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THE DEGRADATION OF β-SITOSTEROL BY PSEUDOMONAS SP. NCIB 10590 UNDER AEROBIC CONDITIONS

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Summary—The bacterial degradation of β-sitosterol by Pseudomonas sp NCIB 10590 has been studied. Major biotransformation products included 24-ethylcholest-4-en-3-one, androsta-1,4-diene-3,17-dione, 3-oxochol-4-en-3-one-24-oic acid and 3-oxopregn-4-en-3-one-20-carboxylic acid. Minor products identified were 26-hydroxy-24-ethylcholest-4-en-3-one, androst-4-ene-3,17-dione, 3-oxo-24-ethylcholest-4-en-26-oic acid, 3-oxochola-1,4-dien-3-one-24-oic acid, 3-oxopregna-1,4-dien-3-one-20 carboxylic acid and 9α-hydroxyandrosta-1,4-diene-3,17-dione. Studies with selected inhibitors have enabled the elucidation of a comprehensive pathway of β-sitosterol degradation by bacteria.

INTRODUCTION

The metabolism of sterols by bacteria has been studied for many years and began in earnest after Tak[1] observed that several species of Mycobacterium could utilise cholesterol as sole carbon source. In the last forty years, therefore, there have been many descriptions of bacteria capable of degrading sterols.

The metabolism of cholesterol by bacteria has been comprehensively documented by Sih *et al.* [2–5] and Owen *et al.* [6, 7]. The metabolism of the phytosterol β-sitosterol, however, is less well documented.

Arima *et al.* [8] showed in 1969 that many species of the genera Arthrobacter, Bacillus, Brevibacterium, Corynebacterium, Microbacterium, Mycobacterium, Nocardia, Protoaminobacter, Serrattia and Streptomyces were capable of accumulating androsta-1,4-diene-3,17-dione (ADD) as side-chain cleavage products of β-sitosterol in the presence of the enzyme inhibitor α,α′-Dipyridyl (α,α′-D). A further study by Nagasawa *et al.* [9] revealed that *Arthrobacter simplex* was capable of giving a good yield (39%) of ADD from β-sitosterol.

Partial side-chain cleavage products of β-sitosterol were not reported until Martin and Wagner [10] studied the metabolism of β-sitosterol by Nocardia sp. M29 and obtained 3-oxo-23,24-dinor-1,4-choladienoic acid and its methyl ester in moderate yield. Arima *et al.* [11] identified the same bisnor acid as a β-sitosterol degradation product using *Norcardia corallina* IFO 3338.

A more detailed description of β-sitosterol degradation has been presented by Knight and Wovcha [12] who have shown the production of the novel intermediates 9-hydroxy-27-nor-4-cholestene-3,24-dione and 9-hydroxy-26,27-dinor-4-cholestene-3,24-dione along with the major product 9-hydroxy-4-androstene-3,17-dione by a mutant of *Mycobacterium fortuitum*.

However the mechanism of degradation of the branched hydrocarbon side chain of β-sitosterol was not very well understood until Fujimoto *et al.* [13] demonstrated that cell free preparations of Mycobacterium sp. NRRL 3805 were capable of converting 3-oxo-24-ethylcholest-4-en-26-oic acid into 3-oxochol-4-en-24-oic acid and androst-4-ene-3,17-dione. It was concluded that the mode of microbial degradation of the β-sitosterol side chain proceeds via hydroxylation at C_{26}, followed by oxidation to 3-oxo-24-ethylcholest-4-en-24-oic acid to androst-4-ene-3,17-dione. Further studies by Fujimoto *et al.* [14] have shown that HCO_3^- is incorporated onto the C_{28} position of β-sitosterol and campesterol prior to carbon–carbon bond fission.

In a previous study [7] we reported in detail the side-chain cleavage of cholesterol by Pseudomonas sp. NCIB 10590 and herein we present evidence for the side-chain cleavage of β-sitosterol by the same organism.

EXPERIMENTAL

Materials

5α-Cholestane and ADD were obtained from Koch-Light (Colnbrook, Bucks, England). β-Sitosterol and α,α′-D were obtained from BDH (Poole, Dorset, England). All reference steroids

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except β-sitosterol were 100% pure as judged by thin-layer chromatography (TLC) and gas–liquid chromatography (GLC). Commercial β-sitosterol was contaminated with campesterol; the impurity being removed by dissolving and recrystallising β-sitosterol from ethereal solution. General reagents were of "Analar grade", purchased from BDH, and all solvents were redistilled before use.

Methods

Analytical. Infra red (i.r.) spectra, ultraviolet (u.v.) spectra, nuclear magnetic resonance (NMR) spectra, mass spectra (MS) and gas–liquid chromatographic–mass spectra (GLC–MS) were obtained as described previously [14]. TLC and GLC methodology was also conducted as described previously [15]. Isolated metabolites obtained by preparative TLC (PLC) were crystallised from the elution solvent (ethyl acetate) and recrystallised from methanol–dichloromethane, 1:9, unless otherwise stated. The biotransformation medium used was identical to that described previously [7] and contained β-sitosterol at 0.05%. All media was sterilised prior to use at 15 lb per square inch for 20 min.

The organism. Pseudomonas sp. 10590 was routinely maintained as previously described [7].

β-sitosterol metabolism in the absence of inhibitors. A 1-litre aerobic fermentation of β-sitosterol (1) by Pseudomonas sp. 10590 was terminated at 48 h by direct solvent extraction of the culture with an equal volume of ethyl acetate (×3). The pooled ethyl acetate extracts were dried over anhydrous magnesium sulphate and evaporated to dryness *in vacuo* to yield 370 mg of residue. The dry residue was dissolved in 10 ml of dichloromethane and was separated by PLC (20 plates) in the solvent system methanol–dichloromethane, 1:19 (v/v) yielding residual β-sitosterol (130 mg) and one neutral metabolite [2].

24-Ethylcholest-4-en-3-one [*2*]. Recrystallisation of metabolite 2 gave white crystals (190 mg), m.p. 79–80°C. i.r. 1682 (3-ketone) and 1621 cm^{-1} (C_4–C_5 double bond); u.v. 242 nm; NMR (δ) 0.70, 1.15 (6H, s, 18-CH_3 and 19-CH_3), 5.60 (1H, s, 4-H); MS, M^+ 412 (45%) ($C_{29}H_{48}O_1$ requires M^+ 412), base peak at *m/e* 124 (4-ene-3-one, 100%), *m/e* 370 ($M^+ - 42$, CH_2CO, 24%), *m/e* 271 ($M^+ - 141$, 10C-side chain, 17%) and at *m/e* 229 (M^+-183, 10C-side chain + Ring D, 44%). GLC, R_f 6.20; TLC, R_f 1.36; after oxidation, R_f 1.36; after acetylation, R_f 1.36 and after reduction, R_f 1.36.

β-Sitosterol metabolism in the presence of inhibitors. The concentrations of the inhibitors α,α′-D and *n*-propanol required for maximum intermediate accumulation in β-sitosterol fermentations by Pseudomonas sp. 10590 were ascertained as described previously [7].

β-Sitosterol metabolism in the presence of α,α′-D (0.6 mM). A 1-litre fermentation of β-sitosterol (0.5 mg/ml) by Pseudomonas sp. 10590 was carried out for 48 h at 28°C. Analysis of a culture aliquot (10 ml) by TLC and GLC revealed the presence of β-sitosterol and metabolite **2** only. α,α′-D (0.6 mM) was added to the medium and incubation was continued for a further 24 h. At the end of the fermentation the medium was extracted with ethyl acetate to yield 316 mg of residue. The dried residue was separated by PLC (16 plates) into a series of fractions from which steroids 2–8 were crystallised. Structural analyses of metabolites 2–8 revealed that they were identical to 24-ethylcholest-4-en-3-one (100 mg), androst-4-ene-3,17-dione (2 mg), androsta-1,4-diene-3,17-dione (22 mg), 3-oxochol-4-en-24-oic acid (14 mg), 3-oxochola-1,4-dien-24-oic acid (2 mg), 3-oxopregn-4-ene-20-carboxylic acid (9 mg) and 3-oxopregna-1,4-diene-20-carboxylic acid (1 mg) respectively [7].

β-sitosterol metabolism in the presence of 2% n-propanol. A 1-litre fermentation of β-sitosterol (0.5 mg/ml) by Pseudomonas sp. 10590 was carried out for 48 h at 28°C. The initial stage of the fermentation was identical to the α,α′-D experiment. After addition of 2% *n*-propanol and incubation for a further 24 h the culture was extracted with ethyl acetate yielding 320 mg of dried residue. The residue was separated by PLC (16 plates) into a series of fractions from which steroids 2,4,5,8–11 were crystallised.

Metabolites **2** (220 mg), **4** (2 mg), **5** (1 mg) and **8** (1 mg) were found to be identical to 24-ethylcholest-4-en-3-one, androsta-1,4-diene-3,17-dione, 3-oxochol-4-en-24-oic acid and 3-oxopregna-1,4-diene-20-carboxylic acid respectively.

26-Hydroxy-24-ethylcholest-4-en-3-one (**9**). Recrystallisation of metabolite **9** gave white crystals (3 mg), i.r. 3445 (C_{26}-hydroxyl), 1685 (3-ketone) and 1630 cm^{-1} (C_4–C_5 double bond); u.v. 242 nm; NMR (δ) 0.70, 1.19 (6H, s, 18-CH_3 and 19-CH_3), 3.44 (dd, J = 7, 11 Hz, 1H, 26 Ha), 3.60 (dd, J = 6, 11 Hz, 1H, 26-Hb), 5.73 (s, 1H, 4-H). M^+ 428 ($C_{29}H_{48}O_2$ requires M^+ 428; *m/e* 124 (base peak, 100%). GLC, R_f 9.72; TLC, R_f 0.89; after oxidation, R_f 0.75; after acetylation, R_f 1.27 and after reduction, R_f 0.89.

3-oxo-24-ethylcholest-4-en-26-oic acid (**10**). Recrystallisation of metabolite 10 gave white crystals (2 mg), i.r. 1740 (C_{26}–COOH), 1670 and 1610 cm^{-1} (4-en-3-one); u.v. 243 nm; NMR (δ) 0.70, 1.19 (6H, s, 18-CH_3 and 19-CH_3), 5.73 (1H, s, 4-H). M^+ 456 ($C_{30}H_{48}O_3$ requires M^+ 456) and *m/e* 124 (base peak, 100%). GLC, R_f 15.0; TLC, R_f 0.75; after oxidation, R_f 0.75; after acetylation, R_f 0.75 and after reduction, R_f 0.75.

Metabolite **11** (1 mg) was identical to 9α-hydroxyandrosta-1,4-diene-3,17-dione and has been previously described [7].

RESULTS

Pseudomonas sp. 10590 as with cholesterol [7] metabolised β-sitosterol completely after 72 h incubation. During the biodegradation period a transient

metabolite appeared which reached a maximum (38%) after 48 h growth. This metabolite (**2**) was isolated as a crystalline solid. The mass spectrum of metabolite **2** showed a low intensity molecular ion at *m/e* 412 and an intense (base peak) ion at *m/e* 124 suggesting a steroidal 4-en-3-one structure [16]. Confirmation of the structure of ring A was provided by the i.r. spectrum (1621 cm^{-1}, αβ-unsaturated ketone), the u.v. spectrum (λ_{max} 242 nm, di-β-substituted αβ-unsaturated ketone in a 6 membered ring, double bond exocyclic) [17] and by the PMR spectrum (one vinylic proton at δ 5.60 ppm). Metabolite **2** could not be oxidised, acetylated or reduced, indicating the absence of hydroxyl groups and unconjugated ketone groups. The mass spectrum also showed a low intensity ion at *m/e* 271 corresponding to the loss of a 10-carbon side-chain from C_{17}. Compound **2** has therefore been assigned the structure 24-ethylcholest-4-en-3-one.

Additional metabolites could not be detected in the culture medium without the addition of selective enzyme inhibitors. As with cholesterol [7] a number of metabolites accumulated in the medium during the fermentation of β-sitosterol by Pseudomonas sp. 10590.

β-Sitosterol fermentation in the presence of α,α′-D. The course of the fermentation was followed as described previously [7] and after the addition of 0.6 mM α,α′-D at 48 h the fermentation was continued for a further 24 h. A 1-litre culture was extracted after 72 h and separation of the extract by PLC yielded a major neutral metabolite (**2**) and two minor neutral metabolites (**3** and **4**) [Fig. 1] with two major acidic metabolites (**5** and **7**) and two minor acidic metabolites (**6** and **8**) [Fig. 2.].

Metabolite 2 was identical to 24-ethylcholest-4-en-3-one whilst metabolites **3–8** were identical to androst-4-ene-3,17-dione, androsta-1,4-diene-3,17-dione, 3-oxochol-4-en-24-oic acid, 3-oxochola-1,4-dien-24-oic acid, 3-oxopregn-4-ene-20-carboxylic acid and 3-oxopregna-1,4-diene-20-carboxylic acid respectively. The compounds have bee: previously described in detail [7].

β-Sitosterol fermentation in the presence of 2% n-propanol. When 2% *n*-propanol was added to the medium during log phase growth (24 h) of Pseudomonas sp. 10590 on β-sitosterol, an accumulation of metabolites occurred (some of which had not been observed previously). A 1-litre culture was extracted after 72 h and separation of the resultant extract into its components yielded a major neutral component (**2**) and three minor neutral components (**4**, **9** and **11**) [Fig. 1], with one major acidic compound (**10**) and two minor acidic compounds (**5** and **8**) [Fig. 2].

Metabolite **2** and two of the minor metabolites (**4** and **11**) were identical to 24-ethylcholest-4-en-3-

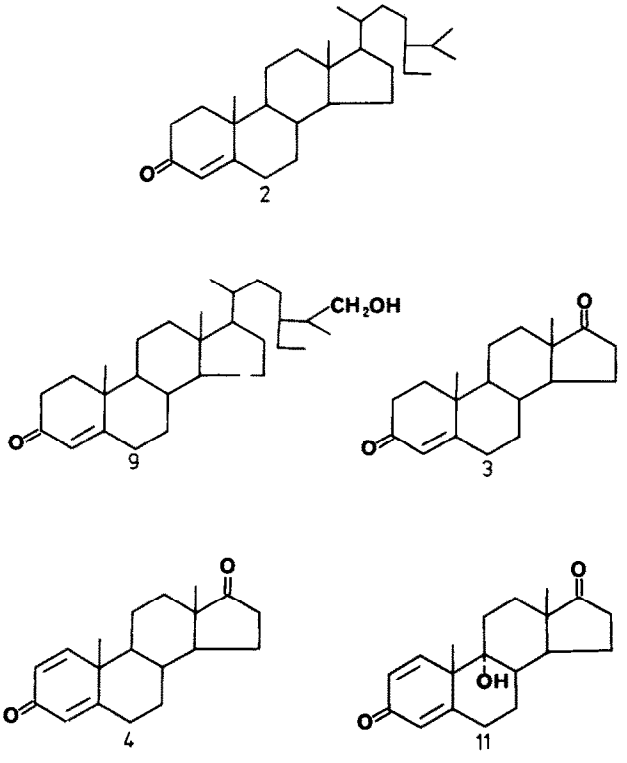


Fig. 1. Neutral metabolites isolated after the aerobic degradation of β-sitosterol by Pseudomonas sp NCIB 10590. **2.** 24-Ethylcholest-4-en-3-one. **3.** Androst-4-ene-3,17-dione. **4.** Androsta-1,4-diene-3,17-dione. **9.** 26-Hydroxy-24-ethylcholest-4-en-3-one. **11.** 9α-Hydroxyandrosta-1,4-diene-3,17-dione.

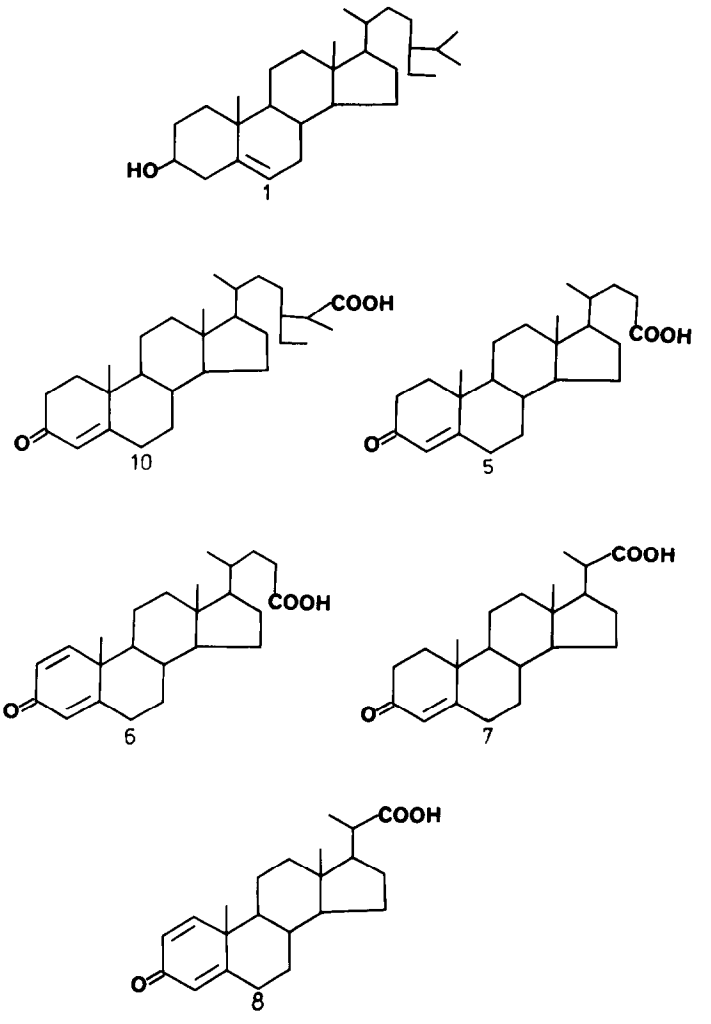


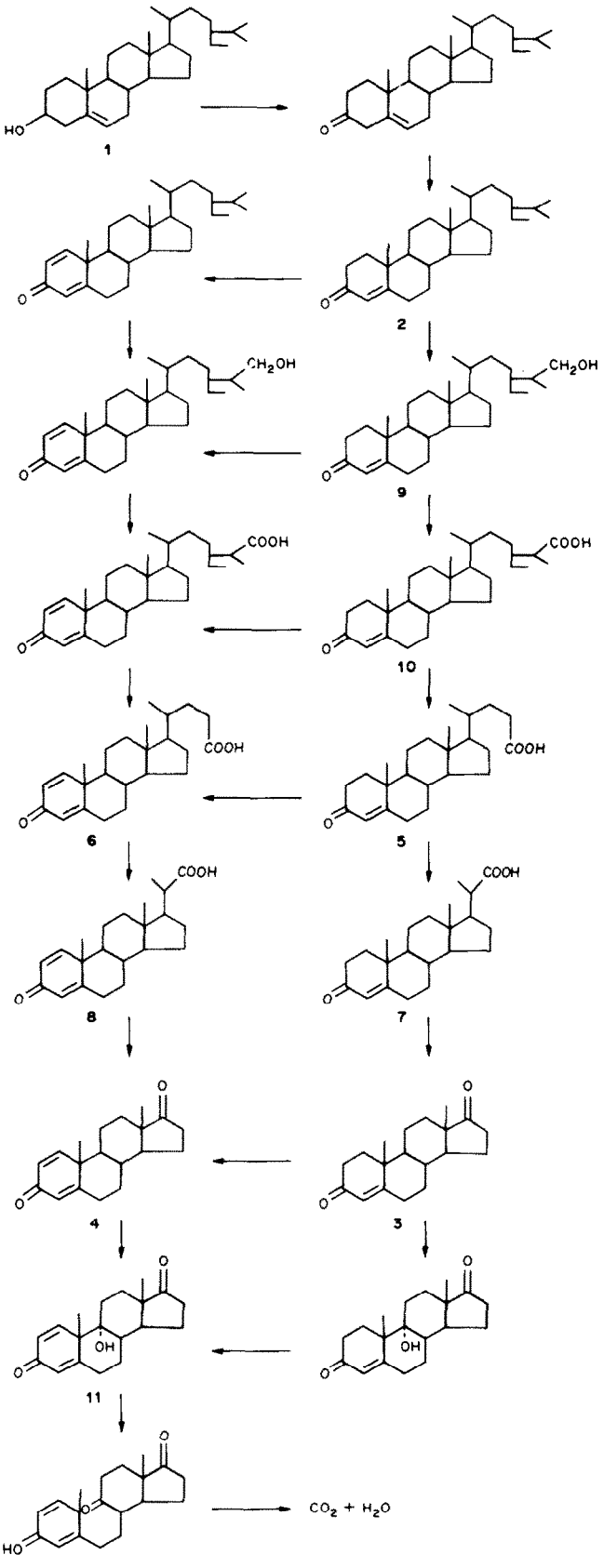
Fig. 2. Acidic metabolites isolated after the aerobic degradation of β-sitosterol (**1**) by Pseudomonas sp NCIB 10590. **1**. β-sitosterol. **5**. 3-Oxochol-4-en-24-oic acid. **6**. 3-Oxochola-1,4-dien-24-oic acid. **7**. 3-Oxopregn-4-ene-20-carboxylic acid. **8**. 3-Oxopregna-1,4-diene-20-carboxylic acid. **10**. 3-Oxo-24-ethylcholest-4-en-26-oic acid.

one, androsta-1,4-diene-3,17-dione and 9α-hydroxyandrosta-1,4-diene-3,17-dione respectively [7].

The minor neutral metabolite (**9**) revealed a low intensity molecular ion at *m/e* 428 and an intense ion at *m/e* 124, suggesting a steroidal 4-en-3-one structure [16]. Confirmation of the structure of ring A was provided by the i.r. spectrum (1630 cm^{-1}, αβ-unsaturated ketone) and u.v. spectrum (λ_{max} 242 nm, di-β-substituted αβ-unsaturated ketone, double bond exocyclic) and by the PMR spectrum (one vinylic proton at 5.73δ). Metabolite **9** could be acetylated with acetyl chloride substantiating the presence of a hydroxyl group, but could not be reduced indicating the absence of an unconjugated ketone group. When metabolite **9** was oxidised with Jones' chromic reagent [18] an acidic steroid was produced which was structurally identical to 3-oxo-24-ethylcholest-4-en-26-oic acid (**10**). Metabolite **9** has therefore been assigned the structure 26-hydroxy-24-ethylcholest-4-en-3-one.

The major acidic metabolite (**10**) was isolated as a crystalline solid. The mass spectrum of the methyl ester showed a low intensity molecular ion at *m/e* 456 with an intense ion at *m/e* 124 indicating a steroidal 4-en-3-one A-ring structure [16]. This was confirmed by the i.r. spectrum (1670 and 1610 cm^{-1}, αβ-unsaturated ketone), the u.v. spectrum (λ_{max} 243 nm) and the PMR spectrum (one vinylic proton at 5.73δ). Metabolite **10** could not be oxidised, acetylated or reduced, indicating a lack of hydroxyl or free ketonic groups. In addition, the melting point (160°C) was almost identical to that described for 3-oxo-24-ethylcholest-4-en-26-oic acid by Fujimoto *et al.* [13].

On the basis of the above data, metabolite **10** has been assigned the structure 3-oxo-24-ethylcholest-4-en-26-oic acid. The two minor acidic metabolites (**5** and **8**) were identical to 3-oxochol-4-en-24-oic acid and 3-oxopregna-1,4-dien-20-carboxylic acid respectively [7].



Scheme 1. Proposed pathway of β-sitosterol (**1**) degradation by Pseudomonas sp NCIB 10590 under aerobic conditions. Compounds 2–11 were isolated during this study.

DISCUSSION

Although the microbial degradation of the phytosterol side-chain has been described in detail by Fujimoto *et al.* [13, 14], the number of intermediates described has remained sparse. The results of this work reveal that Pseudomonas sp. NCIB 10590 is not inhibited by the bulkier side-chain of β-sitosterol and is thus able to metabolise this steroid in a manner analogous to cholesterol degradation [7].

Prior to side-chain cleavage of both bile acids [15, 19–21] and sterols [7] it is evident that the Pseudomonad must initially oxidise the A-ring to at least a 4-en-3-one structure. When this is achieved the substrate is amenable to β-oxidation of the side-chain (bile acids) and to α-oxidation followed by β-oxidation of the side-chain (sterols).

The metabolism of β-sitosterol by Pseudomonas sp. 10590 follows a similar pattern to that described for cholesterol. The terminal methyl group at C_{26} is functionalised by hydroxylation followed by sequential oxidation via the C_{26} aldehyde to the C_{26} carboxylic acid involving alcohol and aldehyde dehydrogenases. The side-chain is then degraded via C_{24} and C_{22} intermediates to C_{19} androstane products. We have not investigated the incorporation of HCO_3^- onto the C-28 position of 3-oxo-24-ethylcholest-4-en-26-oic acid prior to carbon–carbon bond fission. However the results of this study support the findings of Fujimoto *et al.* [14] and confirm that the mode of side-chain cleavage of phyto sterols is via C_{26}-OH, C_{26}-COOH, C_{24}-COOH, C_{22}-COOH metabolites through to C_{19} products. On the basis of these results we wish to propose a pathway (Scheme 1) of β-sitosterol degradation by Pseudomonas sp. 10590 under aerobic conditions.

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