[CONTRIBUTION FROM THE DEPARTMENT OF PHARMACOLOGY AND THE DEPARTMENT OF EXPERIMENTAL PATHOLOGY, MEDICAL SCHOOL, JERUSALEM, ISRAEL]

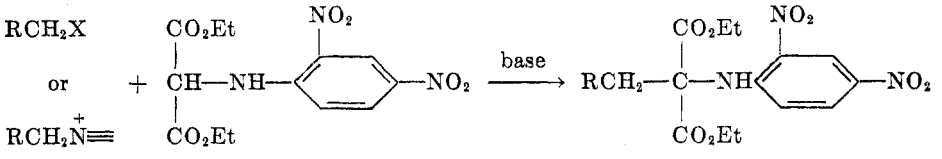
SYNTHESIS OF N-α-(2,4-DINITROPHENYL)-D,L-HISTIDINE

M. HARNIK AND E. MARGOLIASH

Received June 8, 1955

In connection with studies on the structure of cytochrome *c* (1) it became necessary to prepare N-α-(2,4-dinitrophenyl)-D,L-histidine for the purpose of direct comparison on paper chromatograms with dinitrophenylated amino acids (DNP-amino acids) obtained by hydrolysis of cytochrome *c* previously treated with fluoro-2,4-dinitrobenzene. Since Abderhalden and Blumberg (2) isolated a mono-DNP-derivative from a mixture obtained by dinitrophenylation of L-histidine with chloro-2,4-dinitrobenzene but did not specify the structure of this product, it was necessary to synthesize N-α-(2,4-dinitrophenyl)-D,L-histidine (III) by an unequivocal route.

A similar problem of synthesis of mono-DNP-derivatives exists in the case of other natural polyfunctional amino acids. It therefore appeared desirable to develop a more general method of synthesis of similar N-α-substituted D,L-amino acids. For this purpose we prepared diethyl N-(2,4-dinitrophenyl) aminomalonate (I) which might be condensed with alkyl halides or quaternary Mannich bases to yield the substituted malonic esters (II).

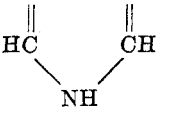


I

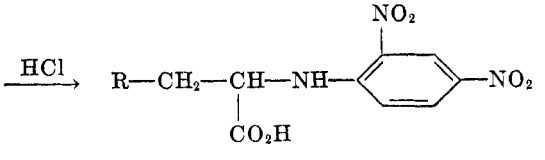
II

X = halogen

IIa: R =



IIb: hydrochloride of IIa



III

In this reaction I would be the analog of acetamino- (3) or formamino- (4) or carbobenzoxyamino- (5) malonic esters, used in D,L-amino acid syntheses. Aminomalonic ester underwent a smooth reaction with fluoro-2,4-dinitrobenzene in the presence of sodium bicarbonate to afford I in 83% yield. Coupling of the sodio derivative of I in absolute ethanol with 4-chloromethylimidazole hydro-

chloride (6), at room temperature, gave a mixture from which the desired product IIa was isolated in 35% yield. The low yield of the product is not surprising in view of the strongly oxidizing nature of aromatic nitro groups in alkaline reaction which destroyed the anion derived from I. Although the correct analytical values for IIa were obtained, paper chromatography showed the presence of some impurities which could be eliminated by conversion of IIa into the hydrochloride (IIb). The optimal time for decarbethoxylation of the pure malonic ester IIb with concentrated hydrochloric acid was about 8 hours as determined by paper chromatography. The isolation of III was best carried out by line paper chromatography with the butanol-formic acid-water mixture of Acher and Crocker (7), elution of the appropriate yellow band from the paper with 30% aqueous acetic acid, and crystallization from water. In this way a 43% yield of III could be separated from the bulk of the impurities. Further purification of the acid by recrystallization was not satisfactory. The compound was also moderately susceptible to decomposition by light. This could be demonstrated by re-chromatographing samples exposed to sunlight, whereupon slow moving reddish spots became visible. The D,L-acid (III was therefore purified by conversion into its methyl ester.

N-α-DNP-D,L-histidine (III) was only very slowly attacked by ethereal or ethereal-methanolic diazomethane. However, by applying the acetyl chloride-methanol method of Hanby, Waley, and Watson (8) the compound was easily esterified and the methyl ester hydrochloride was purified readily.

Comparison by paper chromatography of the D,L-acid (III) as prepared above with the mono-DNP-derivative of L-histidine, prepared according to Abderhalden and Blumberg (2) using both the butanol-formic acid-water solvent of Acher and Crocker (4) and *tert*-amyl alcohol saturated with phthalate buffer, *p*H 6, according to Blackburn and Lowther (9), gave identical R_f values for both compounds. The ultraviolet spectra of these two compounds were also identical, and differed from that of the ring substituted mono-DNP-L-histidine (10) and that of the bis DNP-L-histidine (2). This establishes that the mono-DNP-derivative of Abderhalden and Blumberg (2) is the N-α-substituted histidine. The comparison of the synthetic N-α-DNP-histidine with the presumed DNP-L-histidine obtained from hydrolysates of dinitrophenylated cytochrome *c* will be discussed elsewhere.

EXPERIMENTAL

Analyses by Mrs. Goldstein, Jerusalem, and Drs. Weiler and Strauss, Oxford, England.

N-(2-4-dinitrophenyl)diethyl aminomalonate (I). A solution of 2 g. of fluoro-2,4-dinitrobenzene in 40 ml. of ethanol was added to a solution of 1.75 g. of diethyl aminomalonate and 1 g. of sodium bicarbonate in 20 ml. of water.

The mixture was shaken for 1 hr., then allowed to stand overnight. The crystalline mass was filtered and washed with water. Air-drying afforded 2.82 g. (83%) of yellow needles, m.p. 100.5–102°. A second crop (0.20 g.) of poor quality could be obtained from the filtrate. Two recrystallizations from ethanol raised the m.p. to 102.5–103.5°.

Anal. Calc'd for $C_{13}H_{15}N_3O_8$: C, 45.74; H, 4.43.
Found: C, 45.70; H, 4.46.

Ultraviolet spectrum in ethanol: minima: 243 mμ (ϵ = 8,750); 285 mμ (ϵ = 3,150)
maxima: 260 mμ (ϵ = 9,520); 334 mμ (ϵ = 15,800)

R_f in formic acid-butanol-water (7) = 0.95.

Ethyl 2-(2,4-dinitrophenylamino)-2-carbethoxy-3-imidazolepropionate (II). To a solution of 0.380 g. (0.0164 g.-atom) of sodium in 30 ml. of absolute ethanol was added in one portion 2.796 g. (0.0082 mole) of diethyl N-(2,4-dinitrophenyl) aminomalonate (I) and 1.255 g. (0.0082 mole) of 4-chloromethylimidazole hydrochloride, prepared from fructose or sucrose according to Darby, Lewis, and Totter (6). There was an immediate precipitation of sodium chloride. Next morning 200 ml. of water and 5 ml. of acetic acid were added, and the precipitated oil (3.6 g.) gradually solidified. This could be purified by dissolving in a small quantity of hot ethanol, treating with charcoal, and cooling the filtrate to −20°. The compound crystallized in clusters of rhombic, bright yellow prisms, m.p. 137–138° (dec.); 1.2 g. (35% yield). Recrystallization from xylene or a small amount of ethanol raised the m.p. to 138–139° (dec.). The filtrate yielded an additional 0.6 g., m.p. 127–133°.

Ultraviolet spectrum in ethanol: minima at 245 mμ (ϵ = 8,100); 286 mμ (ϵ = 2,750)
maxima at 256 mμ (ϵ = 8,400); 337 mμ (ϵ = 15,500)

Anal. Calc'd for $C_{17}H_{19}N_5O_8$: C, 48.45; H, 4.54.
Found: C, 48.00; H, 4.77.

Paper chromatography (butanol-formic acid-water) showed the presence of several slow-moving reddish-yellow impurities. The malonic ester derivative was therefore treated with ethanolic hydrogen chloride. Precipitation with ether gave the hydrochloride as bright yellow prisms, m.p. 179–182°. The hydrochloride proved to be uniform on paper; R_f in butanol-formic acid-water = 0.81. Pauly reaction strongly positive.

N-α-(2,4-dinitrophenyl)-D,L-histidine (III). The decarbethoxylation of II with boiling hydrochloric acid in sealed capillaries was followed by paper chromatography. After 8

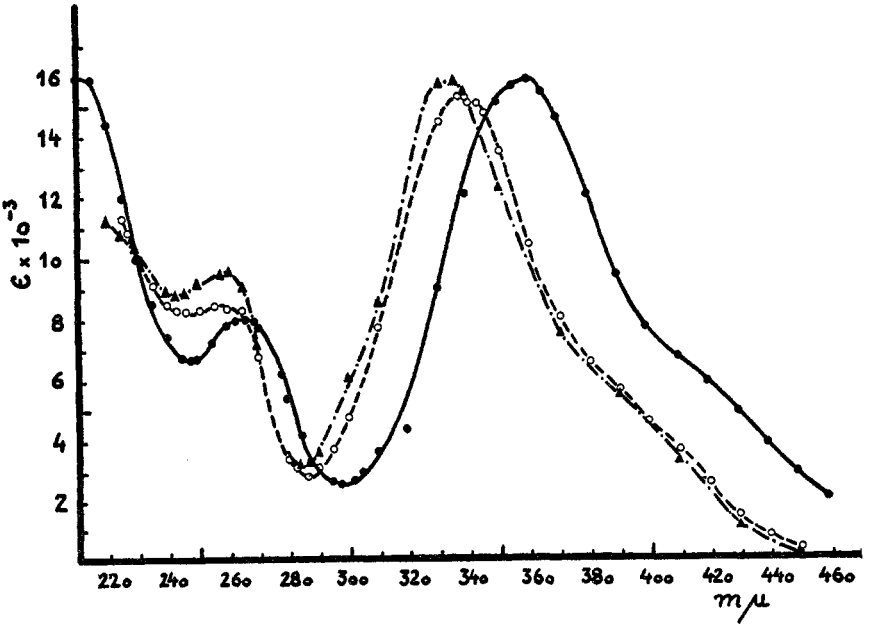


FIG. 1. ULTRAVIOLET SPECTRA: ▲—·—·—▲ N-(2,4-dinitrophenyl)diethyl aminomalonate (I), in 95% ethanol. O- - - - -O Ethyl 2-(2,4-dinitrophenylamino)-2-carbethoxy-3-imidazolepropionate (II), in 95% ethanol. ●————● N-α-(2,4-dinitrophenyl)-D,L-histidine (III), in water.

hours there was almost no starting material left, though after 4 hours' heating less than 20% of the material was still unchanged. The chromatograms showed that complex mixtures were produced. Inspection in ultraviolet light showed several slow moving bands of brilliant green and red fluorescence, while brown and reddish bands were visible in ordinary light. Since the isolation of the required compound from the mixture was otherwise difficult, all the hydrolysate was purified by line chromatography on No. 3 MM Whatman filter paper. In a typical experiment 0.763 g. of the hydrochloride of II was refluxed for 8 hrs. with 3 ml. of concentrated hydrochloric acid. The dark red solution was evaporated to dryness *in vacuo*, and the residue was dissolved in methanol and chromatographed with butanol-formic acid-water on 10 sheets using a line 50 cm. long on each paper. After 18 hours the appropriate bands, $R_f = 0.54$, were cut out and eluted with 30% acetic acid in water in an elution chamber. Evaporation of the solvent and crystallization of the residue from water gave 180 mg. of bright yellow prisms, charring around 250°. The filtrate gave an additional 70 mg. of crystalline material of the same R_f value (total yield: 43%). The substance slowly decomposed when exposed to air as seen by gradual darkening and the appearance of reddish slow-moving bands when chromatographed. Further purification was effected by recrystallization from water, but the results of analysis showed it to be still impure.

Ultraviolet spectrum in water: minima at 247 mμ (ϵ = 6,600); 229 mμ (ϵ = 2,600)
maxima at 265 mμ (ϵ = 8,100); 361 mμ (ϵ = 15,800)

Pauly reaction positive.

The methyl ester hydrochloride of III was prepared by dissolving 157 mg. of the acid in a mixture of 15 ml. of methanol and 5 ml. of acetyl chloride. The following day the solvents were evaporated and the residue was crystallized from methanol, 96 mg., m.p. 195° (dec.)

Anal. Calc'd for $C_{13}H_{14}N_6O_6$: C, 42.01; H, 3.80; N, 18.85.
Found: C, 42.15; H, 4.28; N, 18.70.

R_f in butanol-formic acid-water = 0.62. Pauly reaction positive.

Acknowledgment. The authors wish to thank Prof. F. Bergmann for his interest and help during this work.

SUMMARY

The following compounds have been prepared: diethyl N-(2,4-dinitrophenyl) aminomalonate; ethyl 2-(2,4-dinitrophenylamino)-2-carbethoxy-3-imidazole propionate; N-α-(2,4-dinitrophenyl)-D,L-histidine. It is suggested that the method applied above could be generally employed for the synthesis of N-α-DNP D,L-amino acids, when groups other than the α-amino can undergo dinitrophenylation.

JERUSALEM, ISRAEL

REFERENCES

(1) MARGOLIASH, *Nature*, **175**, 293 (1955).
(2) ABDERHALDEN AND BLUMBERG, *Z. physiol. Chem.*, **65**, 318 (1910).
(3a) SNYDER AND SMITH, *J. Am. Chem. Soc.*, **66**, 350 (1944); (b) ALBERTSON, ARCHER, AND SUTER, *J. Am. Chem. Soc.*, **66**, 500 (1944).
(4) GALAT, *J. Am. Chem. Soc.*, **69**, 965 (1947).
(5) KISSMAN AND WITKOP, *J. Am. Chem. Soc.*, **75**, 1967 (1953).
(6) DARBY, LEWIS, AND TOTTER, *J. Am. Chem. Soc.*, **64**, 463 (1942).
(7) ACHER AND CROCKER, *Biochem. Biophys. Acta*, **9**, 704 (1952).
(8) HANBY, WALEY, AND WATSON, *J. Chem. Soc.*, 3239 (1950).
(9) BLACKBURN AND LOWTHER, *Biochem. J.*, **48**, 289 (1945).
(10) PORTER, *Biochem. J.*, **46**, 304 (1950).