Anal. Calcd. for $C_{18}H_{23}NO_5$: C, 64.89; H, 6.95; N, 4.20; C_2H_5O, 27.0. Found: C, 64.45; H, 7.29; N, 4.02; C_2H_5O, 27.1.

Distillation of this material was accompanied by considerable decomposition and resulted in oils with wide ranges of boiling points, refractive indexes, and ethoxyl content. All efforts to prepare derivatives of VII resulted in intractable oils.

Acid Hydrolysis of VII.—A 2.10-g. sample (0.0063 mole) of the keto diester (VII) was dissolved under nitrogen in 20 ml. of 18% hydrochloric acid and the resulting solution maintained at a slow distillation rate under a 12-inch Vigreux column. In 3 hours, 0.0063 mole of carbon dioxide had been collected at a rate slightly higher than first order and the evolution of the gas had ceased; 64% of two equivalents of alcohol (b.p. and $n^{25}D$) was refractionated from the distillate.

The non-acidic material, 0.51 g., was separated by treatment of the remaining acidic solution with base and extraction with ether. From this 0.13 g. of the piperidone VIII was distilled, b.p. 115–120° (0.3 mm.), $n^{25}D$ 1.5483; hydrochloride, m.p. 155–160°, both alone and after mixing with the hydrochloride of VIII; the residue of this distillation was a tar. The basic solution from which the piperidone VIII was extracted was acidified with hydrochloric acid and evaporated to dryness. The salt residue was extracted with alcohol and the resulting solution evaporated to yield 1.05 g. of a hydrochloride, which, after esterification with alcoholic hydrogen chloride and treatment with sodium bicarbonate, yielded 0.9 g. of the amino diester IV, of which 0.75 g. (40%) was collected on distillation at 140–145° (0.5 mm.); this product gave a methiodide, m.p. 151–153°, both alone and when mixed with an authentic sample of the methiodide of IV.

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[CONTRIBUTION FROM THE STERLING-WINTHROP RESEARCH INSTITUTE]

Asymmetric Enzymatic Synthesis of Amino Acid Anilides

BY NOEL F. ALBERTSON

A number of acyl-DL-amino acids have been converted to acyl-L-amino acid anilides in the presence of papain.

If an acyl derivative of a DL-amino acid, I, is treated with aniline in the presence of papain at the proper *p*H the L-amino acid reacts to form an insoluble anilide, II, whereas the D-form does not react, but remains in solution.[1]

$$\underset{\text{I}}{\text{DL—RCONHCHR'COOH}} + C_6H_5NH_2 \longrightarrow \underset{\text{II}}{\text{L—RCONHCHR'CONHC}_6\text{H}_5} + H_2O + \text{D—RCONHCHR'COOH}$$

It had been suggested that this method might be used as a "general method for resolution of amino acids,"[2] but apparently the first L-amino acid to be obtained by hydrolysis of its acylated anilide was methionine in 1948.[3a,b] The appearance of this paper by Dekker and Fruton prompted us to investigate this method of resolution.

The present paper summarizes the data obtained in the preparation of a number of amino acid anilides. Since all experiments were carried out under comparable conditions, data have been included in Table II even for those anilides which have been previously reported.

It is known that the rate of formation of the anilide, II, is considerably influenced by the nature of R and R' of I. It was noted that when R was phenyl and the *p*H was 5, the rate increased as R' went from hydrogen to methyl to ethyl. The rate for *n*-propyl was about the same as for ethyl, but the rate then decreased when R' was *n*-butyl.

The effect of varying R may be noted in the case of phenylalanine; the formyl derivative gave 0% yield in 96 hours, the acetyl derivative gave 18% yield in 163 hours and the benzoyl derivative gave 93% in 48 hours. The variation in *p*H was not sufficient to account for this difference.

Fox and co-workers[4a,b] pointed out that the *p*H optimum for enzymatic activity may vary with the substrate. Additional confirmatory data, obtained in this investigation, are shown in Table I. It is interesting to note that although benzoylalanine forms no anilide at *p*H 5.6 in 16 hours, benzoyl-2-aminobutyric acid gives a 50% yield under the same conditions. On the other hand, benzoylnorleucine will give a 20% yield in 16 hours at *p*H 6.4 whereas benzoyl 2-aminobutyric acid will not react.

TABLE I

L-Acyl amino acid anilide	Time, hr.	Initial *p*H	Yield, %
Benzoylalanine	22	5.97	0
		5.58	1
		5.13	33
		4.87	48
		4.58	63
Benzoyl-2-aminobutyric	16	6.26	1
		5.72	39
		5.56	54
		5.02	91
Benzoylnorleucine	16	6.38	20
		6.01	36
		5.61	53
		4.73	64
		4.18	25
Acetyltryptophan	66	5.15	20
		4.87	40
		4.60	53
		4.21	36
		3.90	12

Inasmuch as the anilides listed in Table II were prepared from DL-amino acids it has not been shown that they are entirely free of D-isomers. However, incubation of benzoyl-D-alanine with papain and cysteine for one month gave no anilide. On the other hand, the anilide prepared from

(1) M. Bergmann and H. Fraenkel-Conrat, *J. Biol. Chem.*, **119**, 707 (1937).

(2) J. Fruton, G. Irving and M. Bergmann, *ibid.*, **133**, 703 (1940).

(3) (a) C. Dekker and J. Fruton, *ibid.*, **173**, 471 (1948); (b) the referee called our attention to the resolution of 3-fluorotyrosine; C. Niemann and M. Rapport, THIS JOURNAL, **68**, 1671 (1946).

(4) (a) S. Fox and C. Pettinga, *Archiv. Biochem.*, **25**, 13 (1950); (b) S. Fox, C. Pettinga, J. Halverson and H. Wax, *ibid.*, p. 21.

TABLE II

L-ANILIDES

DL-Amino acid	Deriv.	Hr.	*p*H	Yld., %	M. p., °C.	*T*	$[\alpha]T_D$	Nitrogen, % Calcd.	Obsd.
Glycine	bz[a]	116	4.40	87	209–211[b]	..	.....	...	...
Alanine	bz	100	4.57	84	175–176[c]	31	−13.2[d]	...	...
2-Aminobutyric	bz	16	5.02	91	170–171[e]	29	−19	9.92	9.65
2-Aminobutyric	Cbzo[f]	16	5.02	93	149–151.5[g]	26	−30	8.97	9.07
Norvaline	bz	19	5.04	98	184–185	27	−21.7	9.45	9.37
Norvaline	Ac[h]	48	4.73	0	.....	..	....	...	...
Valine	bz	360	4.88	71	217–218[i]	32	−36	9.45	9.30
Norleucine	bz	43	5.70	89	182–183	29	−22	9.03	8.97
Norleucine	Ac	116	4.90	78	191	31	−80	11.28	11.12
Norleucine	[j]	...	5.12	..	152–153	29	−32	8.09	8.12
Leucine	bz	24	5.12	94	216–217[k]	31	−28	...	...
Isoleucine	bz	360	4.94	90	218–218.5	28	−33.6	9.03	8.95
2-Aminodecanoic	Ac	60	6.1	72	151–152	28	−63.5	9.20	9.16
Tryptophan	Ac	165	4.70	77	198–199	31	82.4	13.07	12.93
Threonine	bz	192	5.14	0	.....	..	....	...	...
Allothreonine	bz	500	4.80	58[l]	.....	..	....	...	...
o-Methylthreonine	bz	48	4.80	90	144–145	31	7	8.97	8.93
ω-Chloroallylglycine	bz	17	5.35	59	164–166	29	− 9	8.52	8.48
Ornithine	dibz	43	5.50	90	233–234	31	9.5	10.11	10.08
Lysine	dibz	116	5.40	100	217–218	28	−23	9.78	10.07[m]
Lysine	2-Ac 6-bz	73	4.93	53	195–197	31	−51.2	11.44	[n]
Phenylalanine	HCO	96	3.3 to 5.0	0	.....	..	....	...	...
Phenylalanine	Ac	163	4.98	18	194–195	27	28.6	9.92	9.89
Phenylalanine	bz	48	5.17	90	221–222[p]	31	27	...	...
Phenylalanine	Cbzo	20	5.88	..	157–158.5	25	14	7.48	7.38
Tyrosine	bz	18	5.39	74	208–209[q]	28	21.2	7.77	7.77
Methionine	bz	48	5.70	93	161–161.6[r]	28	−25.1	...	...

[a] Benzoyl. [b] Uncor., m.p. 212.5°, ref. 1. [c] Uncor., m.p. 175–176°, ref. 1. [d] All rotations are for 5% in pyridine, except ornithine which is 5% in acetic acid. [e] All m.p.'s are corrected unless otherwise specified. [f] Carbobenzoxy. [g] Uncor. [h] Acetyl. [i] M.p. 220–221°, ref. 4b. [j] Benzenesulfonyl. [k] M.p. 213°, ref. 1. [l] Yield of crude anilide. [m] Difficulty was experienced in analyzing lysine derivatives for nitrogen, especially by Kjeldahl. [n] Dumas values of 9.76 and 9.33% N were obtained. These results are undoubtedly due to analytical difficulties rather than an impure material. [p] M.p. 219–220°; rotation 27.6°, ref. 1. [q] M.p. 208–208.5°, ref. 4a. [r] Some samples melted at 176–177.5°, but the rotation was the same. The reported m.p. is 159°, ref. 3.

benzoyl-DL-leucine had $[\alpha]^{24}D$ of 7.6 (5% in glacial acetic acid) whereas the benzoyl-L-anilide prepared from a commercial sample of L-leucine had $[\alpha]^{26}D$ of 8.6.[5] The influence of the acyl group in determining the stereochemical specificity of phenylhydrazide formation in the presence of papain has been shown by several groups of workers.[6]

Of the amino acids investigated, the only one whose derivative would not react was threonine. Benzoylallothreonine gave a jelly-like precipitate difficult to purify.[7] That the precipitate was the anilide was indicated by hydrolysis of the residual benzoylallothreonine to give an active allothreonine. It is interesting to note that N-benzoyl-O-methylthreonine reacted fairly rapidly to give a nicely crystalline product.

Certain failures seem worthy of note. Glycyl-DL-norleucine did not react in 9 days at *p*H 4.50 (a diketopiperazine was expected); 2-benzamido ornithine gave no product in seven days at *p*H 4.32 (a piperidone was expected) and 2-benzamido-γ-butyrolactone gave no product in seven days at *p*H 5.23 (benzoyl-1-homoserine anilide was expected). In addition, acetamidocyanoacetic acid (*p*H 4.75) and 2-acetamido-4-cyanobutyric acid (*p*H 5.15) failed to give anilides after several days at 38°. N-Methylaniline, benzylamine, butylamine and aminomalonic ester did not react with hippuric acid but *o*-toluidine and *m*-chloraniline did react. These results are in general agreement with the recent extensive studies on amines made by Waldschmidt-Leitz and Kuhn.[8]

The unreacted D-plus DL-acylamino acids were conveniently racemized by the method of du Vigneaud and Meyer.[9]

Experimental

2-Acetyl-6-benzoyllysine was prepared by acetylation of 6-benzoyllysine; yield 91%; m.p. 143–145°.

Anal. Calcd. for $C_{15}H_{20}N_2O_4$: N, 9.58; neut. equiv., 292. Found: N (D), 9.49; N (K), 6.98, 7.62; N (semimicro Kjeldahl), 9.28; neut. equiv., 292 (*cf.* footnote 1, Table II).

2-Amino-5-chloro-4-pentenoic acid (ω-chloroallylglycine)[10] was prepared in 61% yield by acid hydrolysis of di-

(5) The literature (ref. 1) reports $[\alpha]^{22}D$ 9.0 (5% in glacial acetic acid) with papain as the enzyme. Other enzymes gave values of 8.8 and 9.2.

(6) (a) F. Bennett and C. Niemann, THIS JOURNAL, **70**, 2611 (1948); (b) *ibid.*, **72**, 1798 (1950); (c) H. Milne and C. Stevens, *ibid.*, **72**, 1742 (1950).

(7) The only other anilide which was not originally precipitated in a state of high purity was 2-acetyl-6-benzoyllysine anilide.

(8) E. Waldschmidt-Leitz and K. Kuhn, *Z. physiol. Chem.*, **285**, 23 (1950).

(9) V. du Vigneaud and C. Meyer, *J. Biol. Chem.*, **98**, 295 (1932).

(10) J. Fillman and N. Albertson, THIS JOURNAL, **70**, 171 (1948).

ethyl (3-chloroallyl)-acetamidomalonate. The latter compound was prepared in the usual manner[11] from 1,3-dichloropropene and acetamidomalonic ester; yield 60%; b.p. 135–144° at 1.5 mm.; $n^{25}D$ 1.4728.

Anal. Calcd. for $C_{12}H_{18}ClNO_5$: N, 4.80. Found: N, 4.84.

Ethyl *s*-butylacetamidocyanoacetate was prepared in 62 to 71% yield by condensation of *s*-butyl bromide with acetamidocyanoacetic ester. A sample recrystallized from ethanol melted at 116.5–119.5°.

Anal. Calcd. for $C_{11}H_{18}N_2O_3$: N, 12.38. Found: N, 12.20.

Hydrolysis of the crude condensation product gave a 46% yield of DL-isoleucine. This amino acid was also prepared in 14.5% over-all yield from acetamidomalonic ester. Resolution of the formyl derivative according to the method of Locquin[12] gave formyl-L-isoleucine, m.p. 154.5°, thus confirming the identity of the amino acid.

Papain.—In all of the experiments described, papain from a single batch from the American Ferment Company, Inc., was used. It had stood for about a year at room temperature prior to use. In a few experiments using an experimental papain prepared from frozen fresh papaya latex, yields slightly higher than those recorded here were obtained in the same period of time.

Buffer solutions were prepared from disodium phosphate and citric acid according to the procedure of McIlvaine[13] except only one-tenth as much water was used.

Anilides were prepared by the procedure of Fruton[3] with minor modifications as indicated in the following representative example.

Benzoyl-L-norvaline Anilide.—In an erlenmeyer flask was placed 12.8 g. of benzoyl-DL-norvaline, 58 ml. of normal sodium hydroxide, 10 ml. of aniline, 0.7 g. of cysteine hydrochloride in 20 ml. of water, 70 ml. of buffer (*p*H 4.23) and 300 ml. of distilled water. The enzyme solution, prepared by extracting 3.6 g. of papain with 50 ml. of water, was then added. The *p*H was brought to 5.04 by the addition of about 1.5 g. of citric acid and the flask was placed in an oven at 38° for 19 hours. The anilide was filtered, washed with water and recrystallized from methanol with practically no loss. (See Table II for data.) From the original filtrate, 5.2 g. of crude benzoyl derivative was obtained (81% recovery). This material had $[\alpha]^{32}D$ −25° (1% in one equivalent of sodium hydroxide).

The above stoichiometric ratios were maintained for other acylamino acids. Recovery of the crude benzoyl derivatives usually approached quantitative yields if the concentrated filtrates were extracted with ethyl acetate after acidification.

Hippuryl-*o*-toluidide was prepared in the same manner as the above using hippuric acid and commercial *o*-toluidine. At *p*H 4.32 a 20% yield was obtained in 18 hr.; m.p. 196.6–197.2° cor.[14]

Anal. Calcd. for $C_{16}H_{16}N_2O_2$: N, 10.44. Found: N, 10.45.

Hippuryl-*m*-chloroanilide was obtained in 47% yield in 18 hours. The product, from methanol, melted at 199.6–200.4° cor.

Anal. Calcd. for $C_{15}H_{13}ClN_2O_2$: N, 9.71. Found: N, 9.72.

Racemization of Benzoyl-D-alanine.—To a solution of 23.9 g. of recovered benzoyl D-(and DL)-alanine, m.p. 130–132° ($[\alpha]^{27}D$ −26.6° (1% in 1 equiv. of sodium hydroxide)) in 62 ml. of 2 *N* NaOH and 75 ml. of water was added 124 ml. of acetic anhydride and the solution placed in an oven at 38° for 2 days. It was then concentrated *in vacuo*, dissolved in water and acidified with hydrochloric acid to give 19.3 g., m.p. 155–157° (81% crude yield). It had zero rotation. In similar manner there was racemized acetyl-D-tryptophan (70%), benzoyl-D-norleucine (78%), benzoyl-D-leucine (94%), acetyl-D-decanoic acid (82%) and benzoyl-D-ω-chloroallylglycine (68%).

Partial Racemization of Benzoyl-L-leucine Anilide.—To a solution of 1 g. of sodium in 100 ml. of ethanol was added 3.1 g. of benzoyl-l-leucine anilide (m.p. 215–216°; $[\alpha]^{24}D$ 7.6 (5% in glacial acetic acid)). The solution was allowed to stand for 22 hours at 40°, acidified to congo red with alcoholic hydrogen chloride, and the product isolated. It was no longer soluble to 5% in acetic acid; m.p. 194.8–195.8° cor.; $[\alpha]^{28}D$ 1.0° (2% in acetic acid).

Anal. Calcd. for $C_{19}H_{22}N_2O_2$: N, 9.03. Found: N, 8.96.

Ethyl 2-Acetamido-4-cyanobutyrate.—To a solution of 135 g. of acetamidoacetoacetic ester[15] and 50 ml. of acrylonitrile in 300 ml. of ethanol was added a solution of sodium ethylate until the solution was alkaline to litmus. The temperature slowly rose to 40°. Additional quantities of sodium ethylate were added, as needed, to keep the solution basic. After the temperature started to fall the solution was allowed to stand at least 2 hours and was then made acid to congo paper with alcoholic hydrogen chloride. The product, isolated by distillation, was obtained in yields of 58 to 62%; b.p. 150–152° (0.8 mm.); m.p. 64.2–65.0° cor.

Anal. Calcd. for $C_7H_{14}N_2O_3$: N, 14.14. Found: N, 14.10.

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(15) N. Albertson, B. Tullar, J. King, B. Fishburn and S. Archer, THIS JOURNAL, **70**, 1150 (1948).