[CONTRIBUTION FROM THE NEW YORK STATE AGRICULTURAL EXPERIMENT STATION, CORNELL UNIVERSITY]

A New Stepwise Degradation of Peptides[1]

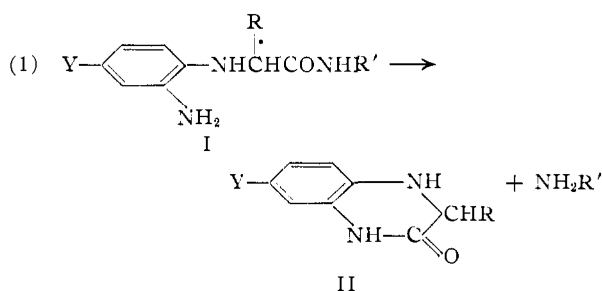
BY ROBERT W. HOLLEY AND ANN D. HOLLEY

RECEIVED JUNE 16, 1952

A stepwise degradation of peptides has been developed in which the N-(4-carbomethoxy-2-nitrophenyl)-peptide (V) is prepared and reduced, and the N-(2-amino-4-carbomethoxyphenyl)-peptide (VI) undergoes lactam formation with removal of the terminal amino acid as a 7-carbomethoxy-3,4-dihydro-2(1H)-quinoxalone (VII). Glycylglycylglycine, glycyl-L-alanyl-L-leucine and L-phenylalanyl-L-leucine have been degraded with an average yield of 84% per amino acid residue.

One of the most difficult problems in the elucidation of the structure of naturally occurring polypeptides is the determination of the sequence of the constituent amino acids. One approach to this problem is stepwise degradation, in which each amino acid is removed and identified as one proceeds along the peptide chain. Procedures of this general type have been described in the literature by Schlack and Kumpf,[2] Abderhalden and Brockmann,[3] Bergmann and Zervas,[4] Edman,[5] Levy[6] and Khorana.[7]

The present paper describes a new stepwise degradation which makes use of the type of reaction shown in equation (1).[8] That this type of reac-



tion might be general and might occur under mild conditions was suggested by the fact that *o*-aminophenylglycine lactamizes so readily that the free amino acid has never been obtained,[9,10] and by the successful removal of the N-chloroacetyl group from N-chloroacetylpeptides by reaction with *o*-phenylenediamine.[10] The latter reaction presumably proceeds by way of I (R = H, Y = H). If lactamization, as in equation (1), is general, conversion of a peptide by some means into an N-*o*-aminophenyl derivative followed by lactamization would afford a means of removing the terminal amino acid from the peptide. This process could then be repeated as a stepwise degradation.

Of the possible methods for the conversion of a peptide to an N-*o*-aminophenyl derivative, such as I, the reduction of the corresponding N-*o*-nitrophenylpeptide seemed most promising. This compound might be prepared by reaction of the peptide with an *o*-nitrofluorobenzene. The particular fluorobenzene chosen for the present work was 4-carbomethoxy-2-nitrofluorobenzene (III). The preparation of N-2,4-dinitrophenylpeptides, using 2,4-dinitrofluorobenzene, is well known.[11] Preliminary investigation indicated, however, that the 2,4-dinitrophenyl derivatives would not be satisfactory in the present work because the reduction products of these derivatives were very susceptible to oxidation in air. The use of 2-nitrofluorobenzene in place of 2,4-dinitrofluorobenzene was not possible because it is insufficiently reactive. 4-Carbomethoxy-2-nitrofluorobenzene (III) although not as reactive as 2,4-dinitrofluorobenzene, gives practically quantitative yields of the derivatives, V, in 24 hours at 35°. Catalytic reduction of the sodium salts of these derivatives proceeds smoothly and quantitatively in aqueous solution, using Adams catalyst, and affords, after neutralization, the N-(2-amino-4-carbomethoxyphenyl)-peptides (VI). These compounds, VI, have the desired N-*o*-aminophenylpeptide type structure (I, Y = $COOCH_3$), and, if the reaction shown in equation (1) is general, these compounds should undergo lactam formation. This was found to be the case. In fact, lactam formation takes place so readily that these compounds (VI) are not stable as the free acids in aqueous solution.[12]

In the degradation procedure, the aqueous reduction mixture is filtered to remove the catalyst, and the sodium salt of VI is neutralized with an equivalent of acid. Lactam formation, that is, formation of the 7-carbomethoxy-3,4-dihydro-2(1H)-quinoxalone (VII) takes place in the aqueous solution, and is complete in 5 hours at room temperature or in 15 minutes at 70°. (The higher temperature is used if necessary to keep the derivative, VI, in solution.) The dihydroquinoxalone, VII, crystallizes from the solution, and is collected by filtration. The filtrate is evaporated to dryness, and the residue, which consists of the peptide or amino acid, VIII, plus sodium chloride, is treated again with 4-carbomethoxy-2-nitrofluorobenzene and the above series of reactions repeated. The degradation procedure is summarized in the equations.

In order to avoid carrying over unreacted peptide from one step to the next and to avoid the accumulation of inorganic salts and by-products as the

(1) Journal Paper No. 899, New York State Agricultural Experiment Station.

(2) P. Schlack and W. Kumpf, *Z. physiol. Chem.*, **154**, 125 (1926).

(3) E. Abderhalden and H. Brockmann, *Biochem. Z.*, **225**, 386 (1930).

(4) M. Bergmann and L. Zervas, *J. Biol. Chem.*, **113**, 341 (1936).

(5) P. Edman, *Acta Chem. Scand.*, **4**, 283 (1950). A modification of this procedure has been published by H. Fraenkel-Conrat and J. Fraenkel-Conrat, *ibid.*, **5**, 1409 (1951).

(6) A. L. Levy, *J. Chem. Soc.*, 404 (1950).

(7) H. G. Khorana, *Chemistry & Industry*, 129 (1951).

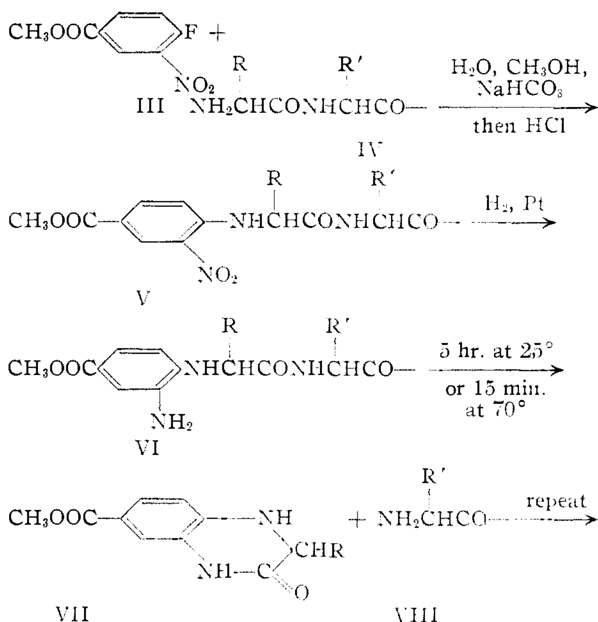
(8) A preliminary account of this work appeared in THIS JOURNAL, **74**, 1110 (1952).

(9) J. Plöchl, *Ber.*, **19**, 6 (1886).

(10) R. W. Holley and A. D. Holley, THIS JOURNAL, **74**, 3069 (1952).

(11) F. Sanger, *Biochem. J.*, **39**, 507 (1945).

(12) No study has been made of the effect of the nature of the substituent Y, or the presence of other substituents, on the ease of lactam formation from I. The choice of Y = $COOCH_3$ may not be the best from the standpoint of stepwise degradation of a peptide.



degradation proceeds, the N-(4-carbomethoxy-2-nitrophenyl)-peptides (V) are isolated at each step.

The dihydroquinoxalones, VII, are nicely crystalline compounds, and are identified by comparison with authentic samples prepared from the amino acids. The properties of the dihydroquinoxalones, VII, prepared from a number of L-amino acids are given in Table I.

TABLE I[16]

7-CARBOMETHOXY-3,4-DIHYDRO-2(1H)-QUINOXALONES (VII)

Prepared from	Vol. methanol for recryst. (ml./100 mg.)	M.p.,[a] °C.	$[\alpha]^{25}D$ (c 1, methanol)	Nitrogen, % Calcd.	Nitrogen, % Found[b]
Glycine	50	232–254[c]	...	13.59	13.31
L-Alanine	3	216–218	+18°	12.72	13.15
L-Valine	2	207–210	+58	11.29	11.25
L-Leucine	2	212–215	+20	10.68	10.50
L-Isoleucine	5	225–227	+52	10.68	10.60
L-Phenylalanine	3	198–201[d]	−99	9.46	9.79
L-Proline	4	182–192[e]	−270[f]	11.38	11.20
Hydroxy-L-proline	6	227–235	−270[g]	10.68	10.45
L-Threonine	(3 ml. H_2O)	85–87[h]	+24	11.20	11.05
L-Aspartic acid	4	209–214	−88[g]	10.60	10.30[i]
L-Glutamic acid	2	170–175[j]	−10	10.07	10.05[k]
L-Asparagine	(40 + 8 ml. H_2O)	288–291	...[l]	15.96	15.75
L-Glutamine	12	232–236[e]	−10[g]	15.15	14.75

[a] All pairs of dihydroquinoxalones with melting points within 10° of each other gave mixed melting points with at least 15° depression below the lower of the two melting points, with the exception that the L-valine and L-leucine dihydroquinoxalones gave a mixed m.p. 204–214°. [b] Analyses by Dr. G. Weiler and Dr. F. B. Strauss, Oxford, England. All samples were dried before analysis. [c] The once-recrystallized derivative of glycine melts at approximately 275–280°. [d] Transition 190–192°. [e] Dec. [f] c 1 or 0.5. [g] c 0.5. [h] Partially solidifies again and melts at 180–185°. [i] Neut. equiv. 267 (calcd. 264). [j] Usually observed as a transition with m.p. 265–270°. [k] Neut. equiv. 284 (calcd. 278). [l] Too insoluble for measurement.

Glycylglycylglycine, glycyl-L-alanyl-L-leucine, and L-phenylalanyl-L-leucine have been degraded using this method. In these experiments, 0.25-millimole quantities of the peptides were degraded in order to have sufficient material to be able to determine the optical rotations of the dihydroquinoxalones. The average yield per amino acid residue was 84%.

Stepwise degradation, if it is to compete with partial hydrolysis as a practical method for the elucidation of the structure of a polypeptide, must be applicable to more than the first few amino acids in a polypeptide. The yields at each step of the degradation must be high if the starting material is to suffice for many steps. In the present instance, the average yield per amino acid residue obtained with simple peptides would permit repetition for ten amino acid residues with an over-all yield of 17%, and the repetition for twenty amino acids with an over-all yield of 3%.

This stepwise degradation, because of the types of reactions involved, cannot be applied to polypeptides which do not have a free α-amino group, and the degradation can be used to degrade only α-amino acid units. In addition to these general limitations, which are inherent in the approach, this degradation may not be applicable to peptides containing all amino acids. It is believed that none of the amino acids listed in Table I will cause difficulty, although only peptides containing the simplest amino acids have been degraded thus far. Some modification of the method will be required if the peptides to be degraded contain cysteine, cystine or methionine. In these instances it may be possible to substitute a chemical reduction for the catalytic reduction or, perhaps better, it may be possible to desulfurize the peptide with Raney nickel[13] or oxidize it with performic acid[14,15] before degradation, and retain catalytic reduction. The choice of procedure will probably depend on the peptide which is being degraded. The degradation of polypeptides containing histidine, lysine, ornithine and tyrosine may involve other problems since there may be side reactions involving the extra 2-amino-4-carbomethoxyphenyl groups substituted on the side chains of these amino acids. Thus far no satisfactory method has been found for recrystallization of the dihydroquinoxalone prepared from L-tryptophan, and no method has been found for the isolation from aqueous solution of the dihydroquinoxalone prepared from L-arginine. All of these problems remain to be investigated.

Experimental[16]

Stepwise Peptide Degradation. General Procedure.—A mixture of 0.25 millimole of peptide, 44 mg. (0.52 millimole) of sodium bicarbonate, and 100 mg. (0.50 millimole) of 4-carbomethoxy-2-nitrofluorobenzene (methyl 4-fluoro-3-nitrobenzoate)[17] was dissolved in 0.5 ml. of water and 1.5 ml. of methanol, and the solution was heated at 35° for 24 hours. The solution was cooled, and was evaporated to dryness *in vacuo* at room temperature. The residue was dissolved in 2 ml. of water and 3 ml. of ether, and the aqueous solution was extracted repeatedly with ether. The aqueous solution was acidified with 0.4 ml. of 1 *N* hydrochloric acid and

(13) *Cf.* R. A. Turner, J. G. Pierce and V. du Vigneaud, *J. Biol. Chem.*, **193**, 359 (1951).

(14) *Cf.* F. Sanger, *Nature*, **160**, 295 (1947); J. M. Mueller, J. G. Pierce, H. Davoll and V. du Vigneaud, *J. Biol. Chem.*, **191**, 309 (1951).

(15) Catalytic reduction proceeds satisfactorily in the presence of cysteic acid. *Cf.* E. B. Maxted, *J. Chem. Soc.*, 1987 (1949).

(16) All melting points were determined on a microscope hot-stage and are corrected. The amino acids were purchased from Nutritional Biochemicals Corp., Cleveland. The peptides are described in ref. 10.

(17) L. S. Fosdick and A. F. Dodds, THIS JOURNAL, **65**, 2305 (1943). This compound should be handled with care since contact with the skin may result in a severe dermatitis.

cooled. The N-(4-carbomethoxy-2-nitrophenyl)-peptide (V) was collected by filtration, or, if not crystalline, it was extracted with ether or ethyl acetate, and the solution was washed with water, dried over magnesium sulfate or sodium sulfate and evaporated to dryness.

The N-(4-carbomethoxy-2-nitrophenyl)-peptide was dissolved in a solution of 24 mg. (0.29 millimole) of sodium bicarbonate in 4 ml. of water, 20 mg. of platinum oxide[18] was added, and the compound was reduced in a micro hydrogenator. The theoretical amount of hydrogen was absorbed in 30 to 40 minutes. After the catalyst was removed by filtration and washed with a little water, the sodium salts in the aqueous solution were neutralized with 0.29 ml. of 1.00 *N* hydrochloric acid. If the N-(2-amino-4-carbomethoxyphenyl)-peptide (VI) remained in solution, the solution was allowed to stand at room temperature; the dihydroquinoxalone, VII, began to crystallize within a few minutes, and the reaction was complete within a few hours. If VI precipitated from the aqueous solution at room temperature it was redissolved by warming the solution to 70° for a few minutes; the dihydroquinoxalone, VII, soon precipitated.

The dihydroquinoxalone, VII, was collected by filtration, recrystallized (for solvent see Table I), and identified by comparison with samples of the dihydroquinoxalones prepared from the amino acids. The filtrate from the dihydroquinoxalone was evaporated to dryness, and the residue, consisting of peptide or amino acid, VIII, was subjected to treatment with 4-carbomethoxy-2-nitrofluorobenzene in the next step of the degradation.

A. **Degradation of Glycylglycylglycine.**—2-Amino-4-carbomethoxyphenylglycylglycylglycine and 2-amino-4-carbomethoxyphenylglycylglycine were soluble in water at room temperature, so dihydroquinoxalone formation was allowed to take place at room temperature. The yields of the crude dihydroquinoxalone (VII, R = H) were: 43 mg. (84% for the first step), m.p. 248–254°; 35 mg. (81% for second step[19]), m.p. 256–260°; 27 mg. (76% for third step[19]), m.p. 247–253°. Recrystallization from methanol gave m.p. 265–271°, 276–284° and 276–284°, respectively, all undepressed when mixed with authentic dihydroquinoxalone from glycine. The 4-carbomethoxy-2-nitrophenyl derivatives of glycylglycine and glycine, obtained as intermediates in the degradation, were shown by m.p. and mixed m.p. to be identical with the derivatives prepared from glycylglycine and glycine.

B. **Degradation of Glycyl-L-alanyl-L-leucine.**—2-Amino-4-carbomethoxyphenylglycyl-L-alanyl-L-leucine was not completely soluble at room temperature so the mixture was heated at 50° for 5 minutes to dissolve the precipitate and initiate dihydroquinoxalone formation. The mixture was then left at 25° overnight. The yield of crude dihydroquinoxalone (VII, R = H) was 49 mg. (95% for first step), m.p. 245–255°. Recrystallization from methanol gave 28 mg. m.p. 278–285°, mixed m.p. with authentic dihydroquinoxalone from glycine undepressed. 4-Carbomethoxy-2-nitrophenyl-L-alanyl-L-leucine was obtained as an oil, which was extracted with ethyl acetate. Dihydroquinoxalone formation from the 2-amino-4-carbomethoxyphenyl-L-alanyl-L-leucine was carried out at 70° for 5 minutes, and the mixture was left overnight at 25°. The yield of crude dihydroquinoxalone (VII, R = CH_3) was 40 mg. (77% for second step[19]), m.p. 195–210°. Recrystallization from 1 ml. of methanol gave 31 mg., m.p. 216–218°; $[\alpha]^{27}D$ +18° (*c* 1.1, methanol); mixed m.p. with authentic dihydroquinoxalone from L-alanine undepressed; mixed m.p. with authentic dihydroquinoxalone from L-leucine 185–210°. The 4-carbomethoxy-2-nitrophenyl-L-leucine was obtained as an oil, which was extracted with ether. The yield of crude dihydroquinoxalone (VII, R = $(CH_3)_2CHCH_2$) was 49 mg. (102% for third step[19]), m.p. 202–215°. Recrystallization from 1 ml. of methanol gave 37 mg., m.p. 212–215°; $[\alpha]^{27}D$ +19° (*c* 1.1, methanol); mixed m.p. with authentic dihydroquinoxalone from L-leucine undepressed; mixed m.p. with authentic dihydroquinoxalone from L-alanine 187–205°.

C. **Degradation of L-Phenylalanyl-L-leucine.**—Dihydroquinoxalone formation from 2-amino-4-carbomethoxyphenyl-L-phenylalanyl-L-leucine was carried out in 2.5 ml. of 50% methanol heated at 70° for 15 minutes. The mixture was cooled, 2 ml. of water was added and the dihydroquinoxalone was collected by filtration. The yield of crude dihydroquinoxalone (VII, R = $C_6H_5CH_2$) was 57 mg. (77% for first step), m.p. 189–199°. Recrystallization from 1.5 ml. of methanol gave 36 mg., m.p. 196–199° (transition 190–193°); $[\alpha]^{23}D$ −99° (*c* 0.5, methanol) mixed m.p. with authentic dihydroquinoxalone from L-phenylalanine undepressed. In the second step, the yield of crude dihydroquinoxalone (VII, R = $(CH_3)_2CHCH_2$) was 39 mg. (77%),[19] m.p. 202–212°. Recrystallization from 0.8 ml. of methanol gave 29 mg., m.p. 213–215°, $[\alpha]^{23}D$ +20° (*c* 1, methanol), mixed m.p. with authentic dihydroquinoxalone from L-leucine undepressed.

N-(4-Carbomethoxy-2-nitrophenyl)-amino Acids.—These were prepared from the amino acids using the same procedure used for the preparation of derivatives of peptides, V. It was found convenient when working with amounts larger than 0.25 millimole to first dissolve the amino acid and sodium bicarbonate in the water. Part of the methanol was added to this solution, and the 4-carbomethoxy-2-nitrofluorobenzene was added in the remainder of the methanol. Usually, if the solutions are warm when mixed, everything remains in solution. The characteristic yellow-orange color of the derivative begins to develop as soon as the reactants are mixed. The reaction mixture was heated at 35° for 24 hours. No indication has been found of saponification of the carbomethoxy group under these conditions. After 24 hours reaction, the excess 4-carbomethoxy-2-nitrofluorobenzene can be recovered unchanged. The yields were 96–100%. The properties of the N-(4-carbomethoxy-2-nitrophenyl)-amino acids are summarized in Table II.

TABLE II[16]

N-(4-CARBOMETHOXY-2-NITROPHENYL)-AMINO ACIDS AND PEPTIDES

Prepared from[a]	Solvent[b] for recryst. (ml./100 mg.)	M.p., °C.	$[\alpha]^{23}D$ (*c* 1, methanol)	Nitrogen, % Calcd.	Found[c]
Glycine	M, 1	203–204	.....	11.02	10.90
L-Alanine	M–W, 0.6–1.5	125–128[d]	+29°[e]	10.44	10.55
L-Valine	B–H, 1–1	128–130	−7	9.46	9.34
L-Isoleucine	B–H, 1–1	122–125	−23	9.03	8.77
L-Proline	M–W, 0.6–0.6	138–141	−690[f]	9.52	9.33
Hydroxy-L-proline	M–W, 0.5–1	171–174	−1140[g]	9.03	8.95
L-Threonine	E–H, 4–4	130–132[h]	+22	9.39	9.26
L-Asparagine	M–W, 2–2	177–180	−12	12.76[i]	12.70
L-Glutamine[j]	M–B–H, 2–4–2	128–136	−90	11.76[k]	11.50
L-Arginine	M–W, 7–7	262–265	....[l]	19.82	19.75
Glycylglycine	M–W, 2–2	211–213	.....	13.50	13.37
Glycylglycylglycine	M–W, 3–3	216–218	.....	15.21	15.08
Glycyl-L-alanyl-L-leucine	M–W, 1.5–1.5	133–139	....[l]	12.78	12.80
L-Phenyl-alanyl-L-leucine	M–W, 0.6–0.4	160–162	....[l]	9.19	9.49

[a] The derivatives of glycine, L-alanine, L-asparagine, L-glutamine, and the four peptides were obtained crystalline when the aqueous solution from the reaction was acidified. The derivative of L-arginine was obtained crystalline without acidification. The derivatives of L-valine, L-isoleucine, L-proline, hydroxy-L-proline and L-threonine were obtained crystalline after extraction into ether or ethyl acetate and removal of the solvent. The derivatives of L-leucine, L-phenylalanine, L-aspartic acid, L-glutamic acid and L-alanyl-L-leucine have not been obtained crystalline. [b] Abbreviations of solvents: B = benzene, E = ether, H = hexane, M = methanol and W = water. [c] Analyses by Dr. G. Weiler and Dr. F. B. Strauss, Oxford, England. All samples were dried before analysis. [d] Also obtained in low-melting modification, m.p. 51–55°. [e] *c* 2. [f] *c* 0.25 or 1. [g] *c* 0.5. [h] Also higher melting modification, m.p. 143–145°. [i] Calcd. for $C_{12}H_{13}N_3O_7 \cdot H_2O$. [j] L-Glutamine was purchased from Dougherty Chemicals, Richmond Hill 18, N. Y. [k] Calcd. for $C_{13}H_{15}N_3O_7 \cdot CH_3OH$. [l] Not determined.

(Reaction of *o*-nitrofluorobenzene with glycine in a similar manner for 47 hours at 25° gave a 3% yield of crude N-*o*-nitrophenylglycine. Reaction at reflux temperature for 2 hours, using ethanol instead of methanol, gave a 42% yield.)

(18) Obtained from The American Platinum Works, Newark, N. J.

(19) The yields given for the second and third steps are calculated assuming that the yield of peptide or amino acid, VIII, in the previous step was the same as the yield of dihydroquinoxalone, VII, isolated in that step.

7-Carbomethoxy-3,4-dihydro-2(1H)-quinoxalones. ("Dihydroquinoxalones") **VII.**—A solution of 0.5 millimole of the N-(4-carbomethoxy-2-nitrophenyl)-amino acid and 55 mg. (0.65 millimole) of sodium bicarbonate in 3 ml. of water was placed in a micro hydrogenator with 20 mg. of platinum oxide. The theoretical amount of hydrogen was absorbed in 30 to 60 minutes. (The hydrogenation mixture turns dark brown during the hydrogenation and becomes colorless, or very nearly so, at the end of the hydrogenation.) The catalyst was removed by filtration, using care to avoid unnecessary exposure of the solution to air. The sodium salts were neutralized with 0.65 ml. of 1.00 *N* hydrochloric acid. The 7-carbomethoxy-3,4-dihydro-2(1H)-quinoxalone precipitated immediately, sometimes as an oil, and the mixture was cooled. If the product had precipitated as an oil, it crystallized at once. The product was collected by filtration, washed with water, and dried *in vacuo*, yield 80–95%. It was recrystallized (recovery 50–85%), usually from methanol (see Table I); if necessary it was decolorized by treatment of the methanol solution with charcoal. The properties of these compounds are summarized in Table I.

The 7-carbomethoxy-3,4-dihydro-2(1H)-quinoxalones (VII) exhibit absorption maxima at 320 mμ, log ε 4.20 and 258 mμ, log ε 4.28, and a minimum at 278 mμ, log ε 3.53 (95% ethanol).

(Catalytic reduction of N-2,4-dinitrophenylglycine using the same procedure gave a purplish-black solid, insoluble in ethanol and ether and very soluble in water. Crystallization from water gave a low yield of black crystals which did not melt below 290°.)

GENEVA, NEW YORK

[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF NORTH TEXAS STATE COLLEGE]

Antitubercular Studies. IV. Derivatives of 1-(4-Nitrophenacyl)-4-alkypyridium Bromides

BY PRICE TRUITT, J. R. BUTTRAM AND RAY HERD

RECEIVED JULY 7, 1952

The synthesis and properties of various compounds containing the 1-(4-alkylpyridinium) and 1-(4-alkylpiperidyl) moieties are described. This group of substances are primarily 4-nitrophenacyl and 4-aminophenacyl derivatives. Although none of the compounds reported in this paper exhibit antitubercular activity, a number of them do have interesting antifungal and amebacidal properties.

Previous work in this Laboratory[1,2] indicated that certain compounds containing the 1-(4-alkylpiperidyl) grouping possess some antitubercular activity. This activity was highest with 1-(diphenylmethyl)-4-alkylpiperidines,[1] but these materials were inactive in the presence of serum. The 4-alkyl-1-(2-hydroxyl-2-phenylethyl)-piperidines[2] were active at 10 mg. % in the presence of serum. Thus, it seemed of interest to prepare other structurally related compounds in order to test their physiological properties as growth inhibitors.

The general methods used for these preparations were similar to those reported by Truitt, Hall and and Arnwine[3] for the preparation of 4-hexyl-1-(4-nitrophenacyl)-pyridinium bromide and the corresponding reduction products. References to other pertinent work are also given in this article and in reference (2).

Physiological Activity[4]

None of the compounds of this series appear to have antitubercular activity. *In vitro* tests with other organism were also negative, except that compound no. 16 exhibited moderate activity against *Strep. hemolyticus*.

Compounds no. 5, 10, 16, 17 and 20 were tested for amebacidal activity. Compound no. 10 was least active. Compounds no. 5 and no. 16 were amebacidal at 1:5,000 dilution and amebastatic at 1:50,000 dilution. Compounds no. 17 and no. 20 were amebacidal at 1:50,000 dilution. However, these more active compounds were not promising *in vivo* in rats at 0.5% concentration in the diet.

1-(4-Aminophenacyl)-4-(octyl)-pyridinium bromide (no. 17) gave complete inhibition of the following fungi: *Candida albicans*, 125 mg./ml.; *Trichophyton interdigitale*, 31.25 mg./ml.; *Nocardia asteroides*, 250 ml./ml.; and *Histoplasma capulatum*, 31.25 mg./ml. Compound no. 22 gave complete inhibition of the same fungi, as follows: *Candida albicans*, 125 mg./ml.; *Trichophyton interdigitale*, 31.25 mg./ml.; *Nocardia asteroides*, 62.5 mg./ml.; and *Histoplasma capsulatum*, 15.6 mg./ml.

Experimental

Procedure I. 1-(4-Nitrophenacyl)-4-alkylpyridinium Bromide.—To a solution of 0.10 mole of 4-nitrophenacyl bromide in 100 ml. of acetone was added 0.103 mole of 4-alkylpyridine. A reaction began almost immediately and the formation of an oil or crystals was noted. The reaction mixture was allowed to stand overnight; at the end of this time the oil (if present) had changed to a light yellow crystalline solid.

The crystals were filtered, washed with ether and recrystallized from the appropriate solvent.

The data for this group of quaternary salts are included in Table I.

Procedure II. 1-(4-Acetylaminophenacyl)-4-alkylpyridinium Bromide.—A mixture of 0.0195 mole of 4-acetylaminophenacyl bromide, 200 ml. of toluene and 0.0195 mole of 4-alkylpyridine was refluxed for 30 minutes. The reaction mixture was allowed to cool and 200 ml. of ether was added. The crystalline solid was removed and recrystallized.

These compounds are listed with pertinent information in Table I.

Procedure III. 1-(4-Aminophenacyl)-4-alkylpiperidines and Hydrobromides.—A solution of 0.015 mole of 1-(4-nitrophenacyl)-4-alkylpyridinium bromide in 100 ml. of ethyl alcohol was hydrogenated at room temperature under 50 p.s.i. hydrogen pressure and with 0.1 g. of platinum oxide. About one hour was required for the theoretical amount (0.09 mole) of hydrogen to be absorbed.

The product, which had precipitated, was removed by

(1) Price Truitt and W. J. Middleton, THIS JOURNAL, **73**, 5669 (1951).

(2) Price Truitt, B. Bryant, W. E. Goode and B. C. Arnwine, *ibid.*, **74**, 2179 (1952).

(3) Price Truitt, R. E. Hall and B. C. Arnwine, *ibid.*, **74**, 4552 (1952).

(4) The testings were arranged for by Dr. Loren Long of Parke Davis and Company.