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Antituberculosis Agents—IV.* Some Dihydrostreptomycin Derivatives

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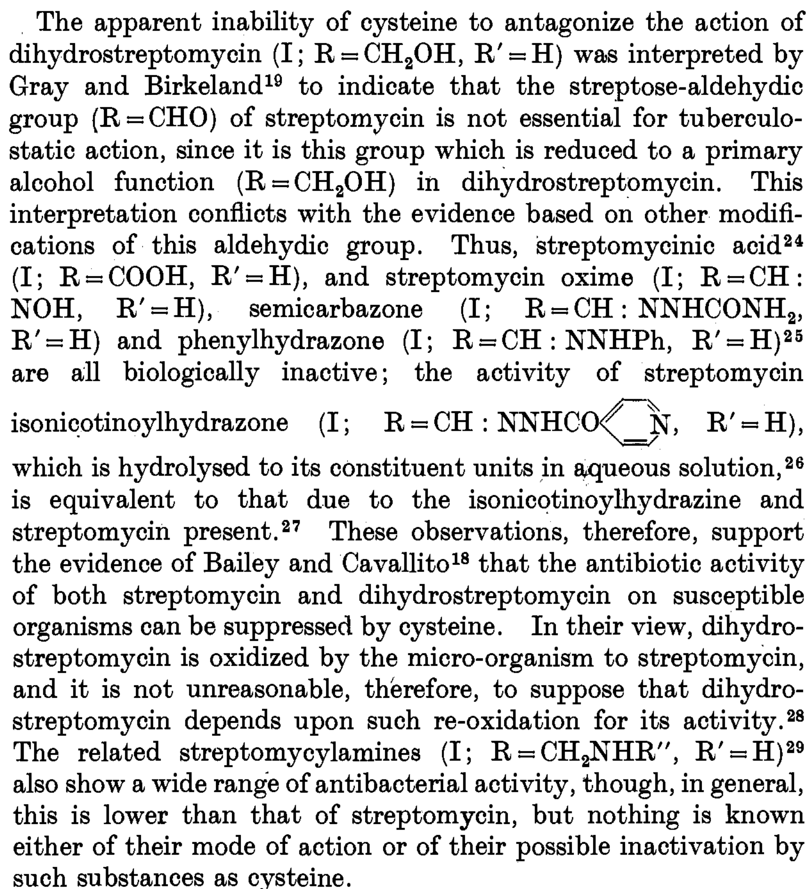
The antibacterial action of streptomycin (I; R = CHO, R′ = H) has been studied widely, and was reviewed in 1951[1] and more recently by Williamson,[2] but the mechanism of its action against *Mycobacterium tuberculosis* is still only incompletely understood. Thus, inhibition of fatty acid oxidation by streptomycin can be traced in *E. coli* to a block in the terminal respiration system at a point which involves a pyruvate–oxalacetate condensation.[3,4] One product of such a metabolic condensation, 2-phospho-4-hydroxycarbonyladipic acid, has been isolated. Its formation is markedly inhibited by streptomycin,[5] and the condensation mechanism is entirely lost in streptomycin-resistant strains. Oxidation of fatty acids in *M. tuberculosis*, on the other hand, occurs by some alternative and unidentified mechanism, which does not involve pyruvate, oxalacetate or other members of the citric acid cycle, and which is only partially inhibited by streptomycin.[6] This accords with the observations of Bernheim[7] on the oxidation of fatty acids by the tubercle bacillus.

In general, young actively-growing cultures are more susceptible to streptomycin than older or resting organisms,[8] and this suggests that streptomycin blocks the synthesis of metabolites essential for growth or cell division. Certain cell constituents such as proteins,[9] nucleoproteins,[10] deoxynucleic acid[10,11] and possibly ribonucleic acid[13] form precipitable streptomycin complexes, and even relatively simple precursors of these substances such as purines and pyrimidines[14] have been reported to antagonize the action of streptomycin against *M. tuberculosis*. The activity of streptomycin is also reduced by inclusion in the culture medium of peptone,[15,16] and the amino acids methionine, cysteine, tyrosine and aspartic acid.[15,17] Of these, antagonism by cysteine is of

* See References.

particular interest, since dihydrostreptomycin is similarly antagonized,[18] despite reports to the contrary.[19, 20]

The inhibition of streptomycin by sulphydryl (—SH) groups was first demonstrated with thioglycollic acid and cysteine.[21] Cavallito[22] has since shown that streptomycin reacts much more readily with sulphydryl compounds which have vicinal amino groups, the affinity of the —SH group for streptomycin being approximately proportional to the separation of the —SH and —NH_2 groups. This explains the failure of cysteine to antagonize streptomycin once the latter has been brought into contact with the bacterial cells.[23]

The apparent inability of cysteine to antagonize the action of dihydrostreptomycin (I; R = CH_2OH, R′ = H) was interpreted by Gray and Birkeland[19] to indicate that the streptose-aldehydic group (R = CHO) of streptomycin is not essential for tuberculostatic action, since it is this group which is reduced to a primary alcohol function (R = CH_2OH) in dihydrostreptomycin. This interpretation conflicts with the evidence based on other modifications of this aldehydic group. Thus, streptomycinic acid[24] (I; R = COOH, R′ = H), and streptomycin oxime (I; R = CH : NOH, R′ = H), semicarbazone (I; R = CH : $NNHCONH_2$, R′ = H) and phenylhydrazone (I; R = CH : NNHPh, R′ = H)[25] are all biologically inactive; the activity of streptomycin isonicotinoylhydrazone (I; R = CH : NNHCON, R′ = H), which is hydrolysed to its constituent units in aqueous solution,[26] is equivalent to that due to the isonicotinoylhydrazine and streptomycin present.[27] These observations, therefore, support the evidence of Bailey and Cavallito[18] that the antibiotic activity of both streptomycin and dihydrostreptomycin on susceptible organisms can be suppressed by cysteine. In their view, dihydrostreptomycin is oxidized by the micro-organism to streptomycin, and it is not unreasonable, therefore, to suppose that dihydrostreptomycin depends upon such re-oxidation for its activity.[28] The related streptomycylamines (I; R = CH_2NHR'', R′ = H)[29] also show a wide range of antibacterial activity, though, in general, this is lower than that of streptomycin, but nothing is known either of their mode of action or of their possible inactivation by such substances as cysteine.

The present work has been concerned with the preparation of derivatives in which the primary alcoholic hydroxyl group R of dihydrostreptomycin (I; $R = CH_2OH$, $R' = H$) is blocked to prevent its ready oxidation by bacilli. Comparison of the tuberculostatic activity of such derivatives should provide a means of substantiating the conclusions of Bailey and Cavallito,[18] and of establishing the importance of the streptose-aldehyde function as a factor contributing to the activity of the molecule as a whole. The problem of obtaining a suitable derivative was beset with numerous synthetic difficulties, not the least of which is the ease with which the streptose–streptidine link in streptomycin and the dihydrostreptose–streptidine link in dihydrostreptomycin is cleaved with acid.[30, 31] A further limitation was also imposed by the known instability of streptomycin which is readily degraded by alkali to maltol.[32] Dihydrostreptomycin, on the other hand, is stable in this respect, but the guanidino groups are unstable to strong alkali, when strepturea dihydrostreptobiosaminide and ultimately streptamine dihydrostreptobiosaminide are formed.[33, 34] The preparation of *N*-acetyltrityldihydrostreptomycin (VI; $R = CH_2OCPh_3$, $R' = H$), therefore, represents a compromise which we recognize departs from ideality in at least two respects. Thus, the trityl ether link is cleaved by hot aqueous acid[35, 36] and probably has no greater stability than that of the streptose–streptidine link. Its stability to alkali, however, permits the easy removal of all protecting acetyl substituents except one, that of the *N*-acetyl-*N*-methylglucosamine fragment. Retention of this group in the final product results from the restriction to mild deacetylation conditions (ammonia-methanol at 0°) imposed by the presence of guanidino substituents. The disadvantage of a test compound in which the *N*-acetyl group is retained on the *N*-methyl-L-glucosamine fragment is perhaps not so serious as would at first appear. Thus, addition of formaldehyde to streptomycin gives the *N*-hydroxymethyl derivative (I; $R = CHO$, $R' = CH_2OH$) which is claimed to exhibit biological activity comparable with that of the parent compound.[37] The choice of *N*-acetyltrityldihydrostreptomycin as the test compound, however, required that it should be compared with *N*-acetyldihydrostreptomycin as well as dihydrostreptomycin.

N-Acetyltrityldihydrostreptomycin (VI; $R = CH_2OCPh_3$) was

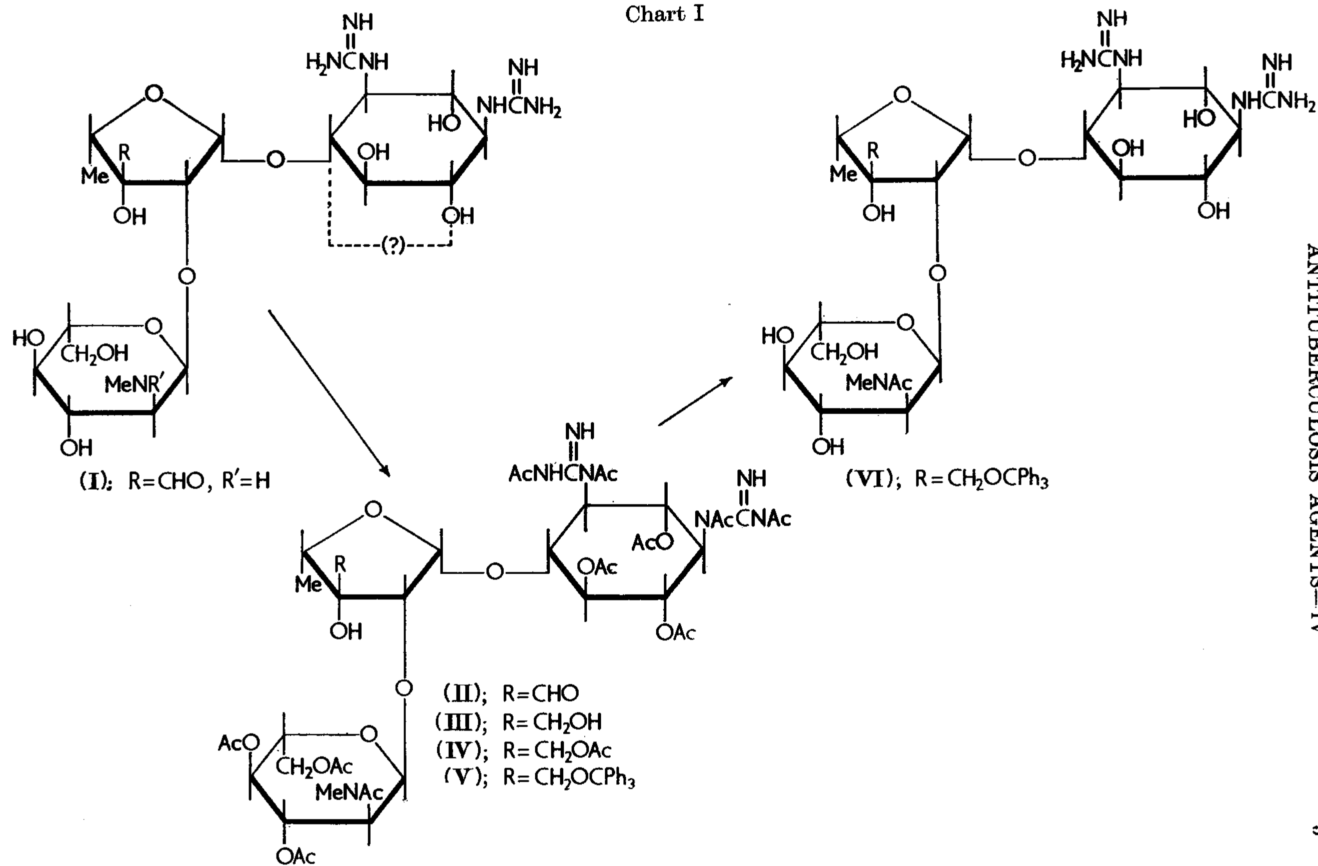
prepared from streptomycin (I; R = CHO, R′ = H) as outlined in Chart I.

Undeca-acetylstreptomycin has been prepared by Wolfrom *et al.*[38] using a two-stage acetylation procedure with acetic anhydride-pyridine, but attempts to apply this method to streptomycin sulphate failed. Addition of fused sodium acetate to the reaction mixture maintained at room temperature for 24 h assisted the removal of sulphate ions and permitted the formation of undeca-acetylstreptomycin in 71 per cent yield. Repeated precipitation from benzene-light petroleum (b.p. 80–100°) gave much higher yields than purification by chromatography.[38]

Attempts to hydrogenate undeca-acetylstreptomycin (II; R = CHO) at a platinum catalyst in methanol or ethanol failed completely, but the aldehyde group was reduced slowly (18 h) when glacial acetic acid was used as the solvent. The catalytic effect of an acidic solvent on the rate of hydrogenation is typical of aliphatic bases.[39,40] The product, undeca-acetyldihydrostreptomycin (III; R = CH_2OH) was characterised by conversion to the known dodeca-acetyldihydrostreptomycin (IV; R = CH_2OAc).

Trityl ethers may be synthesised, (a) from tritanol by reaction with an alcohol in the presence of an acid catalyst,[41] (b) from trityl chloride by reaction with an alkoxide[42] or (c) from trityl chloride and an alcohol. Tritylation of carbohydrates and their acetylated derivatives which are often soluble in dry pyridine is conveniently carried out by the latter method,[43] since the pyridine is then available to assist the removal of hydrogen chloride. The velocity of the reaction is influenced by temperature[44] and can often be accelerated by using an excess of reagent.[45] Reaction of undeca-acetyldihydrostreptomycin in dry pyridine with eight moles of trityl chloride[46] for eight days at room temperature and in the absence of light gave crude undeca-acetyltrityldihydrostreptomycin (V), which on deacetylation with ammonia-methanol at 0° gave the required *N*-acetyltrityldihydrostreptomycin (VI). The product which had m.p. 179–181° and $[\alpha]_D^{21}$ −69·3° gave good hydrogen and nitrogen analyses, but microanalysis for carbon was invariably low. The dipicrate similarly gave good hydrogen and nitrogen, but poor carbon analyses. The ultraviolet absorption maximum of *N*-acetyltrityldihydro-

Chart I



streptomycin at 259 mμ (ε, 975) is typical of the trityl group and in good agreement with that expected by summation of the maximum at 260 mμ of triphenylmethyl chloride (ε, 770) and the absorption at 259 mμ of *N*-acetyldihydrostreptomycin (ε, 240). *N*-Acetylstreptomycin (I; R = CHO, $R' = COCH_3$) and *N*-acetyldihydrostreptomycin (I; $R = CH_2OH$, $R' = COCH_3$) were prepared by deacetylation of undeca-acetylstreptomycin and dodeca-acetyldihydrostreptomycin with ammonia-methanol.

We are indebted to Glaxo Laboratories Ltd. for investigation of the tuberculostatic activity of *N*-acetyltritylstreptomycin and *N*-acetyldihydrostreptomycin against *M. tuberculosis* (human strain 666) in Dubos liquid medium. The results recorded in Tables II and III show that whereas *N*-acetylstreptomycin and *N*-acetyldihydrostreptomycin are somewhat less effective than streptomycin itself, activity is very significantly less in *N*-acetyltrityldihydrostreptomycin. This, then, provides further support for the view that microbiological oxidation of dihydrostreptomycin to streptomycin is a pre-requisite for the activity of the former, and that the streptose-aldehydic group is essential for the activity of the latter. None of the acetylated intermediates showed significant activity.

Experimental*†

Undeca-acetylstreptomycin. Acetic anhydride (50 ml) was added in 5 ml amounts over a period of 50 min, with constant stirring, to streptomycin sulphate (5·1 g) in dry methanol (100 ml) and pyridine (30 ml). Fused sodium acetate (5·24 g) was added and stirring continued for 24 h. The sodium sulphate was filtered off and the filtrate concentrated *in vacuo* below 50° to a semi-solid mass, which was triturated and washed with ether. The product was triturated with dry pyridine (50 ml) and acetic anhydride (50 ml) added in small amounts. The resultant solution was maintained at room temperature for 44 h and then heated to 50° for 5 h. After cooling, the solution was added to crushed ice (750 g) with stirring, and the resulting aqueous solution ex-

* Analyses by Miss M. Buchanan, Mr. W. McCorkindale and Dr. A. C. Syme of this College, and by Drs. Weiler and Strauss, Oxford.

† Melting points are uncorrected.

tracted with chloroform (5 × 50 ml). The chloroform extract was evaporated to a small volume (*ca.* 10 ml) *in vacuo* below 50°, and the syrupy liquid triturated with crushed ice (100 g) to yield a cream coloured solid. A further yield of crude product was obtained by extracting the aqueous liquid with chloroform. The combined products were dissolved in chloroform (50 ml) the solution dried ($CaCl_2$) and the solvent evaporated. The residual semi-solid mass was dissolved in benzene, the solution filtered and poured slowly into light petroleum (b.p. 80–100°). Reprecipitation of the product from benzene-light petroleum gave *undeca-acetylstreptomycin* (5·3 g, 71 per cent) as a colourless solid, m.p. 168–170° (d.), with sintering at 145°, $[\alpha]_D^{21} - 64 \cdot 5°$ (*c*, 1·1 in $CHCl_3$).

Anal. Calcd. for $C_{43}H_{61}N_7O_{23}.2H_2O$: C, 47·8; H, 6·1; N, 9·1. Found: C, 47·9; H, 5·6; N, 9·0.

Undeca-acetyldihydrostreptomycin. Undeca-acetylstreptomycin (1·005 g) in glacial acetic acid was shaken with platinum oxide (0·157 g) and hydrogen at room temperature and atmospheric pressure for 18 h. The solution was filtered and the solvent removed *in vacuo* below 50°, leaving a viscous residue which on trituration with dry ether afforded a white solid (0·95 g). The product, washed thoroughly with dry ether and dried *in vacuo*, gave *undeca-acetyldihydrostreptomycin* as a colourless powder, m.p. 156–158° (d.) with sintering at 130–135°, $[\alpha]_D^{21} - 58 \cdot 4$ (*c*, 1·1 in $CHCl_3$).

Anal. Calcd. for $C_{43}H_{63}N_7O_{23}.2H_2O$: C, 47·7; H, 6·2; N, 9·1. Found: C, 47·1; H, 6·7; N, 9·5.

Dodeca-acetyldihydrostreptomycin. (a) Dihydrostreptomycin sulphate (10·25 g) was acetylated as described in the preparation of undeca-acetylstreptomycin, and gave dodeca-acetyldihydrostreptomycin (11·4 g, 75 per cent), m.p. 152–155° (d.) with sintering at 130°, $[\alpha]_D^{20} - 67 \cdot 6°$ (*c*, 1·086 in $CHCl_3$). Wolfrom, Cron, De Walt and Husband[38] give m.p. 153–155°, $[\alpha]_D^{20} - 67°$.

Anal. Calcd. for $C_{45}H_{65}N_7O_{24}.2H_2O$: C, 48·1; H, 6·2; N, 8·7. Found: C, 48·0; H, 5·9; N, 8·5.

(b) Undeca-acetyldihydrostreptomycin (0·25 g) was treated with acetic anhydride (10 ml) and pyridine (19 ml) at room temperature for 48 h, and then at 50° for 4 h. The solution was concentrated *in vacuo* below 50° to a viscous residue, which on

trituration with dry ether afforded a white amorphous substance. The product was washed thoroughly with dry ether, dried *in vacuo*, dissolved in chloroform (5 ml) and added slowly to petroleum ether (b.p. 60–80°; 50 ml) with stirring. The white amorphous precipitate was dried *in vacuo* to yield, dodeca-acetyldihydrostreptomycin m.p. 152–155° (d.) $[\alpha]_D^{21} - 66 \cdot 9°$ (*c*, 0·88 in $CHCl_3$).

Anal. (After drying for 3 h at 100° *in vacuo*.). Calcd. for $C_{45}H_{65}N_7O_{24}$: N, 9·0. Found: 9·2.

N-*Acetyltrityldihydrostreptomycin.* Undeca-acetyldihydrostreptomycin (3·684 g, 0·0034 mole), dried over phosphorus pentoxide was dissolved in dry pyridine (30 ml) and treated with trityl chloride (7·88 g, 0·028 mole). The resultant solution was kept in the dark for 8 days, when it slowly attained a reddish-brown colour. Pyridine was removed *in vacuo* at room temperature and the residue extracted with ether. The ether-insoluble buff-coloured residue was dissolved in chloroform (50 ml), the solution concentrated to about 20 ml, and cooled to 5° for 2 h when crystals of tritylpyridinium chloride (2·33 g) were collected. The chloroform solution was added slowly to ten times its volume of dry ether, and the precipitate of crude undeca-acetyltrityldihydrostreptomycin (4·6 g) dried *in vacuo*.

The amorphous product was dissolved in dry methanol (20 ml) (previously boiled and cooled to remove carbon dioxide), the solution saturated with dry ammonia at 0°, and then maintained at room temperature for 24 h. The solvent was removed *in vacuo* at room temperature and the residue washed with ether and chloroform. The chloroform-insoluble residue (3·712 g) was dissolved in dimethylformamide (15 ml), the solution filtered from ammonium chloride, and poured into dry ether (150 ml). The product was suspended in ethanol and chromatographed on ethanol-washed alumina (75 g forming a column 30 × 1·9 cm). Elution with ethanol-methanol was followed by measurement of the optical density of each 50 ml fraction at 259 mμ, and gave the four fractions shown in Table I.

Concentration of fraction 2 *in vacuo* below 45° gave O-α-*2*-N-*acetyl*-N-*methylamino-2-deoxy*-L-*glucopyranosyl-(1 → 2)*-O-*β-3*-C-*trityloxymethyl-5-deoxy*-L-*lyxofuranosido-(1 → 4)-1,3-diguanidino-2,4,5,6-tetrahydroxycyclohexane*(N-*acetyltrityldihydrostreptomycin*),

Table I.

Fraction	Solvent	Volume collected (ml)	Weight of product (g)	$E^{1\%}_{1\,cm}$ at 259 mμ in methanol
1	Ethanol	1,000 ml	—	—
2	Methanol–ethanol (1 : 4)	2,100 ml	0·376 g	10·8
3	Methanol–ethanol (1 : 1)	1,250 ml	0·35 g	6·62
4	Methanol–ethanol (4 : 1)	1,150 ml	0·55 g	4·38
5	Methanol	800 ml	0·15 g	3·39

as a colourless solid, m.p. 179–181° (d.) $[\alpha]^{21}_{D} - 69\cdot3°$ (*c*, 1·5 in water).

Anal. Calcd. for $C_{42}H_{57}N_7O_{13}$: N, 11·3. Found: N, 11·25. R_F, 0·16, when chromatographed on Whatman No. 1 paper with *n*-butanol–2·5 per cent aqueous *p*-toluenesulphonic acid, and the spots developed with potassium ferricyanide and sodium nitroprusside solution.[45]

Dipicrate was prepared from an aqueous solution of *N*-acetyltrityldihydrostreptomycin and saturated aqueous picric acid, m.p. 178–180° (from methanol).

Anal. Calcd. for $C_{54}H_{63}N_{13}O_{27}.2H_2O$: N, 13·3. Found: N, 13·3.

N-*Acetyldihydrostreptomycin.* Dodeca-acetyldihydrostreptomycin (1·01 g) was dissolved in dry methanol (10 ml) (previously boiled and cooled to remove carbon dioxide), the solution saturated with dry ammonia at 0°, and then maintained at room temperature for 24 h. The solvent was removed *in vacuo* at room temperature and the residue washed with chloroform. The chloroform-insoluble residue was dissolved in dry methanol (5 ml) and poured into dry ether (50 ml) when N-*acetyldihydrostreptomycin* was obtained as a white precipitate (0·466 g, 82 per cent), m.p. 190–192° (d.) (micro-block), $[\alpha]^{20}_{D} - 91\cdot6°$ (*c*, 1·01 in water).

Anal. Calcd. for $C_{23}H_{43}N_7O_{13}.1\frac{1}{2}H_2O$: C, 42·4; H, 7·1; N, 15·0. Found: C, 42·2; H, 7·1; N, 15·1.

Dipicrate, m.p. 168–170° (d.) (from aqueous methanol).

Anal. Calcd. for $C_{35}H_{49}N_{13}O_{27}.2H_2O$: N, 16·4. Found: N, 16·7.

Table II. Inhibition of *M. tuberculosis* in Dubos' Liquid Medium after incubation at 37° for 14 days

Compound	Concentration μg/ml													
	100	50	25	12·5	6·25	3·12	1·56	0·78	0·4	0·2	0·1	0·05	0·02	0·01
Streptomycin (I; R=CHO, R′=H)	−	−	−	−	−	−	−	−	−	−	++	++	++	++
N-Acetylstreptomycin (I; R=CHO, R′=$COCH_3$)	−	−	−	−	−	−	−	+	+	++	++	++		
N-Acetyldihydrostreptomycin (I; R=CH_2OH, R′=$COCH_3$)	−	−	−	−	−	−	−	+	++	++	++	++		
N-Acetyltrityldihydrostreptomycin (VI; R=CH_2OCPh_3)	−	−	+	+	++	++	++	++	++	++	++	++		
Undeca-acetylstreptomycin	++	++	++	++	++	++	++	++	++	++	++	++		
Dodeca-acetyldihydrostreptomycin	++	++	++	++	++	++	++	++	++	++	++	++		

Table III. Inhibition of *M. tuberculosis* in Dubos' Liquid Medium after incubation at 37° for 28 days

Compound	Concentration μg/ml										
	100	50	25	12·5	6·25	3·12	1·56	0·78	0·4	0·2	0·1
Streptomycin (I; R=CHO, R′=H)	−	−	−	−	−	−	−	−	±	+	++
N-Acetylstreptomycin (I; R=CHO, R′=$COCH_3$)	−	−	−	−	−	−	+	++	++	++	++
N-Acetyldihydrostreptomycin (I; R=CH_2OH, R′=$COCH_3$)	−	−	−	−	±	+	+	++	++	++	++
N-Acetyltrityldihydrostreptomycin (VI; R=CH_2OCPH_3)	−	+	++	++	++	++	++	++	++	++	++

N-*Acetylstreptomycin* was prepared from undeca-acetylstreptomycin (0·824 g) as described for *N*-acetyldihydrostreptomycin, and was obtained as a colourless amorphous product (0·4 g, 81 per cent), m.p. 180–182° (d.) (micro-block), $[\alpha]_D^{20} - 87 \cdot 1°$ (*c*, 1·675 in water).

Anal. Calcd. for $C_{23}H_{41}N_7O_{13}.1\frac{1}{2}H_2O$: C, 42·5; H, 6·8; N, 15·1. Found: C, 42·3; H, 7·15; N, 15·6.

Tuberculostatic Activity

The compounds were dissolved in water (1,000 μg/ml), the solutions diluted in Dubos' liquid medium and the dilution inoculated with *M. tuberculosis* (human strain 666). The results were recorded after 14 days' incubation at 37°C and again after 28 days, and are shown in Tables II and III.

Summary. Protective etherification of the primary alcoholic group in the dihydrostreptose unit of dihydrostreptomycin has been attempted in order to test the hypothesis that the antitubercular activity of the latter is due to its re-oxidation to streptomycin. Cold methanolic ammonia was selected for deacetylation of undeca-acetyltrityldihydrostreptomycin, since the streptidine–dihystostreptose linkage is sensitive to acid and the guanidino groups to strong alkali. The *N*-acetyl group of the glucosamine unit still remained intact under the conditions used. The *in vitro* activity of *N*-acetyltrityldihydrostreptomycin was significantly lower than *N*-acetyldihydrostreptomycin and *N*-acetylstreptomycin. The latter compounds possessed about one-quarter of the activity of streptomycin and the other acetylated intermediates tested were inactive.

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