[CONTRIBUTION FROM THE STAMFORD RESEARCH LABORATORIES OF THE AMERICAN CYANAMID COMPANY]

# Studies in Chemotherapy. IX. Ureylenebenzene and Cyclohexane Derivatives as Biotin Antagonists<sup>1</sup>

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Since a number of important pathogenic organisms probably require preformed biotin,<sup>2</sup> and because of the minute amounts of the vitamin present in most body tissues,<sup>3</sup> an investigation of potential biotin antagonists seemed appropriate.

As a starting point in this study, representative compounds in two closely related series of ureylenebenzene and cyclohexane derivatives indicated in Table I were prepared.



The synthesis of the desired compounds was carried out by procedures designed to ensure the orientation of the final products. The most promising approach to the 2,3-ureylene derivatives appeared to be by way of  $\omega$ -(2-aminophenyl)-butyric or valeric acids so that the relation of the nitrogen adjacent to the side chain was fixed. Previous methods<sup>4</sup> for the preparation of δ-(2-aminophenyl)-valeric acid were not attractive, but the condensation of methyl acetate with o-benzoylaminocinnamaldehyde, which is readily available from quinoline,<sup>5</sup> afforded a more favorable starting point. The subsequent reactions involved in the synthesis of the analog, IV, in which two methylene groups replace the sulfur atom in biotin, are outlined in Chart I.

Conversion of XIV to the acetyl derivative (XVI) was carried out to permit a comparison of our *o*-aminocinnamylidene acetic acid (XV) with that of Diehl and Einhorn.<sup>4</sup> The 1,2,3-orienta-

(1) Presented in part before the Division of Medicinal Chemistry, New York Meeting of the American Chemical Society, September 15, 1944.

(2) Landy, Dicken, Bicking and Mitchell, Proc. Soc. Expll. Biol. Med., 49, 441 (1942).

(3) Taylor, Pollack and Williams, Univ. Texas Pub., No. 4237, p. 41 (1942).

(4) Diehl and Einhorn, Ber., 20, 377 (1887); von Braun, ibid., 40, 1845 (1907).

(5) Reissert and Arnold, ibid., 38, 3415 (1905).

tion of  $\delta$ -(2-acetvlamino-3-nitro-5-bromophenvl)valeric acid (XIX) was established by its oxidation to the corresponding benzoic acid (XX). This acid was identical by melting point and mixed melting point with the same compound prepared from 2-amino-3-nitrobenzoic acid.<sup>6</sup> The intermediate 2-amino-3-nitro-5-bromobenzoic acid obtained in this procedure had the same melting point as the product prepared in a different manner by Adams and Snyder.<sup>7</sup> Although the diamino acid, XXII, was not isolated, its instability in the presence of oxygen and a positive color test with benzil<sup>8</sup> showed it to be an o-phenylenediamine. The presence of an o-ureylene ring in compound III was demonstrated by a comparison of its ultraviolet absorption spectrum<sup>9</sup> with that of o-phenylene urea (Fig. 1).



Fig. 1.—Ultraviolet absorption spectra of: (1) o-phenyleneurea, (2)  $\delta$ -(2,3-ureylenephenyl)-valeric acid, III, and (3)  $\delta$ -(3,4-ureylenephenyl)-valeric acid, VII, in 95% ethanol.

<sup>(6)</sup> James, Kenner and Stubbings, J. Chem. Soc., 775 (1920).

<sup>(7)</sup> Adams and Snyder, THIS JOURNAL, 60, 1412 (1938).

<sup>(8)</sup> Hinsberg, Ann., 237, 327 (1887).

<sup>(9)</sup> We are indebted to Mr. D. Richardson and Mr. B. Costa of the Physics Division for the ultraviolet absorption data in Figs. 1 and 2. A medium Hilger quarts prism spectrograph with a Hilger-Spekker photometer was employed.



After this work was completed, it was found that  $\delta$ -(2-aminophenyl)-valeric acid could be prepared by an extension of the K. F. Schmidt<sup>10</sup> reaction of alkylphenyl ketones and hydrazoic acid as applied to cyclic ketones by Briggs and De Ath.<sup>11</sup> Benzosuberone<sup>12</sup> reacted with hydrazoic acid to form the previously unknown lactam of  $\delta$ -(2-aminophenyl)-valeric acid which was readily hydrolyzed to the acid. The acetyl derivative of this acid had the same melting point as XVII, and a mixed melting point showed no depression. Since Diehl and Einhorn<sup>4</sup> reported no analysis for XVII, the discrepancy in melting points might be explained by considering their product to be a diacetyl derivative.

Compounds IX and X were prepared by the Friedel-Crafts reaction of *o*-phenyleneurea and glutaric anhydride followed by appropriate reductions. Evidence for the orientation of the intermediate  $\gamma$ -(3,4-ureylenebenzoyl)-butyric acid was furnished by a comparison of its ultraviolet absorption spectrum with the spectra of 2,3- and 3,4-ureylenebenzoic acids (Fig. 2). These acids were prepared through 2- and 4-nitro-3-aminobenzoic acids, the structures of which have been well established.<sup>13</sup> Although their esters have not been characterized previously, both of the ureylenebenzoic acids have been prepared by other methods.<sup>14</sup> Attempts to oxidize the side chain of  $\gamma$ -(3,4-ureylenebenzoyl)-butyric acid to the

(12) Borsche and Roth, Ber., 54, 174 (1921).

(14) Griess, ibid., 5, 192 (1872); Zehra, ibid., 23, 3631 (1890).

corresponding benzoic acid with the usual reagents were not successful.

The ultraviolet absorption spectrum of  $\gamma$ -(3,4ureylenebenzoyl)-butyric acid (3) shown in Fig. 2 resembled that of the 3,4-benzoic acid (2) more closely than it did 2,3-ureylenebenzoic acid (1). The shift of the maxima toward longer wave lengths accompanying an increase in the positive character of the substituent group [(3) compared with (2)] was in agreement with the observations of Herold.<sup>16</sup>

Since there are only two possible  $\delta$ -ureylenephenylvaleric acids, final proof of the structure of IX (m. p. 234–36°) was obtained by comparing it with III (m. p. 263–65°). A mixture of these two compounds melted at 195–205°. With the structure of III established by synthesis, only one possibility remains for IX.

 $\gamma$  - (2 - Benzoylaminophenyl) - butyronitrile<sup>16</sup> served as the starting point for the synthesis of compound I. In general, the procedures were similar to those outlined for III beginning with the bromination step XVII. The nitrile was employed at this stage because difficulty was encountered in acetylating  $\gamma$ -(2-aminophenyl)-butyric acid due to its ease of lactamization. Compound VII was prepared by substituting succinic for glutaric anhydride in the procedure used for the synthesis of IX. The evidence for the structure of I and VII was analogous to that described in detail for the corresponding  $\delta$ -ureylenephenylvaleric acids (cf. Figs. 1 and 2).

(16) von Braun, Ber., 40, 1845 (1907).

<sup>(10)</sup> K. F. Schmidt, Ber., 57, 704 (1924).

<sup>(11)</sup> Briggs and De Ath, J. Chem. Soc., 456 (1937).

<sup>(13)</sup> Kaiser, ibid., 18, 2943 (1885).

<sup>(15)</sup> Herold, Z. physik. Chem., B18, 265 (1932).

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Like biotin,<sup>17</sup> the cyclohexane derivatives possess three asymmetric carbon atoms. In view of the limitation in isomeric forms resulting from low temperature catalytic reduction of benzene derivatives in general,<sup>18</sup> it seems unlikely that the present compounds are mixtures of all possible isomers. We have found, for example, that hydrogenation of o-phenyleneurea at  $25^{\circ}$  produces a different (cis?) form of hexahydro-ophenyleneurea than that obtained by Einhorn and Bull.<sup>19</sup> Apparently homogeneous products were also obtained in the catalytic reduction of the 3,4-ureylenebenzene derivatives. An unsuccessful attempt was made to separate isomeric forms of compound VIII by fractional precipitation of the acid from an aqueous solution of its potassium salt.<sup>20</sup> This procedure applied to the 2,3-derivatives, however, resulted in the separation of two isomers (syn and anti?) in each case (II-A, II-B, IV-A, IV-B). Microscopic examination and infrared absorption spectra established the fact that these isomers were actually different.21

Desthiobiotin has been found to replace biotin for a strain of yeast, but not for *L. casei*,<sup>22</sup> and Dittmer, Melville and du Vigneaud<sup>23</sup> have reported that the yeast strain converted the desthio derivative to biotin. These investigators and Lilly and Leonian<sup>24</sup> also found that desthiobiotin inhibited the growth of *L. casei* by interfering with its utilization of biotin. Similarly, biotin sulfone and imidazolidone-caproic acid have been shown to exhibit antibiotin activity with this organism, while the sulfone and imidazolidonevaleric acid possessed a slight growth-promoting action for yeast.<sup>25</sup>

With the exception of the benzoic acid derivatives, all of the substances in the present group, both in the benzene and cyclohexane series, proved to be biotin antagonists for both *L. casei* and yeast. Several of the compounds inhibited the growth of *L. arabinosus* as well, and in all cases the effect could be reversed by appropriate concentrations of biotin. None of the products tested showed any growth-promoting activity for any of these organisms. The antibiotin activity of the compounds for *L. casei* and yeast, and their molar inhibition ratios, are recorded in Table II.

As might be anticipated, the phenyl derivatives were less active than the corresponding cyclohexanes. When *L. casei* was the test organism,

(17) Cf. du Vigneaud, Science, 96, 455 (1942); Harris, Wolf, Mozingo and Folkers, *ibid.*, 97, 447 (1943).

(18) For a discussion of this point with numerous references see Linstead, Doering, Davis, Levine and Whetstone, THIS JOURNAL, 64, 1986 (1942).

(19) Einhorn and Bull, Ann., 295, 209 (1897).

(20) Cf. Linstead and Doering, THIS JOURNAL, 64, 2001 (1942).

(21) We are indebted to Dr. A. F. Kirkpatrick for the microscopic studies, and to Dr. R. C. Gore for the infrared absorption spectra.

(22) Melville, Dittmer, Brown and du Vigneaud, Science, 98, 497 (1943).

(23) Dittmer, Melville and du Vigneaud, ibid., 99, 203 (1944).

(24) Lilly and Leonian, *ibid.*, **99**, 205 (1944).

(25) Dittmer and du Vigneaud, ibid., 100, 129 (1944).



Fig. 2.—Ultraviolet absorption spectra of: (1) 2,3ureylenebenzoic acid, (2) 3,4-ureylenebenzoic acid, and (3)  $\gamma$ -(3,4-ureylenebenzoyl)-butyric acid in 95% ethanol.

the position of the side chain with respect to the ureylene group seemed to be less important for maximum activity than the total number of carbon atoms (including the carbocyclic ring) separating the ureylene and carboxyl groups. With yeast, however, quite the reverse situation was found. Here the position of the side chain appeared to be more significant than the number of

#### TABLE II

ANTIBIOTIN ACTIVITY AND MOLECULAR INHIBITION RATIOS WITH L. casei AND YEAST

	M. D., °C	M. E. C. X 104ª		Inhib. ratio × 10 <sup>-50</sup>	
Compound	(cor.)	L. casei	Yeast	L. casei	Yeast
I	299-300	100	0.625	250	3.1
II-A	218-220	50	0.003	125	0.015
II-B	192-194	25	0.003	62.5	0.015
III	263-265	25	5.0	62.5	25.0
IV-A	222-226	0.125	0.006	0.31	0.03
IV-B	183–184	0.125	0.006	0.31	0.03
VII	253.5-255	6.25	12.5	15.0	62.5
VIII	137-139	0.016	0.31	0.04	1.56
$\mathbf{IX}$	234 - 236	3.1	3.1	7.5	15.6
x	212 - 214	0.125	0.31	0.31	1.56

<sup>a</sup> M. E. C. indicates the smallest amount in moles/liter which produces greater than 50% inhibition of growth of *L. casei* after seventy-two hours in a medium containing the minimum concentration of biotin (4.1  $\times$  10<sup>-10</sup> *M*) for normal growth. With yeast, readings were taken at forty hours, and  $2 \times 10^{-10}$  *M* biotin was used. <sup>b</sup> Moles of antagonist required to inhibit the growth-promoting action of one mole of biotin. carbon atoms it contained. Wherever a separation of geometric isomers was possible, there appeared to be practically no difference in their antibiotin activity. It will be of interest to attempt to resolve the optical isomers. Further studies on the possible applications of the more potent of these biotin antagonists as chemotherapeutic agents are in progress.

## Experimental<sup>26</sup>

Compounds I and II (cf. Table I).—As indicated in the preceding discussion,  $\gamma$ -(2-benzoylaminophenyl)-butyronitrile served as an intermediate in the synthesis of these compounds. The nitrile was obtained by benzoylation of tetrahydroquinoline<sup>27</sup> followed by reaction with phosphorus pentachloride to yield  $\gamma$ -(2-benzoylaminophenyl)propyl chloride.<sup>28</sup> The chloro compound was converted to the corresponding iodo derivative m. p. 122–124°,<sup>29</sup> and thence to the nitrile.<sup>4</sup>  $\gamma$ -(2-Aminophenyl)-butyric acid hydrochloride was obtained by refluxing 40 g. of the nitrile with 200 cc. of 20% hydrochloric acid for nineteen hours.<sup>30</sup> Attempts to acetylate this product in acetic anhydride with sodium acetate or by using acetamide gave only the lactam.<sup>31</sup> Consequently,  $\gamma$ -(2-benzoylaminophenyl)-butyronitrile was employed in several subsequent steps before converting it to the acid.

 $\gamma$ -(2-Benzoylamino-5-bromophenyl)-butyronitrile.—A solution of 1.70 g. (0.011 mole) of bromine in 5 cc. of glacial acetic acid was added to a solution of 2.64 g. (0.01 mole) of  $\gamma$ -(2-benzoylaminophenyl)-butyronitrile in 20 cc. of glacial acetic acid over a four-hour period. The solution was then poured into 100 cc. of water, and the light yellow precipitate was filtered off, washed with water, and dried yield 3.3 g. (96%), m. p. 127–133°. Three crystallizations from aqueous alcohol gave a colorless material melting at 140–141°.

Anal. Calcd. for C<sub>17</sub>H<sub>15</sub>ON<sub>2</sub>Br: N, 8.1. Found: N, 8.1, 8.2.

 $\gamma$ -(2-Benzoylamino-3-nitro-5-bromophenyl)-butyronitrile.—A solution of 125.cc. of fuming nitric acid in 125 cc. of glacial acetic acid was added rapidly to a solution of 13.8 g. of  $\gamma$ -(2-benzoylamino-5-bromophenyl)-butyronitrile in 125 cc. of glacial acetic acid at about 60°. This temperature was maintained for two hours. The reaction mixture was then poured into 1500 cc. of ice and water, and the precipitated yellow powder was filtered off, washed with water and dried; yield 12.8 g. (82%); m. p. 134-139°. Two crystallizations from aqueous alcohol gave pale yellow needles, m. p. 142-144°.

Anal. Calcd. for  $C_{17}H_{14}O_3N_3Br$ : N, 10.8. Found: N, 11.0, 10.7.

 $\gamma$ -(2-Benzoylamino-3-nitro-5-bromophenyl)-butyric Acid.—A suspension of 1.7 g. of the above nitrile in 25 cc. of 20% hydrochloric acid was refluxed for twenty-two hours, cooled and filtered. The solid was taken up in bicarbonate and filtered from a little unchanged starting material. Acidification of the filtrate gave 1.2 g., m. p. 150-166°. Two crystallizations from 50% alcohol gave 0.96 g. (58%), m. p. 167-170°.

Anal. Calcd. for  $C_{17}H_{16}O_5N_2Br$ : C, 50.0; N, 6.9. Found: C, 49.5, 49.5<sup>32</sup>; N, 7.0, 7.2.

(I)  $\gamma$ -(2,3-Ureylenephenyl)-butyric Acid.—Seven hundred milligrams of  $\gamma$ -(2-benzoylamino-3-nitro-5-bromo-

(26) All melting points are corrected. Microanalyses were carried out in these Laboratories under the direction of Dr. J. A. Kuck, to whom we are indebted for these data.

(27) We are indebted to Dr. K. W. Saunders of the Chemical Research Division for this material nD 1.5892 ca. 96% pure.

- (28) German Patent 164,365, Friedländer, 8, 1055 (1905-1907).
- (29) von Braun<sup>4</sup> reported a m. p. of 112-113°.
  (30) von Braun<sup>4</sup> specifies the use of concentrated hydrochloric acid in a sealed tube at 125°.

(31) Galat and Elion, THIS JOURNAL, 65, 1566 (1943).

(32) Micro Van Slyke wet combustion.

phenyl)-butyric acid<sup>33</sup> was dissolved in 80 cc. of 5% sodium carbonate and the solution was stirred in an atmosphere of nitrogen during the subsequent operations. The solution was warmed to 55° and 140 g. of 2% sodium amalgam was added in the course of two and a half hours. The original orange colored solution soon became colorless but quickly colored again if exposed to the air. When the amalgam had reacted, the mixture was cooled in an icebath and treated with phosgene until the solution was acid. The precipitate was redissolved by the addition of alkali and the solution was decanted from the mercury, decolorized with Norit, and acidified. Three hundred milligrams (79%), m. p. 280-283°, was obtained. Two crystallizations from glacial acetic acid raised this to 299-300°.

Anal. Calcd. for  $C_{11}H_{12}O_3N_2$ : N, 12.7. Found: N, 12.7, 13.0.

(II)  $\gamma$ -(2,3-Ureylenecyclohexyl)-butyric Acid.—Four hundred thirty milligrams of I and an equal weight of Adams platinum oxide catalyst (Baker) was suspended in 50 cc. of glacial acetic acid and shaken with hydrogen at a pressure of 50 lb. per square inch for six hours. The residue, after filtration and evaporation *in vacuo*, was crystallized from boijing water (Norit) to give a product melting at 195–208°. Another recrystallization from water narrowed the range to 198–208°. No further change was caused by crystallization from water or aqueous alcohol.

Anal. Calcd. for  $C_{11}H_{15}O_3N_2$ : C, 58.4; H, 8.0; N, 12.4. Found: C, 58.4, 58.4<sup>32</sup>; H, 8.0, 8.2; N, 12.6, 12.6.

By a process of fractional acidification<sup>20</sup> II was separated into II-A (high melting) and II-B (low melting). After a pilot run in which ten cuts were made, the following process was used. Five hundred mg. of II (m. p. 198– 208°) was dissolved in 1.5 cc. of N/7 aqueous potassium hydroxide and precipitated in fractions by the daily addition of a portion of 0.5 N hydrochloric acid. The twentyfour-hour interval between acidification and collection was adopted to permit equilibration of the precipitate and the solution. The first five fractions were removed by filtration. Fraction 6 was obtained by evaporation, extraction with isopropanol and evaporation of the extract.

Fraction	HCl, g.	Wt., mg.	M. p., °C
1	0.66	29	209-218
$^{2}$	1.26	142	212-219
3	0.43	47	209-218
4	0.45	49	1926
5	1.32	162	192-6
6	0.16	57	172 - 84

#### Total 486 (97%)

Fractions 1, 2 and 3 were combined and crystallized twice from water to give 131 mg. of II-A, rosets, m. p. 218–220°;  $n_1 = 1.597$ ;  $n_2 = 1.480$ . It was soluble in boiling water to the extent of about 15 mg. per cc.

Anal. Calcd. for  $C_{11}H_{18}O_{2}N_{2}$ : C, 58.4; N, 12.4. Found: C, 58.1<sup>32</sup>; N, 12.5.

Fractions 4 and 5 were combined and crystallized three times from water to give 176 mg. of II-B, lath-shaped crystals, m. p. 192–194°;  $n_1 = 1.563$ ;  $n_2 = 1.536$ . About 25 mg. dissolved in 1 cc. of boiling water.

Anal. Caled. for  $C_{11}H_{18}O_2N_2$ : C, 58.4; N, 12.4. Found: C, 58.2<sup>32</sup>; N, 12.7.

Compounds III and IV.—The steps employed in the synthesis of these derivatives are outlined in Chart I. Technical quinoline, purified by the method of Wyler,<sup>84</sup>

(33) This compound was utilized in the reaction before it was recognized that the benzoyl group was still present, enough amine having been formed to give a positive diazo test. Debenzoylation probably occurred in the alkaline medium. A comparable resistance to acid hydrolysis and susceptibility to basic hydrolysis is shown by a number of o-nitroacylamino compounds. Cf. Verkade and Witjens, Rec. trav. chim., **62**, 200 (1943).

(34) Wyler, Ber., 60, 398 (1927).

gave yields of XIII equally as good as those of Reissert and Arnold,<sup>5</sup> who used synthetic quinoline only. It was unnecessary to distill or dry the quinoline after freeing it from the recrystallized zinc chloride complex. The aldehyde, XIII, was purified by continuous extraction with dry acetone to give material of m. p. 196-197°.

XIV. Methyl-o-benzoylaminocinnamylidene Acetate.-Conditions of the Claisen reaction<sup>25</sup> were used in this reaction: 2.4 g. (0.11 g. atom) of sodium was very finely powdered under xylene or toluene in a 50-cc. test-tube. The solvent was decanted and 35 cc. of methyl acetate was added. The test-tube was immersed in a bath at  $-8^{\circ}$ and 11.1 g. (0.044 mole) of XIII was added with vigorous stirring in about two and a half hours, maintaining the temperature at  $-8^{\circ}$  throughout. After about 0.5 g. of the aldehyde had been added, it was necessary to add five drops of methanol to start the reaction, which was sig-naled by the appearance of a red color. When all of the aldehyde had been added, the thick, red mixture was stirred for an additional thirty minutes and then treated with 6 cc. of glacial acetic acid. Ether was added and the yellow solid was triturated and filtered. The material was washed with water, filtered and dried; 7.75 g. (57%), m. p. 166-167° was obtained. In repeating the reaction, yields of 40-65% were obtained. The only factors of determined importance were (a) purity of the aldehyde, (b) fineness of the sodium sand, (c) presence of a trace of methanol, (d) size of the run, larger scale reactions giving much lower yields. The material was not purified further and was used directly for the next reaction.

XV. o-Aminocinnamylideneacetic Acid.—The hydrolysis of the methyl ester, XIV, was actually carried out in two steps (cf. Chart I), since a purer product appeared to be obtained in this manner. Twenty-six grams (0.085 mole) of XIV was boiled for five to ten minutes with 150 cc. of 10% sodium hydroxide and the solution was decanted from the insoluble tar. Upon cooling, the sodium salt of o-benzoylaminocinnamylideneacetic acid crystallized. Solution of this salt in water and acidification gave 22.9 g. (52%) of the acid as a light yellow powder melting with effervescence at 270–275°. Acidification of the hydrolysis mother liquor gave 4.33 g. (total crude yield 69%) of brown solid, melting from 260–267° with decomposition. This could be purified further by conversion to the sodium salt.

A solution of 6.0 g. (0.02 mole) of the benzoyl derivative in 70 cc. of 20% sodium hydroxide was refluxed for eight and a half hours. After dilution with water the solution was made strongly acid with hydrochloric acid and ex-tracted with ether to remove the benzoic acid. The solution was then neutralized and extracted with ether. This ether solution of the amino acid exhibited a greenish fluorescence which made the end-point of the extraction easy to observe. After exhaustion with ether at neutrality the solution was made progressively more acid and extracted with ether at each step until no more amino acid was removed. The optimum pH for this operation seemed to lie between 4 and 5. After washing with water, the ether solution was dried with sodium sulfate and evaporated to The solid residue was washed with water and drvness. dried; yield 2.81 g. (73%); m. p. 172°36 (dec.). XVI. o-Acetylaminocinnamylideneacetic

XVI. o-Acetylaminocinnamylideneacetic Acid.—Refluxing of the amino acid with acetic anhydride as directed by Diehl and Einhorn<sup>36</sup> was found to be unnecessary. When 0.5 g. of XV was added to 7 cc. of acetic anhydride, the amino acid dissolved at once and a precipitate began to separate almost immediately. After chilling, the solid was collected and dried; yield 0.51 g. (83%), m. p. 260– 262° (effervescence). A m. p. of 253° (dec.) is reported in the literature.<sup>36</sup>

**XVII.**  $\delta$ -(2-Acetylaminophenyl)-valeric Acid.—3.0 g. of XVI was shaken in 200 cc. of methanol with 0.2 g. of Adams catalyst (Baker) at 45 lb. of hydrogen pressure for two and

(35) Marvel and King, "Organic Syntheses," Coll. Vol. I, 296 (1932).

(36) Diehl and Einhorn, Ber., 18, 2326 (1885), reported m. p. 176.5° (dec.) after crystallization from water or aqueous alcohol.

a half hours. The suspension was filtered and the filtrate evaporated to dryness. The residue was crystallized from water; yield 2.4 g. (79%); m. p. 126-128°.<sup>37</sup>

Anal. Calcd. for C<sub>13</sub>H<sub>17</sub>O<sub>3</sub>N: equiv. wt., 235. Found: equiv. wt., 236, 236.

XVIII.  $\delta$ -(2-Acetylamino-5-bromophenyl)-valeric Acid. —To a solution of 2.8 g. (0.012 mole) of the acetylamino acid, XVII, in 22 cc. of glacial acetic acid, was added a solution of 1.96 g. (0.012 mole) of bromine in 6 cc. of glacial acetic acid during one and two-thirds hours. After standing for twenty minutes, the red solution was poured into 100 cc. of water and the almost colorless precipitate was filtered off, washed with water and dried; yield 3.34 g. (89%); m. p. 146-148°. Crystallization from aqueous alcohol gave white needles, m. p. 152-153°.

Anal. Calcd. for C13H16O3NBr: N, 4.5. Found: N, 4.6.

XIX.  $\delta$ -(2-Acetylamino-3-nitro-5-bromophenyl)-valeric Acid.—4.18 g. of XIX was suspended in 40 cc. of glacial acetic acid and treated with a solution of 53 cc. of fuming nitric acid (d. 1.50) in 40 cc. of glacial acetic acid. The mixture was warmed at 50-60° for two hours. The solution was then cooled and added to ice and water. The precipitate was collected and dried; yield 4.20 g. (88%); m. p. 205-207°.

Anal. Caled. for  $C_{13}H_{15}O_{5}N_{2}Br$ : N, 7.8; Br, 22.3. Found: N, 7.8, 7.5; Br, 22.4.

XX. 2-Acetylamino-3-nitro-5-bromobenzoic acid was obtained by the oxidative degradation of XIX as follows: One hundred eighty milligrams (0.0015 mole) of magnesium sulfate, 0.24 g. (0.015 mole) of potassium permanganate, 0.050 g. (0.00014 mole) of XIX and 11.9 cc. of water were added in that order to a ground glass jointedtest-tube and the mixture was heated on a steam-bath. After four hours, the manganese dioxide was filtered and the pale yellow solution concentrated on the steam-bath to 1 cc. The product was precipitated with dilute hydrochloric acid and taken up in ether. The ether extracts were united, washed twice with water and evaporated to dryness to yield yellowish crystals. After two recrystallizations from 40% acetic acid, the product was very pale yellow in color and melted at 199-201° (micro-block).

A sample of XX was prepared from 2-amino-3-nitrobenzoic acid<sup>6</sup> by dissolving 0.24 g. (0.0013 mole) of this acid in 1.0 cc. of boiling glacial acetic acid and treating it dropwise with 0.30 cc. (0.0013 mole) of a solution of 1.00 g. of bromine in 1.0 cc. of glacial acetic acid. A crystalline precipitate appeared near the end of the addition; 1.0 cc. of glacial acetic acid and 0.05 cc. of bromine solution were added and the mixture heated briefly to obtain solution and ensure complete bromination. After cooling, the crystals were centrifuged, washed and recrystallized from glacial acetic acid. The resulting product was washed with acetic acid, then water, and dried at 100°; it weighed 0.14 g. and melted sharply at 246–248° (micro-block). Adams and Snyder<sup>7</sup> synthesized this compound by another method and gave the m. p. as 245–247°.

To 0.5 cc. of boiling acetic anhydride, was added 0.07 g. of 2-amino-3-nitro-5 bromobenzoic acid. After cooling, 0.01 cc. of concentrated sulfuric acid was added and the solution allowed to stand for fifteen minutes. The mixture was poured into 1.0 cc. of water to precipitate the product and decompose the anhydride. The solid was centrifuged and washed consecutively with glacial acetic acid and water. Recrystallization from 40% acetic acid, followed by several washings with fresh solvent and a final aqueous wash yielded 2-acetylamino-3-nitro-5-bromobenzoic acid which melted sharply at 197-199° (micro-block). The melting point of a mixture of this product and XX obtained from XIX was 198-200° (micro-block).

**XXI.**  $\delta$ -(2-Amino-3-nitro-5-bromophenyl)-valeric Acid. —A 1.4-g. sample of XIX and 200 cc. of 1:1 hydrochloric acid were refluxed together for two and three-quarters hours. There was a clear solution in one-half hour.

(37) A melting point of  $151^{\circ}$  has been reported<sup>4</sup> for an acetylated acid obtained after boiling  $\delta$ -(2-aminophenyl)-valeric acid with acetic anhydride for several hours.

After cooling, the solid was taken up in ether and the solution was extracted three times more with ether. The ether solutions were combined, washed with water, dried with sodium sulfate and evaporated. The oil (1.17 g.95%) solidified on scratching and melted at  $119-121^\circ$ . This product was used directly in the next step.

III.  $\delta$ -(2,3-Ureylenephenyl)-valeric Acid.—This compound, 0.77 g. (95%), m. p. 253-257°, was obtained from 1.1 g. of XXI (see Compound I). It was purified for reduction by treatment with Norit in alkaline solution and crystallization from acetic acid; m. p. 263-265°.

Anal. Calcd. for  $C_{12}H_{14}O_3N_2$ : N, 12.0. Found: N, 12.0.

IV.  $\delta$ -(2,3-Ureylenecyclohexyl)-valeric Acid.—Two hundred milligrams of III was reduced with 250 mg. of Adams platinum oxide catalyst (Baker) in 75 cc. of glacial acetic acid in a manner similar to the reduction of I to II. The reduction required forty hours, and 155 mg. of crude material, m. p. 201–205° was obtained. Crystallization from water gave 104 mg. (51%) m. p. 212–215°. This m. p. was not raised by another crystallization.

Anal. Calcd. for  $C_{12}H_{20}O_{1}N_{2}$ : C, 60.0; H, 8.4; N, 11.7. Found: C, 60.0, 60.3<sup>32</sup>; H, 8.7, 8.6; N, 11.4, 11.5.

The isomeric forms IV-A and IV-B were prepared from IV by a process analogous to that used in the separation of II-A and II-B. Five hundred milligrams of IV was dissolved in 15.5 cc. of 0.135 N potassium hydroxide and the solution was fractionally precipitated with 0.517 N hydrochloric acid with the following results

Fraction	HCl, g.	Wt. ppt., mg.	M. p., °C.
1	0.51	35	197 - 216
<b>2</b>	1.32	179	209 - 219
3	0.47	50	211 - 222
4	. 39	47	208 - 219
5	.20	23	204 - 218
6	21	25	179-183
7	.96	34	182 - 193
8		91	180-183
		warmer .	

## Total 484 (97%)

Fraction 8 resulted when the filtrate from 7 was evaporated, the residue extracted with hot isopropanol and this evaporated.

IV-A was obtained when fractions 2-5 were combined and recrystallized three times from water to give 160 mg., m. p. 222-226°. This m. p. was not sharpened by crystallization from alcohol. (Purification of a small amount as the quinine salt gave a melting point of 228-229°.) nav. >1.560, <1.565.

Anal. Calcd. for  $C_{12}H_{20}O_4N_2$ : C, 60.0; H, 8.4. Found: C, 60.1, 60.2; H, 8.4, 8.2.

IV-B was obtained by combining fractions 6 and 7 and crystallizing twice from 3 cc. of water; wt. 42 mg., m. p. 183°; *n* av. >1.530, <1.535.

Anal. Calcd. for  $C_{12}H_{20}O_{3}N_{2}$ : C, 60.0; H, 8.4. Found: C, 60.0; H, 8.3.

**Compounds V, VI, XI and XII.**—2-Nitro-3-amino and 3-amino-4-nitrobenzoic acids, both of which were obtained by nitration of *m*-acetylaminobenzoic acid,<sup>18</sup> served as intermediates for the synthesis of these compounds. The separation of the isomeric nitro-amonobenzoic acids is described here since Kaiser<sup>13</sup> reported no details of his procedure.

The nitration products obtained from 49 g. of *m*-acetylaminobenzoic acid and 100 g. of fuming nitric acid were separated by taking up the precipitate in 300 cc. of hot water with an excess of barium carbonate and filtering while hot. Acidification of the filtrate gave 26 g. (42%) of 2-nitro-3-acetylaminobenzoic acid, m. p.  $215-230^{\circ}$ . Crystallization from water raised the m. p. to  $240-241^{\circ}$ , identical with Kaiser's value. The 4-nitro isomer was obtained as a mixture of acetyl and free amino derivatives from the relatively insoluble barium salt (filtered off with the excess barium carbonate) by acidification of the suspension in hot water. The yield was 18 g. (29%) melting at 178-184°. Kaiser reported the m. p. of the pure material as  $205-206^{\circ}$ . Our low m. p. was due to partial deacetylation. Hydrolysis of the two acetylamino nitrobenzoic acids was effected by boiling barium hydroxide.

The 2,3- and 3,4-diaminobenzoic acids<sup>13</sup> were prepared by substituting stannous chloride for tin in the method of Schilling.<sup>33</sup> From 5 g. of 2-nitro-3-aminobenzoic acid, 20 g. of SnCl<sub>2</sub>·2H<sub>3</sub>O and 50 cc. of concentrated hydrochloric acid, 3 g. (71%) of 2,3-diaminobenzoic acid was obtained. The product, which darkened on standing, decomposed at 201° after sintering at 191°. Schilling<sup>38</sup> reported the m. p. as 190-191°.

In a similar manner 8.1 g. of 3-amino-4-nitrobenzoic acid yielded 6.2 g. (62%) of the hydrochloride of 3,4-diaminobenzoic acid decomposing at 249–250°.

V. 2,3-Ureylenebenzoic Acid (Griess<sup>14</sup>).—The procedure of Zehra<sup>14</sup> for XI was employed in the preparation of this compound. Three grams of 2,3-diaminobenzoic acid was suspended in 25 cc. of acetic acid and treated with 10 cc. of phosgene-saturated benzene. The reaction mixture was poured into ice and water and the product removed by filtration. It was decolorized by treatment with Norit in bicarbonate solution and crystallized from acetic acid in which it was only slightly soluble hot. It did not melt below 305°.

Anal. Calcd. for  $C_8H_6O_3N_2$ : C, 54.0; H, 3.4; N, 15.7. Found: O, 54.1, 53.9; H, 3.4, 3.5; N, 15.4, 15.7.

The methyl ester of V was obtained by treatment of an ether suspension of 400 mg. with ethereal diazomethane.<sup>39</sup> Evaporation of the ether gave the ester, which after crystallization from absolute ethanol had a m. p. of 260–263°; yield 300 mg.

Anal. Calcd. for C<sub>9</sub>H<sub>3</sub>O<sub>3</sub>N<sub>2</sub>: C, 56.3; H, 4.2; N, 14.6. Found: C, 55.8, 55.9; H, 3.9, 4.2; N, 14.4.

VI. 2,3-Ureylenecyclohexanecarboxylic Acid.—The reduction of V was carried out as described in the preparation of II. From 500 mg. of V, reduced with 500 mg. of Adams catalyst in 1500 cc. of glacial acetic acid, there was obtained 340 mg. (66%) of VI. After three recrystallizations from absolute ethanol, the compound decomposed at  $204-205^{\circ}$ .

Anal. Calcd. for  $C_8H_{12}O_8N_2$ : C, 52.2; H, 6.6; N. 15.2. Found: C, 52.2; H, 6.5; N, 15.2, 15.0.

XI. 3,4-Ureylenebenzoic Acid.—The method of Zehra<sup>14</sup> was used as described under V. From 5 g. of 3,4-diaminobenzoic acid, 3 g. (76%) of XI was obtained. It was purified in sodium bicarbonate solution by treatment with Norit. The product did not melt below 300° and was insoluble in common solvents.

The methyl ester of XI was prepared as described for the ester of V. The reaction was much slower, possibly because of the lower solubility of the acid in ether. From 600 mg. of XI, after crystallization from ethanol, 500 mg. (77%) of the methyl ester, m. p.  $312-313^{\circ}$ , was obtained.

Anal. Calcd. for C<sub>9</sub>H<sub>5</sub>O<sub>4</sub>N<sub>2</sub>: N, 14.6. Found: N 14.6, 14.7.

XII. 3,4-Ureylenecyclohexanecarboxylid Acid.—(See VI). Reduction of 1.00 g. of XI required 2.00 g. of Adams catalyst, and gave 0.4 g. of unreduced material which was separated by virtue of its insolubility in acetic acid. The reduced product was isolated by evaporation of the acetic acid. After four recrystallizations from alcohol, 0.5 g. (45%) m. p. 206-207°, was obtained.

Anal. Calcd. for  $C_8H_{12}O_3N_2$ : N, 15.2. Found: N, 15.4, 15.2.

**Hexahydro**-o-phenyleneurea.—Low-pressure catalytic reduction of o-phenyleneurea<sup>40</sup> resulted in the formation of a hexahydro derivative which differed from the product

<sup>(38)</sup> Schilling, Ber., 34, 904 (1901).

<sup>(35)</sup> "Organic Syntheses." Coll. Vol. 11, John Wiley and Sons. New York, N. Y., 1943, p. 165.

<sup>(40)</sup> Kym, J. prakt. Chem., [2] 75, 323 (1907).

(m. p. 230-231°) prepared in a different manner by Einhorn and Bull.<sup>19</sup> The lower melting form obtained in this study was prepared as follows: 1.8 g. of *o*-phenyleneurea in 175 cc. of absolute alcohol and 4 cc. of hydrogen chloride-saturated alcohol was hydrogenated at 45 pounds pressure and room temperature with 0.1 g. of Adams catalyst. After fourteen hours the catalyst was filtered and the solution was concentrated. After treatment with water and filtration from the insoluble starting material (0.33 g., m. p. 307-310°) the solution was clarified with Norit and concentrated to dryness. The gummy residue solidified on scratching and was extracted with 100 cc. of hot benzene. Concentration of the benzene solution yielded 0.72 g. (38%) of crystalline solid, m. p. 143-146°. Two crystallizations from benzene-hexane gave colorless needles, m. p. 147-149°.

Anal. Calcd. for  $C_7H_{12}ON_2$ : C, 60.0; H, 8.6; N, 20.0. Found: C, 60.3, 60.3; H, 8.4, 8.5; N, 19.7, 20.0.

 $\beta$ -(3,4-Ureylenebenzoyl)-propionic Acid.—To a stirred mixture of 16 g. (0.12 mole) of *o*-phenyleneurea, 12.0 g. (0.12 mole) of succinic anhydride, and 630 cc. of *s*-tetra-chloroethane was added 64.0 g. (0.48 mole) of aluminum chloride (Mallinckrodt analytical reagent) at room temperature. The bath temperature was raised to 100° over a period of two and three-quarters hours and was then held at 100–110° for one hour and at 110–120° for one-half hour. After cooling, the reaction mixture was decomposed with ice and hydrochloric acid and the solvent was steam distilled. A considerable quantity of black tar was filtered off and the filtrate was concentrated to a small volume. Upon cooling, a red-brown powder precipitated which was purified by treatment with Norit in bicarbonate solution. Acidification gave 3.55 g. (13%) of a light brown powder, m. p. 291° (dec.), and 2.45 g. (9%) of small white needles, m. p. 294° (dec., uncor.), was obtained on crystallization from glacial acetic acid.

Anal. Caled. for  $C_{11}H_{10}O_4N_3$ : C, 56.4; H, 4.3; N, 12.0. Found: C, 56.2, 56.4; H, 4.2, 4.5; N, 11.8, 11.8.

VII.  $\gamma$ -(3,4-Ureylenephenyl)-butyric Acid.—To 20 g. of amalgamated zinc was added 40 cc. of water, 40 cc. of concentrated hydrochloric acid, and 0.45 g. of recrystallized  $\beta$ -(3,4-ureylenebenzoyl)-propionic acid (when unrecrystallized keto acid was used, the yield and the purity of the reduced product was much less satisfactory). The solution was refluxed for five hours, 10 cc. of concentrated hydrochloric acid being added after two hours. The solution was decanted from the zinc and after thorough cooling 0.33 g. (78%) of a white powdery solid, m. p. 251–254°, had separated. The m. p. could be raised to 253.5–255° by treatment with Norit in bicarbonate solution and crystallization from aqueous alcohol.

Anal. Calcd. for  $C_{11}H_{12}O_3N_2$ : C, 60.0; H, 5.5; N, 12.7. Found: C, 60.4, 60.3; H, 5.6, 5.3; N, 12.4, 12.6.

VIII.  $\gamma$ -(3,4-Ureylenecycloheryl)-butyric Acid.—0.54 gram of VII, 0.5 g. of Adams catalyst, and 150 cc. of glacial acetic acid were shaken with hydrogen at 40 lb. pressure and room temperature for five and one-half hours. The catalyst was filtered and the acetic acid was removed at reduced pressure. Crystallization of the residual gum from water gave 0.24 g. (43%) of a white powder melting 128-132°. A sample melting at 137-139° was obtained by further crystallization from water.

Anal. Calcd. for  $C_{11}H_{18}O_{3}N_{2}$ : C, 58.4; H, 8.1; N, 12.4. Found: C, 58.2, 58.1; H, 7.9, 8.0; N, 12.6, 12.7.

 $\gamma$ -(3,4-Ureylenebenzoyl)-butyric Acid.—To a stirred mixture of 13 g. (0.097 mole) of o-phenyleneurea, 11 g. (0.096 mole) of glutaric anhydride, and 650 cc. of s-tetrachloroethane was added 52 g. (0.39 mole) of aluminum chloride. The reaction was run as in the case of succinic anhydride and worked up in the same manner. Treatment with Norit in bicarbonate solution followed by two crystallizations from acetic acid gave 1.25 g. (5%) of a white solid melting at 280–282°.

Anal. Calcd. for  $C_{12}H_{12}O_4N_2$ : N, 11.3. Found: N, 11.3, 11.0.

IX.  $\delta$ -(3,4-Ureylenephenyl)-valeric Acid.—The Clemmensen reduction of the keto acid was carried out in the same manner as in the case of the preparation of VII. From 1 g. of the keto acid there was obtained 0.84 g. (89%) of the reduced acid melting at 231-235°. Two recrystallizations from aqueous alcohol gave 0.66 g. melting at 234-236°. A mixture of III and IX had a m. p. of 195-205°.

Anal. Calcd. for  $C_{12}H_{14}O_8N_2$ : C, 61.5; H, 6.0; N, 12.0. Found: C, 61.3; H, 5.7; N, 11.8, 12.0.

The same acid was obtained by catalytic reduction with Adams catalyst in acetic acid, but for some unexplained reason this material could not be obtained in a state of sufficient purity to enter into the following reaction.

X.  $\delta$ -(3,4-Ureylenecyclohexyl)-valeric Acid.—Reduction of 1.3 g. of IX in glacial acetic acid with 1.3 g. of Adams catalyst according to the procedure for VIII gave, after purification by crystallization from alcohol, 361 mg. (27%) of fine crystals melting at 212-214°.

Anal. Calcd. for  $C_{12}H_{20}O_3N_2$ : C, 60.0; H, 8.4; N, 11.7. Found: C, 60.0, 60.2; H, 8.4, 8.2; N, 11.8.

Biochemical Results.—The antibiotin activity of the various compounds was determined against *Lactobacillus casei* (A. T. C. C. 7469). The basal medium was the improved biotin-free medium of Shull and Peterson.<sup>41</sup> However, a "vitamin-free" casein hydrolyzate (SMA Co.) was substituted for their peroxide-treated casein hydrolyzate. Blank and maximum titrations were unchanged by this substitution. Unless otherwise stated, the biotin concentration employed was 0.1  $\mu$ g. per liter (4.1  $\times$  10<sup>-10</sup>M).

Compounds to be tested were dissolved in the basal medium at the maximum concentration desired and diluted in a series of two-fold steps. The test solutions were prepared just before use and were sterilized by autoclaving. Sterilization of II-A, II-B, IV-A and IV-B by filtration gave results identical with the autoclaved compounds. For most series a final volume of 5 cc. in an 18  $\times$ 150 mm. test-tube was used. To permit the assay of smaller quantities during the separation of isomers, a 1.0 cc. volume in  $13 \times 100$  mm. tubes was chosen. Check series showed the results with the two volumes to be identical within the error of the method, *i. e.*,  $\pm$  one tube. Growth was checked visually or turbidimetrically at forty and sixty-four hours and acid production determined by titration at seventy-two hours. The minimum effective concentrations of the compounds were chosen as those levels at which growth or acid production was less than 50% of that in the control tubes. Since the end-points as determined by titration were somewhat more reproducible, only these values are given.

In Table III the data of a typical test are presented for two compounds. In this series a titration of 3.10 cc. of 0.1 N alkali represented 50% inhibition. Thus, in this experiment the minimum effective concentrations were  $3.13 \times 10^{-6}M$ for VIII and  $2.5 \times 10^{-5}M$  for X. These values are higher than the average of several experiments recorded in Table II.

Table IV illustrates the reversal of one of the analogs (IV) by biotin. The inhibition ratio was (41) Shull and Peterson, J. Biol. Chem., 151, 201 (1943).

3.45

4.55

5 25

5.30

	Lactobacillus casei <sup>a</sup>	
Compound	$M/1 \times 10^{5}$	Cc. of 0.1 N acid per 5 cc.
	Uninoculated	0.75
Controls	Inoculated	5.35 5.40
	Inoculated, biotin omittee	1 1.10
VIII	25	0.70
	12.5	. 85
	6.25	.75
	3.13	. 70
	1.56	3.90
	0.78	5.00
х	50	1.00
	25	* 1.45

12.5

6.25

3 13

1.56

TABLE III

INHIBITORY EFFECT OF COMPOUNDS VIII AND X ON

<sup>a</sup> Biotin concentration  $4.1 \times 10^{-10} M$ .

of the same magnitude throughout the forty-fold range, but the values rose slightly with the higher biotin levels. This was also noted in several similar experiments with X.

### TABLE IV

TITRATION OF BIOTIN AGAINST COMPOUND IV° WITH Lactobacillus casei

Biotin concn. $\times$ 10 <sup>-10</sup> M	M. E. C. $\times$ 10 <sup>4</sup> $M^a$	Inhibition ratio $\times$ 10 <sup>-5b</sup>
0.8	0.03	0.38
4,1	0.125	0.31
8.2	0.5	0.6
16	2.0	1.25
32	4.0	1.25

<sup>a</sup> and <sup>b</sup> See Table II. <sup>o</sup> Mixture of IV-A and IV-B before separation of isomers.

Compounds VI, XII, o-phenyleneurea and ohexahydrophenyleneurea were also tested. Of these only o-hexahydrophenyleneurea inhibited L. casei,  $3 \times 10^{-4}M$  per liter being the minimum effective concentration. None of the compounds reported has shown any ability to replace biotin for the growth of L. casei.

To measure the antibiotin activity of the analogs with yeast, a similar procedure employing the technique of Hertz<sup>42</sup> was used. The inoculum was a suspension of yeast cells (S. cerevisiae 139) taken directly from an agar slant (less than four weeks old) and diluted to the desired concentration. Biotin was added at  $2.0 \times 10^{-10}$ M. No growth stimulation of the yeast was observed with any of the compounds recorded in Table II.

Several of the compounds were tested against L. arabinosus 17-5 using the technique and basal medium of Wright and Skeggs.43 Å biotin concentration of  $1 \times 10^{-9} M$  was employed. The m. e. c. was determined as for L. casei. Compound VII inhibited growth at  $3 \times 10^{-4} M$ . II, IV and IX were active only at  $1 \times 10^{-2} M$  while X was inactive at this concentration.

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## Summary

The synthesis of representative compounds in two closely related series of ureylenebenzene and cyclohexane derivatives is described. Evidence for the structure of these compounds is presented. The separation of isomeric forms in the ureylenecyclohexane series has been effected in some cases.

With few exceptions, these substances have been found to possess antibiotin activity when assayed with L. casei or yeast. In general, the cyclohexane derivatives are shown to be more potent as biotin antagonists than the corresponding benzene compounds. When geometric isomers can be separated, two different forms are found to have approximately equal antibiotin activity.

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<sup>(42)</sup> Hertz, Proc. Soc. Exptl. Biol. Med., 52, 15 (1943).

<sup>(43)</sup> Wright and Skeggs, ibid., 56, 95 (1944).