SYNTHESIS OF BOTH ^{14}C- AND ^{3}H- LABELLED TROSPECTOMYCIN AND THE ASSESSMENT OF THEIR UTILITY IN BIOLOGICAL STUDIES.

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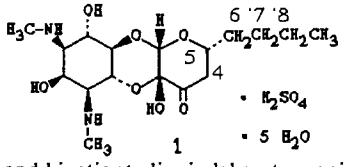
SUMMARY

Three radiolabelled forms of trospectomycin (6'-n-propylspectinomycin sulphate hydrate) have been synthesised, namely [8'-^{14}C]-, [6',7'-^{3}H]-, and [5',6',7'-^{3}H]-trospectomycin. Although all three forms of the drug are metabolically stable and therefore suitable for use in animal studies the ^{14}C compound is produced in poor yield as is the [6',7'-^{3}H]trospectomycin. On the other hand, the [5',6',7'-^{3}H]-form is produced in good yield and appears to be the labelled form of choice. Following subcutaneous administration of any of the labelled forms to rats, more than 50% of the dose was excreted in the urine as unchanged drug during the first 6h after dosing. Approximately 80% and 10% of the dose appeared in the urine and faeces respectively after 6 days.

KEYWORDS: [^{3}H]trospectomycin, [^{14}C]trospectomycin, ^{3}H-n.m.r., metabolic stability.

INTRODUCTION

Trospectomycin 1 as the sulphate salt is a semi-synthetic, parenteral, broad spectrum antibiotic derived from spectinomycin [1]. It has activity against Gram positive (Staphylococcus, Streptococcus) and Gram negative bacteria (Neisseria gonorrhoeae and Haemophilus influenzae).



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In order to expedite metabolic and kinetic studies in laboratory animals and man the radioactively labelled compound has been prepared. The synthesis of both ^{14}C and ^{3}H labelled trospectomycin is reported here together with preliminary studies to assess the suitability of each labelled form.

EXPERIMENTAL

General. Liquid scintillation counting was performed on an LKB 1218 Rackbeta liquid scintillation counter (L.K.B. Instruments Ltd., Croydon, Surrey, U.K.) with Fisofluor 1 as scintillant (Fisons Scientific Apparatus plc., Loughborough, U.K.) using the external standard method to correct for quenching. Thin layer chromatograms obtained on plates coated with Kieselgel 60 F_{254}, thickness

0362–4803/90/070763–07$05.00

Received November 20, 1989
Revised January 30, 1990

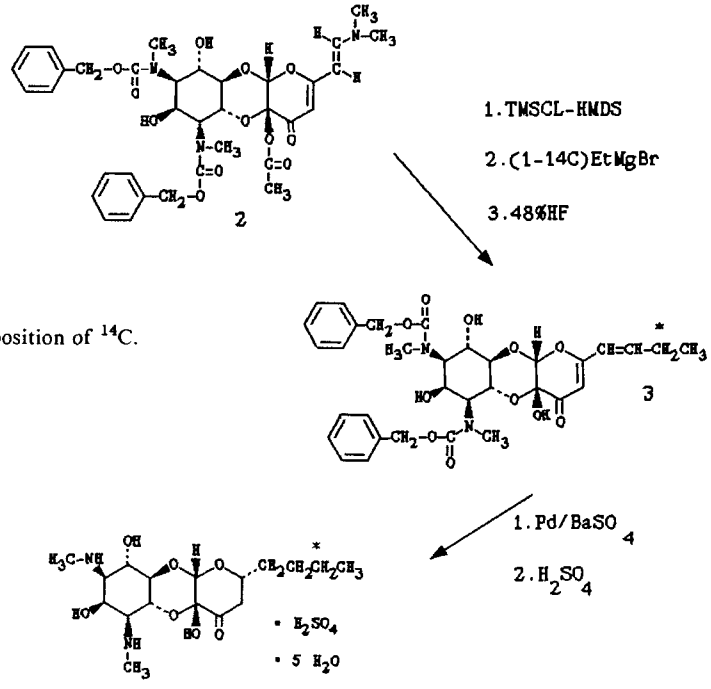
0.25 mm (E.Merck A.G., Darmstadt, W.Germany) were scanned with a plate scanner (ESI-Panax, Rotheroe and Mitchell Ltd., Ruislip, Middx., U.K.). In addition compounds were detected by either autoradiography using Kodak X-Omat x-ray film, U.V. light or visualised as yellow spots by spraying with 2,4-dinitrophenylhydrazine (0.5% w/v in 2M HCl). Tritium NMR spectra were obtained on a Bruker WH-90 spectrometer (Bruker, Karlsruhe, W. Germany). Mass spectra were determined by means of a Finnigan 4600 mass spectrometer (Finnigan MAT Ltd., Hemel Hempstead, Herts., U.K.) using a DCI probe in the CI mode with ammonia as the reagent gas. HPLC was conducted on an APS Hypersil-NH_2 column, 3um (250x4.6 mm) (Shandon Southern Products Ltd., Runcorn, U.K.) with a mobile phase of CH_3CN:0.1M KH_2PO_4, pH 2 (70:30 v/v) at a flow rate of 2.0 mL/min with detection at 215 nm. Kodak X-Omat x-ray film was used for autoradiography.

Materials. [1-^{14}C]Ethyl bromide of specific radioactivity 250 μCi/mmol and radiochemical purity >98%, as determined by radio-GLC was purchased from the Physics and Radioisotope Services, Petrochemicals and Plastics Division, ICI plc, Billingham, U.K.). Tritium gas was obtained from Amersham International plc., Aylesbury, U.K.

Synthesis of [8'-^{14}C]trospectomycin 1a.

The method used was that of White and Cain[2] (Scheme. 1).

SCHEME 1



* Denotes position of ^{14}C.

[1-^{14}C]Ethylmagnesium Bromide. Dry diethyl ether (5 mL), magnesium turnings (83.5 mg) and a small crystal of iodine were placed in a 15 mL test tube with side-arm, equipped with a magnetic stirrer, reflux condenser and cotton wool guard tube. The whole procedure was conducted whilst passing nitrogen through the side-arm. To the warmed reaction mixture (35°C) was added dropwise [1-^{14}C]ethyl bromide (a total of 379 mg consisting of 111 mg of [1-^{14}C]ethyl bromide (250 μCi) and 268 mg of non-radioactive ethyl bromide) at a rate to keep the ether at a steady reflux. After addition of the alkyl bromide the mixture was heated to reflux with stirring for a further hour and then cooled to room temperature. This [1-^{14}C]ethylmagnesium bromide was then ready for addition to the silylated enamine (see below).

[8'-^{14}C]N,N'-dicarbobenzyloxy-6'-propylidine-4',5'-didehydrospectinomycin (dienone) 3 . The enamine 2 (0.5 g) (N,N'-dicarbobenzyloxy-2'-O-acetyl-6'-((dimethylamino) methylene)-4',5'-didehydrospectinomycin[2]) dissolved in dry tetrahydrofuran (5 mL) was placed in a 20 mL pear-shaped flask equipped with a side-arm and magnetic stirrer. Hexamethyldisilazane (1 mL) and trimethylsilylchloride (1 mL) were added and the cloudy solution was stirred under N_2 at 55°C for 2.5 h. TLC of the product ($CH_3CN/CHCl_3$; 1:4 v/v) showed a major product of R_f 0.33 (U.V. lamp) whereas the original enamine did not move from the origin. The mixture was poured into toluene (10 mL), and concentrated to a foam in vacuo to remove excess silanising reagents. The material was dissolved in toluene (15 mL) and placed in a 50 mL two necked flask equipped with a magnetic stirrer and N_2 inlet. After cooling to 0°C the [1-^{14}C]ethylmagnesium bromide in diethyl ether was added dropwise with stirring and the mixture was then brought to room temperature and stirred for a further 1.5 h. TLC in the above system and location (autoradiography and U.V. light) showed a major radioactive, less polar product (the disylylated dienone 3 [2]). The mixture was poured into ethyl acetate (15 mL), glacial acetic acid (0.6 mL) and satd. NaCl (5 mL), shaken, the organic phase separated and the aqueous phase washed with ethyl acetate. The combined ethyl acetate extracts were dried over anhyd. Na_2SO_4 and taken to dryness (foam) in vacuo. The foam was dissolved in acetonitrile (7.5 mL), cooled to 0°C, treated with aqueous HF (48%)(0.75 mL) and left overnight in the refrigerator. TLC in $CH_3CN/CHCl_3$ (1:4 v/v) showed that the major product had become more polar (R_f 0.26; U.V. light and autoradiography) and had the same R_f as authentic dienone. However there was a major radioactive component at R_f 0.36 with a number of other minor contaminants. The solution was poured onto a mixture of satd. brine (15 mL), ethyl acetate (10 mL) and toluene (5.0 mL). After separation the organic extracts were dried over Na_2SO_4 and concentrated in vacuo. A small chromatography column (20 x 1.5 cm) fitted with a coarse sinter was dry packed with silica gel (kieselgel 60, 70-230 mesh, E.Merck A.G.) (10 mL). After conditioning the column by passing 20 mL of $CHCl_3$ through it the impure dienone dissolved in $CHCl_3$ (1 mL) was placed on the top of the column and eluted with the following solvents.

1. $CHCl_3$, 20 mL.

2. $CH_3CN/CHCl_3$, (5:95 v/v), 20 mL

3. $CH_3CN/CHCl_3$, (1:9 v/v), 20 mL.

4. $CH_3CN/CHCl_3$, (1:4 v/v), 20 mL.

5. $CH_3CN/CHCl_3$, (1:1 v/v), 20 mL.

Fractions (5 mL) were collected and samples (10 uL) of each chromatographed (TLC - $CH_3CN/CHCl_3$-see above) and scanned with the TLC plate scanner in order to locate the ^{14}C-dienone. Fractions 4,5 and 6 contained the largest proportion of the dienone and were therefore combined and evaporated to dryness in vacuo to give a pale yellow oil which solidified. The mass spectrum of the product (34.46 mg) obtained in an identical synthesis of the non-radioactive compound had a molecular ion of 639 as had that of an authentic sample. This corresponds to the molecular ion + 1 m/z unit.

[8'-^{14}C]Trospectomycin 3. The ^{14}C-dienone (34.46 mg) plus non radioactive dienone (165.54 mg) was dissolved in propan-2-ol (16 mL) and placed in a 50 mL round bottomed flask equipped with a septum and magnetic stirrer. To this solution was added pyridine (0.2 mL) and 10% $Pd/BaSO_4$ (0.2 g). The mixture was hydrogenated at 1 atmosphere for 2 h 20min. The catalyst was removed by centrifugation (2,000 rpm, 5 min, M.S.E. Bench Top Centrifuge, M.S.E., Crawley, U.K.), the filtrate concentrated in vacuo (26°C), dissolved in water (3 mL), treated with 0.62 mL of 0.5 M H_2SO_4 and filtered.

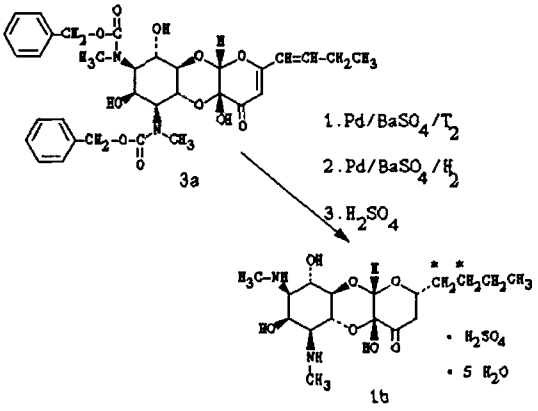
The filtrate was lyophilised overnight, dissolved in water and treated with acetone until the solution became cloudy. The mixture was cooled to 0°C for 1 h and the white crystalline solid that appeared was filtered. TLC of the product in $CH_3OH/CHCl_3/NH_4OH$ (S.G. 0.88) (4:3:2 v/v) gave a major radioactive spot at R_f 0.63 the same as authentic trospectomycin. The spot could be visualised as an orange spot by spraying with the 2,4-dinitrophenylhydrazine reagent as can the trospectomycin. A minor contaminant radioactive spot could be observed at R_f 0.95 and was probably the dienone, a sample of which on chromatography ran at the same R_f. The product was therefore recrystallised from H_20/acetone and then kept at 53% relative humidity for two days to give 33.14 mg of [8'-^{14}C]trospectomycin sulphate (specific radioactivity 0.025 μCi/mg) which chromatographed (TLC) as a single spot (spray reagent, autoradiography, scanning) in $CH_3OH/CHCl_3/NH_4OH$ (see above) and BuOH/HOAc/H_2O (60:12:24 v/v) (R_f 0.63 and 0.32 respectively). Mass spectrometry of the radiolabelled compound gave the same molecular ion (m/z 374) as the authentic compound.

Synthesis of [6',7'-^3H]trospectomycin 1b.

The non-radioactive dienone 3a (100 mg) was reduced (at Amersham International plc., Cardiff,

U.K.) (scheme 2) under the same conditions as the ^{14}C-dienone, with 10 Ci of tritium gas to give after removal of labile tritium by freeze drying, 5.1 Ci of product. TLC in the same systems used for the analysis of the [^{14}C]trospectomycin revealed a complex pattern of peaks on scanning. This suggested that the dienone had been incompletely reduced. Therefore, 48 mCi of the Amersham product were taken up in propan-2-ol, mixed with 200 mg of the dienone and reduced with hydrogen gas as above. TLC in the same systems revealed a major peak (ca. 60% of the total activity) of the same R_f as authentic trospectomycin. Recrystallisation from methanol/H_2O and equilibration at 53% relative humidity for two days gave [^{3}H]trospectomycin sulphate 1b (32mg; specific radioactivity 80.0 μCi/mg). Analysis by HPLC and TLC (see above) showed the compound to be >98% pure. The NMR spectrum showed the tritium to be present in two non-equivalent sites, chemical shift 1.53 and 1.28 ppm, with approximately equal intensity.

SCHEME 2

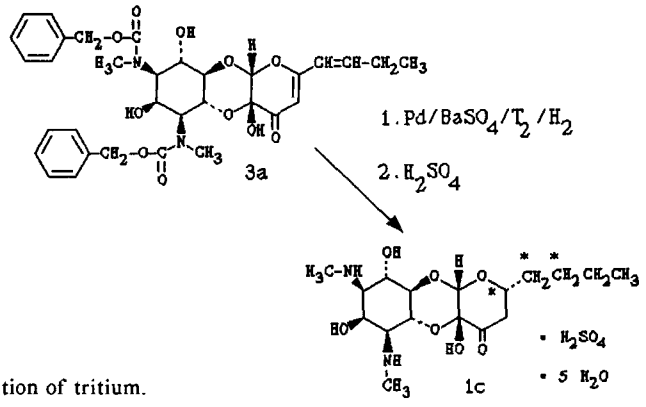


* Denotes position of tritium.

Synthesis of [5',6',7'-^{3}H]trospectomycin 1c.

The dienone 3a (20 mg) was reduced in exactly same way as the Amersham compound with the exception that a mixture of tritium and hydrogen gases was used (5.1 mL; 1Ci). TLC of the reaction mixture and scanning revealed a major peak (80% of total activity) in both TLC systems (see above). The sulphate salt was made as above and recrystallised together with 36.4 mg of nonradioactive trospectomycin to give after hydration 36.41 mg of [^{3}H]trospectomycin sulphate of specific activity 416.5 μCi/mg and a purity of >98% (TLC and HPLC). Tritium NMR revealed three non-equivalent resonance peaks at 1.28,1.53 and 3.82 ppm.

SCHEME 3.



* Denotes position of tritium.

RESULTS AND DISCUSSION

[8'-^{14}C]Trospectomycin was synthesised from the trimethylsilyl derivative of the enamine 2 by reaction with [1-^{14}C]ethylmagnesium bromide, desylylation with hydrofluoric acid to give compound 3 which was reduced with hydrogen and palladium on barium sulphate catalyst. The overall yield was poor for the following reasons: The reaction of the ethylmagnesium bromide could only have theoretically given a yield of 20%. In practice this was reduced to 1.6%. The yield at the reduction stage was poor due to the purification step.

[6',7'-^{3}H]Trospectomycin. It had been anticipated that three tritium atoms would be incorporated into the trospectomycin by saturation of the two double bonds of 3a (scheme 2). However only two resonances were apparent in the tritium NMR spectrum at 1.53 and 1.28 ppm. These correspond to the 6' and 7' positions of the alkyl side chain and the 4',5' double bond had not been reduced leading to a lower specific activity and lower yield.

[5',6',7'-^{3}H]Trospectomycin. The same resonances in the tritium NMR were apparent as in the [6',7'-^{3}H]trospectomycin. However, there was an additional resonance at 3.82 corresponding to the 5' position in the pyranobenzodioxin ring. The tritium which would have been in the 4' position is enolisable and would have been lost during the work-up. The radiochemical yield using a mixture of tritium and hydrogen gas was considerably better than that obtained using a suboptimal volume of tritium gas alone.

Animal Experiments

After rats were dosed subcutaneously with either [8'-^{14}C]- or [6',7'-^{3}H]- or [5',6',7'-^{3}H]-trospectomycin more than 50% of the dose was excreted in the first six hours after dosing. Over six

days approximately 70 to 90 % of the dose was excreted in the urine and 10 to 20 % in the faeces. Little $^{14}CO_2$ was detected in the expired air of the animal dosed with the ^{14}C-labelled compound nor was any $^{3}H_2O$ detected in the expired air of the animals dosed with the 6',7'-^{3}H compound. The urines from the rats dosed with the ^{3}H-labelled compounds were subjected to freeze drying (X2) and there was no significant loss of radioactivity which would have corresponded to $^{3}H_2O$ produced by metabolism of [^{3}H]trospectomycin.

Although the ^{14}C compound is metabolically stable it would not be of any use for metabolism studies due to the poor yields. Similarly, the 6', 7'-^{3}H compound, although metabolically stable, is neither easily synthesised nor can it be produced with high specific activity, therefore the [5',6',7'-^{3}H]trospectomycin is the labelled form of choice.

REFERENCES.

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2. White, D.R. and Cain, G.A. Tetrahedron Lett. 30, 1469 (1989).