[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF HARVARD UNIVERSITY, DEPARTMENT OF SURGERY OF BETH ISRAEL HOSPITAL AND HARVARD MEDICAL SCHOOL]

Synthesis of Peptides and Esters of Phenylalanine as Chromogenic Substrates for Chymotrypsin[1]

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The synthesis of acetylphenylalanine β-naphthyl ester, phthalylphenylalanine β-naphthyl ester, nicotinylphenylalanyl-β-naphthylamide and nicotinylphenylalanyl-*p*-anisidide are described. Enzymatic hydrolysis with chymotrypsin was observed with the two esters and with the naphthylamide derivative. The naphthol or naphthylamine obtained by enzymatic hydrolysis of the substrates could be converted to azo dyes. Therefore, these substrates appear to possess properties on which to base a method for the colorimetric determination of chymotrypsin.

The structural requirements of substrates susceptible to enzymatic hydrolysis by chymotrypsin have been defined.[2] These are a phenylalanine or tyrosine residue (Ia), a primary peptide or ester link which is subject to hydrolysis by the enzyme (Ib, where R is NH_2 or NHR′ or OR′), and a secondary peptide link (Ic), which is not attacked by the enzyme and consists of an aroyl group (I, R′ = methyl, phenyl or pyridyl), protecting the amino group of phenylalanine. The esters undergo enzymatic hydrolysis with chymotrypsin at least 100 times more rapidly than the peptides.[2]

In order to make possible the development of highly colored azo dyes from the hydrolysis product of the substrate, for the colorimetric determination or histochemical demonstration of enzymatic activity, analogous compounds were prepared which liberate naphthol or naphthylamine on hydrolysis. An analog was also made with *p*-methoxyaniline, which can be converted to a colored pigment with potassium 1,2-naphthoquinone-4-sulfonate. The principles involved were similar to those used earlier in developing methods for a variety of enzymes.[3-10]

On the basis of earlier studies[2] with synthetic substrates, it was expected that N-acetylphenylalanine β-naphthyl ester (II) would be sufficiently specific and readily hydrolyzed to demonstrate chymotryptic activity. It was expected that the peptides, nicotinylphenylalanyl-β-naphthylamide (VI) and nicotinylphenylalanyl-*p*-anisidide (XI) would be even more specific but would be hydrolyzed much more slowly by chymotrypsin. The methiodide derivative (VII) would be more soluble in water, but its susceptibility to enzymatic hydrolysis could not be predicted from available data.[2] The ester, phthalylphenylalanine β-naphthyl ester (VIII), and the urea derivative (XIV) were not expected to be hydrolyzed by chymotrypsin.[2]

Preliminary experiments[11] have shown that at *p*H 7.8 and 37°, homogenates of fresh pancreatic tissue (rat), which contain lipase and esterase, in 2 hours failed to hydrolyze compounds VI, VII, VIII, XI or XIV. Hydrolysis of compound II was moderate. After conversion of chymotrypsinogen to chymotrypsin in these homogenates by the addition of trypsin, and in other experiments with crystalline chymotrypsin,[12] hydrolysis was observed to be extensive with the ester (II), moderate with the peptide (VI) and the ester (VIII), and not at all with the peptide (XI) and the methiodide (VII), or the urea derivative (XIV). Crystalline trypsin, carboxypeptidase and pepsin failed to hydrolyze any of these substrates. Enzymatic hydrolysis was indicated by the appearance of naphthol or the aromatic amines in excess of the small amount due to spontaneous hydrolysis at *p*H 7.8 in control experiments.

N-Acetyl-D,L-phenylalanine β-naphthyl ester (II) was prepared by converting acetylphenylalanine to the acid chloride with oxalyl chloride followed by treatment with naphthol in pyridine. The acid chloride could also be prepared, although in small yield by the treatment of acetylphenylalanine with phosphorus pentachloride and precipitation of the acid chloride by ligroin. Nicotinyl-D,L-phenylalanine-β-naphthylamide (VI) and its methiodide (VII) were readily prepared through the use of the peptide synthesis of Sheehan and Frank.[13] Phthalylphenylalanyl chloride[13] (III) reacted with β-naphthylamine in pyridine to give the amide (IV) and with β-naphthol to give the ester (VIII). The amide (IV) was refluxed with one mole of hydrazine to give phenylalanylnaphthylamide (V) and phthalic acid hydrazide. Since the solubility of V in acid is slight, advantage was taken of the ready solubility of phthalic acid hydrazide in alkali to remove the latter from the reaction product. The free amine (V) could be made to react with the hydrochloride of nicotinic acid chloride[14] in the presence of pyridine, to afford the diamide (VI). This was converted to its methiodide (VII) by reaction with methyl iodide for several days at room temperature. Nicotinyl-

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(2) H. Neurath and G. W. Schwert, *Chem. Revs.*, **46**, 69 (1950).

(3) M. L. Menten, J. Junge and M. H. Green, *J. Biol. Chem.*, **153**, 471 (1944).

(4) L. H. Manheimer and A. M. Seligman, *J. Nat. Cancer Inst.*, **9**, 181 (1948).

(5) M. M. Nachlas and A. M. Seligman, *ibid.*, **9**, 415 (1949).

(6) M. M. Nachlas and A. M. Seligman, *Anat. Record*, **105**, 677 (1949).

(7) M. M. Nachlas and A. M. Seligman, *J. Biol. Chem.*, **181**, 343 (1949).

(8) A. M. Seligman, M. M. Nachlas, L. H. Manheimer, O. M. Friedman and G. Wolf, *Ann. Surg.*, **130**, 333 (1949).

(9) A. M. Seligman and M. M. Nachlas, *J. Clin. Invest.*, **29**, 31 (1950).

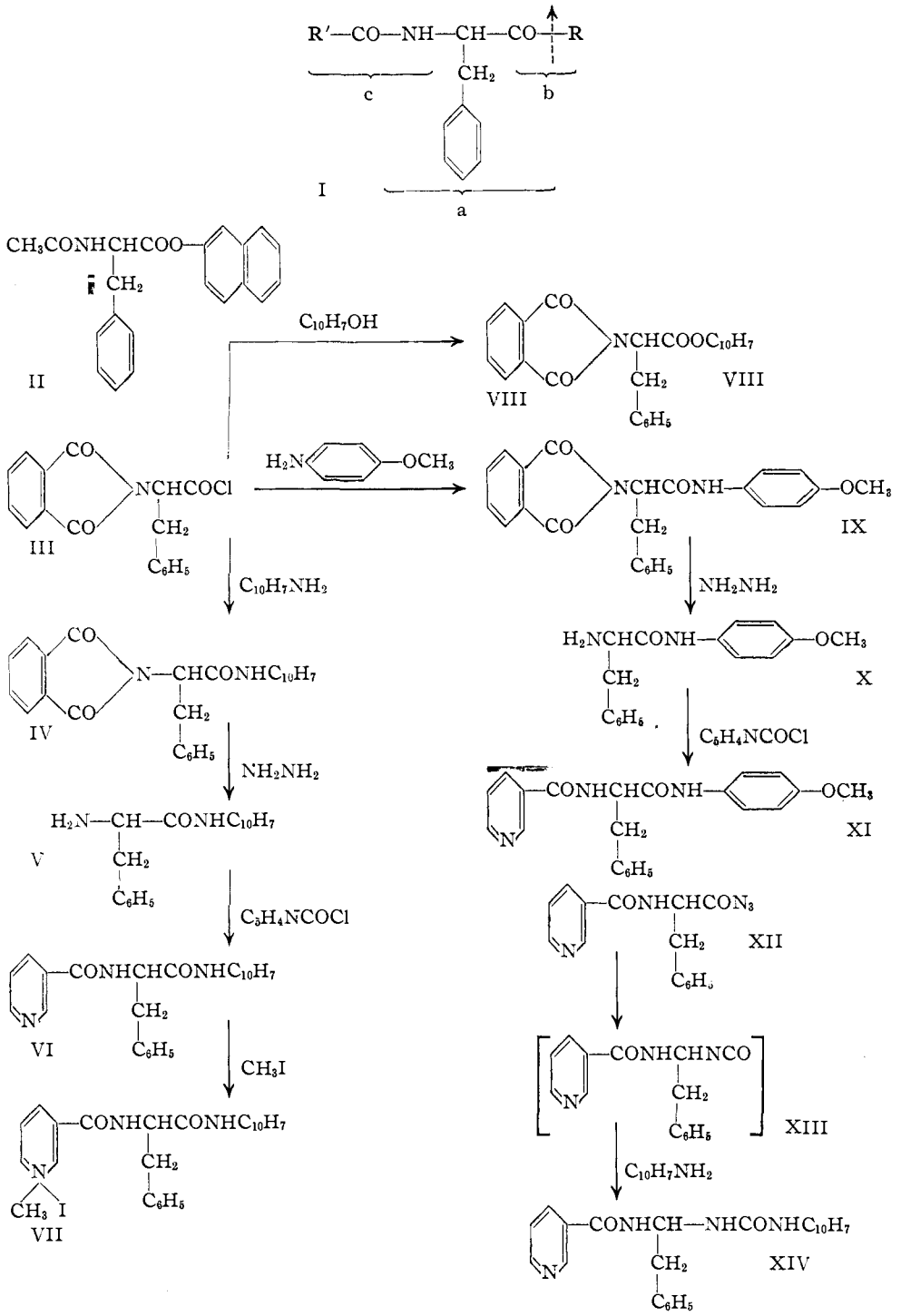
(10) G. Wolf and A. M. Seligman, THIS JOURNAL, **72**, 2080 (1950).

(11) H. A. Ravin and A. M. Seligman, *J. Biol. Chem.*, in press.

(12) Obtained from Syn-Zyme Laboratories, Inc., 78 West 12th Street, New York City.

(13) J. C. Sheehan and V. S. Frank, THIS JOURNAL, **71**, 1856 (1949).

(14) E. Späth and H. Spitzer, *Ber.*, **59**, 1477 (1926).



D,L-phenylalanyl-*p*-anisidide (XI) was prepared in similar manner from the acid chloride (III).

Various unsuccessful attempts were made to prepare nicotinylphenylalanylnaphthylamide (VI) from nicotinylphenylalanine *via* the acid chloride. Nicotinylphenylalanine was obtained in small yield by the action of nicotinic acid chloride upon the sodium salt of phenylalanine in aqueous solution and in good yield by saponification of nicotinylphenylalanine ethyl ester, which was obtained by treatment of phenylalanine ethyl ester with nicotinic acid chloride in pyridine. The methyl ester of nicotinylphenylalanine has been prepared by a slightly different method.[15] However, no recognizable products could be obtained by the action of thionyl chloride or phosphorus pentachloride upon nicotinylphenylalanine under various conditions. Treatment of nicotinylphenylalanine ethyl ester with hydrazine gave the hydrazide, which was readily transformed into the azide (XII). Instead of the expected reaction of the azide with β-naphthylamine to give the amide

(15) B. M. Iselin, H. T. Huang, R. V. MacAllister and C. Niemann, THIS JOURNAL, **72**, 1729 (1950).

(VI), it appeared to undergo a Curtius rearrangement to yield the isocyanate (XIII) which then reacted with naphthylamine, resulting in the formation of N-(benzylnicotinamidomethyl)-N′-β-naphthylurea (XIV).

Experimental[16]

Acetylphenylalanine-β-naphthyl Ester (II).—To a cooled solution of acetylphenylalanine[17] (1 g.) in anhydrous dioxane (5 cc.) was added oxalyl chloride (0.5 cc. or 1.15 moles). The solution was kept at room temperature with exclusion of moisture for 1 hour and then concentrated to about one-half its volume in order to remove any excess oxalyl chloride. To the resulting solution was added a solution of β-naphthol (0.7 g.) and anhydrous pyridine (0.4 cc.) in benzene (10 cc.). The mixture was heated on the steam-bath for 30 minutes, concentrated to a small volume under reduced pressure, taken up in benzene and washed first with dilute acid, then with two portions of *N* sodium hydroxide solution mixed with ice, finally with water until neutral, and dried. The solvent was removed and the product crystallized by solution in a large amount of ether, addition of some petroleum ether, careful evaporation of the ether until the appearance of slight cloudiness and dropwise addition of ether until the cloudiness just disappeared. Upon cooling, crystals of the product separated and more could be obtained if the solution was kept overnight. The ester crystallized from ether–petroleum ether in long, silky, colorless needles, 0.5 g. (31%), m.p. 127°.

Anal. Calcd. for $C_{21}H_{19}O_3N$: C, 75.68; H, 5.75. Found: C, 75.80; H, 5.86.

Acetylphenylalanyl chloride could also be prepared, although in small yield, by the addition of acetylphenylalanine (1 g.) to a solution of phosphorus pentachloride (1 g.) in anhydrous benzene (50 cc.). The solution was kept at room temperature for 1 hour, until all the acetylphenylalanine had gone into solution. The benzene was then removed at room temperature under reduced pressure and ligroin was added to the residue. The precipitated acid chloride was collected by filtration.

Phthalyl DL-phenylalanyl-β-naphthylamide (IV).—To a solution of phthalylphenylalanyl chloride[13] (III) (2 g.) in benzene (20 cc.) was added a solution of β-naphthylamine (0.91 g.) and anhydrous pyridine (0.53 cc.) in benzene (20 cc.). The mixture was heated on the steam-bath for 5 minutes, then cooled in ice. The amide crystallized from the reaction mixture and was collected by filtration. More of the product was obtained by concentrating the filtrate to a small volume. The total yield was 2.4 g. (89%). It crystallized from alcohol in short colorless needles, m.p. 220°.

Anal. Calcd. for $C_{27}H_{20}O_3N_2$: C, 77.03; H, 4.81. Found: C, 76.55; H, 4.82.

Phenylalanyl-β-naphthylamide (V).—Hydrazine (0.23 cc.) was added to a suspension of phthalylphenylalanylnaphthylamide (IV) (3 g.) in 95% alcohol (300 cc.) and the mixture was refluxed for 2 hours. The residual solid, after removal of the solvent under reduced pressure, was digested with *N* sodium hydroxide solution (50 cc.) at 50° for 5 minutes and the mixture was filtered. The filtrate contained the sodium derivative of phthalic acid hydrazide. The residue was dissolved in hot alcohol and crystallized by careful addition of water. It was obtained in colorless flakes, 1.35 g. (65%), m.p. 113°. Although the crystals were sparingly soluble in acid, when dissolved in alcohol and precipitated by addition of water, it could be redissolved by dilute acid.

Anal. Calcd. for $C_{19}H_{18}ON_2$: C, 78.47; H, 6.26. Found: C, 77.99; H, 6.34.

Nicotinyl-DL-phenylalanyl-β-naphthylamide (VI).—Nicotinic acid chloride hydrochloride could be made by the method of Späth and Spitzer.[14] It was found to be simpler, however, to prepare the compound by solution of nicotinic acid (2 g.) in thionyl chloride (25 cc.). The mixture was refluxed on the steam-bath for 30 minutes, concentrated to a small volume and the acid chloride hydrochloride was precipitated by addition of anhydrous ether (80 cc.); yield 2.25 g. (78%).

To a solution of phenylalanylnaphthylamide (V) (2 g.) in anhydrous pyridine (20 cc.) was added a slight excess of nicotinic acid chloride hydrochloride (1.35 g.). The solution was heated on the steam-bath for 15 minutes, the solvent was evaporated under reduced pressure and the residue was dissolved in a large volume of ethyl acetate. This solution was washed first with a small volume of *N* hydrochloric acid, then with dilute sodium carbonate solution and finally with water. The solution was dried and concentrated when nicotinylphenylalanylnaphthylamide crystallized in clusters of colorless needles, 2.0 g. (73%), which could be recrystallized from benzene, m.p. 199°.

Anal. Calcd. for $C_{25}H_{21}O_2N_3$: C, 75.91; H, 5.40. Found: C, 75.66; H, 5.55.

Nicotinyl-DL-phenylalanyl-β-naphthylamide Methiodide (VII).—To a solution of the free base (VI) (2 g.) in benzene (500 cc.) and methanol (10 cc.) was added an excess of methyl iodide (0.6 cc.). This solution was kept at room temperature for 4 days. Crystals of the methiodide appeared and were collected by filtration. The compound, 1.6 g. (62%) crystallized from absolute alcohol in the form of lemon-yellow prisms, m.p. (with decomposition) 173°.

Anal. Calcd. for $C_{26}H_{24}O_2N_3I$: C, 58.10; H, 4.57. Found: C, 57.88; H, 4.73.

Phthalyl-DL-phenylalanine-β-naphthyl Ester (VIII).—To a solution of phthalylphenylalanyl chloride[13] (III) (2 g.) in benzene (20 cc.) was added β-naphthol (0.92 g.) and anhydrous pyridine (0.53 cc.) in benzene (20 cc.). The mixture was refluxed for 1 hour, whereupon a precipitate of pyridine hydrochloride appeared. After cooling, the solution was shaken first with *N* hydrochloric acid, then with two portions of *N* sodium hydroxide solution, and finally with water until neutral. The benzene solution was then concentrated and upon addition of 95% alcohol and cooling, crystals appeared, 1.6 g. (59%), which could be recrystallized from alcohol and were obtained as thick, colorless needles, m.p. 173°.

Anal. Calcd. for $C_{27}H_{19}O_4N$: C, 77.08; H, 4.85. Found: C, 77.67; H, 5.36.

Phthalyl-DL-phenylalanyl-p-anisidide (IX).—To a solution of phthalylphenylalanyl chloride[13] (III) (1.8 g.) in benzene (10 cc.) was added a solution of *p*-anisidine (0.71 g.) and anhydrous pyridine (0.46 cc.) in benzene (10 cc.). The procedure was the same as for the preparation of the naphthylamide (IV). The collected crystals, 2.1 g. (92%), were recrystallized from benzene and appeared in thin, colorless needles, m.p. 182°.

Anal. Calcd. for $C_{24}H_{20}O_4N_2$: C, 71.90; H, 5.14. Found: C, 71.51; H, 5.53.

Nicotinyl-DL-phenylalanyl-p-anisidide (XI).—Phthalylphenylalanylanisidide (IX) (2.6 g.), dissolved in 95% alcohol (200 cc.), was refluxed with hydrazine (0.21 cc.) for 2 hours. The solvent was then removed under reduced pressure, the residue digested at 50° with *N* sodium hydroxide solution, cooled and filtered. The filtrate contained phthalic acid hydrazide. The residue, DL-phenylalanyl-*p*-anisidide (X), was recrystallized from water and obtained in colorless flakes, 1.1 g. (69%), m.p. 76°. To a solution of this compound (1 g.) in anhydrous pyridine (10 cc.) was added powdered nicotinic acid chloride hydrochloride (0.73 g.) (prepared as described above). The mixture was heated on the steam-bath for 30 minutes, and evaporated to dryness under reduced pressure. The residue was treated with cold *N* hydrochloric acid and collected by filtration. The hydrochloride of nicotinyl-DL-phenylalanyl-*p*-anisidide so obtained could be recrystallized from alcohol by the addition of benzene and appeared in the form of colorless prisms, 1.1 g. (68%), m.p. (with decomposition) 215–218°.

Anal. Calcd. for $C_{22}H_{21}O_3N_3 \cdot HCl$: C, 64.11; H, 5.46. Found: C, 63.89; H, 5.98.

Nicotinyl-DL-phenylalanine.—To a solution of phenylalanine ethyl ester (2.4 g.) in chloroform (25 cc.) was added a solution of nicotinic acid chloride hydrochloride (2.3 g.) and anhydrous pyridine (2.0 cc.) in chloroform. The resulting solution was kept at room temperature for 15 minutes, washed with dilute sodium carbonate solution and water. The solvent was then evaporated and the nicotinylphenyl-

(16) Microanalyses by Mrs. Shirley Golden. Melting points uncorrected.

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

alanine ester was saponified by the addition of methanol (75 cc.) and 2 *N* potassium hydroxide solution (12.2 cc.). The methanol was evaporated on the steam-bath and the *p*H of the aqueous residue adjusted to about 6.5 by the addition of dilute acid. A precipitate of nicotinylphenylalanine was obtained which crystallized from water in the form of short, colorless needles, 2.9 g. (86%), m.p. 198°.

Anal. Calcd. for $C_{15}H_{14}O_3N_2$: C, 66.71; H, 5.22. Found: C, 66.63; H, 5.23.

Nicotinylphenylalanine was also prepared by adding powdered nicotinic acid chloride hydrochloride (3.2 g.) to a solution of phenylalanine (2.7 g.) in 2 *N* sodium hydroxide solution (8.23 cc.) and water (100 cc.), slowly and with stirring, simultaneously with a solution of sodium carbonate (4 g.) in water (50 cc.). The mixture was stirred for a further 30 minutes, the *p*H adjusted to about 7 and the solution concentrated to about 20 cc. under reduced pressure. The solid which crystallized on cooling was collected and digested with boiling alcohol and filtered. The residue consisted of phenylalanine. Nicotinylphenylalanine 1.8 g. (43%) crystallized from the filtrate upon concentration and cooling.

Nicotinyl-DL-phenylalanylhydrazide.—Nicotinylphenylalanine ethyl ester (1 g.) was heated on the steam-bath for 1.5 hours with 85% hydrazine hydrate (10 cc.). Ether (50 cc.) was then added to the cooled reaction mixture, whereupon the hydrazide precipitated. It was collected by filtration and recrystallized from a mixture of alcohol and benzene, 0.7 g. (72%), m.p. 185°.

Anal. Calcd. for $C_{15}H_{16}O_2N_4$: C, 63.36; H, 5.68. Found: C, 63.42; H, 6.28.

N-(Benzylnicotinamidomethyl)-N'-β-naphthylurea (XIV).—To an ice-cold solution of nicotinylphenylalanylhydrazide (0.5 g.) in 2 *N* hydrochloric acid (20 cc.) was added slowly a cooled solution of sodium nitrite (0.15 g.) in water (5 cc.). The resulting solution was kept at room temperature for 15 minutes, cooled in ice and neutralized by the addition of solid sodium bicarbonate. The precipitated nicotinylphenylalanyl azide (XII) was extracted with ethyl acetate, washed with water and dried over anhydrous sodium sulfate. This solution, after addition to it of β-naphthylamine (0.25 g.), was kept at room temperature for 48 hours. The solvent was then removed under reduced pressure and the solid residue digested with boiling water and filtered hot. The filtrate contained unreacted naphthylamine. The residue was recrystallized from alcohol. The urea derivative (XIV) crystallized in colorless flakes, 0.35 g. (48%), m.p. 227°.

Anal. Calcd. for $C_{25}H_{22}O_2N_4$: C, 73.14; H, 5.41. Found: C, 73.12; H, 4.99.

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The Synthesis of Chloramphenicol Analogs

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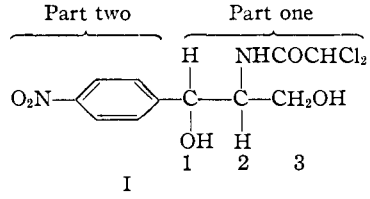
The acid related to chloramphenicol by oxidation at the primary alcoholic grouping has been synthesized. As a by-product in the saponification of N-dichloroacetyl-O-acetyl-*p*-nitrophenylserine ethyl ester, the alkaline soluble α-dichloroacetamido-*p*-nitrocinnamic acid ethyl ester was obtained. A homochloroamphenicol, 1-(*p*-nitrophenyl)-2-dichloroacetamido-2-methyl-1,3-propanediol, was prepared. By reduction of 1,3-diphenyl-2-phenylhydrazono-1,3-propanedione and subsequent transformations, 1,3-di-(*p*-nitrophenyl)-2-dichloroacetamido-1,3-propanediol was synthesized. Four compounds containing a *p*-nitrophenyl grouping and a polyhydroxy side chain were also prepared. None of these synthetic analogs of chloramphenicol showed any antibiotic activity.

The characterization of chloramphenicol (I) and the elaboration of two methods for its synthesis by Parke, Davis and Co. chemists[1,2,3,4] makes this antibiotic the first one in which the relation between structure and biological action can be thoroughly explored. For purposes of discussion one may divide the chloramphenicol molecule (I) into two parts; first, the stereochemically specific 2-acylamido propanediol side chain, which can be regarded as a grouping native to physiological systems in the same sense that penicillin is peptide-like and streptomycin is carbohydrate-like and, second, the nitrobenzene grouping which is more in the nature of the classical chemotherapeutic agent. Controulis, *et al.*,[2] have already shown that only one of the four possible side chain stereoisomers of chloramphenicol is active. Changes in the side chain considered in this paper result in complete loss of antibiotic activity.

On the other hand it is becoming evident that such high structural specificity in the aromatic grouping is not necessary for antibiotic activity. Long, *et al.*, Bambas, *et al.*,[5] and Buu-Hoï[6] have reported that the *m*- and *o*-nitro isomers of I and the analogs with a halogen replacing the nitro group exhibit appreciable activity. Forthcoming work from this Laboratory will further substantiate this generalization.



I

The first change in the side chain of I to be discussed is the replacement of the primary alcohol group at C_3 by a carboxyl group. The starting material used was the DL-phenylserine (II) of Erlenmeyer[7] formed as the major diastereomer in the reaction between glycine and benzaldehyde in alkaline solution. Although the configuration of this amino acid was allegedly shown to be "*trans*" by Forster and Rao,[8] thus corresponding to the *threo*-configuration of I, the proof is not conclusive. To demonstrate that I and II are of the same configuration, we have reduced the methyl ester of II with lithium aluminum hydride to an amino alcohol identical with DL-*threo*-1-phenyl-2-amino-1,3-propanediol which can be converted to I as described by Long and Troutman.[3] The com-

(1) M. C. Rebstock, H. M. Crooks, Jr., J. Controulis and Q. R. Bartz, THIS JOURNAL, **71**, 2458 (1949).

(2) J. Controulis, M. C. Rebstock and H. M. Crooks, Jr., *ibid.*, **71**, 2463 (1949).

(3) L. M. Long and H. D. Troutman, *ibid.*, **71**, 2469 (1949).

(4) L. M. Long and H. D. Troutman, *ibid.*, **71**, 2473 (1949).

(5) L. M. Long and N. Jenesel, *ibid.*, **72**, 4299 (1950); L. L. Bambas, H. D. Troutman and L. M. Long, *ibid.*, **72**, 4445 (1950).

(6) Buu-Hoï, Hoán, P. Jacquignon and N. H. Khoi, *Compt. rend.*, **230**, 662 (1950).

(7) E. Erlenmeyer, Jr., and E. Früstück, *Ann.*, **284**, 36 (1895).

(8) M. O. Forster and K. A. N. Rao, *J. Chem. Soc.*, 1943 (1926).