Biosynthesis of Aristeromycin: The Role of (1*R*,2*R*,3*S*,4*S*)-1-Hydroxymethylcyclopentane-2,3,4-triol

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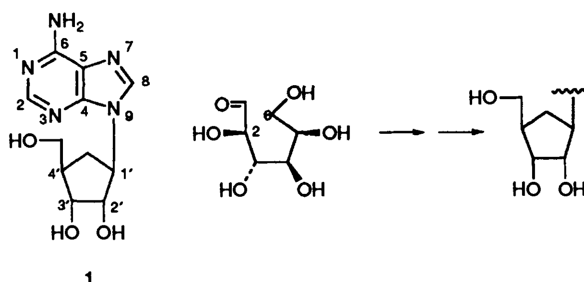
(1*R*,2*R*,3*S*,4*S*)-1-Hydroxymethylcyclopentane-2,3,4-triol **2**, a possible precursor of aristeromycin **1** was synthesised in tritiated form and administered to *Streptomyces citricolor*. Radioactivity was incorporated into aristeromycin, but degradation showed that incorporation of radioactivity was non-specific, indicating that the tetrol **2** is not an immediate precursor of aristeromycin **1**.

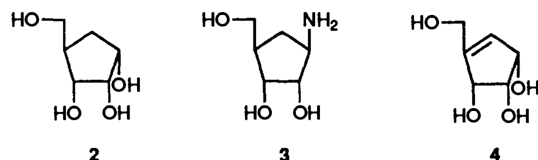
Aristeromycin **1** is an antibiotic produced by *Streptomyces citricolor*.[1] Because of its interesting biological properties[2–7] and the antiviral or antitumour properties of analogues[8–11] there has been considerable interest in its biosynthesis.[12–14]

Radiolabelled glucose was incorporated into aristeromycin **1**[12] in such a way that formation of the carbocyclic bond between C-2 and C-6 of glucose was indicated (Scheme 1).



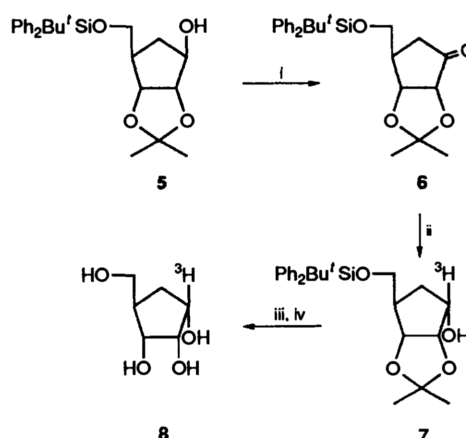
Scheme 1

Several putative intermediates have been shown to be produced by *S. citricolor*. Thus (1*R*,2*R*,3*S*,4*S*)-1-hydroxymethylcyclopentane-2,3,4-triol **2**[13] and (1*R*,2*R*,3*S*,4*R*)-4-amino-1-hydroxymethylcyclopentane-2,3-diol **3**[14] were shown by isotope dilution experiments to be produced by *S. citricolor*. (1*S*,2*S*,3*R*)-4-hydroxymethylcyclopent-4-ene-1,2,3-triol **4** has recently been isolated from the same organism.[15] However, as yet, there is no firm evidence regarding the proximate cyclopentane precursor of the carbocyclic system.



We have studied the incorporation of the tetrol **2** into *S. citricolor*. The [4-^{3}H]-labelled form of this, tetrol **8**, was prepared as shown in Scheme 2 in 85% overall yield and with a specific activity of 19.07 mCi mmol^{-1}.† The labelled tetrol was administered to a 24 h culture of *S. citricolor*. Preliminary experiments had shown that under the incubation conditions employed, aristeromycin production began 30 h after inoculation of the culture medium. Aeration was extremely important. With 500 cm^3 of culture medium in 2 dm^3 shake flasks, aristeromycin production was extremely low, whereas in

† 1 Ci = 3.7 × 10^{10} Bq.



Scheme 2 *Reagents:* i, pyridinium chlorochromate; ii, [^{3}H$_4$]NaBH$_4$; iii, Bu$_4$NF; iv, Amberlyst 15/MeOH

a fermenter with controlled oxygen tension, aristeromycin levels reached 330 mg dm^{-3} (Fig. 1). In shake flasks, improved aeration was achieved using 250 cm^3 flasks containing 30 cm^3 culture medium. Incorporations were low (see Table 1) owing, at least in part, to low uptake of the tetrol by the cells. However, aristeromycin of specific activity sufficiently high to permit location of the label was obtained. The radiochemical purity of the labelled aristeromycin was confirmed by conversion into the 2′,3′-isopropylidene derivative, which in a typical case had the same specific activity (21.9 μCi mmol^{-1}) as the aristeromycin (21.3 μCi mmol^{-1}). The problem that remained was to determine if incorporation was specific. No simple method for location of label that might have been present at C-1′ in the labelled aristeromycin **1** could be devised. Accordingly, the simpler objective of determining the distribution of label between the pseudo-sugar and base components of aristeromycin **1** was attempted. Hydrolytic methods were not applicable. Oxidative removal of the pseudo-sugar (periodate followed by permanganate) proved unsuccessful. Accordingly, attention was turned to the purine base. A tritium label in the base could only have been located at C-8 or C-2. A selective method for eliminating tritium at C-8 was, in principle, possible by enzymatic conversion of aristeromycin into the hypoxanthine analogue **9** by adenosine deaminase, followed by oxidation at C-8 using xanthine oxidase (Scheme 3). In the event, although deamination of aristeromycin proceeded smoothly, the hypoxanthine analogue proved not to be a substrate for xanthine oxidase.

Accordingly, a different approach was explored. It had been reported that direct bromination of adenosine under controlled

Table 1 Incorporation of [4-^{3}H]-(1*RS*,2*RS*,3*SR*,4*SR*)-1-hydroxymethylcyclopentane-2,3,4-triol **8*** into aristeromycin by *S. citricolor*[a]

% Incorporation
0.039[b]
0.19[b]
0.025[b]
0.019[b]
0.0085[c]

[a] For each incubation, 51.5 μCi labelled tetrol **8** (0.38 mg) was administered. [b] 32 h incubation. [c] 6 h incubation.

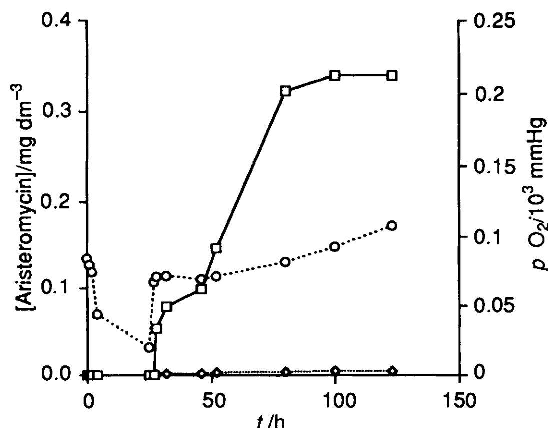
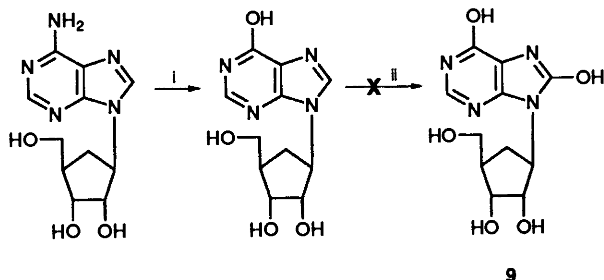
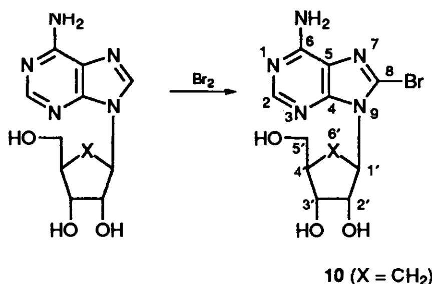


Fig. 1 Aristeromycin production by *Streptomyces citricolor* showing the effect of aeration under shake flask and fermenter conditions; —□— fermentation; ··· ◇ ··· 2 dm^3 shake flask; ··· ○ ··· pO_2



Scheme 3 *Reagents:* i, adenosine deaminase; ii, xanthine oxidase



Scheme 4 *Reagents:* Br_2

conditions in aqueous solution gave 8-bromoadenosine (Scheme 4, X = O).[16] Accordingly, bromination of aristeromycin was explored (Scheme 4, X = CH_2). By careful experimentation, it was found that the best conversions (95% of aristeromycin converted) were obtained between pH 4 and 7 (Fig. 2). It was convenient to operate at pH 4 (pH meter). It was found that determination of the radioactivity of the 8-bromoaristeromycin **10** (X = CH_2) produced was subject to large experimental errors. Accordingly, the labelled aristeromycin was brominated and the amount of tritium released into the aqueous medium was quantitatively determined by adsorption of the purine base products on a cation exchange resin and measurement of the activity of the eluted water. The released radioactivity was corrected for unconverted aristeromycin (determined by HPLC) and was found to be 39 ± 6% of the total radioactivity of the original labelled aristeromycin. Complete randomisation of the tritium over the 10 carbon-bound hydrogen atoms of aristeromycin would be expected to give 10% of the total label at each site. The location of over one third of the total label at one site in the purine base strongly argues against the specific incorporation of the tetrol **2** into aristeromycin.

* Strictly speaking the stereochemical assignment at C-3 for the [4-^{3}H]-labelled tetrol **8** should be *RS*. For ease of comparison with the unlabelled tetrol **2**, C-3 of **8** has been assigned *SR* as for the unlabelled derivative.

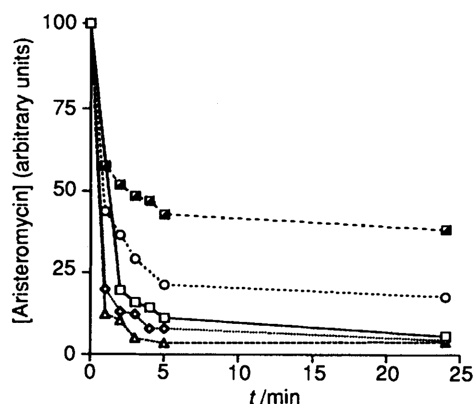


Fig. 2 Bromination of aristeromycin: pH optimisation; —□— pH 4; ··· ◇ ··· pH 5; ··· ○ ··· pH 6.1; --- △ --- pH 7.2; --- ◪ --- H_2O

Experimental

Mass spectra were determined on a Kratos MS80 spectrometer. NMR spectra were determined on Bruker WH-400 or Bruker ACF-250 spectrometers. All coupling constants *J* are quoted in Hz. ^{13}C NMR spectra were determined at 62.9 MHz using a Bruker ACF-250 spectrometer. Sodium [$^3H_4$]boranuide ([$^3H_4$]$NaBH_4$), NCS tissue solubiliser and OCS scintillation cocktail were obtained from Amersham International. TLC was carried out using silica gel 60 F_{254} plates. Flash chromatography and preparative column chromatography were both carried out using silica gel 60 for dry column chromatography and column chromatography (30–70 mesh), respectively.

Growth Medium.—This consisted of D-glucose (20 g), soluble starch (30 g), soybean flour (10 g), corn steep liquor (10 g), Bactopeptone (2.5 g), sodium chloride (1.5 g) and calcium carbonate (5 g). All of the ingredients except the calcium carbonate were dissolved in water. The solution was brought to pH 7 (1 mol dm^{-3} NaOH), the volume was adjusted to 1 dm^3, the calcium carbonate was added and the mixture was heated in an autoclave at 121 °C for 25 min at 1.03 bar.†

Assay System for Aristeromycin **1**.—Aristeromycin and neplanocin A were assayed by HPLC using a 25 cm reverse phase column (Techsphere 5C8) and with linear gradient

† 1 bar = 10^5 Pa.

elution with methanol–water (10–25%) over 20 min at 1 cm^3 min^{-1} and with UV detection at 257 nm. Detection limits were of the order of 1 μg cm^{-3}.

Synthesis of [4-^{3}H]-(1RS,2RS,3SR,4SR)-1-*Hydroxymethylcyclopentane*-2,3,4-*triol* **8**.—To a mixture of pyridinium chlorochromate (65 mg, 4 equiv.) and powdered activated 4 Å molecular sieves (105 mg) was added, under argon, the alcohol **5**[13] (30 mg) as a solution in CH_2Cl_2 (2 cm^3). The mixture was stirred for 15 min, when TLC (EtOAc–light petroleum, 1:3) showed that no starting material remained (R_fs: starting material **5**, 0.23; ketone **6**, 0.41; epimeric alcohol **7**, 0.23). Diethyl ether (3 cm^3) was added and the mixture was filtered through a short column of silica gel. The column was washed with diethyl ether and the filtrate plus washings were evaporated under reduced pressure to give the ketone **6**, which was used in the next step without further purification. Thus, to the ketone **6** (26.8 mg) in 90% aqueous ethanol (2 cm^3) was added sodium [$^3H_4$]boranuide (*ca.* 20 mg, 100 mCi) at 0 °C. The mixture was stirred at this temperature for 30 min when TLC as before indicated that the reaction had gone to completion. Saturated aqueous ammonium chloride solution (1 cm^3) was added and the mixture was evaporated under reduced pressure. The residue was dissolved in $CHCl_3$ (5 cm^3) and the solution was washed with water (4 cm^3). The $CHCl_3$ solution was evaporated to dryness under reduced pressure and the residue of protected tetrol **7** (27 mg) was dissolved in tetrahydrofuran (3 cm^3). Tetrabutylammonium fluoride (1 mol dm^{-3} in tetrahydrofuran; 0.2 cm^3) was added and the mixture was stirred overnight, when TLC ($CHCl_3$–EtOH, 9:1) indicated that the reaction had gone to completion. The mixture was evaporated under reduced pressure and the residue was purified by chromatography on silica gel (CH_2Cl_2–EtOH, 9:1). Fractions containing the product (R_f 0.35) were combined and evaporated to dryness under reduced pressure to give the labelled diol (12 mg, *ca.* 100%). This was dissolved in MeOH (3 cm^3) containing Amberlyst 15 cation exchange resin (H^+ form; 5 mg). The solution was stirred at room temperature for 80 h after which time TLC (CH_2Cl_2–EtOH, 9:1) indicated that the reaction had gone 50% towards completion. The solution was heated at 60 °C for 4 h after which time TLC [light petroleum (b.p. 40–60 °C)–ethyl acetate, 3:1] indicated the presence of tetrol with only a trace of starting material and of another product of higher R_f. (R_fs: diol, 0.5; tetrol **8**, 0.16; higher R_f compound, 0.57). The mixture was filtered and the resin was washed with water. The filtrate plus washings were evaporated under reduced pressure and the labelled tetrol was purified by chromatography on silica gel ($CHCl_3$–MeOH, 3:1) to give the tetrol **8** (8 mg, 85% overall), pure by TLC (^{1}H NMR data as for the unlabelled material[17]). The radiochemical purity of the tetrol was determined by TLC [light petroleum (b.p. 40–60 °C)–ethyl acetate, 3:1). Bands (1 cm) were removed from the plate and their radioactivities were determined. The radiochemical purity was found to be 98%, specific activity 19.07 mCi $mmol^{-1}$.

Incorporation of Labelled Tetrol **8** *into Aristeromycin.*—Two flasks (250 cm^3) containing full growth medium (30 cm^3) were inoculated with spores of *S. citricolor.* The flasks were incubated with shaking (28 °C, 200 r.p.m.) for 24 h. Labelled tetrol (51.5 μCi, 0.38 mg) in water (0.5 cm^3) was added to the two flasks and incubation was continued for a further 32 h. The culture was centrifuged (20 min, 3500 *G*).* The cells were washed by suspension in aqueous NaCl (0.2 mol dm^{-3}; 10 cm^3). The cells were spun down and the washing process was repeated a further four times. The washed cells (*ca.* 1 g wet weight) were solubilised in NCS solubiliser (6 cm^3). The solution was diluted with OCS scintillation cocktail (16 cm^3) and the radioactivity was determined. The radioactivity of the cells from the two flasks was 0.055 μCi and 0.079 μCi, respectively, indicating the presence of 0.26% of the administered activity. Unlabelled aristeromycin (20 mg) was added to the supernatant solutions (above). The solutions were adjusted (pH meter) to pH 5 (1 mol dm^{-3} HCl) and applied to a column of Dowex 50W-X8 ion exchange resin (H^+ form). The column was washed with water until no more radioactivity could be detected in the washings. The column was eluted with aqueous ammonia (1 mol dm^{-3}) and fractions containing aristeromycin as determined by TLC ($CHCl_3$–MeOH, 3:1) were combined and evaporated under reduced pressure in the presence of silica gel (1 g). The silica gel was packed on to the top of a silica gel column which was eluted with $CHCl_3$–MeOH (3:1). Fractions containing aristeromycin were combined and the radioactivity of the aristeromycin was determined. Each aristeromycin sample was converted into the isopropylidene derivative[18] and the radioactivity of the derivative was determined. For material from the two flasks, the activities of the aristeromycin were 20.1 and 22.7 μCi $mmol^{-1}$, and the activities of the corresponding isopropylidene derivatives were 21.7 and 22.1 μCi $mmol^{-1}$, respectively.

Bromination of Aristeromycin: Optimisation.—To five solutions of aristeromycin (100 mg in 5 cm^3, 0.5 cm^3) in 3 cm^3 of buffer solution [pH 4.0 (acetate), pH 5.0 (acetate), pH 6.1 (phosphate), pH 7.2 (phosphate)] was added 1 cm^3 of a solution of bromine (1 cm^3) in 100 cm^3 of each of the above buffer solutions. At intervals, samples were withdrawn, the reaction was quenched by the addition of aqueous sodium hydrogen sulfite (50 mm^3) and the progress of the bromination was followed by HPLC (isocratic, 30% methanol in water). The best conversion with the least formation of by-products was observed at pH 4 (95% conversion). When isolation of bromoaristeromycin was attempted, decomposition apparently occurred and pure material could not be obtained. However, in the aromatic region of the ^{1}H NMR spectrum, the signal attributable to 8-H was diminished whereas the signal attributable to 2-H [δ([$^2H_6$]DMSO) 8.12] in both aristeromycin and 8-bromoaristeromycin remained unchanged. To ascertain that bromination had proceeded as expected, the reaction was run in D_2O with 250 MHz observation (^{1}H). Under these conditions, the reaction proceeded cleanly with complete disappearance of the signal attributable to 8-H.

Preparative Bromination of Aristeromycin.—To a solution of aristeromycin (50 mg, 0.19 mmol) in water (5 dm^3) bromine (1 cm^3) was added dropwise, with stirring. The reaction mixture was allowed to stand overnight when by TLC (butan-1-ol–H_2O–conc. ammonia, 85:1:1) it was found that approximately 10% of the aristeromycin remained unchanged. The solution was evaporated under reduced pressure and the residue was dissolved in water (1 cm^3). Silica gel (0.5 g) was added and the mixture was evaporated to dryness under reduced pressure. The silica gel was applied to the top of a column of silica gel (10 g) which was eluted with propan-2-ol–H_2O (9:1). Fractions containing the product were evaporated to dryness to give 8-bromoaristeromycin **10** (X = CH_2) (52 mg, 80% as a foam). δ_H(250 MHz; [$^2H_6$]DMSO) 2.01 (3 H, m, 4′-H, 5′-H_2), 3.30–3.50 (2 H, m, 6′-H_2), 3.85 [1 H, br s (apparent), 3′-H], 4.6–4.8 (4 H, 1′-H, 2′-H, 6′-OH, 2′- or 3′-OH), 4.93 (1H, d, *J* 6.0, 2′- or 3′-OH), 7.36 [2 H, br s (apparent), NH_2] and 8.07 (1 H, s, 2-H). δ_C 27.34 (C-6′), 45.51 (C-4′), 62.31 (C-1′), 63.24 (C-5′), 71.43 (C-3′), 72.42 (C-2′), 119.77 (C-5), 127.83 (C-8), 150.57 (C-6), 152.28 (C-2) and 154.99 (C-4). *m/z* (CI, NH_3) 346 [$(M + 1)^+$ (Br^{81}), 78%], 344 [$(M + 1)^+$ (Br^{79}), 84], 266 (35), 246 (19), 216 (35), 136 (100), 113 (19), 95 (8), 85 (14) and 61 (8).

* 1 $G = 6.673 \times 10^{-11}$ m^3 kg^{-1} s^{-2}.

Bromination of Labelled Aristeromycin.—The labelled aristeromycin (12.7 mg) was dissolved in water (1.5 cm^3) and two samples (each 600 mm^3) of this solution were each treated with a solution of bromine (0.65 cm^3) in acetate buffer (0.5 mol dm^{-3}, 100 cm^3, pH 4.0). The solutions were stirred at room temperature for 24 h. The excess of bromine was removed by the addition of aqueous sodium hydrogen sulfite and the solutions were applied to columns of Dowex 50W-X8 ion exchange resin (H^+ form, 2.5 g). The columns were washed with water and the radioactivities of the eluates were determined. Elution was continued until no more radioactivity could be detected. The radioactivity of each fraction was determined, and each fraction was examined by HPLC for the presence of aristeromycin or of bromoaristeromycin. The amounts of aristeromycin and of bromoaristeromycin eluted were found to be negligible. From the quantities eluted it was calculated that the radioactivity corresponding was two orders of magnitude lower than background. The radioactivity of each initial sample of aristeromycin was 3123 d.p.m. The total activity in the eluted water following bromination was for sample A, 2313 d.p.m. and for sample B, 2350 d.p.m. Conversions of aristeromycin into the 8-bromo derivative were found to be 81.6 and 96.2%, respectively. Correction of the radioactivity of eluted water to take into account residual aristeromycin gave values of 1357 and 1103 d.p.m. for samples A and B, respectively. The percentage of initial radioactivity released was therefore 43.5 and 35%, respectively. As a check on the procedure, the bases were eluted from the columns with aqueous ammonia solution (1 mol dm^{-3}). The amounts eluted corresponded to 2313 and 2350 d.p.m., respectively, for samples A and B. The total activity accounted for by eluted water and eluted bases in the two samples was 109% in each case.

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