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Microbial transformation of β-sitosterol and stigmasterol into 26-oxygenated derivatives

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The genetically modified Mycobacterium *sp. BCS 396 strain has been used to transform sterols with stigmastane side chain in order to obtain 26-oxidized metabolites.* β-*Sitosterol* ***(I)*** *was transformed to 4-stigmasten-3-one* ***(II)****, 26-hydroxy-4-stigmasten-3-one* ***(III)****, and 3-oxo-4-stigmasten-26-oic acid* ***(IV)****, while stigmasterol* ***(V)*** *was converted to 4,22-stigmastadien-3-one* ***(VI)****, 6*β*-hydroxy-4,22-stigmastadien-3-one* ***(VII)****, 26-hydroxy-4,22-stigmastadien-3-one* ***(VIII)****, 3-oxo-4,22-stigmastadien-26-oic acid methyl ester* ***(IX)****, and 3-oxo-1,4,22-stigmastatrien-26-oic acid methyl ester* ***(X)*** *with that strain. In both* β*-sitosterol and stigmasterol, 26-oxidation generates the* R*-configuration on C-25. (Steroids* **60**:621–625, 1995)

Keywords: β-sitosterol; microbial side chain degradation; mechanism of C-26-oxidation

Introduction

Our research group has long dealt with the cleavage of sterol side chain by several *Mycobacterium* species. Initially, we inhibited the steroid skeleton degradation by 8-hydroxyquinoline in order to obtain selective removal of the sterol side-chain.[1,2]

In the 1970s Marsheck et al.[3] and Wovcha et al.[4] obtained mutants from sterol-consuming *Mycobacterium* strains, which lost their property to degrade the steroid skeleton, by different mutagenic treatments. Recently, 17-ketosteroids have been produced as intermediates in the syntheses of a wide variety of steroid drugs by such mutants from phytosterols.

In the last years we used genetically modified *Mycobacterium* strains in microbial transformations in order to afford partial side-chain cleavage of sterols.[5] One of our aims was to obtain derivatives of 17(20)-dehydro-23,24-dinorcholanoic acids, from which novel syntheses of corticosteroid intermediates bearing the pregnane side-chain were elaborated.[6–8]

In the course of that work we also isolated 24(25)-dehydro-26-oxygenated derivatives from β-sitosterol. The main product of this type of metabolite was 9α-hydroxy-3-oxo-4,24(25)-stigmastadien-26-oic acid in which the configuration of 24(25)-double bond was determined as being E.[9,10] Since Sih et al. proposed that 3-oxo-4-stigmasten-26-oic acid is degraded by the β-oxidation mechanism,[11,12] we supposed the latter compound to be the precursor of 9α-hydroxy-3-oxo-4,24(25)-stigmastadien-26-oic acid. The X-ray study of 3-oxo-4-stigmasten-26-oic acid which was also isolated in that experiment, revealed that the newly formed C-25 chiral center has the *R*-configuration.[9]

In this paper we report on the microbial transformation of β-sitosterol and stigmasterol with the genetically modified *Mycobacterium* sp. BCS 396 strain, which accumulates products of initial steps in the side-chain degradation to a higher extent.

Experimental

Microbial transformation of β*-sitosterol (I) with* Mycobacterium *sp. BCS 396 strain*

Mycobacterium sp. BCS 396 strain was obtained by the technique of spheroplast fusion[5] and deposited in the National Collection of Agricultural and Industrial Microorganisms (Budapest) under No. NCAIM B(P) 000348. Cultures of this strain were cultivated for 7 days at 32°C on agar slants. The agar medium contained (in g/L of distilled water): Lab lemco meat extract, 8.0; glycerol, 5.0; NaCl, 5.0; agar, 15.0.

β-Sitosterol transformations were carried out in 500 mL flat-bottomed flasks containing 100 mL culture medium. The microbial transformation medium used consisted of (grams per liter of distilled water): glycerol, 5.0; meat extract, 8.0; yeast extract, 1.0; Tween 80, 1.0, adjusted to pH 6.5. A fine suspension of 15 g of β-sitosterol, 2 g Tween 80, and 6 g polypropylene glycol 2000

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was dispersed by heating and added to 1 L of the culture medium before autoclaving at 121°C for 20 min. The sterile culture media in 10 flasks were inoculated with the *Mycobacterium* cells and shaken on a rotary shaker (deflection 2.5 cm, 320 rotations min^{-1}) at 32°C for 168 h.

After incubation, the mycobacterial cells were filtered off and extracted three times with 0.15 L ethyl acetate. The filtrate was also extracted three times with 0.2 L ethyl acetate. The extracts were combined and the solvent was evaporated under reduced pressure. The residue (19 g) was chromatographed on a silica gel column filled with 300 g of Kieselgel 60 (Reanal Ltd, Budapest) using *n*-hexane and ethyl acetate mixtures of gradually increasing ethyl acetate content as eluents. The three transformation products identified earlier were eluted in this way. Elution with 10% ethyl acetate in hexane yielded 305 mg 4-stigmasten-3-one (**II**; m.p.: 89–90°C),[13] 30% ethyl acetate in hexane gave 405 mg 26-hydroxy-4-stigmasten-3-one (**III**) (m.p.: 108–114°C), and 40% ethyl acetate in hexane resulted in 4.1 g 3-oxo-4-stigmasten-26-oic acid (**IV**; m.p.: 164–168°C).[9,11]

Microbial transformation of stigmasterol (V) with Mycobacterium *sp. BCS 396 strain*

Biotransformation of stigmasterol was carried out in 10 × 500 mL flasks containing 100 mL of the same culture medium as described in β-sitosterol conversion, but stigmasterol was transformed with grown microbial suspension. A solution of 200 mg stigmasterol in 5 mL ethanol was added to each flask, when the culture was in exponential growth phase (48 h). Then the microbial transformation of stigmasterol was continued at 32°C for 192 h.

After incubation, the products were extracted from the broth as described previously for β-sitosterol transformation. The crude product (3.1 g) obtained by evaporation of the combined extracts was chromatographed on a silica gel column prepared from 50 g of Kieselgel 60 using the eluent system that was applied in the separation of β-sitosterol transformation products.

Elution of the column with 15% ethyl acetate in hexane produced 90 mg of the known 4,22-stigmastadien-3-one (**VI**); m.p.: 122–124°C after recrystallization from acetone.[14]

Characteristic spectral data. IR (in KBr pellet): ν_{CH} 2930, 2865; $\nu_{C=O}$ 1680; $\nu_{C=C}$ 1620; ^1H NMR ($CDCl_3$, δ_{TMS} = 0.0 ppm): 4.95–5.25 (m, 2 H, H-22 + H-23); 5.73 (bs, 1 H, H-4); ^{13}C NMR ($CDCl_3$, δ_{TMS} = 0.0 ppm): 123.8 (C-4); 129.5 (C-23); 138.2 (C-22); 171.7 (C-5); 201.0 (C-3);
MS (EI, 70 eV, see Figure 1): $[M]^+$ 410 (100%, $C_{29}H_{46}O$); m/z 367 (43%, $C_{26}H_{39}O$); m/z 298 (41%); m/z 271 (53%, $C_{19}H_{27}O$); m/z 269 (40%, $C_{19}H_{25}O$); m/z 245 (20%, $C_{17}H_{25}O$); m/z 124 (29%, $C_8H_{12}O$).

Further elution with 20% ethyl acetate in hexane yielded 510 mg of 3-oxo-4,22-stigmastadien-26-oic acid methyl ester (**IX**); m.p.: 153–155°C after recrystallization from acetone, $[\alpha]_D$ = +58° (c = 1, chloroform).

Characteristic spectral data. IR (in KBr pellet): ν_{CH} 2965, 2940, 2870; $\nu_{C=O}$ 1745, 1670; $\nu_{C=C}$ 1610; ^1H NMR ($CDCl_3$, δ_{TMS} = 0.0 ppm): 3.65 (s, 3 H, OCH_3); 4.93 (dd, 1 H, H-23); 5.27 (dd, 1 H, H-22); 5.73 (bs, 1 H, H-4);
^{13}C NMR ($CDCl_3$, δ_{TMS} = 0.0 ppm): 51.3 (OCH_3); 123.7 (C-4); 127.8 (C-23); 139.7 (C-22); 171.5 (C-5); 176.8 (C-26); 199.5 (C-3);
MS (EI, 70 eV, see Figure 1): $[M]^+$ 454 (100%, $C_{30}H_{46}O_3$); m/z 367 (44%, $C_{26}H_{39}O$); m/z 366 (37%, $C_{26}H_{38}O$); m/z 297 (40%); m/z 271 (60%, $C_{19}H_{27}O$); m/z 269 (47%, $C_{19}H_{25}O$); m/z 245 (28%, $C_{17}H_{25}O$); m/z 210 (12%, $C_{13}H_{22}O_2$); m/z 183 (21%, $C_{11}H_{19}O_2$); m/z 124 (12%, $C_8H_{12}O$); m/z 123 (62%, C_9H_{15}); m/z 95 (54%, C_7H_{11}).

The fractions collected from the elution of the column with 25% ethyl acetate in hexane yielded 120 mg of 3-oxo-1,4,22-stigmastatrien-26-oic acid methyl ester (**X**); m.p.: 129–135°C after recrystallization from acetone, $[\alpha]_D$ = −8° (c = 1, chloroform).

Characteristic spectral data. IR (in KBr pellet): ν_{CH} 2930, 2870; $\nu_{C=O}$ 1740, 1665; $\nu_{C=C}$ 1625, 1605; ^1H NMR ($CDCl_3$, δ_{TMS} =

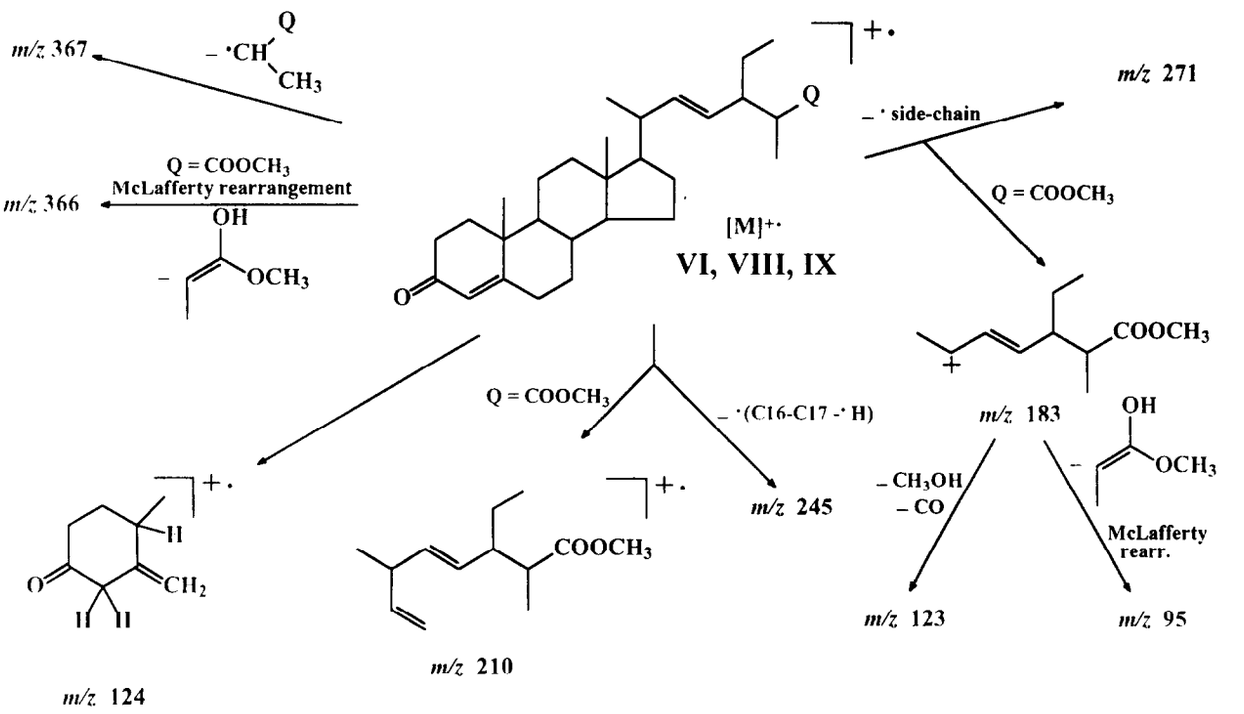

Figure 1 Mass spectral fragmentation of compounds **VI**, **VIII**, and **IX**.

0.0 ppm): 3.65 (s, 3 H, OCH_3); 4.93 (dd, 1 H, H-23); 5.25 (dd, 1 H, H-22); 6.05 (bs, 1 H, H-4); 6.22 (dd, 1 H, H-2); 7.05 (d, 1 H, H-1);
^{13}C NMR ($CDCl_3$, δ_{TMS} = 0.0 ppm): 51.3 (OCH_3); 123.8 (C-4); 127.4 (C-2); 127.9 (C-23); 139.7 (C-22); 155.9 (C-1); 169.3 (C-5); 176.8 (C-26); 186.4 (C-3);
MS (EI, 70 eV): $[M]^+$ 452 (66%, $C_{30}H_{44}O_3$); m/z 183 (60%, $C_{11}H_{19}O_2$, [side chain]$^+$); m/z 123 (43%, C_9H_{15}, [183 − CH_3OH − CO]$^+$); m/z 122 (100%, $C_8H_{10}O$, [ring A + C-6 + 2H]$^+$); m/z 121 (60%, C_8H_9O, [ring A + C-6 + H]$^+$); m/z 95 (39%, C_7H_{11}, see Figure 1).

Further elution with 30% ethyl acetate in hexane produced 25 mg of 26-hydroxy-4,22-stigmastadien-3-one **(VIII).**

Characteristic spectral data. IR (in KBr pellet): ν_{OH} 3420; ν_{CH} 2935, 2870; $\nu_{C=O}$ 1670; $\nu_{C=C}$ 1615; ^1H NMR ($CDCl_3$, δ_{TMS} = 0.0 ppm): 3.47 (dd, 2 H, H-26); 5.08 (dd, 1 H, H-23); 5.24 (dd, 1 H, H-22); 5.72 (bs, 1 H, H-4);
^{13}C NMR ($CDCl_3$, δ_{TMS} = 0.0 ppm): 67.4 (C-26); 123.9 (C-4); 128.2 (C-23); 139.1 (C-22); 171.7 (C-5); 199.7 (C-3);
MS (EI, 70 eV, see Figure 1): $[M]^+$ 426 (89%, $C_{29}H_{46}O_2$); m/z 367 (16%); m/z 297 (62%); m/z 271 (66%); m/z 269 (61%); m/z 245 (38%, $C_{17}H_{25}O$); m/z 124 (38%).

Further elution with 40% ethyl acetate in hexane yielded 50 mg of the known 6β-hydroxy-4,22-stigmastadien-3-one **(VII);** m.p.: 212–214°C after recrystallization from acetone.[14]

Analytical methods

Melting points were determined on a Boetius apparatus, the $[\alpha]_D$ values on an Opton polarimeter. IR spectra were measured on a Bruker IFS-85 FT-IR spectrometer. NMR spectra were obtained on a Bruker AC-250 NMR spectrometer. Mass spectra were taken on a Finnigan MAT 8430 mass spectrometer; elemental compositions of the ions were determined by high resolution (R = 10000) mass measurements using perfluorokerosene (PFK) as the reference standard.

The transformation of the substrates as well as the isolation and purification of the transformation products were followed by thin-layer chromatography (TLC). The conditions and the results of TLC analysis were the following: adsorbent layer: silica gel 60 F_{254} (Sheet No. 5554, E. Merck, Darmstadt, Germany); developing solvent: ethyl acetate-*n*-hexane (3:7, v/v); distance of development: 16 cm; detection: by scanning the sheets with a CAMAG TLC scanner II (CAMAG, Muttenz, Switzerland) i) in UV light at 240 nm, ii) at 450 nm after spraying the sheet with H_2SO_4: EtOH (1:1, v/v) and heating at 110°C for 3 min; R_f values of the compounds: **I:** 0.55; **II:** 0.80; **III:** 0.35; **IV:** 0.25; **V:** 0.58; **VI:** 0.80; **VII:** 0.20; **VIII:** 0.35; **IX:** 0.70; **X:** 0.55.

In the course of the bioconversions the evaporation residues of the ethyl acetate extracts of the broth samples were analyzed. Incubations of the substrates (**I, V**) carried out in the absence of *Mycobacterium* BCS 396 strain in the culture medium were similarly followed up.

Table 1 Selected bond lengths (with ESD-s in parentheses) for the side chain of **IX**

Carbon numbers	Bond lengths (Å)	Carbon numbers	Bond lengths (Å)
C(17)–C(20)	1.520(9)	C(25)–C(26)	1.524(12)
C(20)–C(21)	1.527(11)	C(25)–C(27)	1.527(12)
C(20)–C(22)	1.516(10)	C(28)–C(29)	1.495(12)
C(22)–C(23)	1.309(9)	C(26)–O(2)	1.169(9)
C(23)–C(24)	1.513(9)	C(26)–O(3)	1.311(10)
C(24)–C(25)	1.504(11)	C(30)–O(3)	1.445(11)
C(24)–C(28)	1.538(11)		

X-ray crystal structure analysis of **IX:** Crystals of **IX** were grown from acetone. Data were collected on a Rigaku AFC6S diffractometer. The structure was solved using the teXsan program package (Molecular Structure Corporation, 1992) running on a Silicon Graphics R3000 workstation. Hydrogen atoms were generated. The fractional coordinates with their estimated standard deviation (ESD) values, full lists of bond lengths, bond angles, and thermal parameters will be deposited at the Cambridge Crystallographic Data Centre. The applied weighing scheme was $w = 1/\sigma^2(F_o)$.
$C_{30}H_{46}O_3$, M_W = 454.69, a = 12.267(5) Å, b = 30.630(3) Å, c = 7.365(6) Å, V = 2767(1) Å^3, $P2_12_12_1$, Z = 4, D_{calc} = 1.091 gcm^{-3}, N_{tot} = 3299, N_{obs} = 1328 ($I > 1.5\sigma$ (I)), R = 0.055, R_W = 0.101, $2\Theta_{max}$ = 150°, λ = 1.5418 Å, μ = 5.26 cm^{-1}.
Selected bond lengths, bond angles, and torsion angles are given in Tables 1–3.

Results and discussion

In this paper we compare the microbial transformation of β-sitosterol and stigmasterol by *Mycobacterium* sp. BCS 396 strain, which lost its ability to accomplish β-oxidation because of the damage of the respective enzyme system due to genetic modification.

The first step in the microbial transformation of β-sitosterol (**I**) is the formation of 4-en-3-one structure in ring A of the steroid skeleton, as was proven by the isolation of 4-stigmasten-3-one (**II**) from the fermentation broth. The stigmastane side-chain was subsequently hydroxylated at C-26, and the resulting 26-hydroxy-4-stigmasten-3-one (**III**) was transformed into 3-oxo-4-stigmasten-26-oic acid (**IV**), identical with that described earlier.[9,11] This initial degradation of the stigmastane side chain fits into the mi-

Table 2 Selected bond angles (with ESD-s in parentheses) for the side chain of **IX**

Carbon numbers	Bond angles (degrees)	Carbon numbers	Bond angles (degrees)
C(17)–C(20)–C(21)	113.7(6)	C(24)–C(25)–C(27)	112.3(7)
C(17)–C(20)–C(22)	111.2(6)	C(24)–C(28)–C(29)	114.5(8)
C(20)–C(22)–C(23)	126.8(8)	C(25)–C(24)–C(28)	111.7(7)
C(21)–C(20)–C(22)	108.1(6)	C(26)–C(25)–C(27)	107.3(8)
C(22)–C(23)–C(24)	126.8(8)	C(25)–C(26)–O(3)	111.3(8)
C(23)–C(24)–C(25)	110.8(6)	C(25)–C(26)–O(2)	124.9(9)
C(23)–C(24)–C(28)	108.2(7)	O(2)–C(26)–O(3)	123.8(9)
C(24)–C(25)–C(26)	111.4(7)	C(26)–O(3)–C(30)	115.1(8)

Table 3 Selected torsion angles (with ESD-s in parentheses) for the side chain of **IX**

Carbon numbers	Torsion angles (degrees)
C(13)–C(17)–C(20)–C(21)	−61.9(9)
C(13)–C(17)–C(20)–C(22)	175.7(6)
C(17)–C(20)–C(22)–C(23)	−129.6(9)
C(18)–C(13)–C(17)–C(20)	−46.5(9)
C(20)–C(22)–C(23)–C(24)	−176.2(7)
C(22)–C(23)–C(24)–C(25)	−127.5(9)
C(22)–C(23)–C(24)–C(28)	109.8(10)
C(23)–C(24)–C(25)–C(26)	−178.6(7)
C(23)–C(24)–C(25)–C(27)	60.8(9)
C(23)–C(24)–C(28)–C(29)	−67.0(9)
C(24)–C(25)–C(26)–O(3)	132.2(9)
C(25)–C(26)–O(3)–C(30)	179.3(10)

crobial degradation pathway elucidated by Sih and colleagues.[11,12]

The microbial transformation products formed from stigmasterol (**V**) were 4,22-stigmastadien-3-one (**VI**), 6β-hydroxy-4,22-stigmastadien-3-one (**VII**), 26-hydroxy-4,22-stigmastadien-3-one (**VIII**), 3-oxo-4,22-stigmastadien-26-oic acid methyl ester (**IX**), and 3-oxo-1,4,22-stigmastatrien-26-oic acid methyl ester (**X**), as shown in Figure 2.

With respect to the structure elucidation of the isolated products, mass spectrometry was very informative and gave the first characteristic data on the compounds studied.[15,16]

The mass spectral fragmentation of 4,22-stigmastadien-3-one (**VI**) and its 26-oxygenated derivatives (**VIII** and **IX**) is shown in Figure 1 as an example. Besides the well-known fragmentation pattern of 4-en-3-ones, i.e., the formation of ion m/z 124 from ring A + C-6 by double H-rearrangement as well as the loss of the side chain (also with a double H-rearrangement) characteristic for (side-chain unsaturated) sterols and elimination of C-16–C-17 (with H-rearrangement) from ring D observed in various 17-substituted steroids, the presence of the carbo(metho)xyl group at C-26 of the unsaturated side-chain of compound **IX** results in some unique features, which are depicted in Figure 1. These are: i) the loss of C-25–C-27 in a McLafferty rearrangement from both the molecular ion and the side chain ion m/z 183, yielding ions m/z 366 and m/z 95, respectively; ii) the salient abundance of the side-chain ion m/z 183 and its daughter ions m/z 123 and m/z 95; iii) the occurrence of ion m/z 210, representing the C-16–C-17 portion of ring D (with the side-chain) in moderate abundance which is normally not observed in steroid mass spectra. These features are also observed in the mass spectrum of compound **X** (see in Experimental).

Most of the stigmasterol metabolites have 4-en-3-one structure in ring A of steroid skeleton, but a part of compound **IX** was dehydrogenated between C-1 and C-2 yielding 3-oxo-1,4,22-stigmastatrien-26-oic acid methyl ester (**X**).

We also observed the formation of 6β-hydroxy-4,22-stigmastadien-3-one from stigmasterol. Similar hydroxylation was also found by Mahato and Banerjee during transformation of stigmasterol by a soil pseudomonad,[14] and by Katsui et al. who isolated 6β-hydroxy-4,22-stigmastadien-3-one (**VII**) from roots of kidney beans.[17]

Control experiments were carried out under conditions similar to the bioconversions, but in the absence of the microorganism, to investigate the possibility of autooxidation of β-sitosterol (**I**) and stigmasterol (**V**) by ambient air. In these experiments, no transformation of the sterol substrates could be detected.

X-ray diffraction studies on 3-oxo-4,22-stigmastadien-26-oic acid methyl ester (**IX**) formed as the main product from stigmasterol indicated that it has the *R*-configuration at C-25 (see Figure 3) similarly to that of the corresponding β-sitosterol metabolite **IV**.

Thus, it can be concluded that the microbial oxidation of the sterol side-chain at C-26, the initial step of the degradation process, comprises a stereoselective reaction in both of these substrates.

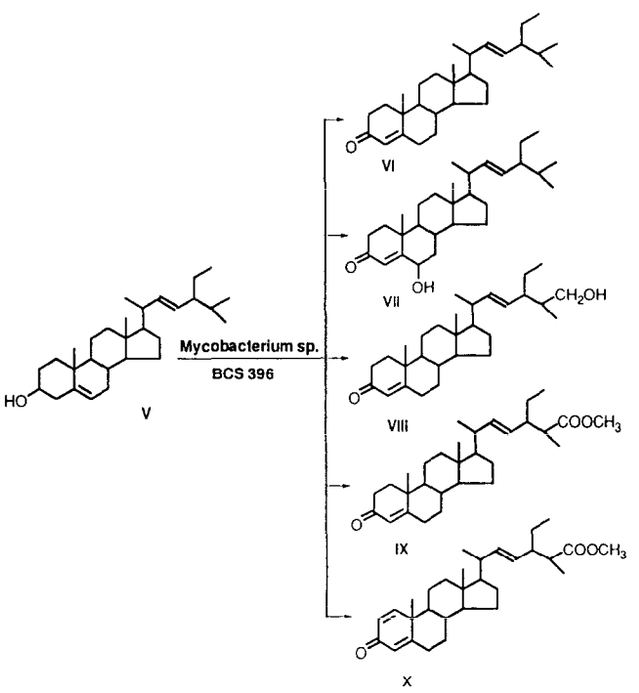

Figure 2 Microbial transformation of stigmasterol obtained by *Mycobacterium* sp. BCS 396 strain.

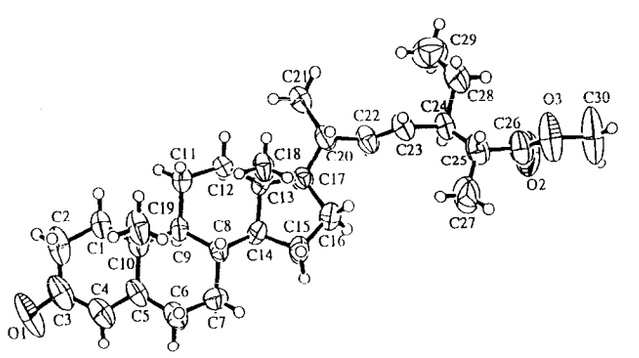

Figure 3 X-ray structure of 3-oxo-4,22-stigmastadien-26-oic acid methyl ester (**IX**).

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