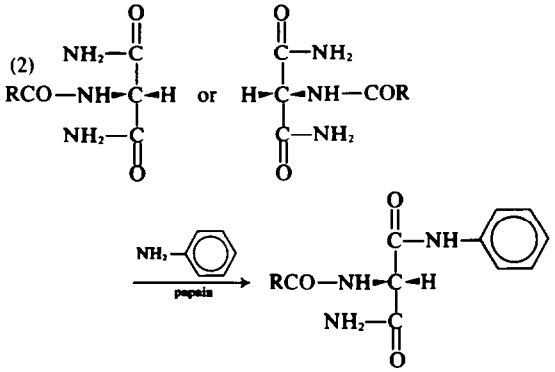
Abstract—Resolution of Z-DL-alanine amide has been achieved through papain-catalyzed reactions with aniline, the three anisidines, the three aminophenols and the three fluoroanilines. Most of the resultant anilides displayed better than a 95% resolution of the racemic amide. The four methyl esters of hippuric acid, Z-glycine, Z-L-alanine and Z-DL-alanine were prepared by means of a recently reported catalytic dehydrator in the presence of excess dry methanol. Subsequent treatment with ammonia produced the amides. Three N-acylaminomalonic amides were synthesized by a different route than previously used, from ethyl aminomalonate hydrochloride. Trial asymmetric syntheses were unsuccessful for papain catalysis of such achiral amides with aniline. A few organic solutes were tested for their effects on the activity of papain during anilide synthesis. The pH dependence of yield was studied for papain catalysis of reactions between aniline and hippuric amide and then aniline and Z-glycine amide.

The only amide of an N-acylamino acid that has been used for amide exchange with aniline under papain catalysis is hippuric amide.[1] It was the purpose of this research, first to establish the ability of papain to resolve Z-DL-alanine amide‡ through papain-catalyzed reactions with aniline and substituted anilines.

Four substrates, hippuric amide, Z-glycine amide, Z-L-alanine amide and Z-DL-alanine amide, were prepared in excellent yield from the corresponding N-acylamino acids by a route not previously employed. Each acylated amino acid was stirred at room temperature with excess anhydrous methanol and a catalytic dehydrator,[7] recently



Second, with the assurance that papain would show stereochemical preference for Z-L-alanine amide, experimental conditions were explored for a papain-catalyzed asymmetric synthesis, involving achiral N-acylaminomalonic amides. Although α-chymotrypsin has been used for an asymmetric hydrolysis of diethyl acetamidomalonate,[2,3] the basicity of the solution caused an early racemization, which prevented isolation of an optically active product. Papain has never been used for an asymmetric synthesis. The rationale of such an asymmetric synthesis is that the chirality and conformation of papain[4] designate a preference for Z-L-alanine[1] and therefore Z-L-alanine amide as shown in Eqn (1). Preferential positioning of the achiral N-acylaminomalonic amide in a similar manner, demonstrated in Eqn (2), would be predicted on the basis of known mechanistic features[5,6] at the crevice of the active site of this dilohal, spheroidal enzyme. A reaction should cause an asymmetric synthesis to occur. Acidity of the buffered medium and insolubility of products should prevent racemization and allow isolation of an optically active product.



reported, which consisted of Dowex H-Form resin and anhydrous $CaSO_4$. Removal of the resin and salt mixture was followed by treatment of the methanolic solution of the ester with dry ammonia. Yields are given in Table 1.

Table 1. The use of a catalytic dehydrator in producing amides of N-acylamino acids

Amide	M.p.	% Yield
Hippuric amide	182–184°	80
Z-Glycine amide	136–138°	79
Z-L-Alanine amide	128–130°	83
Z-DL-Alanine amide	124–125°	84

†This research was supported by a Frederick Gardner Cottrell Grant from the Research Corporation, Grants from the Society of the Sigma Xi and Grant No. 233H-264 from the California State Polytechnic University, Pomona.

‡Z is the currently accepted abbreviation for N-(benzyloxycarbonyl)-.

Amides of the Z-amino acids were subjected to papain-catalyzed reactions with aniline, the three aminophenols, the three anisidines and the three fluoroanilines. Data concerning reaction products are summarized in Table 2. In most instances, resolutions calculated from optical rotations of products amounted to 95% or greater. Phosphoric hexamethyltriamide (PHMT) was used as a solubilizing agent for Z-L-alanine amide and also for Z-DL-alanine amide, buffered at pH 4·25.

The dependence of yield on pH was established for representative reactions between aniline and Z-glycine amide (pH optimum 4·25) and then hippuric amide (pH optimum 5·0). Since solubilities of certain amides are low, potential solubilizing agents were qualitatively tested for their effects on the activity of papain during anilide formation. It is known that papain retains activity for catalytic hydrolysis even in a 40% urea solution.[8] In the current study, aniline was used with hippuric acid or Z-glycine amide, with the potential solubilizing agents: dimethyl sulfoxide (DMSO), phosphoric hexamethyltriamide (PHMT), urea and dimethylformamide (DMF). DMSO caused deactivation of the enzyme, presumably through oxidation of its essential mercapto group at the active site. Ordinary mercaptans are known to be oxidized to disulfides by DMSO.[9,10]. PHMT and urea were the best agents for retention of activity.

Table 2. Anilides and substituted anilides from amides of N-(benzyloxycarbonyl)amino acids produced by papain catalysis

Product		% Yield After 48 hr	% L-Enantiomer	M.p.	$[\alpha]_D^{25°}$ in Pyridine	% N[d] Found	Requires
Z-glycine[a]							
o-Hydroxyanilide	ZGoH[b]	13·0	Achiral	174–176°	Achiral	9·43	9·33
m-Hydroxyanilide	ZGmH	37·3	Achiral	160–162°	Achiral	9·49	9·33
p-Hydroxyanilide	ZGpH	3·3	Achiral	177–179°	Achiral	9·13	9·33
Z-L-alanine							
anilide	ZLAA	61·8	100	161–162°	−36·35°		
o-Hydroxyanilide	ZLAoH	37·5	100	173–174°	−37–36°	8·76	8·91
m-Hydroxyanilide	ZLAmH	46·5	100	182–183°	−41·34°	8·73	8·91
p-Hydroxyanilide	ZLApH	57·9	100	198–199°	−40·77°	9·03	8·91
Z-alanine							
anilide[c]	ZAA		99·5	161–163°	−36·00°	Mixture m.p. no change	
o-Hydroxyanilide	ZAoH		99·6	172–173°	−37·05°	Mixture m.p. no change	
m-Hydroxyanilide	ZAmH		99·2	183–184°	−40·64°	Mixture m.p. no change	
p-Hydroxyanilide	ZApH		99·3	196–198°	−40·23°	Mixture m.p. no change	
Z-L-alanine							
o-Anisidide	ZLAoAs	26·6	100	100–101°	−41·85°	8·43	8·53
m-Anisidide	ZLAmAs	34·3	100	117–120°	−43·97°	8·76	8·53
p-Anisidide	ZLApAs	42·8	100	164–166°	−39·94°	8·30	8·53
Z-alanine							
o-Anisidide[c]	ZAoAs		96·7	100–101°	−38·23°	Mixture m.p. no change	
m-Anisidide	ZAmAs		85·7	117–119°	−41·40°	Mixture m.p. no change	
p-Anisidide	ZApAs		95·8	162–164°	−36·60°	Mixture m.p. no change	
Z-L-alanine							
o-Fluoroanilide	ZLAoF	29·0	100	152–154°	−34·36°	8·78	8·86
m-Fluoroanilide	ZLAmF	35·9	100	150–153°	−34·52°	9·03	8·86
p-Fluoroanilide	ZLApF	42·3	100	168–170°	−33·48°	9·00	8·86
Z-alanine							
o-Fluoroanilide	ZAoF		96·5	151–153°	−33·18°	Mixture m.p. no change	
m-Fluoroanilide	ZAmF		95·8	150–153°	−31·64°	Mixture m.p. no change	
p-Fluoroanilide	ZApF		94·0	167–170°	−30·36°	Mixture m.p. no change	

[a] Z is the abbreviation for N-(benzyloxycarbonyl)-.

[b] Abbreviations used in the experimental part.

[c] Products from a racemic reactant contain some D-enantiomer. No percent yields are given. Mixture m.ps showed no change with products from L-reactants. Hence N-analyses were unnecessary.

[d] All nitrogen analyses were run by Mr. C. F. Geiger, Ontario, California.

Table 3. Diethyl N-(alkoxycarbonyl)aminomalonates and their derived N-(alkoxycarbonyl)aminomalonic amides

Alkoxy group	M.p.	% Yield[a]	% N Found	% N Requires
Esters				
Methoxy[12]	45·5–46·0°	92	6·17	6·01
Ethoxy	58·0–58·5°	67	5·87	5·67
Benzyloxy[11]	32–33°[11]	(Used as an oil for amide synthesis)		
Amides				
Methoxy	194–195°	70	23·65	23·99
Ethoxy	187–188°	72	22·46	22·21
Benzyloxy	193–194°	80	16·68	16·40

[a] Yield of amide based on weight of ester used.

Synthesis of N-(alkoxycarbonyl)aminomalonic amides involves a different approach than had been used by others[11-13] for preparation of the required intermediate esters. Addition of MgO and the alkyl chloroformate to aqueous diethyl aminomalonate hydrochloride produced the diethyl(N-alkoxycarbonyl)aminomalonate. Passage of ammonia into a methanolic solution of the ester gave rise to the amide. Information for the esters and amides is displayed in Table 3. Acetamidomalonic amide was similarly formed from diethyl acetamidomalonate and dry ammonia.

These diamides were exposed to a vast array of experimental conditions in attempting to achieve a papain-catalyzed asymmetric reaction with aniline. In no instance could a reaction be induced. On examination of a space-filled model of the enzyme, Smolarsky concluded[14] that the diamides actually could fit properly at the crevice of the enzyme. Due to the small size of the N-acyl groups of acetamidomalonic N-(methoxycarbonyl)aminomalonic and N-(ethoxycarbonyl)aminomalonic amides, possibly there is insufficient binding at a hydrophobic region of the enzyme.[15] The second amide group of these diamides is more polar than the methyl group of Z-L-alanine amide. Inhibition of proper binding by this second amide group could further contribute to a failure of a reaction to take place.

EXPERIMENTAL

Catalytic dehydration for the formation of methyl esters of N-acylamino acids, and then amides. The dehydrator components, Drierite and 20–50 mesh Dowex 50 W-X8 H-Form resin, were prepared by the method of Vesley and Stenberg.[7] A mixture of 10 g of dry hippuric acid, 23·3 g of Drierite and 10 g of dry Dowex H-Form resin was stirred with 125 ml anhyd. MeOH in a sealed flask for 2 hr at room temp. The mixture stood for 3 days with occasional shaking. Resin and calcium salts were removed, solids extracted with MeOH, and the combined, refiltered filtrate was cooled in an ice bath. Dry ammonia was passed in for 10 min. The mixture was sealed and allowed to stand 12 hr at room temp. Cooling and passage of ammonia was repeated, followed by standing 2 days at room temp. After cooling in an ice bath, the solid was rapidly removed by filtration and the filtrate was further worked up to give a total of 8 g of hippuric amide. It was recrystallized from anhyd MeOH with m.p. 183–185°. A mixture m.p. with known hippuric amide[16] caused no depression. Nearly identical procedures were used for Z-glycine amide and Z-L-alanine amide.

For Z-DL-alanine amide, the dehydration mixture was allowed to stand 5 days after stirring for 2 hr. After similar twice treatment with ammonia, in the cold, it was maintained 6 days at room temp. Evaporation to dryness and recrystallization from EtOH yielded 8·3 g of the amide, m.p. 124–125° (Found: N, 12·70. requires: 12·59%). Significant details are itemized in Table 1.

N-(Alkoxycarbonyl)aminomalonic amides and acetamidomalonic amide. The three N-(alkoxycarbonyl) groups that were incorporated were N-(methoxycarbonyl)-, N-(ethoxycarbonyl)- and N-(benzyloxycarbonyl)-. The procedure has been used very effectively during the synthesis of amides of ordinary *Z*-amino acids,[17,18] with hydrochlorides of esters of Z-amino acids. The appropriate alkyl chloroformate and MgO were added to an aqueous soln of diethyl aminomalonate hydrochloride[19] over chloroform. Isolation of the diethyl N-(alkoxycarbonyl)aminomalonate was followed by treatment of an ice-cold methanolic soln of the ester with ammonia as described elsewhere in this research. The amides were recrystallized from MeOH. From 10·5 g of the original ester hydrochloride there was isolated 7·7 g of N-(ethoxycarbonyl)- aminomalonic amide. Pertinent information is summarized in Table 3. Two of the esters have been reported by different synthesis.[11,12] The reported m.p. for diethyl Z-aminomalonate is 32–33°. It was obtained as an oil in this work and used directly for conversion to the amide.

An ice cold methanolic soln of diethyl acetamidomalonate was treated with dry ammonia in the usual manner. Recrystallization of the amide resulted in a 93% yield, m.p. 203–207°. Acetamidomalonic amide had been prepared previously[20] from this ester by means of aqueous ammonia under pressure.

pH Dependence of yield for anilides from amides of N-acylamino acids. Each 100 ml of 0·5 M buffered soln contained 0·0100 mole hippuric amide, 0·0100 mole aniline, and 0·250 g each active papain and L-cysteine·HCl·H_2O from a common stock soln. After a 24 hr incubation period at 40°, these weights of hippuric anilide resulted, with pH preceding the weight: 3·0–0·0025 g; 3·5–0·048 g; 3·75–0·12 g; 4·00–0·18 g; 4·25–0·16 g; 4·50–0·19 g; 5·0–0·35 g; 5·5 = 0·17 g. The pH optimum for hippuric amide was 5·0.

For Z-glycine amide, each 100 ml of soln also contained 10 g PHMT. After 48 hr, these results were obtained: 3·50–0·30 g; 4·00–0·47 g; 4·25–0·48 g; 4·50–0·35 g; 4·75–0·33 g; 5·00–0·26 g. The pH optimum was 4·25.

The effect of solutes on yields of anilides. The four solutes tested for effects on the activity of papain were DMSO, PHMT, urea and DMF. A blank with no added solute permitted the qualitative estimate of activity change for the papain. DMSO destroyed the activity of papain. DMF retained only moderate activity. Results are given for PHMT and urea. Anilide products were identified by m.ps and mixture m.ps with known compounds. Incubation temp was 40°.

For hippuric acid, 100 ml of 0·5 M buffer pH 4·75 was mixed with the designated weight of solute. Then 90 ml of the mixture was used to dissolve 0·0100 mole of aniline and 0·0100 mole of hippuric acid. Ten ml of a common stock soln that contained 0·250 g each of papain and L-cysteine·HCl·H_2O was added. Weights of solutes are given first, then weights of product formed at 0–24 hrs and 24–48 hrs. PHMT: 0·00 g, 0·28 g, 0·20 g; 2·5 g, 0·40 g, 0·21 g; 5·0 g, 0·29 g, 0·26 g; 10·0 g, 0·38 g, 0·20 g; 20·0 g, 0·19 g, 0·09 g. Urea: 0·00 g, 0·27 g, 0·23 g; 2·5 g, 0·31 g, 0·27 g; 5·0 g, 0·25 g, 0·22 g; 10·0 g, 0·27 g, 0·22 g; 20·0 g, 0·26 g, 0·21 g.

For Z-glycine amide, urea was used as before at pH 4·25. Weights of Z-glycine anilide are recorded for 0–24 hr and 24–28 hr. Urea: 0·00 g, 1·1 g, 0·52 g; 5·0 g, 0·47 g, 0·22 g; 10·0 g, 0·33 g, 0·17 g; 15·0 g, 0·26 g, 0·13 g; 20 g, 0·20 g, 0·15 g.

A single run was made with Z-glycine amide at pH 4·25 for a solute mixture of 5·0 g each of urea and PHMT: 0–24 hr, 1·65 g; 24–48 hr, 0·035 g.

Resolutions of Z-DL-alanine amide. Aniline and nine substituted anilines yielded various anilides or substituted anilides through papain-catalyzed reactions with both Z-L-alanine amide and Z-DL-alanine amide. The chief features of the experiments are summarized in Table 2. Abbreviations for products that are given in Table 2 are used here. Eastman spectrograde pyridine was used for all optical rotations at 25°, measured in a Rudolph Model-80 High Precision Polarimeter, in a 1 dm water-jacketed polarimeter tube. In all cases for these two amides, reaction mixtures included 2·22 g (0·0100 mole) of Z-L-alanine amide or 2·22 g of the racemic amide and 0·0100 mole of aniline or substituted aniline, which were dissolved in 90 ml of acetic acid buffer that contained 5 g of PHMT. The pH was 4·25, except for the aminophenols for which the pH was 4·75. The soln was heated on a steam bath to dissolve the amide, then place in an incubator at 40°. A mixture of 0·250 g active papain and 0·250 g L-cysteine·HCl·H_2O was dissolved in 10 ml of 0·5 M acetic acid buffer and placed in the incubator. When both solns reached 40°, they were mixed and incubated for 48 hr. At the end of this time, anilide was removed by suction filtration, dried and weighed. It was treated with carbon in MeOH, suction filtered 4 times and the soln was allowed to evaporate to dryness under the hood. The solids were dried over P_2O_5 in a vacuum desiccator and optical rotations were measured (Table 2). Mixture m.ps were determined for each pair of anilides formed from the racemic amide and the L-amide with each aniline reactant. No depression or change was shown in any instance. Known Z-L-alanine anilide[21] prepared from Z-L-alanine melted at 161–162°. A mixture m.p. with this same product from the L-amide caused no depression.

In recording data, the abbreviation for each anilide (Table 2) is followed by the weight of anilide produced for 0–48 hr incubation

at 40°. Data for Z-glycine *o*-, *m*- and *p*-hydroxyanilides are also given, for which 100 ml of 0·5 M buffer solution, pH 4·75, was used without any added PHMT, with 0·250 g each of L-cysteine·HCl·H_2O and active papain, and 0·0100 mole each of the aminophenol and Z-glycine amide.

ZGoH 0·39; ZGmH 1·12 g; ZGpH 0·10 g; ZLAA 1·83 g; ZAA 1·22 g; ZLAoAs 0·90 g; ZAoAs 0·72 g; ZLAmAs 1·16 g; ZAmAs 0·87 g; ZLApAs 1·44 g; ZApAs 0·84 g; ZLAoF 0·91 g; ZAoF 0·40 g; ZLAmF 1·13 g; ZAmF 0·64 g; ZLApF 1·33 g; ZApF 0·79 g; ZLAoH 1·80 g; ZAoH 0·79 g; ZLAmH 1·46 g; ZAmH 0·74 g; ZLApH 1·82 g; ZApH 0·75 g.

The trial asymmetric syntheses. The three N-acylaminomalonic amides (Table 3) and acetamidomalonic amide were submitted to various reactions with aniline in attempting to induce papain-catalyzed asymmetric syntheses. Filtration preceded incubation to be certain that any undissolved reactants were removed. A water-jacketed funnel was often used, with the filter flask in constant temp bath, to maintain incubator temp prior to incubation. Variations of reaction conditions included usage of 10 ml or 15 ml of PHMT, mixtures of urea and PHMT, variations of pH from 4·0 to 5·0, increased concentration of active papain up to 0·750 g per 100 ml of mixture, with 0·250 g of L-cysteine·HCl·H_2O, temp at 40° or 45° for incubation, an increase in volume of soln per 0·0100 mole of amide and 0·0100 mole of aniline used, and use of substituted anilines. Also, the ratio of aniline to amide was set at 0·0150 mole to 0·0100 mole on occasion. The incubation period was often maintained up to a 5 day period. Desired enzyme-catalyzed reactions were not observed.

A typical procedure for a trial reaction is given. Phosphoric hexamethyltriamide (10 g) was used to dissolve 0·010 mole of N-(ethoxycarbonyl)aminomalonic amide. 0·50 M Buffer at pH 4·5 (85 ml) that contained 0·010 mole of aniline was slowly added to the soln at 45°. The soln was suction filtered at 45° with a water-jacketed funnel and a filter flask in a constant temp bath. Then 0·250 g of active papain and 0·250 g of L-cysteine·HCl·H_2O were dissolved in 5 ml of the buffer at 45° and mixed with the previous soln. Incubation was carried out at 45° for a period of 2 days. Essentially no ppt formed during this period.

Preparation of active papain. Dried papaya latex was generously donated by the Wallerstein Company, Deerfield, Illinois, imported from the African Congo region. This was used for the preparation of dried, activated papain.[6]

REFERENCES

[1]M. Bergmann and H. Fraenkel-Conrat, *J. Biol Chem.* **119**, 707 (1937).
[2]S. G. Cohen and L. Altschul, *Nature* **183**, 1687 (1959).
[3]S. G. Cohen and L. H. Klee, *J. Am. Chem. Soc.* **82**, 6038 (1960).
[4]J. Drenth, J. N. Jansonius, R. Koekoek and B. G. Wolthers, *Adv. Protein Chem.* **25**, 79 (1971).
[5]A. Berger, I. Schechter, R. Benderly and N. Kurn, *Proc. 10th Eur. Peptide Sympos.* 290 (1971).
[6]J. L. Abernethy, R. Boebeck, A. Ledesma and R. Kemp, *J. Org. Chem.* **38**, 1286 (1973).
[7]G. F. Vesley and V. I. Stenberg, *Ibid.* **36**, 2548 (1971).
[8]L. A. A. Sluyterman, *Biochem. Biophys. Acta* **139**, 418, 430 (1967).
[9]C. N. Yianniso and J. V. Kabinos, *J. Org. Chem.* **28**, 3246 (1963).
[10]T. J. Wallace, *J. Am. Chem. Soc.* **86**, 2018 (1964).
[11]M. Frankel, M. Harnik and Y. Levin, *Ibid.* **74**, 3873 (1952).
[12]M. Frankel, M. Harnik, Y. Levin and Y. Knobler, *Ibid.* **75**, 78, (1953).
[13]A. Berger, M. Smolarsky, N. Kurn and H. R. Bossard, *J. Org. Chem.* **88**, 457, (1973).
[14]M. Smolarsky, private communication, Department of Biophysics, Weizmann Institute of Science, Rehovot, Israel, September 9 (1973).
[15]G. Lowe, *Phil. Trans. Roy. Soc.* **B257**, 240 (1970).
[16]Beilstein, *Vierte Auflage*, Volume IX, System No. 920, p. 236 (1935).
[17]B. F. Erlanger and E. Brand, *J. Am. Chem. Soc.* **74**, 3873 (1951).
[18]J. P. Greenstein and M. Winitz, *Chemistry of the Amino Acids*, Vol. 2, p. 1111. Wiley, New York (1961).
[19]W. H. Hartung, J. H. R. Beaujon and G. Cocolas, *Org. Synth.* Coll. Vol. V, 376 (1973).
[20]M. Ishidate, Y. Sakurai and I. Aiko, *Chem. Abstr.* **55**, 1877 (1961).
[21]D. G. Doherty and E. A. Popenoe, Jr., *J. Biol. Chem.* **189**, 447 (1951).