

Microbial Oxidation of Sterol Side-Chains[†]

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Several sterol metabolising microorganisms, *Pseudomonas convexa*, *P. stutzeri*, *Micrococcus* sp., *Cephalosporium longisporum* and *Moraxella* sp. were isolated from soil on 2-cyclopentyl-6-methyl heptane and cholestane as carbon sources. All these organisms were shown to have the capacity of oxidizing the eight carbon side chain of cholesterol. Excepting *P. convexa*, the organisms needed a chelating agent for prevention of steroid ring degradation. The *Moraxella* sp. proved to be the most versatile as it degraded cholesterol derivatives such as 3 β -methoxy cholest-5-ene, 3 β -chloro cholest-5-ene, 3,5-cyclocholestan-6-one and potassium cholesteryl sulphate to the corresponding 17-keto steroids in 10-50% yield in shake flasks. It also gave high yields of estrone from both 19-hydroxy-3 β -acetoxy cholest-5-ene and 19-hydroxy-3 β -acetoxy sitost-5-ene. Two new enzymes one carrying out the oxidative O-demethylation of 6 β -methoxy 3,5-cyclocholestan-6-one and the other, the isomerisation of *i*-steroids to normal 3 β -hydroxy- Δ^4 steroids were discovered.

Immobilized *Moraxella* cells in agar carried out the side chain degradation of cholesteryl sulphate to yield 3 β -hydroxy androst-5-en-17-one sulphate and the 19-hydroxy-3 β -acetoxy derivatives of both cholesterol and sitosterol to estrone in high yields. The half life of entrapped cells was estimated to be 28 days.

It is estimated that the current turnover of estrone based oral contraceptives in India is Rs. 4 crore per annum. Although methods have been developed in several laboratories and particularly this department for the total synthesis of estrone and steroid analogues, the Indian steroid industry largely depends on imported estrone as it finds the costs involved in large scale manufacture abnormally high.

For the last eight years we have been trying to develop a microbial process to eliminate the C₁₇ side-chain of cholesterol (1) and sitosterol (2) and their derivative to obtain useful steroidal intermediates such as C-19, 3 β -hydroxy-androst-5-en-17-one (3), for anabolic steroids and the C-18 estrone (4) for oral contraceptives (Chart 1).

The standard approach which has been overwhelmingly used by other workers in the area is to select microorganisms from natural sources e.g. soil, sewage, silage, etc. on cholesterol or some of its derivatives as the sole source of carbon with inorganic nutrients. A vast majority of such organisms have as a predominating activity the oxidation of the steroid ring system, initiated through the dehydrogenation of the 3 β -hydroxyl group. Only a few organisms have pronounced side-chain degrading ability. Typical pathways for steroid degradation are shown in Chart 2¹⁻³.

In most organisms cholest-4-en-3-one (5) is the immediate metabolite of cholesterol. The elimination of side-chain occurs at this stage with the major

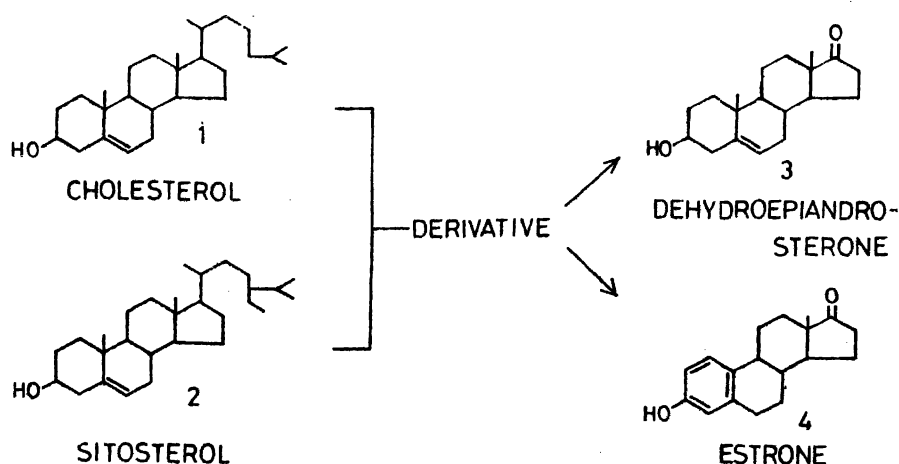


Chart 1
Objectives

[†] Acharya P. C. Ray Memorial Lecture (1979) delivered under the auspices of the Indian Chemical Society, on December 27, 1983 at Cuttack.

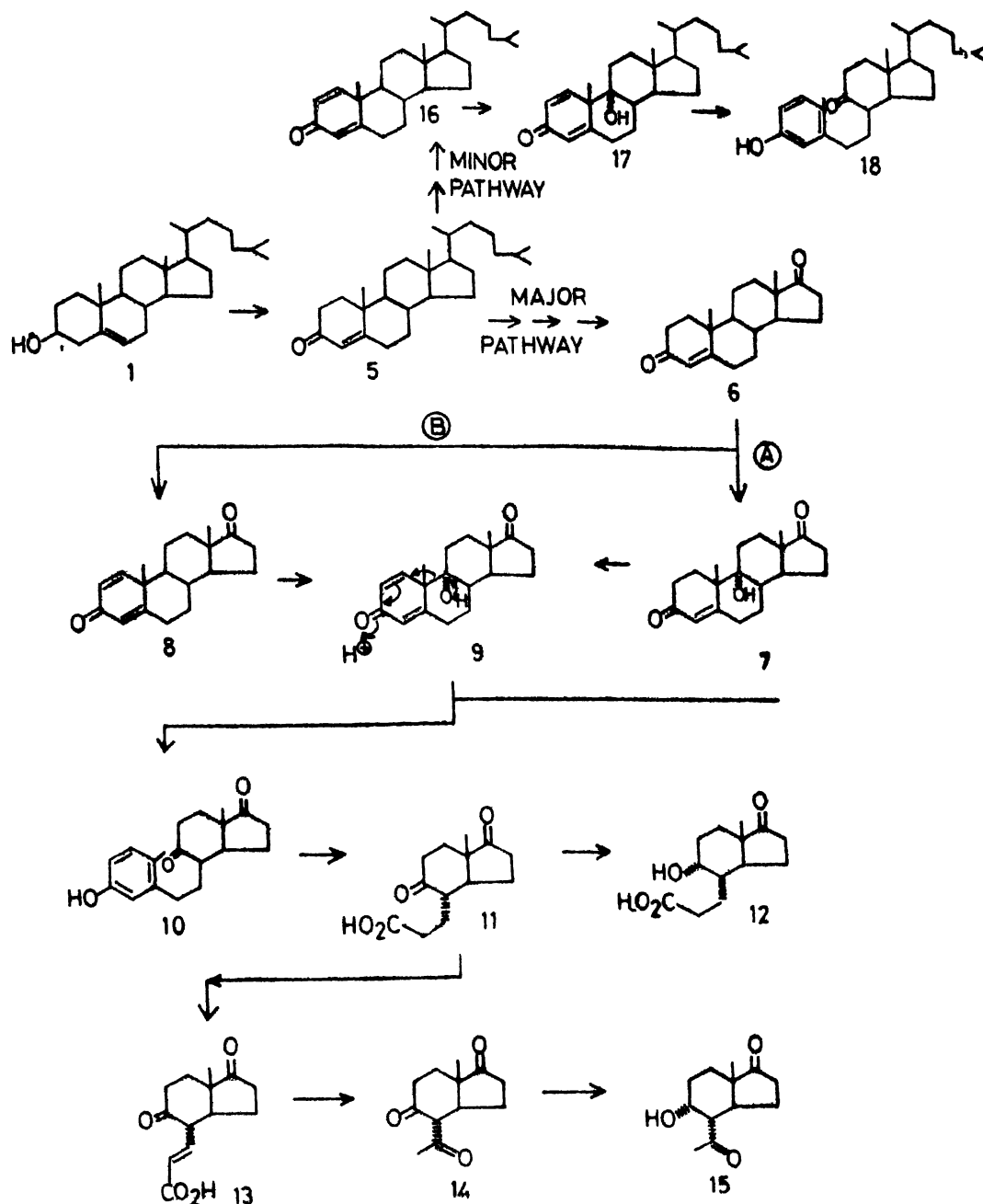


Chart 2

Steroid ring cleavage by microorganisms

rity of sterol degrading microbes such as *Nocardia*, *Pseudomonas*, *Mycobacteria*, *Bacillus*, *Arthrobacters*, etc⁴⁻⁶. However, 1,2-dehydrogenation (16), 9 α -hydroxylation (17) or *seco*-phenol (18) formation have also been known to occur without any degradation or attack on the side chain⁷⁻¹². Besides the mode of B-ring degradation as shown in Chart 2, occasionally ring A degradation has been noted particularly between C-3 and C-4 and C-4 and C-5¹³.

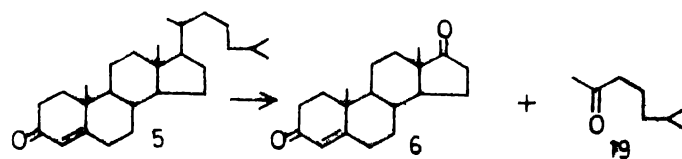
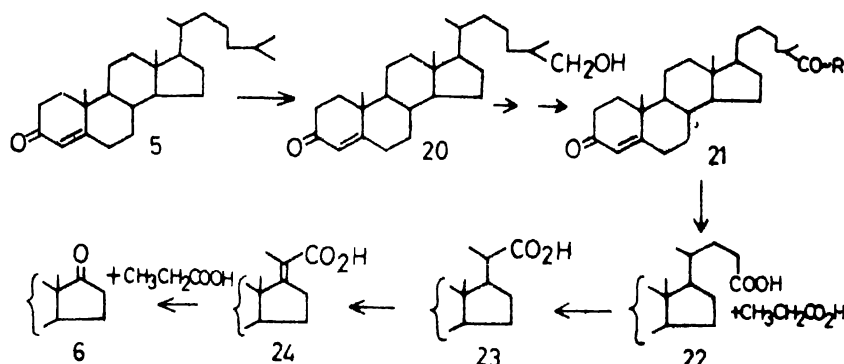
Elimination of side chain :

Different microbes have atleast two pathways for cleavage of side-chains. Horvath and Kramli¹⁴

isolated 6-methyl-heptan-2-one (19) from fermentation of cholesterol indicating a direct cleavage between C-17 and C-20 sterols. Our data also indicate (vide *Supra*^{4,6}) that a similar pathway exists in *Pseudomonas convexa*. However, this is a rare pathway and most organisms prefer to use step-wise degradation-terminal oxygenation (28) followed by progressive β -oxidation (Chart 3).

In *Mycobacteria* and other species the terminal hydroxylation at C-26 of cholesterol has been clearly established^{9,15,16}. The oxidation of 26-hydroxy-cholesterol (20) to the carboxylic acid (21) followed

A) 17,20 CLEAVAGE

B) STEPWISE CLEAVAGE (β -OXIDATION)

SITOSTEROL SIDE CHAIN OXIDATION

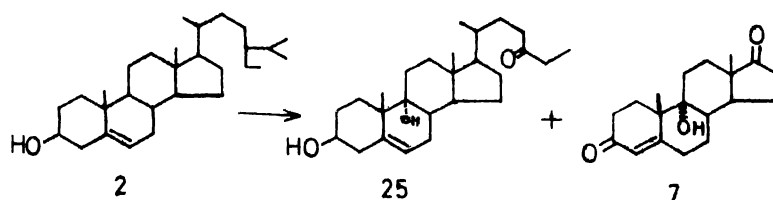


Chart 3

Side chain cleavage by microorganisms

by progressive β -oxidation through the C-24 acid (22) with elimination of propionic acid and the C-22 acid (23) was proposed by Sih and co-workers as a logical pathway^{6,6,12,17}. The last stage of elimination of a 3-carbon piece as propionic acid from 23 has been shown to occur through dehydrogenation at the 17-20 bond (24). This can undergo a reverse aldolisation to the 17-ketone and has been shown to occur under anaerobic conditions¹².

Data on the mechanism of elimination of the ten-carbon side-chain of sitosterol (2) are scanty. However, the isolation of 9 α -hydroxy-27-nor-cholest-4-ene-3,24-dione (25) and 9 α -hydroxy androst-4-ene-3,17-dione (9) in the fermentation of sitosterol, stigmasterol and campesterol by a mutant of *Mycobacterium fortuitum* SCM-I indicates a β -oxidative pathway for ten-carbon chains¹².

In most strains the Δ -3-oxo structure is preferred for the side-chain cleavage reactions⁹. However, the reported degradation of 3 β -methoxy cholest-5-en (24) to 3 β -methoxy androst-5-en-17-one¹⁹ as well as our data on modified steroids^{4,2} (vide *Supra*) indicate that this is not an absolute requirement.

Ring-A aromatization :

Sih and Wang reported²⁰ the degradation of 19-hydroxy cholest-4-en-3-one (26) as well as 19-hydroxy stigmaster-4-en-3-one (27) by *Nocardia restrictus* and other species to estrone (4). This was probably inspired by an earlier report by Dodson and Muir²¹ of aromatization of 19-hydroxy androst-4-en-3,17-dione by a *Pseudomonad*. Later, it was established that 3 β -acetoxy derivative of 19-hydroxy cholesterol (28), an intermediate in the chemical synthesis of (26), was a better substrate than 26 and yields of 75% of estrone could be obtained from this substrate in *Nocardia* sp. ATCC 19170²². These developments have made the microbial process of estrone from sterols a formidable competitor against the synthetic processes.

The mechanism of ring A aromatization² follows the well known dienone-phenol rearrangement with elimination of the angular hydroxymethyl as formaldehyde. In most organisms the side chain elimination is believed to take place before aromatization can occur, as 19-nor-3-hydroxy-cholesta-1,3,5(10) triene (29) is not metabolized²³. On the other hand,

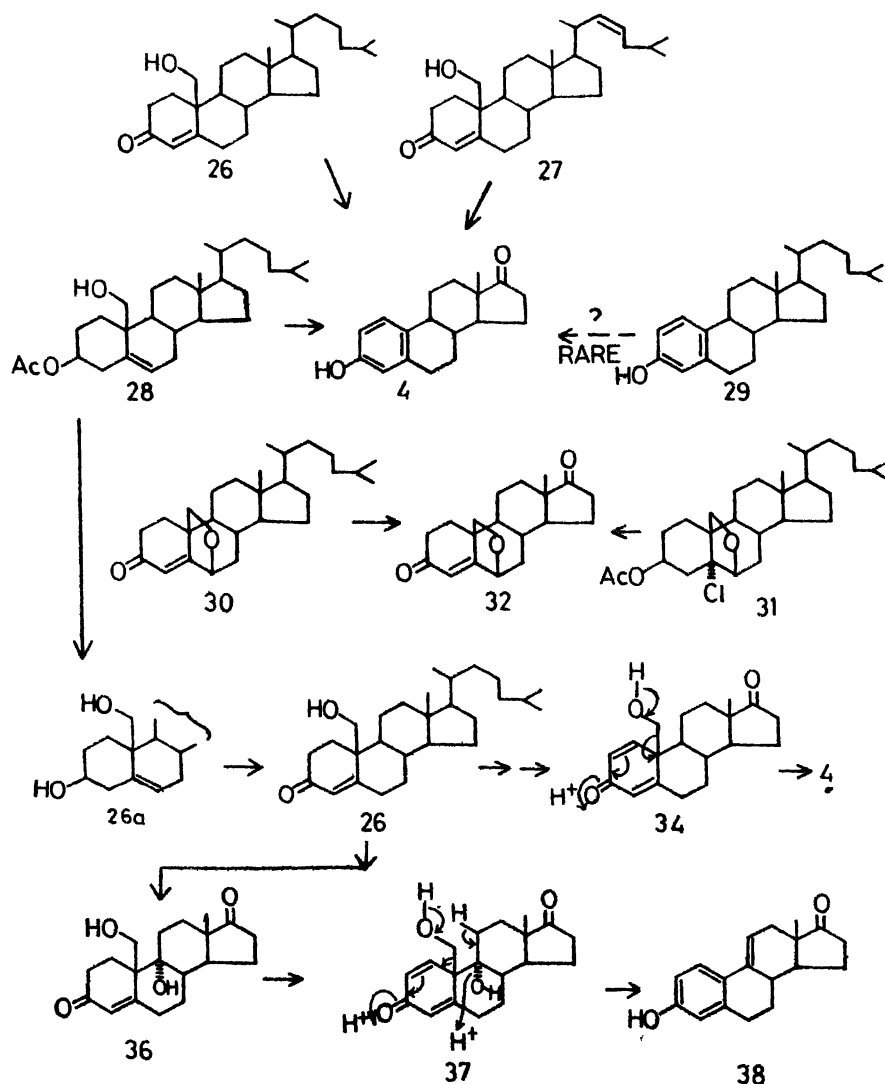


Chart 4

Ring A aromatization with side chain cleavage

at least in one organism the side-chain can be eliminated after ring A aromatization²³. The metabolism of the 19,6 β -oxido steroids (30, 31) gave good yields of the corresponding 6 β ,19-oxido androst-4-ene-3,17-dione (32) (Chart 4).

Interestingly enough, 19-hydroxy-cholesta-4,7-dien-3-one (33) gave estrone and not the expected equilenin in the same organism²⁴. But with a *Mycobacteria*, 33 yielded a mixture of estrone, equilin and equilenin. It is believed that the presence of the 19-hydroxy methyl group prevents 9 α -oxygenation. This is true of most strains excepting in *Corynebacter* which produces Δ^9 -estrone (38)²⁵, presumably from the 9 α -hydroxy intermediate (37).

Several strategies have been employed to suppress the 9 α -hydroxylation: (i) restriction of metals such as iron which forms the integral part of hydroxylating enzymes⁴ with different chelating agents, phase

restriction etc., (ii) mutation of sterol metabolising organism, and (iii) chemical modification of steroid structures for blocking the ring A metabolism.

In the fermentation of free sterol chelating agents such as α,α' -bipyridyl or phenanthroline, EDTA and other sequestering agents, 8-hydroxy quinolines²⁶⁻²⁸, and lipophilic micellar sequestering of substrates²⁸ have been generally used with moderate success. But these reagents also invariably decrease the activity of the iron containing C-26 hydroxylase resulting in sluggish metabolism. So far the mutational studies have been promising²⁴⁻²⁶ but are yet to lead to a viable commercial process for C-19 steroids.

Chemical modifications of steroids have met with success in the prevention of ring degradation. Particularly noteworthy is the fermentation of 3 α ,5 α -cyclo-6 β -hydroxy sterols (44) and their methyl ethers (45) to yield the corresponding 17-keto derivatives in 60% yield in *Mycobacterium phlei* KNGSF 70²⁷.

The 6 β ,19-oxido derivatives of sterols have also been converted to the 17-oxo steroids by *Arthrobacter* species³⁸. Successful side-chain elimination from 3 β -methyl ether of sterols as well as 4 β -5 β oxide cholestan-3-one³⁹ was observed in the presence of enzyme inhibitors³⁹.

Present studies :

Our studies began about 8 years ago with a primary emphasis on strain selection. Instead of using cholesterol as the sole source of carbon for selecting microorganisms, we used a two pronged approach to use 2-cyclopentyl-6-methyl heptane (38) which is considered to have only the ring D and the side chain of cholesterol, and the hydrocarbon, cholestane (41), which lacks the primary point of attack, the 3 β -hydroxyl, as carbon sources in a mineral salt medium. Our previous experience had shown that microbes selected on cholesterol show predominantly the ring degradation activity.

38 proved to be rather refractory for sustaining the growth of soil microorganisms as compared to the more complex cholesterol (1) or even the hydrocarbon cholestane (41). It took several months of trial to get a strain of *Pseudomonas convexa* to grow feebly on 38. This organism was rather unusual as it refused to grow on any energy rich carbon source such as carbohydrates, Krebs cycle acids or glutamate but grew feebly with 80-100° petroleum ether. After a week's growth in shake flask in 38, both cyclopentanone (39) and cyclopentanol (40) could be isolated as metabolites.

The organism refused to grow on cholesterol but on incubation of 7 days old resting cells grown on 38 in phosphate buffer cholesterol yielded only one product, 3 β -hydroxy androst-5-en-17-one (3). From many respects *P. convexa* seemed to be the ideal organism for side chain degradation. Comparative tlc indicated also the formation of 6-methyl heptan-2-one (19) in very small quantities. But the structure could not be confirmed due to paucity of material (Chart 5).

Unfortunately all efforts to make *P. convexa* grow better on any substrate failed.

In our next selection procedure we primarily selected our strain on the hydrocarbon, cholestane (41), and those of the selected strains which also grew on 38 were used for further studies. Only three organisms identified as a *Pseudomonas stutzeri*, a *Micrococcus* sp. and a filamentous mould, *Cephalosporium longisporum* survived the double selection procedure.

On cholestane all the three isolates yielded the same 17-oxo-5- α -androstane (43) demonstrating their side-chain degrading abilities. However on cholesterol (1) without any inhibitor all accumulated cholest-4-en-3-one (5) with small amounts of androst-4-ene-3,17-dione (6).

In presence of enzyme inhibitors such as α, α' -bipyridyl more accumulation of 17-oxo steroids such as (3) and (6) were observed.

More promising results were obtained with a mixture of all the three cultures in presence of bipyridyl and the yield of the dione (6) was 23% and that of (3) was 6%. β -Sitosterol (2) was degraded to the dione (6) in 6% yield (Chart 6).

Even though the results were better with mixed cultures, the reproducibility was poor with such cultures.

In the mean time, two more cultures were isolated on *iso*-octyl cyclopentane (38). They were found to grow very satisfactorily on cholesterol. These strain were identified as a *Pseudomonas* sp. and a *Moraxella* sp., respectively. The *Moraxella* sp. was selected for further studies as it accumulated some amounts of androst-4-ene-3,17-dione (6) and androsta-1,4-diene-3,17-dione (7) even without inhibitors. In presence of inhibitors, substantial amounts of 6 and 7 were produced from cholesterol.

The *Moraxella* strain had also the advantage of growing fast on cholesterol and the modified analogues (Chart 7). The data in Chart 7 has been obtained with 0.5% steroid substrates for shake flask fermentation for 72 hr at 28°. The problem with sterol substrates is their poor solubility excepting for cholesteryl sulphate. It is noteworthy that in all

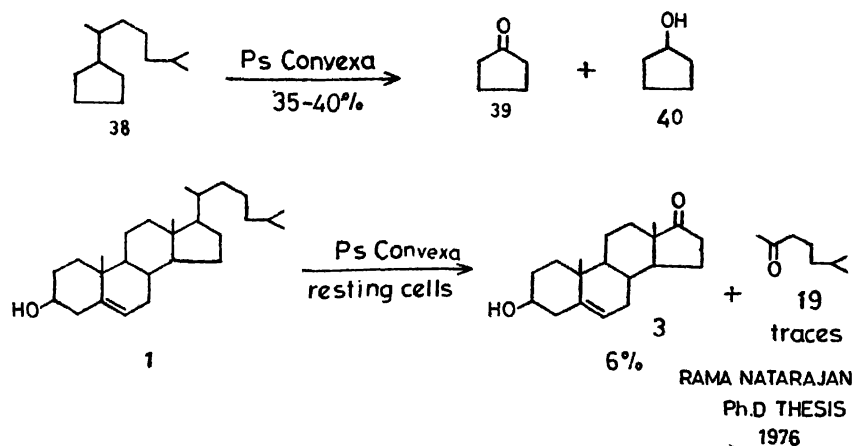
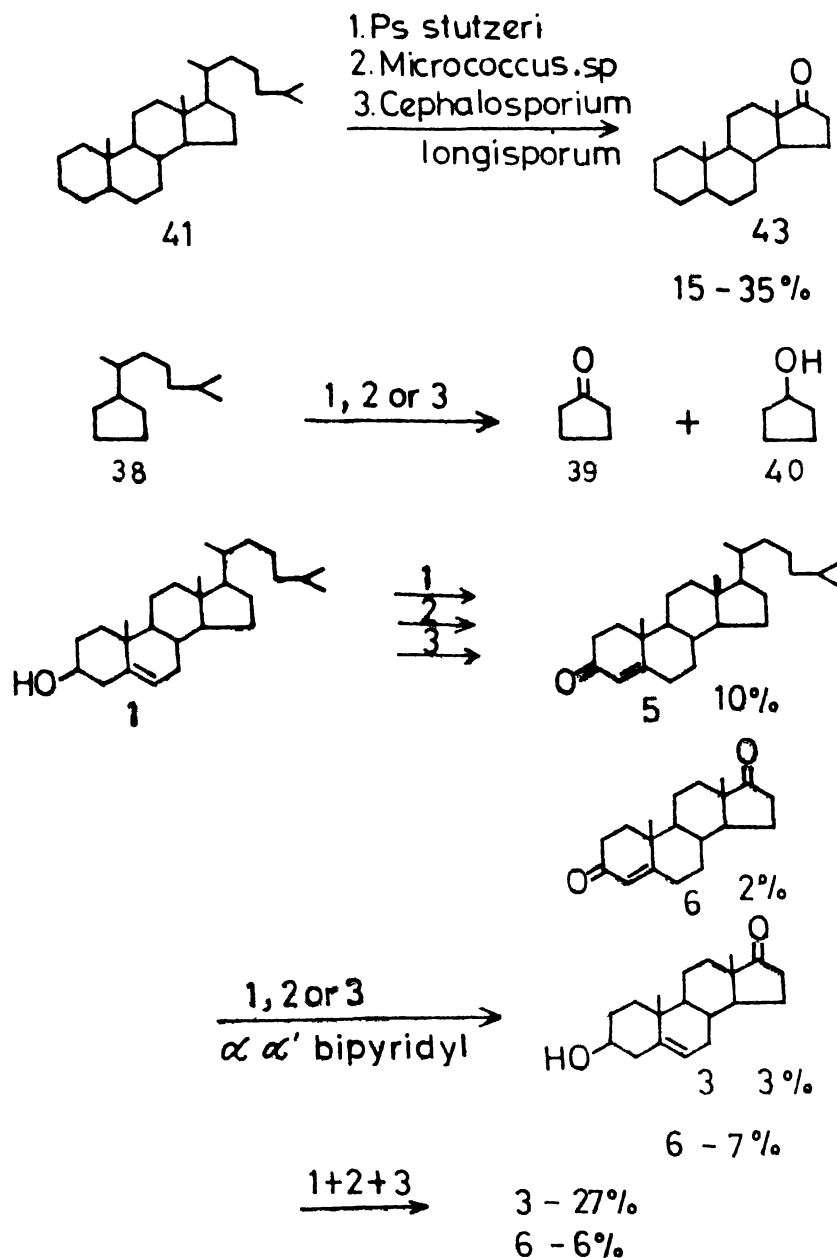


Chart 5

A strain selected on *iso*-octyl cyclopentane



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1978 Ph.D Thesis

Chart 6

Strains selected on cholestane and *iso*-octyl cyclopentane

the substrates the corresponding 17-keto steroids were obtained as products.

The most unexpected results was obtained with *i*-cholesterol (44) and *i*-cholesterol-methyl ether (45) which yielded cholesterol and other products.

i-Cholesterol isomerase :

The enzyme was partially purified about 2.2 fold from the sonicates of *i*-cholesterol grown cells by

ammonium sulphate (40-70%) precipitation. An attempt was made to purify the enzyme further by affinity chromatography with 6 β -methoxy-3,5-cyclo-androstan-17-ol which was prepared from 3 β -hydroxy androst-5-ene-17-one (3) through the intermediates 58, 59 (Chart 8). The affinity column adsorbed the enzyme quantitatively but elution proved difficult. The enzyme had surprisingly a high pH optimum, 8.4, as the normal chemical conversion from *i*-sterol

to normal 3β -hydroxy- Δ^5 -sterols requires high acidity. The optimal substrate concentration for the enzyme was 0.77μ moles with a K_m of $1.43 \mu M$. The enzyme was fairly stable at -20° but lost its activity rapidly at room temperature.

The enzyme for oxidative demethylation of 6β -methoxy-*i*-cholestane could not be demonstrated at the cell-free level^{4,5}.

Immobilization of whole cells :

Although microbial reactions in steroids with immobilized cells have been carried out since 1970 in the 11β -hydroxylation of compound S (61) to cortisol (62)^{4,8} the growth of a viable technology has been rather slow because of some inherent difficulties of poorly soluble hydrophobic substrates with diffusion and permeability problems. Non-

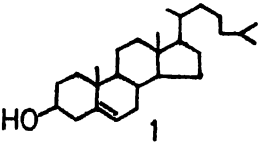
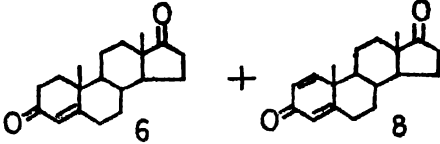
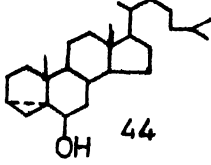
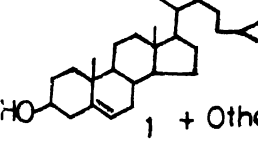
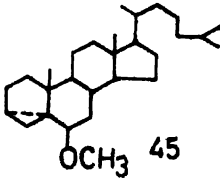
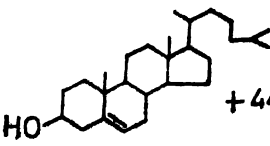
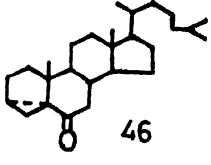
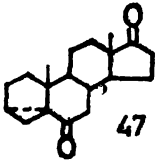
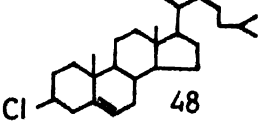
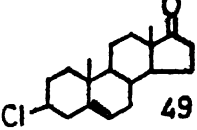
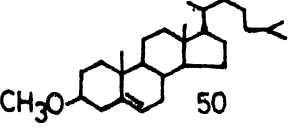
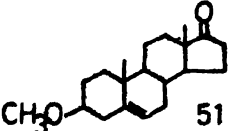
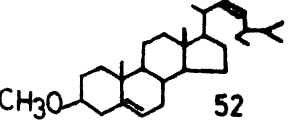

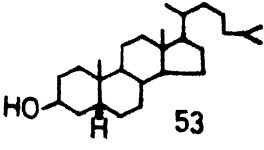
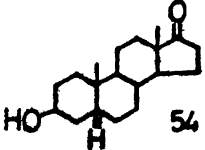
Substrate at 0.05% level (shake flask)	Product after 72hour	Yield (%)
		6 (4%) 8 (2%) Unprotected fermentation 6 (23%) 8 (7%) with α - α Bipyridyl ₃
	 + Other products	—
	 + 44 + Other products	—
		15
		11
		30
		30
		30

Chart 7

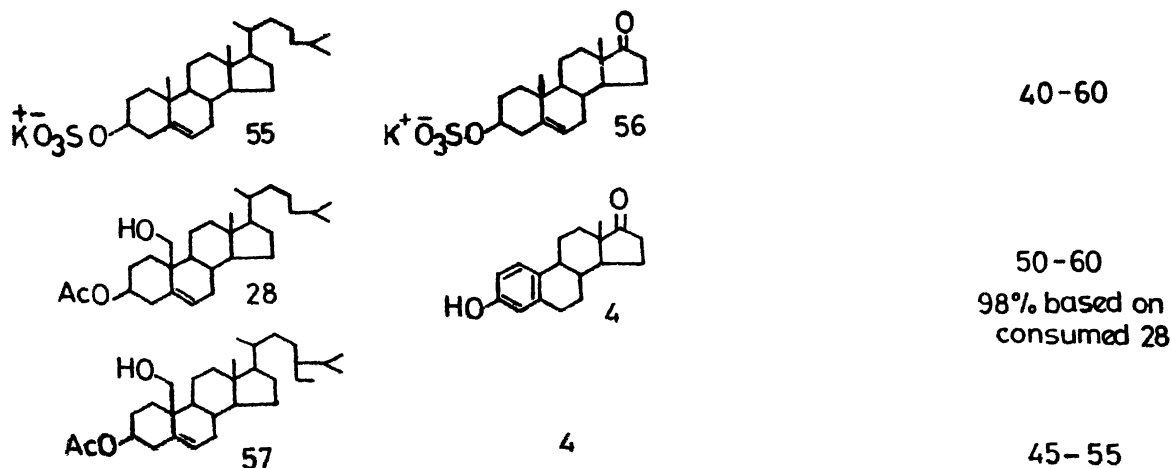


Chart 7a

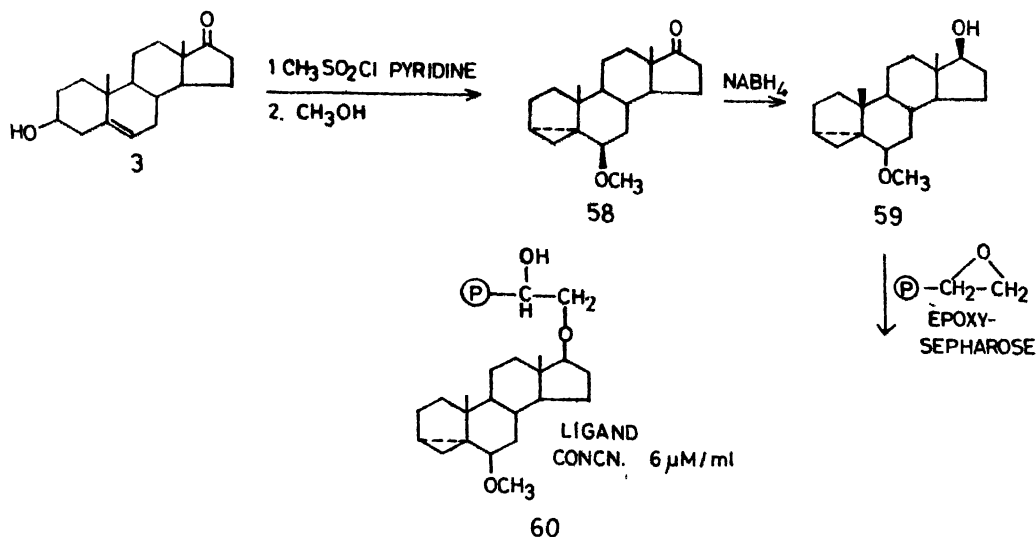
Modification of substrates for sterol side chain degradation by *Moraxella* sp.

Chart 8

Preparation of affinity polymers for sequestering α -sterol isomerase

aqueous solvents invariably cause rapid loss of viability of the entrapped cells. Mention may be made of the work of Monato⁴⁴ who converted 15% ethanolic solution of hydrocortisone (62) to prednisolone (63) with collagen-entrapped *Mycobacterium rhodocrous* in carbon tetrachloride and hydrocarbons. Typical examples of diverse chemical activities of entrapped cells are dehydrogenation of steroid alcohols, ring dehydrogenation (1,2 position), 11 α -hydroxylation, 16 α -hydroxylation, hydrogenation of 1,2-double bond and reduction of steroid ketones⁴⁵⁻⁴⁹ (Chart 9).

There are some reports on side chain degradation of cholesterol by entrapped *Nocardia erythropolis*⁵⁰. To overcome the solubility problem, cholest-4-en-3-(-O-carboxymethyl)oxime which is more water soluble was degraded smoothly by polyacrylamide encapsulated *Mycobacterium phlei* to the corresponding androst-4-en-3-(-O-carboxymethyl)oxime-17-one⁵⁰.

So far, ours is the first attempt to obtain estrone (4) by entrapped cells of *Moraxella*. We attempted entrapments with four different supports. The viability of washed resting cells used for entrapment was about 30% of that in live fermentation for estrone production from 19-hydroxy-cholesteryl acetate. These cells were entrapped in (i) molecular sieve with titanium tetrachloride as fixing agent, (ii) entrapment in polyacrylamide gel, (iii) covalent grafting to adipoylated polystyrene and (iv) entrapment in agar.

The retention of activity of the organism in different modes of entrapment are presented in Table 1. Losses obviously occur in the chemical treatment of the cells during the grafting process. Free radical reagents used for acrylamide polymerization also cause damage to the cells. The agar entrapped cells proved to be the best, retaining 80-90% of the free cell activity. The half-life of these cells was also good. After 28 days of continu-

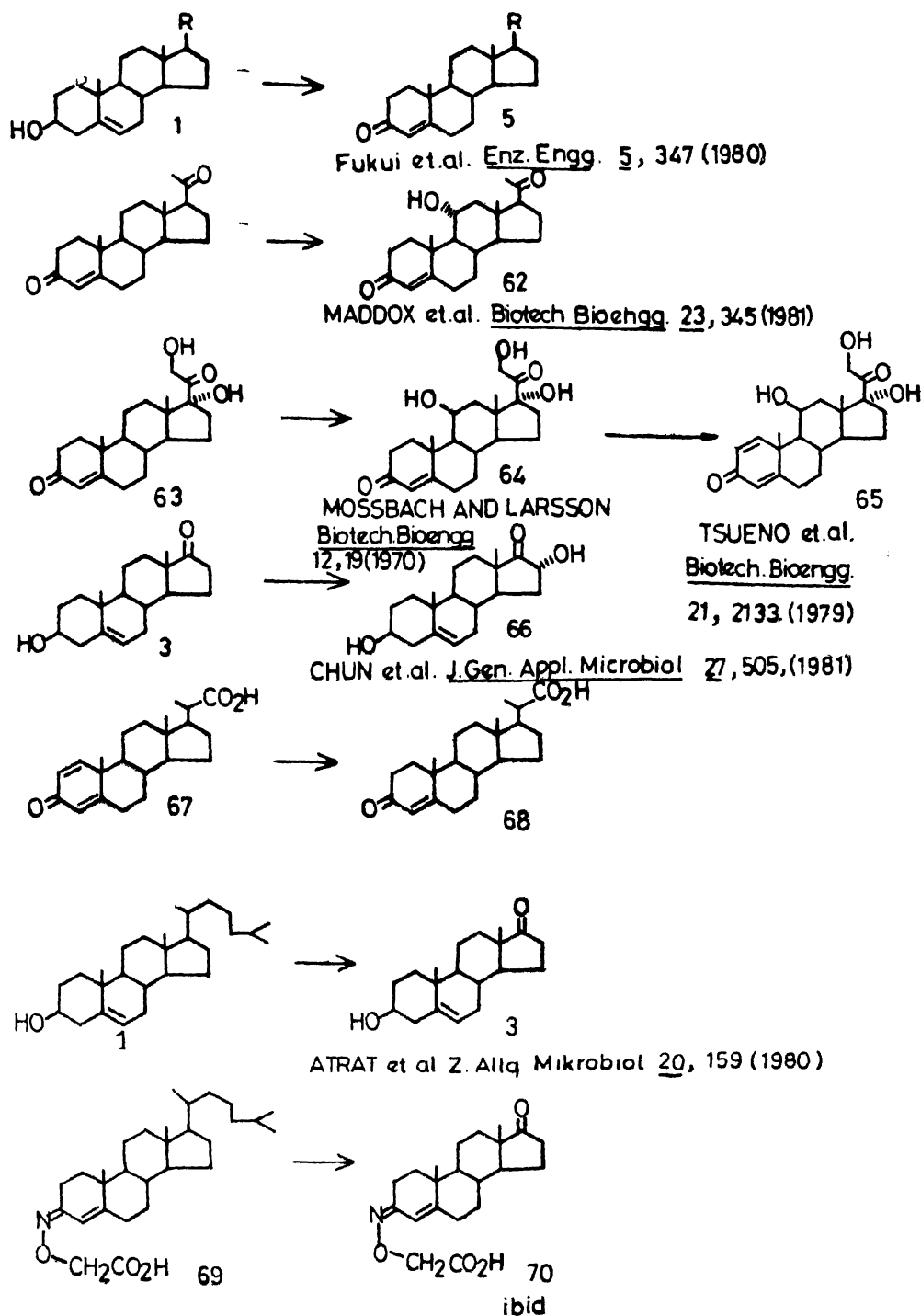


Chart 9
Steroid transformations by immobilized microbial cells

ous fermentation the cells retained 50% of their activity.

One advantage of working with entrapped cells is that virtually no side reaction could be detected and the yields of ring cleaved products were 93-100%

based on consumed substrate which can be recovered and recycled by simple procedure.

Experimental

The melting points and boiling points reported are uncorrected. The uv-spectra were taken on a

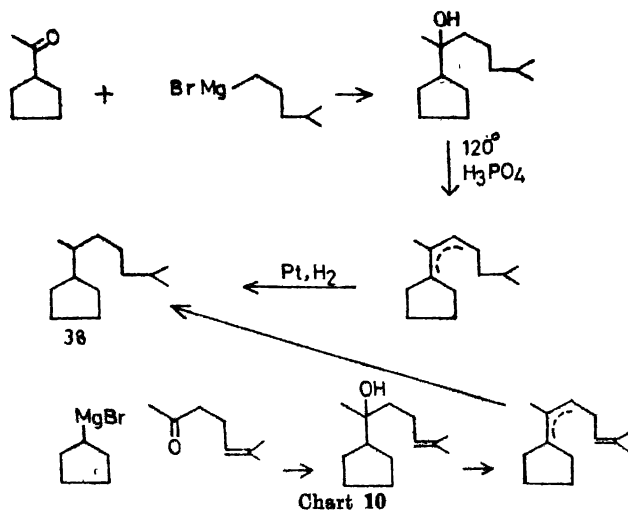
TABLE 1

Sl. No.	Type of immobilization	Support	Substrate	Product	Yield (%)	Retention (%)
1.	Chemical binding	Molecular sieve	19-Hydroxy cholesteryl acetate	Estrone	5	90
2.	Chemical binding	Adipoylated polystyrene	19-Hydroxy cholesteryl acetate	Estrone	15	50
3.	Entrapment	Polyacrylamide	19-Hydroxy cholesteryl acetate	Estrone	3	10
4.	Entrapment	Agar	19-Hydroxy cholesteryl acetate	Estrone	24	80
5.	Entrapment	Agar	Potassium cholesteryl sulphate	Dehydroepi-androsterone sulphate	45	90

* Percent retention of the free cell activity.

Hitachi Model 551 spectrophotometer and the ir spectra on a Perkin Elmer Model 397 spectrometer. The nmr spectra were recorded on Varian T-60 spectrometer. The HPLC analyses were made on a Waters 440 Model HPLC with μ -bondapak C-18 column using uv detector at 254 nm as well as refractive index detector.

2-Cyclopentyl-6-methyl-heptane³⁸ (38): Isohexyl bromide was prepared from 4-methyl-1-pentanol (8 ml)(Aldrich Chemical Co., Milwaukee, Wisc., USA) with phosphorus tribromide (9 ml) with initial stirring below 5° and then on a water bath for 30 min. The oily layer was treated with conc. sulfuric acid (3 ml) in the cold (0°) and the mixture extracted with ether. After bicarbonate (5%) and water washing and drying (anhydrous MgSO₄), the ether was evaporated and the residue distilled on an oil bath (6.0 g), b.p. 145°/712 mm. IR: ν_{\max} 1470, 1385, 1375, 1215, 1020, 930 cm⁻¹.



2,6-Dimethyl heptyl cyclopentane (iso-octyl cyclopentane)

The Grignard reagent was prepared from *iso*-hexyl bromide (6.0 g) in anhydrous ether (50 ml) and magnesium turnings (0.99) and methyl cyclo-

pentyl ketone (3.5 g) was added in 25 ml dry benzene. After 3 hr of refluxing the complex was decomposed with saturated ammonium chloride and worked up to yield the crude 2-cyclopentyl-6-methyl heptan-2-ol which was purified by chromatography over 30 g neutral alumina and eluted with 1:1 chloroform and hexane (3.5 g). IR neat: ν_{\max} 3450 (OH), 1460, 1370, 1300, 1160 and 920 cm⁻¹.

The carbinol was dehydrated with 5 ml ortho-phosphoric acid in an oil bath (120-130°, 2 hr). The mixture of olefins was recovered in ether (75 ml) and hydrogenated with platinum oxide (75 mg) in glacial acetic acid (5 ml). The mixture was filtered, neutralised with bicarbonate and product recovered in ether, dissolved in hexane and clarified by passage through a short column of neutral alumina column (5 g) in hexane. Removal of hexane yielded the pure product (38) (2.0 g). IR neat: ν_{\max} 1470, 1365, 1375 cm⁻¹; nmr (CDCl₃); 0.9 (d, 9H-3CH₃), 1.0-2.2 (m, 17H, 7CH₂-3-CH) ppm.

Cholestane (41) was prepared according to Chang⁵¹, 5 α -androstan-17-one (42) according to Tokes *et al*⁵² and 5-methyl heptan-2-one by the hydrogenation of 5-methyl heptan-5-ene-2-one with platinum. Androst-4-ene-3,17-dione, 3 β -hydroxy androst-5-en-17-one and sitosterol were obtained as gifts from M/s Organon India, Limited. Cholesterol and cholestanone were gifts from M/s CIPLA Laboratories, Bombay. 1,4-Androstadiene-3,17-dione and estrone were from Sigma Chemical Co., St. Louis, Missouri, USA.

i-Cholesterol was prepared according to Koswer and Winsten⁵³ and 6-oxo-3 α ,5 α -cholestane by the method of Hasthorn⁵⁴. The 6 β -methyl ether of 3 α ,5 α -cyclocholestane was obtained according to Patel and Peal⁵⁵. Cholesteryl and sitosteryl-O-methyl ether was prepared according to Narayanan and Iyer⁵⁶. Cholesteryl chloride⁵⁹, cholestanol⁵⁸, 19-hydroxy-3 β -acetoxy cholest-5-ene⁵⁹ and 19-hydroxy-3 β -acetoxy sitost-5-ene were obtained by literature methods. Potassium cholesteryl sulphate was made by a minor modification of the Goto method⁶⁰

in which the reaction mixture of cholesterol, pyridine and chlorosulphonic acid was taken up in water and precipitated by a saturated solution of potassium chloride. The precipitate was recrystallized from 50% ethanol acetone to yield 86% of the product.

Microbiological methods :

Strain selection : Seubert's mineral salts medium⁶¹ with 0.02% 2-cyclopentyl-6-methyl heptane (added after sterilization), 0.05 % cholestane or 0.05 % cholesterol (added in 50 % acetone solution before sterilization) were used for strain selection. The soil samples used were fresh soil from garden or soil pretreated with cholesterol (5 g/sq. m per week for 4 weeks). Soil samples (2-3 g) were then added to 100 ml aliquots of the enrichment medium in 500 ml Erlenmeyer flasks with cotton plugs and the flasks incubated for 15 days on a rotary shaker at 29-30°. After 15 days, aliquots from the cultures were transferred to 100 ml of fresh enriched media every week and the agitation and incubation continued. Usually after 7 to 15 such transfers growth was detected in the enriched media as evidenced by appearance of turbidity and sometimes colour. The cultures were then plated out in enriched agar media (2% agar), colonies were picked out and transferred on enrichment medium agar slants. Colonies from slants which showed growth were then retested in liquid medium. Cross adaptation studies were carried out on hydrocarbon (38) grown cells on cholestane (41) and cholesterol enriched media. Five organisms were found to be viable and retained their chemical activities through these operations. They were identified following standard procedures⁶²⁻⁶⁴. The fungus was identified by prof. K. S. Gopalkrishnan of Biochemical Engineering Department, Indian Institute of Technology, Delhi.

P. convexa isolated on 2-cyclopentyl-6-methyl heptane (38) grew feebly on this substrate and on 80-100° petroleum ether. It did not grow on cholesterol, carbohydrates, Krebs cycle acids or glutamic acid. *P. stutzeri* isolated on cholestane (41) grew moderately on 38 and cholesterol. *Micrococcus* sp. isolated on cholestane grew on 38 and cholesterol. *Cephalosporium longisporum* isolated on cholestane grew feebly on 38 and cholesterol. *Moraxella* sp. isolated on 38 grew well on cholesterol and other sterols.

Maintenance : *P. convexa* was maintained on nutrient agar slants containing 0.01 % hydrocarbon, 38. *P. stutzeri* was maintained on nutrient agar, *Micrococcus* sp. on tryptone agar with 0.01 % cholesterol, *Cephalosporium* on nutrient agar as well as on potato-dextrose agar and the *Moraxella* sp. on enrichment medium agar containing 0.05 % cholesterol and 0.1 % yeast extract. Before fermentation the isolates were grown on liquid medium in shake flasks containing 2-cyclopentyl-6-methyl heptane for *P. convexa* and cholestane for *P. stutzeri*, *Micrococcus* sp. and *C. longisporum* enriched with 0.01% yeast extract. All showed optimum growth at pH 7 and 29°.

Fermentation :

Pseudomonas convexa behaved differently from the other strains as it failed to grow on cholesterol even after 21 days of incubation. Aliquots of 100 ml of sterile mineral salts medium containing 0.01 % yeast extract were distributed in 10 flasks. To each of these flasks 2-cyclopentyl-6-methyl heptane (38) was added (50 mg per flask). The flasks were inoculated with 1 ml of a 96 hr liquid culture of *P. convexa* on the same medium and incubated for 7 days on a rotary shaker at 28°. The cells were then harvested by centrifugation at 10,000 g for 20 min and washed with 0.05 M phosphate buffer, pH 7.0 followed by centrifugation.

The combined cells were resuspended in phosphate buffer (100 ml ; 0.05 M, pH 7.0) and shaken on a rotary shaker for 72 hr at 29°, after the addition of 100 mg cholesterol in 1 ml acetone. The supernate from the centrifuged cells was saturated with sodium chloride and extracted with ether. The ether layer was worked up to yield 22 mg of pungent smelling residue which was identified as a 4:1 mixture of cyclopentanone (39) and cyclopentanol (40) by comparative tlc on silica gel with 10% ethyl acetate in hexane as developing solvent.

No ether soluble acidic products could be detected in the aqueous layer after acidification.

The cholesterol incubation mixture with resting cells was extracted after 72 hr with chloroform after acidification with dil. hydrochloric acid and saturation with sodium chloride. The organic layer was filtered through a layer of hyflosupercel and washed with 5% aqueous sodium bicarbonate. The neutral steroids were recovered after evaporation of the solvent. On tlc (10% ethyl acetate in hexane) two spots of R_f 0.89 and 0.3 were observed. These components were separated by preparative tlc. The fast moving component (9 mg) was identified as cholesterol and the polar component was identified as 3 β -hydroxy androst-5-en-17-one (3) (m. m. p. 139°, superimposable ir spectra). The bicarbonate layer after acidification and chloroform extraction yielded only a trace amount of acids. Besides, there was a faint spot, R_f 0.78, corresponding to that of methyl heptan-2-one. However, not enough material could be obtained for a spectral comparison.

Fermentation of cholestane with *P. stutzeri*, *Micrococcus* sp. and *Cephalosporium longisporum* :

The method used was the same as above. The substrate used was at the level of 25 mg/flask. The inoculum was a 72 hr culture of each organism in a liquid mineral salt medium containing 0.025% cholestane and 0.01% yeast extract.

Pooled fermented broth from 50 flasks was extracted in chloroform and processed in the usual manner and separated by preparative tlc (5 % ethyl acetate in hexane) into a nonpolar fraction (R_f 0.98) (1.3 g, fat and cholestane) and a moderately polar fraction (R_f 0.8) m.p. 121° identified as 5 α -androst-17-one (42) from comparative ir and nmr spectra and m.m.p.

The yield of **42** was 3 mg from *P. stutzeri*, 6 mg from *Micrococcus* and 2.8 mg from the fungus.

Fermentation of cholesterol with *P. stutzeri* and *Cephalosporium longisporum* :

P. stutzeri was grown in 20 shake flasks in sterile nutrient broth with cholestane (50 mg/flask) as inducer. After 24 hr at 29° the pooled cultures were centrifuged (5000 g ; 30 min). The precipitate washed thrice with cold phosphate buffer (0.05 M, pH 7.0), distributed into three 500 ml Erlenmeyer flasks with 100 ml mineral salts medium and 80 mg cholesterol, incubated on a shaker at 29°. After 24 hr interval one flask was removed, extracted before and after acidification, with chloroform (2×50 ml) and examined by tlc.

All the extracts from the 24, 48 and 72 hr incubation mixtures showed three spots in tlc corresponding to cholest-4-en-3-one (**5**), cholesterol and a faint spot corresponding to androst-4-ene-3,17-dione (**6**).

The extracts were pooled and cholestenone (**5**) was separated (60 mg) as a major metabolite by preparative tlc. The polar material corresponding to **6** in mobility could not be prepared in sufficient quantity for an identification.

C. longisporum was grown on Czapek Dox medium⁶⁶ in 5 flasks for 20 hr at 29°. Cholesterol (50 mg) was added to each flask.

After 72 hr incubation, the cultures were filtered, the mycelia extracted with acetone, and the acetone-aqueous layer was extracted into chloroform. The chloroform extract after bicarbonate washing yielded cholesterol and cholest-4-en-3-one (65 mg) after preparative tlc.

Fermentation of cholesterol with mixed cultures in presence of metabolic inhibitors :

The strains of *P. stutzeri*, *Micrococcus* sp. and *C. longisporum* were all inoculated into cholestane-mineral salts medium and incubated for 72 hr. This broth (5 %) was now used to seed 4 flasks each containing nutrient broth and cholestane (10 mg/flask). The mixed cells after 24 hr growth at 29° were centrifuged, washed thrice with 0.05 M phosphate buffer, pH 7 at 0° and resuspended in two flasks (50 ml each containing 125 mg cholesterol). After 2 hr on a rotary shaker 60 mg of α,α' -bipyridyl was added to each flask and the fermentation continued upto 70 hr. The mixed culture was then processed in the usual manner and extracted with 3×50 ml of ethyl acetate. The ethyl acetate extracts showed four tlc spots and were resolved by preparative tlc in 25 % ethyl acetate in hexane. The fastest moving band was identified as cholest-4-en-3-one (5 mg) followed by unreacted cholesterol and bipyridyl. The next fraction (56 mg) was purified by a second preparative tlc in 35 % ethyl acetate and hexane and identified by comparative ir spectroscopy, m.m.p. (171°) and nmr spectroscopy as androst-4-ene-3,17-dione (**6**). The fourth fraction had identical mobility as that of 3 β -hydroxy androst-5-en-17-one (**3**) but could not be completely characterized due to paucity of material.

Fermentation of sitosterol by the mixed culture under the same condition, yielded sitosten-4-one (8 mg) androst-4-en-3,17-dione (7 mg) and traces of 3 β -hydroxy androst-5-en-17-one (**3**) (tlc identification). Cholest-4-en-3-one also yielded **6** (8 mg) and **3**.

The reasons for poorer metabolism of cholestenone could not be established. No significant amount of acids could be isolated from any of the experiments from the bicarbonate layers.

On large scale experiments with mixed cultures yields of **6** from cholesterol varied widely in presence of α,α' -bipyridyl ranging from 3 % to 35 %.

Fermentation with *Moraxella* species :

Moraxella sp. proved to be the most viable and versatile of all the species isolated. It grew well and fermented a large number of sterol analogues. However, it was found that the presence of 0.5 % glucose and 0.1% yeast extract along with mineral salts medium and steroids gave a faster growth without materially affecting the conversion. The general procedure used was as follows.

The resting cells were prepared in 10 flasks in glucose (0.5 %) yeast extract (0.1 %) mineral salts medium containing 0.005% cholesterol (in the case of *i*-steroids 3 $\alpha,5\alpha$ -cyclocholestan-6 β -ol was used as the primary substrate). After 24 hr the cells were harvested by centrifugation, washed with buffer. The cells (5.0-5.5 g) were resuspended in 100 ml buffer containing 0.005% α,α' -bipyridyl in case of experiments with cholesterol (50 mg) which was added in a Tween 80-acetone (1 ml ; 20 : 80) solution. The flask was incubated on a shaker for 72 hr at 28-30°. The extraction and separation procedure was different from those in earlier experiments.

The broth was extracted with methylene chloride and the methylene chloride extract was washed with 5% ferrous sulphate to remove bipyridyl. The residue after evaporation was subjected to a modified Craig's distribution between hexane and 90% methanol. Hexane fraction contained most of the unreacted cholesterol. The methanol fraction was vacuum evaporated and extracted into methylene chloride. After removal of solvent the products were monitored and separated by tlc.

In control experiments run without bipyridyl, only cholest-4-en-3-one (5 g) m.m.p. 77-79° (comparative ir spectra) and unreacted cholesterol (20 mg) were isolated.

In the presence of α,α' -bipyridyl (0.1 mM) the product showed three components in tlc (in 5 % acetone in methylene chloride, R_f 0.8, 0.7 and 0.6) which were purified by preparative tlc and identified as cholest-4-en-3-one (2 mg), androsta-1,4-dien-3,17-dione (8.0 mg, m.m.p. 138-139°, comparative ir spectroscopy) and androst-4-en-3,17-dione (**6**) (5 mg, m.p. 141-142°, ir and nmr spectroscopy).

Cholesteryl acetate fermentation with bipyridyl also yielded the same three products.

Fermentation of *i*-cholesterol (44) :

Fermentation of *i*-cholesterol was carried out without bipyridyl in the manner described for cholesterol. From the polar methanol fraction only one product was isolated (5 mg) and identified as cholesterol by m.m.p. 138-140°, comparative ir and nmr spectroscopy.

In blank experiments without the organism *i*-cholesterol was recovered unchanged.

6 β -Methoxy-3 α ,5 α -cyclocholestane :

Fermentation of 6 β -methoxy-3 α ,5 α -cyclocholestane, (without bipyridyl) recovery and separation of the polar fractions in the same procedure as with cholesterol yielded *i*-cholesterol (8 mg) and cholesterol (20 mg).

***i*-Cholestanone :**

The polar crude material from the fermentation showed two components in tlc (solvent benzene ; R_f 0.35 and 0.23). These were separated by preparative tlc in benzene. The fast moving component was the starting material (18 mg). The polar component (4 mg) had a m.p. 180-182° and showed $[\alpha]_D^{25} +113^\circ$ (chloroform 0.2%). IR (nujol) : ν_{\max} 1725, 1675, 1290, 1150, 720 cm⁻¹, nmr : (δ ppm) 0.97 (s, 3H, C-18 methyl), 1.02 (s, 3H, C-19 methyl), 0.78 (m, 3H, 3 β -H 9Hz, on cyclopropane), 1.2-2.7 (m, 18H ring methylene and ring methine). Based on lit. data²⁸ the product was identified as 3 α ,5 α -cycloandrostan-6,17-dione. Increase in the fermentation time to 5 days gave higher yields of the product (10 mg).

3 β -Chloro-cholest-5-ene :

The product in tlc (benzene) showed only one spot (R_f 0.6) other than the starting material (R_f 0.7). Purification by preparative tlc gave a crystalline solid (2 mg), m.p. 154-156°, $[\alpha]_D^{25} +17^\circ$ (ethanol). IR ν_{\max} (nujol) : 1725, 1000, 870, 820, 760, 730 cm⁻¹; nmr : (δ ppm), 0.9 (s, 3H, C-18 methyl), 1.016 (s, 3H, 19 methyl), 1.6-2.8 (m, ring methylenes and methines), 3.7 (broad m, 1H, C-3 α -H) and 5.4 (t, 1H, C₆-vinyl H). The compound contained chlorine. It was identified as 3 β -chloro-androst-5-en-17-one from literature data²⁷.

3 β -Methoxy-cholest-5-ene :

Fermentation of cholesteryl methyl ether without Δ^2, Δ^3 -bipyridyl yielded besides the starting material [tlc R_f 0.7 (benzene)], only one product (R_f 0.3) which was purified to a crystalline solid (12 mg), m.p. 139-140°. IR (CHCl₃) : ν_{\max} 1730, 1190, 1000, 950, 770, 610 cm⁻¹; nmr : (δ ppm) 0.9 (s, 3H, C-18 methyl), 1.06-2.2 (m, ring methylene and methines), 3.0 (broad m, 1H, C-3 α -H), 3.33 (s, 3H, OCH₃), 5.35 (1H, C₆-vinyl H). It was identified as 3 β -methoxy-androst-5-en-17-one from literature data²⁷.

3 β -Methoxy-stigmast-5-ene :

Fermentation of 3 β -methoxy stigmast-5-ene also yielded 3 β -methoxy-androst-5-en-17-one (11 mg).

Cholestanol :

Fermentation of cholestan-3 β -ol without bipyridyl and separation of products yielded 15 mg of 5 α -androstane-3,17-dione, m.p. 130-132° which was identified by comparative ir spectroscopy.

19-Hydroxy-3 β -acetoxy cholest-5-ene :

Thin layer chromatographic resolution (methylene chloride) gave only one product (R_f 0.15) besides starting material, crystallized from acetone, m.p. 251-253° (18 mg). UV λ_{\max} 289 nm identified as estrone by comparative ir and nmr spectroscopy.

Potassium cholesteryl sulphate :

The products were extracted after fermentation and acidification into methylene chloride. On evaporation the gummy residue showed no movement on tlc plates. The residue was kept overnight in 10 ml methanolic KOH (90 %) diluted with 50 ml water neutralized with 5 % hydrochloric acid and reextracted with methylene chloride. TLC (methylene chloride) showed only one product (R_f 0.15) besides cholesterol (R_f 0.3). This was isolated by preparative tlc (18 mg), m.p. 140-141°, identified as 3 β -hydroxy androst-5-en-17-one (3) by comparative ir and nmr spectroscopy.

***i*-Cholesterol isomerase :**

The partial purification and characterization of *i*-cholesterol isomerase will be reported elsewhere.

Estimation of 3 β -hydroxy-androst-5-en-17-one :

The product was estimated after hydrolysis with a Waters Model 440 HPLC using μ -Bondapak C-18 column with acetonitrile as the mobile phase at a flow rate of 2.2 ml/min. A 254 nm uv detector was used for detecting 3 β -hydroxy-androst-5-en-17-one, while refractive index detector was used for the estimation of cholesterol. The retention times for cholesterol and 3 were 7.5 and 3.0 min, respectively. The amount of sterols was estimated from their respective peak heights with reference to authentic samples. Estrone was separated by tlc and estimated spectrophotometrically at 289 nm.

Immobilization of whole cells :

Moraxella sp. was grown for 48 hr in shake flasks in glucose yeast extract salts medium containing 0.005 % cholesterol. The cells were harvested by centrifugation, washed with 0.7 % saline containing 0.02 % Tween-80 (pH 7.0) or 0.1 M phosphate buffer depending on the type of immobilization.

The free cell activity was tested after stirring the cells for 24, 48, 72, 96 hr at room temperature after incubating them with 0.05 % 19-hydroxy cholesteryl acetate for 24 hr.

Polyacrylamide entrapment :

Saline-Tween-80 washed *Moraxella* cells (5 g) in 20 ml saline (0.7 %) were treated with acrylamide (4 g), N,N'-methylene bis acrylamide (0.2 g) and N, N,N',N'-tetramethyl ethylene diamine (10 μ l). Polymerization was initiated by potassium persulphate (B. D. H.) (2.5 ml of a 2.5 % solution). The

mixture was vigorously stirred below 30° and poured out into petri dishes to give 2-4 mm thick slabs. These were allowed to set for 15 min. The stiff gel was then cut into 2-3 mm cubes, washed from free cells with saline (0.7 %) and stored at 4°.

Agar :

Agar (1.3 g) was dissolved in 0.1 M phosphate buffer (pH 7.0) (50 ml) by steaming. The molten agar was cooled to 40-45° in a warm water bath and washed cells (7 g) were poured into it as a thick slurry in 0.1 M phosphate buffer with rapid mixing. A mixture (d 1.0) of toluene (824 ml) and tetrachloroethylene (176 ml) was taken into a column (36 m × 1000 mm) and the cell agar suspension was added dropwise in such a manner that uniform spherical pellets settled at the bottom (about 30 min). The solvent was drained off, the pellets repeatedly washed with phosphate buffer and stored at 4°.

Polystyrene grafting :

Pieces of adipoylated polystyrene (2-4 mm strips) containing 0.3 equiv of carboxylate per g was suspended in 0.1 M phosphate buffer (pH 7.0) (100 ml) and 1-ethyl-3-(3-dimethylamino propyl)carbodiimide (Sigma) (10 g) (10 equiv/equiv of CO₂H) was added. After 10 min stirring a suspension of 10 g of wet cells in 20 ml buffer was added and the stirring was maintained for 4 hr. The polymer was washed free of unbound cells and stored at 4°. The washings were centrifuged, washed and the unbound cells were suspended in 0.1 M phosphate buffer (100 ml) containing 19-hydroxy cholesteryl acetate (50 mg in 1 ml 20 % Tween-80 in acetone) and incubated for 24 hr for viability studies.

Molecular sieve :

The cylindrical pellets of molecular sieve type 3A-(BDH) were ground in a mortar and sieved to collect 212-300 μ particles. They were washed with dil. nitric acid and activated with titanium tetrachloride²⁸.

The activated particles (5 g) were stirred into a suspension of cells (5 g) in 0.1 M buffer (50 ml) for 15 min and the mixture allowed to stand for 24 hr at 4°. The sieve containing bound cells were separated from free cells by repeated decantation and washing with buffer and stored in buffer at 4°.

Transformations by immobilized cells :

The APS and acrylamide entrapped cells were loaded on columns 1.5 cm × 40 cm; for molecular sieve a glass column of 0.5 cm × 20 cm was used and for agar pellets a larger diameter column (3.0 cm × 30 cm) was necessary.

19-Hydroxy-3 β -acetoxy cholest-5-ene or 19-hydroxy-3 β -acetoxy sitost-5-ene (50 mg) were homogenised in 1 ml Tween-80 acetone (1 : 4 w/w) and added to 100 ml 0.1 M phosphate buffer (pH 7.0). Potassium cholesteryl sulphate (25 mg) was directly dissolved in 100 ml buffer. These substrates were fed to the column at the rate of 0.1 ml/ml bed volume/hr.

The effluents were extracted with methylene chloride and separated by preparative tlc into substrate and product. In every case 95% of the steroids were recovered and the conversions based on utilized substrate were more than 90 %.

Acknowledgement

The authors record their appreciation for the free gift of steroids to M/s Organon India, Limited and to CIPLA Laboratories. Thanks of the authors are due to University Grants Commission for financial support

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