materials, the indazole oxide was obtained in 15% yield as yellow, cottony needles, m.p. 202–203° from ethanol.

Anal. Calcd. for $C_{16}H_{13}N_3O_2$: C, 68.81; H, 4.69; N, 15.05. Found: C, 69.11; H, 4.66; N, 15.11.

4'-Ethoxyazoxybenzene-2'-carboxylic Acid (VII, R = H, R' = OC_2H_5).—The oxidation of the above indazole oxide by the usual method afforded a 20% yield of small yellow spears of the acid, m.p. 134–135°, after recrystallization from heptane.

Anal. Calcd. for $C_{15}H_{14}N_2O_4$: C, 62.92; H, 4.93; N, 9.79. Found: C, 62.60; H, 5.21; N, 9.80.

4'-Ethoxyazoxybenzene.—Decarboxylation of the acid was accomplished by the usual procedure. A 50% yield of yellow flakes, m.p. 76–76.5° after two recrystallizations from hexane, was obtained. A 50–50 mixture of this compound with 4-ethoxyazoxybenzene melted at 47–64°.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY OF THE OHIO STATE UNIVERSITY]

Configuration of the Glycosidic Unions in Streptomycin[1]

BY M. L. WOLFROM, M. J. CRON,[2] C. W. DEWALT[2] AND R. M. HUSBAND[2]

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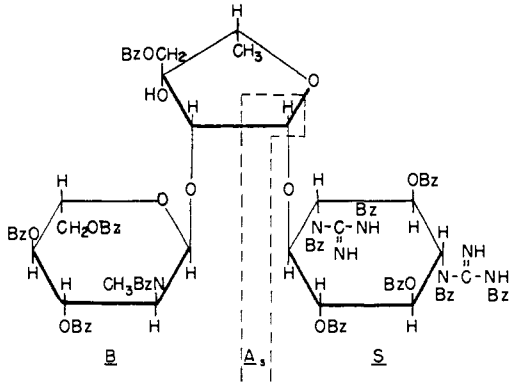
Measurements of the optical rotation of an amorphous but chromatographically homogeneous preparation of dodecabenzoyl- (and acetyl)-dihydrostreptomycin and of that of a crystalline methyl pentabenzoyldihydrostreptobiosaminide, together with other rotatory data, allow calculations to be made which demonstrate that the streptose-streptidine linkage in streptomycin is in all probability β-L and that the hexosamine-streptose linkage is in all probability α-L. It is pointed out that the hydroxyls in streptidine on C-4 and C-6, one of which is involved in the streptose linkage, are not sterically equivalent; this configurational point in the streptomycin molecule remains to be elucidated.

The anomeric structure of the streptidine–streptose linkage in streptomycin was calculated to be of the α-L-type[1a] on the assumption, at that time reasonable, that a symmetrical point of attachment (C-5, see IV) of the aglycon streptidine, a *meso*-form, was such as to maintain its optical inactivity. It has since been determined[3] that the streptidine aglycon is unsymmetrically attached (C-4 or C-6). It therefore follows that the streptidine moiety is optically active by substitution. This requires a revision in the above calculation.

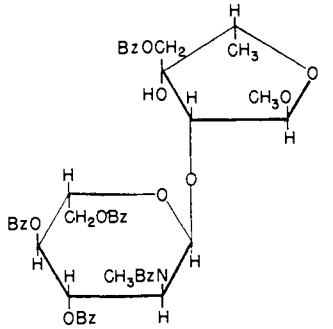
Folkers and co-workers[4] have recorded the rotation for an amorphous dodecabenzoyldihydrostreptomycin (I).



I, Dodecabenzoyldihydrostreptomycin

This preparation was repeated in our laboratories, under milder acylating conditions, with the result that a product with a somewhat higher rotation was obtained. The corresponding acetyl derivative was also prepared. The molecular rotation, [M] = (1833) (+69°) = +126,500,[5] of the benzoate should be the sum of the component parts *S*, A_s and *B*, wherein *S* can be considered to approximate closely the molecular rotation [(991) (+58°) = +57,500] of heptabenzoylstreptidine,[4] A_s is the rotatory contribution of the streptose glycosidic carbon in the streptidine–streptose linkage, and *B* that of the remainder of the benzoylated dihydrostreptomycin, whence (A_s + *B*) = +69,000.

Methanolysis of dihydrostreptomycin with subsequent benzoylation yielded a crystalline methyl pentabenzoyldihydro-L-streptobioseaminide (II) wherein [*M'*] = (874) (−10°) = −8,700. In this glycoside the aglycon, CH_3OH, is optically inactive and should the glycosidic carbon have the same configuration as in streptomycin, then its molecular rotation, A'_s + *B*, should closely approximate that of A_s + *B* = +69,000. Since this is not the case, these glycosides are anomeric with II being the α-L-form on the Hudson classification[6] (it is known that streptose is an L-sugar[7]). In this approximation the size of the aglycon CH_3OH is hardly com-



II, Methyl pentabenzoyldihydro-α-L-streptobioseaminide

(1) Preliminary communications: (a) R. U. Lemieux, C. W. DeWalt and M. L. Wolfrom, THIS JOURNAL, **69**, 1838 (1947); (b) M. L. Wolfrom, M. J. Cron and R. M. Husband, Abstracts Papers Am. Chem. Soc., **118**, 7R (1950); (c) M. L. Wolfrom, paper presented at the Symposium on Antibiotics and Vitamins, Cleveland, Ohio, Meeting, Am. Assoc. for the Advancement of Science, Dec. 29, 1950.

(2) Bristol Laboratories Research Associate (R. M. H.) and Research Fellow of The Ohio State University Research Foundation (Project 224).

(3) F. A. Kuehl, Jr., R. L. Peck, C. E. Hoffhine, Jr., Elizabeth W. Peel and K. Folkers, *ibid.*, **69**, 1234 (1947); F. A. Kuehl, Jr., R. L. Peck, C. E. Hoffhine, Jr., and K. Folkers, *ibid.*, **70**, 2325 (1948).

(4) R. L. Peck, F. A. Kuehl, Jr., C. E. Hoffhine, Jr., Elizabeth W. Peel and K. Folkers, *ibid.*, **70**, 2321 (1948).

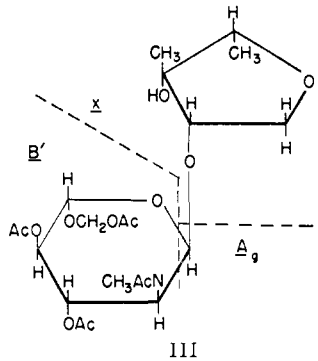
(5) All rotations are recorded in chloroform solution at 25 ± 5° with $c < 5$ and λ = 5892.5 Å.

(6) C. S. Hudson, THIS JOURNAL, **31**, 66 (1909).

(7) J. Fried, Doris E. Walz and O. Wintersteiner, *ibid.*, **68**, 2746 (1946).

parable with that of a cylohexane derivative. However, Hudson[6] has shown that the molecular rotations of glycosides, homologous in the aglycon portion, are enhanced with an increase in the molecular size of the aglycon. The value of A_{OCH_3} for the acetylated methyl D-glucopyranosides is +26,950[8] while that of $A_{OC_6H_{12}}$ for the acetylated cyclohexyl-D-glucopyranosides is +31,300.[9] This group size effect is then favorable to the above calculation and would enhance the value of +69,000. Thus the configuration, A_s, of the streptose glycosidic carbon in the streptomycin molecule is in all probability β-L. The Hudson rules of isorotation[6] are not mathematically rigid but have proved in the past to be entirely valid for assigning anomeric configurations. The above calculations are rendered somewhat hazardous in that they involve benzoate rather than acetate groups but are favored in that streptose is configurationally *trans*, rather than *cis*, on the two centers immediately adjoining its glycosidic carbon.

The molecular rotation of tetraacetyldidesoxydihydro-L-streptobiosamine (III) was established as $[M'']$ = (475.5)(−86°) = −40,900.[10,11] This



III

Tetraacetyldidesoxydihydro-L-streptobiosamine (α-L-anomer)

value may be expressed as $[M''] = A_g + B' + x$, wherein A_g is the rotatory contribution of C-1 of the acetylated N-methyl-L-glucosamine component, B' that of the remainder of the N-methyl-L-glucosamine portion and x that of the optically active didesoxystreptose moiety. The value of x is not known, but it can be assumed to be of low magnitude comparable with the low value (+4,200) exhibited by didesoxydihydrostreptose,[12] in which case

$$A_g + B' = -45{,}100 \qquad (1)$$

Applying the isorotation rules of Hudson[6] to the known anomers of pentaacetyl-N-methyl-L-glucosamine (pyranoid), we find

$$[M]_\alpha = A + B' = (403)(-102°) = -41{,}100^{13,14}$$

$$[M]_\beta = -A + B' = (403)(-16.5°) = -6{,}600^{14,15}$$

whence B' = −23,850 and A = −17,250. The latter value does not differ significantly (opposite sign) from that found for the analogous derivatives of D-glucosamine (A = +18,000)[8] and of D-glucose (A = +19,000)[8]; thus the substitution of an amino or of an N-methylamino group for the hydroxyl group in C-2 of glucose has little effect upon the rotation of C-1. Substituting the value −23,850 in (1) above, A_g = −21,250. This value falls in line with those calculable for a glucose glycosidic bond situated between two optically active units (Table I). Its negative sign and the known fact that the hexosamine component of streptomycin is an L-sugar,[13] indicates that the glycosidic bond joining the N-methyl-L-glucosamine component to streptose is in all probability α-L. This result is verifiable by an alternative calculation. The value −45,100 of equation (1) should approximate that of the anomeric form of pentaacetyl-N-methyl-2-amino-2-desoxy-L-glucopyranose which has the same configuration on C-1 as that found in C-1 of the hexosamine component of streptomycin. As stated previously, both of these anomers are known and the molecular rotation of the α-L-form is indeed −41,100.

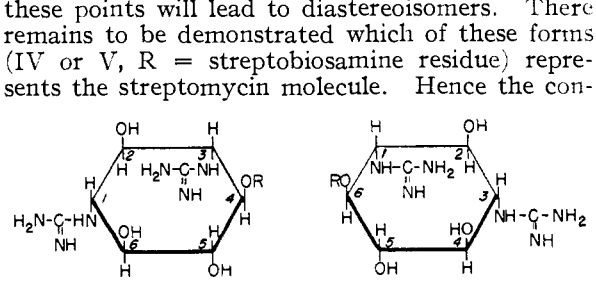
TABLE I

ROTATORY VALUE (A) OF A D-GLUCOPYRANOSE GLYCOSIDIC BOND BETWEEN TWO OPTICALLY ACTIVE UNITS

Substance	$[M]^{20}D$ (c < 5, $CHCl_3$)[a]	A
α-D-Maltopyranose octaacetate	+83,000°	27,600
α-D-Cellobiopyranose octaacetate	+27,800	
β-D-Maltopyranose octaacetate	+42,500	26,200
β-D-Cellobiopyranose octaacetate	−9,900	
Methyl heptaacetyl-β-D-maltopyranoside	+34,900	25,700
Methyl heptaacetyl-β-D-cellobiopyranoside	−16,500	
Heptaacetyl-α-D-maltopyranosyl chloride	+104,100	28,150
Heptaacetyl-α-D-cellobiopyranosyl chloride	+47,800	
levo-Menthyl α-D-glucopyranoside	+20,400[b]	25,100
levo-Menthyl β-D-glucopyranoside	−29,800[b]	

[a] Data from ref. 8. [b] Measured in ethanol solution.

It is to be noted that positions 4 and 6 (IV and V) of the streptidine molecule are not configurationally equivalent and that substitution of the same optically active moiety for the hydroxyl hydrogens at these points will lead to diastereoisomers. There remains to be demonstrated which of these forms (IV or V, R = streptobiosamine residue) represents the streptomycin molecule. Hence the con-



(R optically active)

(8) C. S. Hudson, *Sci. Papers Bur. Standards*, **21**, No. 533, 241 (1926).

(9) E. Pacsu, THIS JOURNAL, **52**, 2568 (1930).

(10) F. A. Kuehl, Jr., E. H. Flynn, N. G. Brink and K. Folkers, *ibid.*, **68**, 2096 (1946).

(11) I. R. Hooper, L. H. Klemm, W. J. Polglase and M. L. Wolfrom, *ibid.*, **68**, 2120 (1946); *ibid.*, **69**, 1052 (1947).

(12) N. G. Brink, F. A. Kuehl, Jr., E. H. Flynn and K. Folkers, *ibid.*, **68**, 2405 (1946).

(13) F. A. Kuehl, Jr., E. H. Flynn, F. W. Holly, R. Mozingo and K. Folkers, *ibid.*, **68**, 536 (1946); *ibid.*, **69**, 3032 (1947).

(14) M. L. Wolfrom and A. Thompson, *ibid.*, **69**, 1847 (1947).

(15) H. E. Stavely, O. Wintersteiner, J. Fried, H. L. White and M. Moore, *ibid.*, **69**, 2742 (1947).

figurational elaboration of the formula of streptomycin is not yet complete.

Experimental[16]

Dodecabenzoyldihydrostreptomycin (I).—This compound has been prepared by Folkers and co-workers,[4] who used a higher reaction temperature and isolated the product by means of a chromatographic procedure.

Dihydrostreptomycin sulfate (1.3 g.) was added to a flask containing 10 ml. of pyridine, 10 ml. of benzoyl chloride and 30 ml. of chloroform (dry and alcohol-free). After shaking at room temperature for 24 hr. the solution was poured with stirring into 300 ml. of cold water containing 20 g. of sodium bicarbonate. The aqueous layer was decanted and the chloroform solution was washed successively with 2% sulfuric acid and water. The sirup obtained on solvent removal from the dried chloroform solution was dissolved at 65° in 360 ml. of 95% ethyl alcohol, allowed to cool with stirring and the precipitate which formed was removed by filtration. The filtrate was heated to 65°, 70 ml. of water added, and the mixture was again allowed to cool. The precipitate which formed, 1.33 g., was removed by filtration; $[\alpha]^{25}D$ +59° (*c* 1.34, chloroform). The filtrate was again heated to 65°, 75 ml. of water added, and after allowing to cool with stirring, 0.86 g. of a white amorphous product separated; $[\alpha]^{25}D$ +69° (*c* 2.3, chloroform). This fraction was reprecipitated from chloroform with petroleum ether (b.p. 30–60°), filtered and dried. The specific rotation did not change.

Anal. Calcd. for $C_{21}H_{29}N_7O_{12}$ $(C_6H_5CO)_{12}$: C, 68.81; H, 4.90; N, 5.35. Found: C, 69.08; H, 4.82; N, 5.48.

A 4-mg. sample of this material in 2 ml. of benzene was added at the top of a 100 × 14 mm. (diam.) column (adsorbent dimensions) of Silene[17]–Celite[17] (5:1 by wt.) which had been pretreated with 20 ml. of 6% *t*-butyl alcohol in benzene. The chromatogram was developed with 8 ml. of the same solution. Ultraviolet light or an alkaline permanganate streak[18] on the extruded column showed only one zone 74–80 cm. from the top.

Dodecaacetyldihydrostreptomycin.—Dihydrostreptomycin (2.8 g.) was dissolved in 75 ml. of dry methanol and 15 ml. of pyridine was added. With vigorous stirring, 25 ml. of acetic anhydride was added in 2 ml. amounts over a period of 20 min. After standing at room temperature for 1 hr., the solution was concentrated under reduced pressure to a white sludge. Ether was added and the white precipitate which formed was recovered by centrifugation, washed twice with ether and dried under reduced pressure. Solution was effected on shaking the dried material with 30 ml. of pyridine and 30 ml. of acetic anhydride. The resultant solution was maintained at room temperature for 40 hr. and was then heated at 50° for 20 hr. On pouring into 500 g. of cracked ice with stirring, 3.2 g. of a yellow precipitate formed. Extraction of the aqueous solution with chloroform afforded an additional 1.0 g. of yellow powder. Extraction of the combined products with benzene (30 ml.) followed by precipitation of the benzene-soluble portion with petroleum ether (300 ml., b.p. 80–90°) yielded a cream colored amorphous powder; yield 3.4 g., $[\alpha]^{25}D$ −59° (*c* 1, chloroform). An 800-mg. portion of this material in benzene was added at the top of a 50 × 20 mm. (diam.) column of Magnesol–Celite (5:1) and the chromatogram was developed with 2 liters of 2% ethanol in benzene. The column was extruded and a 2-cm. fluorescent (with ultraviolet light) band (which also reduced alkaline permanganate[18]) was detected just below a highly fluorescent band at the column top. The 2-cm. zone was eluted with acetone and the product obtained on solvent removal was precipitated from chloroform–petroleum ether as a white, amorphous solid; yield 210 mg., m.p. 153–155° (sintered 130°), $[\alpha]^{22}D$ −67° (*c* 1, chloroform).

Anal. Calcd. for $C_{19}H_{26}N_2O_5(C\text{-}CH_3)(NCOCH_3)_5(OCOCH_3)_7$: C, 49.67; H, 6.02; N, 9.01; C–CH_3, 32.1; mol. wt., 1088. Found: C, 49.81; H, 6.06; N, 8.84; C–CH_3,[19] 31.9; mol. wt. (Rast), 1100.

Methyl Pentabenzoyl-α-L-dihydrostreptobiosaminide (II).—The mixture of methyl anomers of dihydrostreptobiosaminide prepared by methanolysis of a 2.6-g. portion of dihydrostreptomycin sulfate according to the procedure of Brink, Kuehl, Flynn and Folkers,[20] was placed in a flask containing 20 ml. of anhydrous pyridine and 100 ml. of dry alcohol-free chloroform. After chilling to 0°, 5 ml. of benzoyl chloride was added and the mixture was stirred at this temperature for 24 hr. The solution was then poured into 400 ml. of ice and water, the chloroform layer separated, washed successively with dilute hydrochloric acid, water, sodium bicarbonate solution and water and then dried over sodium sulfate. After removal of the solvent, the residue was crystallized from chloroform–petroleum ether; yield 250 mg., m.p. 153–161°.

A 165-mg. portion of this material in 5 ml. of benzene was added at the top of a 190 × 45 mm. (diam.) column of a mixture (95 g.) of Silene[17]–Celite[17] (5:1 by wt.) which had been prewashed with 950 ml. of 6% *t*-butyl alcohol in benzene. The chromatogram was developed with 170 ml. of the same solution. The column was extruded and a zone 133–163 cm. from the top was detected by the alkaline permanganate[18] streak reagent. The sectioned zone was eluted with acetone and the residue which remained on solvent removal was recrystallized from chloroform–petroleum ether; yield 70 mg., m.p. 160–161°, $[\alpha]^{25}D$ −10° (*c* 1.14, chloroform). X-Ray powder diffraction pattern: 4.81[21] − 10,[22] 5.79–9, 3.49–8, 2.83–7, 2.71–6.

Anal. Calcd. for $C_{49}H_{47}O_{14}N$: C, 67.34; H, 5.42; N, 1.60. Found: C, 66.98: H, 5.08; N, 1.83.

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(16) We are indebted to Chas. Pfizer Co., Inc., New York, N. Y., for generously furnishing the dihydrostreptomycin used in this investigation.

(17) L. W. Georges, R. S. Bower and M. L. Wolfrom, THIS JOURNAL, **68**, 2169 (1946).

(18) W. H. McNeely, W. W. Binkley and M. L. Wolfrom, *ibid.*, **67**, 527 (1945).

(19) R. U. Lemieux and C. B. Purves, *Can. J. Research*, **B25**, 485 (1947).

(20) N. G. Brink, F. A. Kuehl, Jr., E. H. Flynn and K. Folkers, THIS JOURNAL, **68**, 2557 (1946).

(21) Interplanar spacing in Å., CuK_α radiation, 1.5418 Å.

(22) Relative intensity, visually estimated; strongest band = 10.