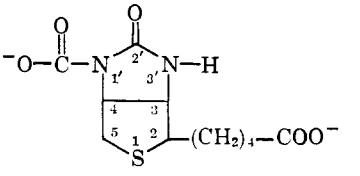
Structure of the Bis-*p*-bromoanilide of Carbon Dioxide Biotin*

Claudie Bonnemere,† Jean A. Hamilton, L. K. Steinrauf, and J. Knappe

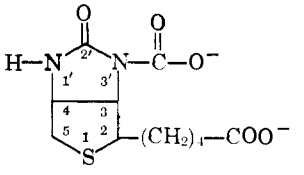
ABSTRACT: The structure of the di-*p*-bromoanilide of CO_2-biotin has been determined by crystallographic analysis. The CO_2 has attached to the biotin on the nitrogen atom farthest from the valeric acid side chain.

The structure was found to have three possible hydrogen bonds, and to have close packing of nearly parallel planar ring systems.

The mechanism of carboxylation and transcarboxylation reactions catalyzed by biotin enzymes involves the intermediary formation of the carboxylated enzymes. The site of CO_2 attachment has recently been investigated in various cases by degrading the intermediates after esterification of the carboxyl group with diazomethane. After digestion with pronase or with trypsin and papain, ϵ-*N*-(methoxycarbonyl biotinyl)-lysine has been obtained, which released methoxycarbonyl biotin after digestion with hog kidney biotinidase (Knappe *et al.*, 1963; Lane and Lynen, 1963; Wood *et al.*, 1963; Numa *et al.*, 1964). Three possible structures might be considered for the combination of CO_2 with biotin:



I



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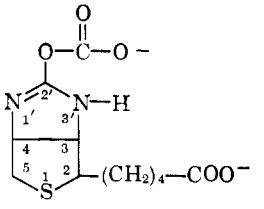

III

From the reaction of biotin methyl ester with methyl chloroformate (Knappe *et al.*, 1961), two products, C_I and C_{II}, were obtained in a relative abundance of 100:7. These were shown to be isomers of methoxycarbonyl biotin methyl ester, and compound C_I was found to be identical to the dimethyl ester of CO_2-biotin obtained from biological systems. It was postulated that the nearness of the valeric acid side group would prove a hinderance to any reaction involving the 3′ position. Therefore the lesser abundant isomer C_{II} was identified with structure II, and the more abundant C_I with structure I. Thus the naturally occurring CO_2-biotin was assumed to be represented by structure I. However, direct and unequivocal evidence has been lacking. It is intended to provide such evidence in this paper by the X-ray analysis of the *p*-bromoanilide.

Materials and Methods

Chemical Synthesis. *N*-Methoxycarbonyl biotin methyl ester (750 mg), mp 132°, isomer C_I (Knappe *et al.*, 1961) was treated with 50 mmoles *p*-bromoanilidomagnesium iodide in 200 ml of methylene chloride. The mixture was refluxed for 4 hours, then ice and aqueous HCl were added. The aqueous layer was extracted several times with ethyl acetate, and this extract was combined with the organic phase and dried over sodium sulfate. The residue obtained after evaporating the solvent to dryness was dissolved in warm ethyl acetate and filtered through a column containing 30 g of Al_2O_3. The filtrate, combined with the ethyl acetate washings of the column, gave 680 mg of slightly colored crystals. These were recrystallized from 100 ml of propanol with decolorizing carbon and gave 650 mg of crystals, mp 226–227°.

Anal. Calcd. for $C_{23}H_{24}O_3N_4SBr_2$ (mw 596): C, 46.32; H, 4.06; N, 9.40; Br, 26.80; S, 5.38. Found: C, 46.25; H, 4.32; N, 9.02; Br, 26.44; S, 4.99.

The ultraviolet spectrum ($\epsilon^{max}_{252m\mu}$ = 51,500 M^{-1} cm^{-1} measured in propanol) corresponded closely to the sum of the spectra of *N*-acetyl-*p*-bromoaniline and *N*-*p*-bromocarbanilidoethyleneurea.

Crystallographic Data. Small white prismatic crystals elongated along *c* were obtained for the X-ray analysis

* From the Chemistry Department, University of Illinois, Urbana (C.B., J.A.H., and L.K.S.), the Department of Biochemistry, Indiana University Medical Center, Indianapolis (J.A.H. and L.K.S., present address), and the Organisch-Chemisches Institut, Heidelberg University, Germany (J.K.). *Received September 28, 1964.* Supported by grants (AM-06198 and AM-0834) from the National Institutes of Health.

† Present address, Apt. 405, Residence Universitaire, Antony, Seine, France.

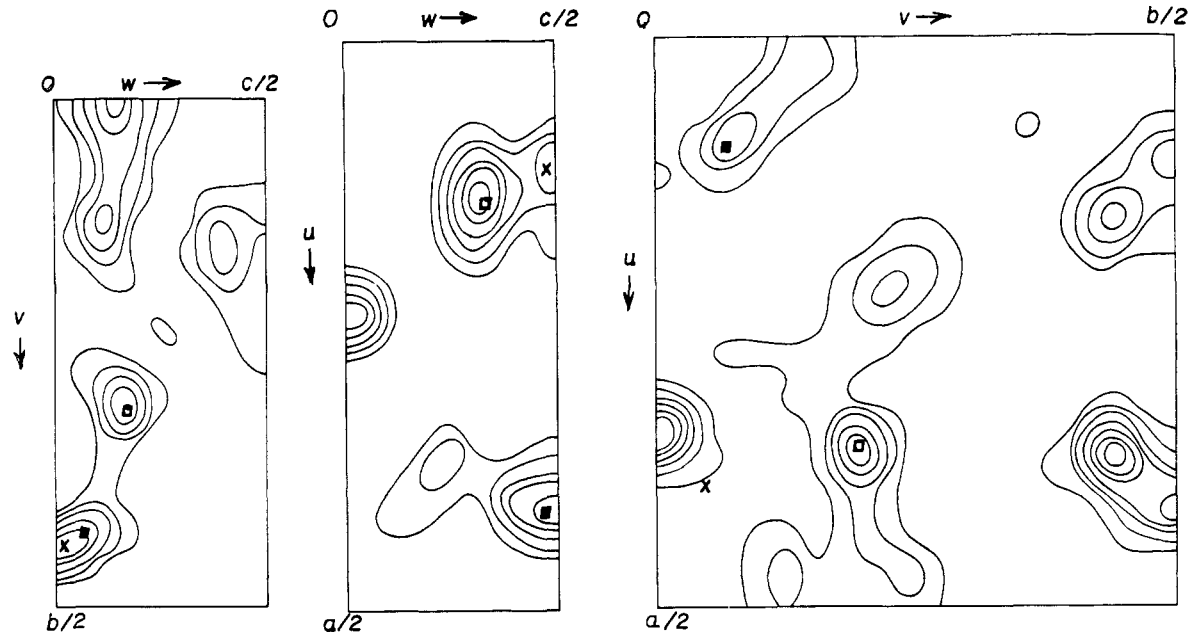

FIGURE 1: The three Harker sections at (a) $\frac{1}{2}a$, (b) $\frac{1}{2}b$, and (c) $\frac{1}{2}c$, respectively. Contours are at arbitrary intervals. The vectors from heavy atoms are indicated by □ for Br1, ■ for Br2, and × for S. The positions indicated are those calculated from the final coordinates in Table I.

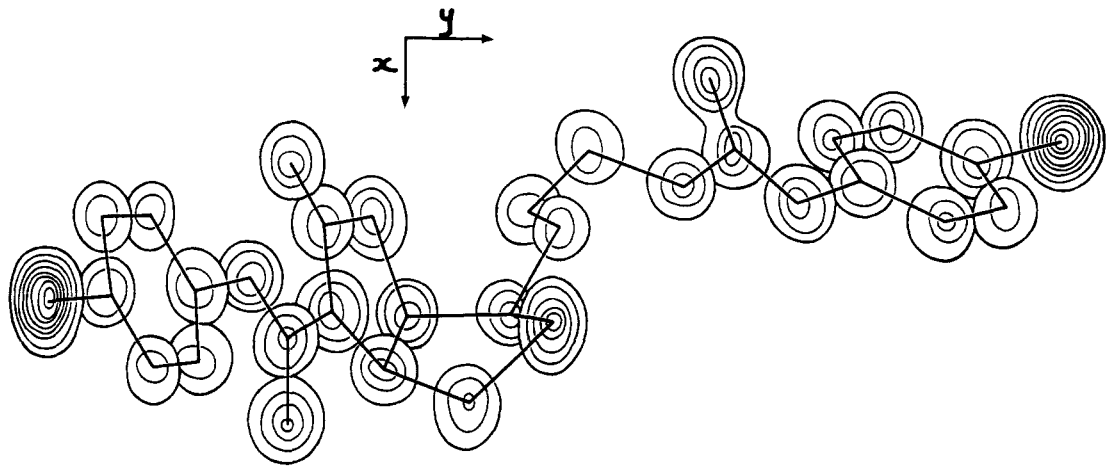

FIGURE 2: A view of the final electron density synthesis looking down the *z* axis. Contour intervals for the bromine sulfur, and lighter atoms are 4, 3, and 2 electrons per cubic Angstrom, respectively.

by recrystallization from dimethylformamide. Precession photographs with molybdenum radiation showed the crystal to be orthorhombic with unit cell dimensions: $a = 19.34 \pm 0.01$ A, $b = 17.23 \pm 0.02$ A, $c = 7.04 \pm 0.01$ A. Systematic absences were observed for the odd numbers of the $h00$, $0k0$, and $00l$ reflections. This requires the space group to be $P2_12_12_1$ (D_2^4), and to have four molecules per unit cell. From the size of the unit cell, the number of molecules, and the molecular weight, the density of the crystals may be calculated to be 1.688 g/cc, in reasonable agreement with the experimental value of 1.65 ± 0.05 g/cc obtained from flotation using bromoform-chloroform mixtures.

No special precautions were taken in the data collection, since we were interested only in the elucidation of the structure. Three-dimensional X-ray data were obtained from visual estimation of nonintegrated Weissenberg photographs using filtered copper radiation. No corrections were made for absorption. From a crystal rotated about the *c* axis the reflections for the $hk0$ through $hk4$ levels were recorded. A second crystal rotated about the *a* axis produced data for the $0kl$ through $6kl$ levels. These two data sets were then scaled together. Out of a total of about 2700 reflections possible, 2254 were recorded.

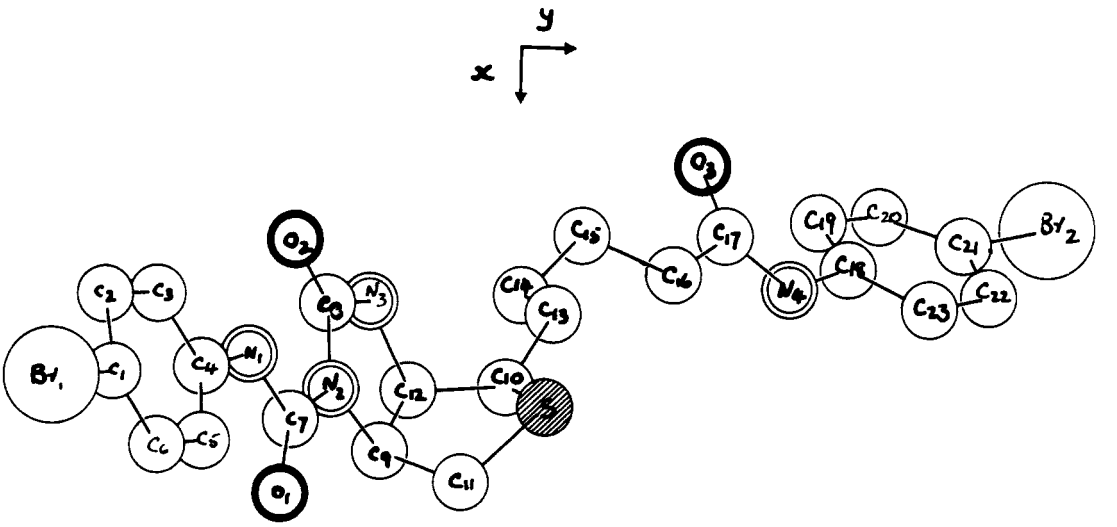

FIGURE 3: A view down the z axis of a ball and stick model with the numbering system for the atoms superimposed.

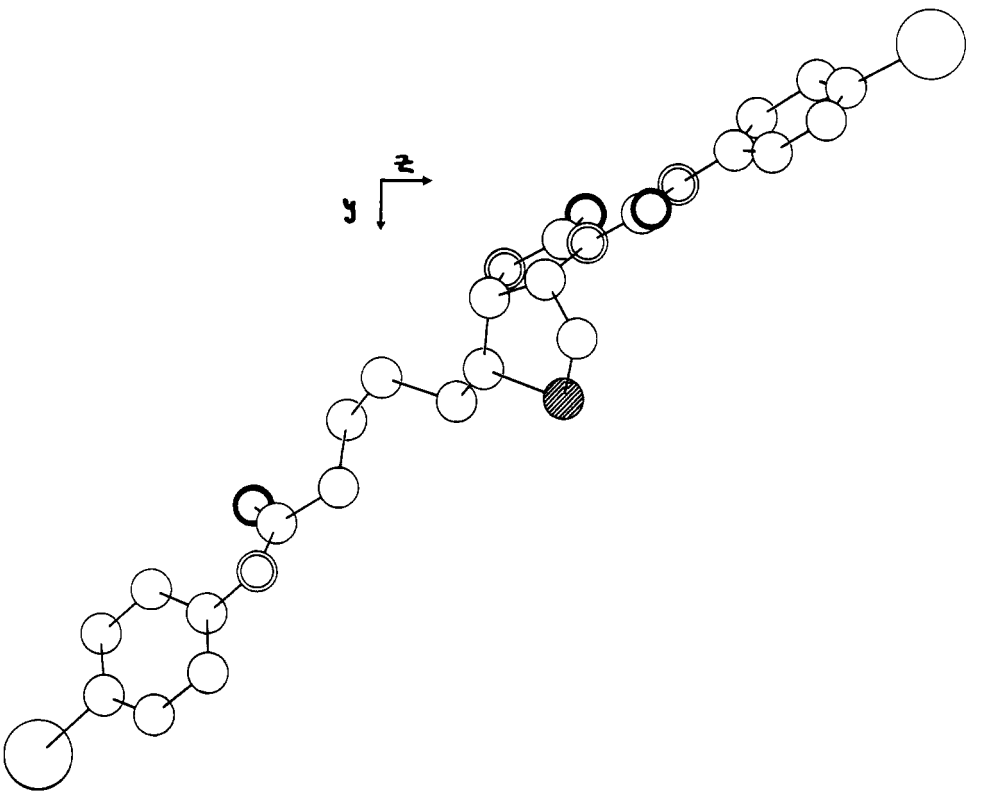

FIGURE 4: A view down the x axis.

Results and Discussion

Determination of the Structure. The structure factor amplitudes obtained after the application of Lorentz and polarization corrections were used to calculate a three-dimensional Patterson synthesis. From inspection of the Harker sections, Figure 1, the approximate coordinates of either bromine atom could be deduced immediately. We then used the coordinates of one of the bromine atoms, calculated the coordinates of the same bromine atom in the three other symmetry-related molecules of the unit cell, and used these four positions to calculate a 4-fold Patterson superposition synthesis. This synthesis clearly showed the position of the other bromine atom in the molecule as well as the sulfur atom. The orientation of the benzene rings could be seen, but the individual atoms were not re-

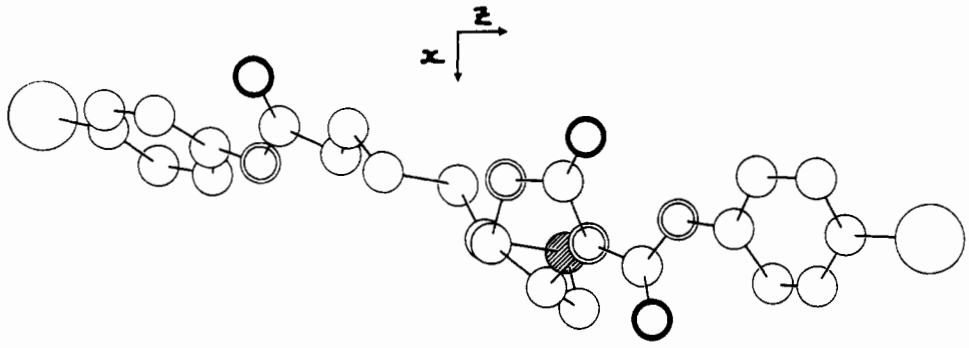

FIGURE 5: A view of the model down the y axis. Note the boat configuration made by the ureido and thio rings.

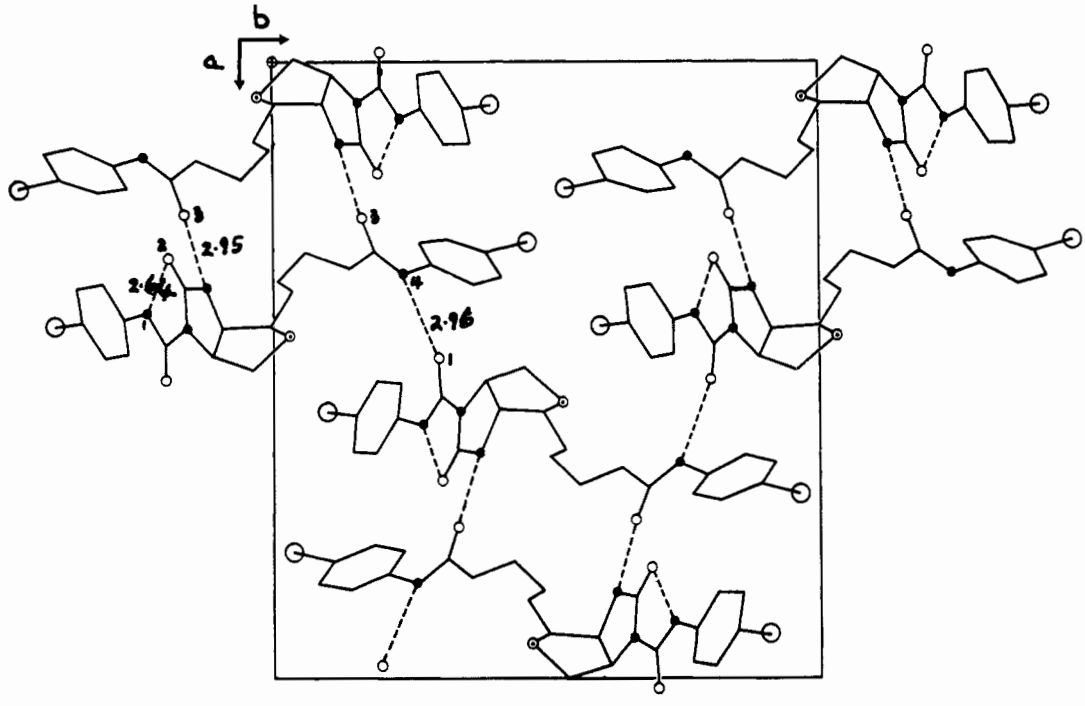

FIGURE 6: Packing diagram looking down the z axis. Hydrogen bonds are indicated by dotted lines.

solved. The lighter atoms were obscured because some false symmetry was produced from the nearness of some of the coordinates of the heavy atoms to special positions (0 or $^1/_2$). This caused no difficulty in recognizing the bromine or sulfur positions, however, and since each of the heavy atoms was near a different special position, a combination of all three heavy atoms should be free of false symmetry. Therefore an electron density synthesis was computed based on three heavy atoms per molecule. The value of R for this was 0.437, where R is $\Sigma||F_o| - |F_c||/\Sigma|F_o|$, the conventional expression for the fit between the observed structure amplitudes F_o, and those calculated F_c from the assumed model of the molecule. This synthesis showed quite clearly the locations of all the atoms, except hydrogen, in the molecule. The structure was refined by four cycles of difference syntheses, at the end of which $R = 0.223$. Finally six cycles of least squares were run and the R was reduced to 0.165.

The final value of R was obtained by allotting the bromine and sulfur atoms individual anisotropic temperature parameters and the carbon, oxygen, and nitrogen atoms individual isotropic parameters, while the hydrogen atoms were not included. All unobserved reflections were included at threshold value. A listing of the final values of the 2254 calculated and observed structure factors may be obtained on request from the Department of Biochemistry, Indiana University Medical Center, Indianapolis.

Description of the Structure. A final electron density synthesis was calculated at the end of the refinement. This is shown in Figure 2 as viewed down the z axis. Figure 3 shows the corresponding ball and stick model of the molecule with our own numbering system

superimposed. Figures 4 and 5 show the molecule along the other two principal axes, y and z. Figure 6 is a packing diagram along the z axis. The positional and temperature parameters are listed in Table I, and the more important bond distances and angles in Tables II and III. From the residuals in the least-squares refinement, the standard deviations in the bond lengths have been calculated to be 0.04 A for those bonds involving only C, N, and O, and 0.03 A for those bonds which involve a bromine or a sulfur atom.

TABLE I: Atomic Coordinates and Temperature Factors.

Atom	x/a	y/b	z/c	B
Br1	0.4291	0.5978	0.5875	5.8[a]
Br2	0.2956	0.4650	0.9652	6.0[a]
S	0.4453	0.0305	0.5100	4.4[a]
O1	0.5181	0.8009	0.7625	4.7
O2	0.3188	0.8077	0.5747	4.5
O3	0.2540	0.1626	0.5942	4.4
N1	0.4083	0.7711	0.8429	3.3
N2	0.4343	0.8426	0.5786	3.4
N3	0.3648	0.8762	0.3341	3.7
N4	0.3489	0.2401	0.6126	4.7
C1	0.4233	0.6517	0.3376	4.1
C2	0.3649	0.6421	0.2551	4.2
C3	0.3622	0.6883	0.0801	4.9
C4	0.4193	0.7281	0.0143	4.5
C5	0.4791	0.7317	0.1235	4.0
C6	0.4837	0.6907	0.2826	4.2
C7	0.4589	0.8048	0.7388	3.3
C8	0.3668	0.8369	0.5056	3.9
C9	0.4826	0.8875	0.4522	3.2
C10	0.4308	0.9958	0.2651	2.7
C11	0.5059	0.9579	0.5505	3.9
C12	0.4352	0.9099	0.2855	2.7
C13	0.3724	0.0351	0.1923	3.4
C14	0.3600	0.0062	0.9692	4.1
C15	0.3093	0.0584	0.8606	4.4
C16	0.3388	0.1391	0.8450	3.7
C17	0.3079	0.1823	0.6642	3.2
C18	0.3304	0.2926	0.4600	3.3
C19	0.2946	0.2646	0.2932	3.5
C20	0.2874	0.3172	0.1455	3.0
C21	0.3112	0.3907	0.1596	3.5
C22	0.3492	0.4169	0.3090	3.7
C23	0.3598	0.3640	0.4666	3.7

[a] These three atoms showed anisotropic vibration in the direction of the z axis. The three were assigned the following anisotropic temperature parameters from the least squares refinement:

Atom	β_{11}	β_{22}	β_{33}
Br1	0.00593	0.00499	0.01322
Br2	0.00415	0.00563	0.02652
S	0.00422	0.00343	0.01452
	β_{12}	β_{13}	β_{23}
Br1	−0.00143	−0.00128	0.00374
Br2	−0.00095	−0.00839	0.00858
S	0.00013	−0.00326	−0.00081

TABLE II: Bond Lengths in Angstrom Units.

Br1-C1	1.96	C5-C6	1.31
Br2-C21	1.88	C9-C11	1.46
S-C10	1.81	C9-C12	1.52
S-C11	1.71	C10-C12	1.47
O1-C7	1.16	C10-C13	1.44
O2-C8	1.16	C13-C14	1.67
O3-C17	1.21	C14-C15	1.46
N1-C4	1.41	C15-C16	1.49
N1-C7	1.33	C16-C17	1.58
N2-C7	1.39	C18-C19	1.43
N2-C8	1.37	C18-C23	1.35
N2-C9	1.48	C19-C20	1.38
N3-C8	1.40	C20-C21	1.35
N3-C12	1.50	C21-C22	1.35
N4-C17	1.31	C22-C23	1.45
N4-C18	1.43		
C1-C2	1.31	Hydrogen Bonds	
C1-C6	1.37		
C3-C4	1.38	N3-O3	2.95
C2-C3	1.46	O2-N1	2.64
C4-C5	1.37	N4-O1	2.96

It is now quite clear that the isomer of CO_2-biotin which we have obtained in this structure analysis is that of structure I, and therefore CO_2 must attach to the 1′-N of biotin in biological reactions.

Perhaps the only unexpected feature in the structure is the fully extended form of the molecule. Indeed, the distance from Br1 to Br2 (from now on using the numbering in Figure 3) within the molecule is 23.9 A. Each molecule forms intermolecular hydrogen bonds to four neighbors as shown in Figure 5. The network made by the hydrogen bonds from N4 to O1 is seen clearly. In this projection it seems that the hydrogen bond from N3 to O3 links two molecules together in a dimer. Actually the molecules above and below are involved and this network forms a spiral up through the crystal structure. There is probably a third hydrogen bond, intramolecular this time, from N1 to O2 forming a six-membered, slightly bent ring. Except for the hydrogen, which cannot be located accurately, the atoms of this ring deviate from a common plane by an average of 0.41 A. Four other ring systems appear in the molecule. The atoms of the lower-numbered benzene ring deviate on the average 0.023 A from the common plane, those of the higher-numbered benzene ring by 0.033 A, those of the ureido ring by 0.002 A, and those of the thio ring by 0.015 A. In the thio ring the sulfur atom was found to be 0.733 A out of the plane; however this was to be expected and the sulfur was not included in the averages given above. If one were to think of the thio ring as being the bottom of a boat, the sulfur atom is the stern making a plane with C10 and C11 that comes up at 37.6° to the bottom of

TABLE III: Bond Angles.

C10-S-C11	91°	C11-C9-C12	109°
C4-N1-C7	126°	C12-C10-C13	123°
C7-N2-C8	125°	S-C10-C12	105°
C7-N2-C9	122°	S-C10-C13	108°
C8-N2-C9	113°	S-C11-C9	109°
C8-N3-C12	110°	N3-C12-C9	105°
C17-N4-C18	124°	C9-C12-C10	110°
Br1-C1-C2	114°	N3-C12-C10	111°
Br1-C1-C6	118°	C10-C13-C14	110°
C2-C1-C6	129°	C13-C14-C15	114°
C1-C2-C3	111°	C14-C15-C16	113°
C2-C3-C4	122°	C15-C16-C17	114°
C3-C4-C5	118°	O3-C17-C16	121°
N1-C4-C3	115°	N4-C17-C16	113°
N1-C4-C5	126°	O3-C17-N4	126°
C4-C5-C6	122°	N4-C18-C19	120°
C1-C6-C5	118°	N4-C18-C23	117°
O1-C7-N1	126°	C19-C18-C23	121°
N1-C7-N2	115°	C18-C19-C20	116°
O1-C7-N2	119°	C19-C20-C21	123°
O2-C8-N2	129°	C20-C21-C22	122°
N2-C8-N3	109°	Br2-C21-C20	123°
O2-C8-N3	123°	Br2-C21-C22	115°
N2-C9-C12	103°	C21-C22-C23	118°
N2-C9-C11	110°	C18-C23-C22	120°

the boat, the ureido ring is the bow of the boat coming up at 62.0°, and the valeric acid chain is an outrigger coming up and away to the right.

It is quite possible that these parallel ring systems make a significant contribution toward the stability of the crystal structure. The lower-numbered benzene ring is only 5.5° from being parallel to the ureido ring of the next molecule along in the *z* direction. The average distance between the two rings is 3.46 A, which is quite similar to the 3.2 A distance between the planes of graphite. The higher-numbered benzene ring and the thio ring are 22.2° away from being parallel to each other and are not nearly so close; the average separation is 4.3 A.

The results obtained from this structure analysis are in good agreement with those of Traub (1956; W. Traub, personal communication, 1964), who has recently completed a somewhat more thorough structure determination of the unsubstituted biotin.

Green (1962) has obtained spectroscopic evidence for the participation of tryptophan residues in the binding of biotin by avidin, a protein from egg white. Perhaps there is an association between the planar rings of biotin and the planes of the tryptophan residues of avidin. If so, the binding to avidin would be reduced by any change to the biotin that would disrupt or cause the planar parts of the molecule to become less planar. This would be consistent with the further observation by Green (1963) that the binding could be reduced by either disrupting the ureido ring of biotin or by oxidation of the tryptophan residues of avidin.

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