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Microbial Degradation of Plant Sterol to Steroidol Intermediates by a Mutant of *Mycobacterium* sp.

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Mycobacterium sp. 변이주에 의한 식물스테롤의 스테로이드 중간체로의 미생물적 분해

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A mutant of *Mycobacterium* sp. has been isolated which is capable of degrading cholesterol and plant sterol to androst-4-ene-3,17-dione and 9-hydroxyandrostene-3,17-dione. Also this mutant hydroxylated the steroidal nucleus at the 9 α position. No ring degradation inhibitory agents are required for these processes.

Microorganisms, including Mycobacterium and Norcardia, are capable of completely degrading sitosterol and cholesterol (1). This microbial process was shown to involve 2-and 3-carbon fragmentations, forming acetic and propionic acids. The degradation pathway involve the forming of intermediates with C-22 and C-24 carboxylic acid side chains (2).

Because most microorganisms are capable of degrading both the sterol side chain and the steroidal nucleus, a series of soil isolates were screened to find microorganisms capable of selectively degrading only C-17 alkyl side chain of sterols without concomitant attacking the steroidal nucleus.

In order to cleave the side chain selectively, the degradation of the steroid nucleus has to be selectively blocked. This selective blockade has been achieved by various method (5): 1) chemical modification of the substrates, 2) the addition of agents which selectively inhibit the activity of some enzymes, 3) mutation of the microorganism to block the 9α hydroxylation or 1,2-dehydrogenation activities without completely inactivating of β-oxidation activities by the enzymes.

Wovcha *et al.* (6) isolated a series of mutants from the sterol degrading *Mycobacterium fortuitum* ATCC6842, which were blocked at vairous stages of the sterol degradation pathway. A number of intermediate compounds were isolated. These intermeidates included: androst-4-ene-3,17-dione (AD); androst-1,4-diene-3,17-dione (ADD); a ring A-degraded tricyclic compound and various 9α-hydroxy steroids. Knight *et al.* (2) also has demonstrated that a mutant of *Mycobacterium fortuitum* is selective in its ability to degrade both the steroid nucleus and sterol side chains of sterols that are branched at the C-24 position. Bioconversion of sitosterol by this mutant resulted in the accumulation of novel 24-oxo intermediates: 9-hydroxy-27-nor-4-cholestene-3,24-dione and 9-hydroxy-26,27-dinor-4-cholestene-3,24-dione. Cholesterol, under the same conditions, was degraded to 9 hydroxy-4-androstene-3,17-dione (9αOHAD).

Marsheck *et al.* (3) described the microbial degradation of sterol to 17-keto steroids by two newly

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isolated *Mycobacterium* sp. (NRRL B-3683 and NRRL B-3805). *Mycobacterium* sp. NRRL B-3683 was produced by U.V. irradiation of a soil *Mycobacterium*. It was capable of converting sitosterol predominantly ADD. Further irradiation of *Mycobacterium* sp. NRRL B-3683 produced *Mycobacterium* sp. NRRL B-3805, which produced predominantly AD from sitosterol. This *Mycobacterium* sp. lacks the ability to dehydrogenate steroid at the C-1 (2) position.

A program was initiated to screen soil microorganisms for the following properties: 1) The ability to degrade sitosterol in good yields to predominantly 3α -hydroxy androsta-5-ene-17-one (DHIA). 2) The ability to selectively degrade the sterol side chain, without concomitant conversion of the steroidal 3-α-hydroxy-5-ene system to a 3-keto-4-ene system.

A microorganism from soil samples was isolated, which is capable of selectively degrade sitosterol to 9α-OHAD.

Materials and Method

Microorganism

From a program to screen microorganisms capable of converting sitosterol to DHIA, a soil bacterium was isolated. This microorganism degraded sitosterol to AD. (TLC plate Fig. 1). This microorganism was isolated from the third transfer of a shake tube culture which incubated in a sitosterol enriched medium (medium A). The isolate was transferred to fresh media every week, and was incubated on a rotary shaker. On the ninth transfer, the microorganism degraded sitosterol to 9α OHAD (TLC plate Fig. 1). Subsequent purfication of the mixed culture showed that a *Mycobacterium* sp. was responsible for the production of 9α OHAD from sitosterol.

Culture media

In general two different media were used in these sterols and steroidal transformation experiments. Medium A contained (per liter of tap water) NH_4NO_3, 1g; K_2HPO_4, 0.25g; $MgSO_4 \cdot 7H_2O$, 0.25g; $FeSO_4 \cdot 7H_2O$, 0.001g; pH 7.0. Medium B contained (per liter of tap water) peptone, 5g; beef extract, 3g; yeast extract, 1g; glucose, 1g; pH 7.0.

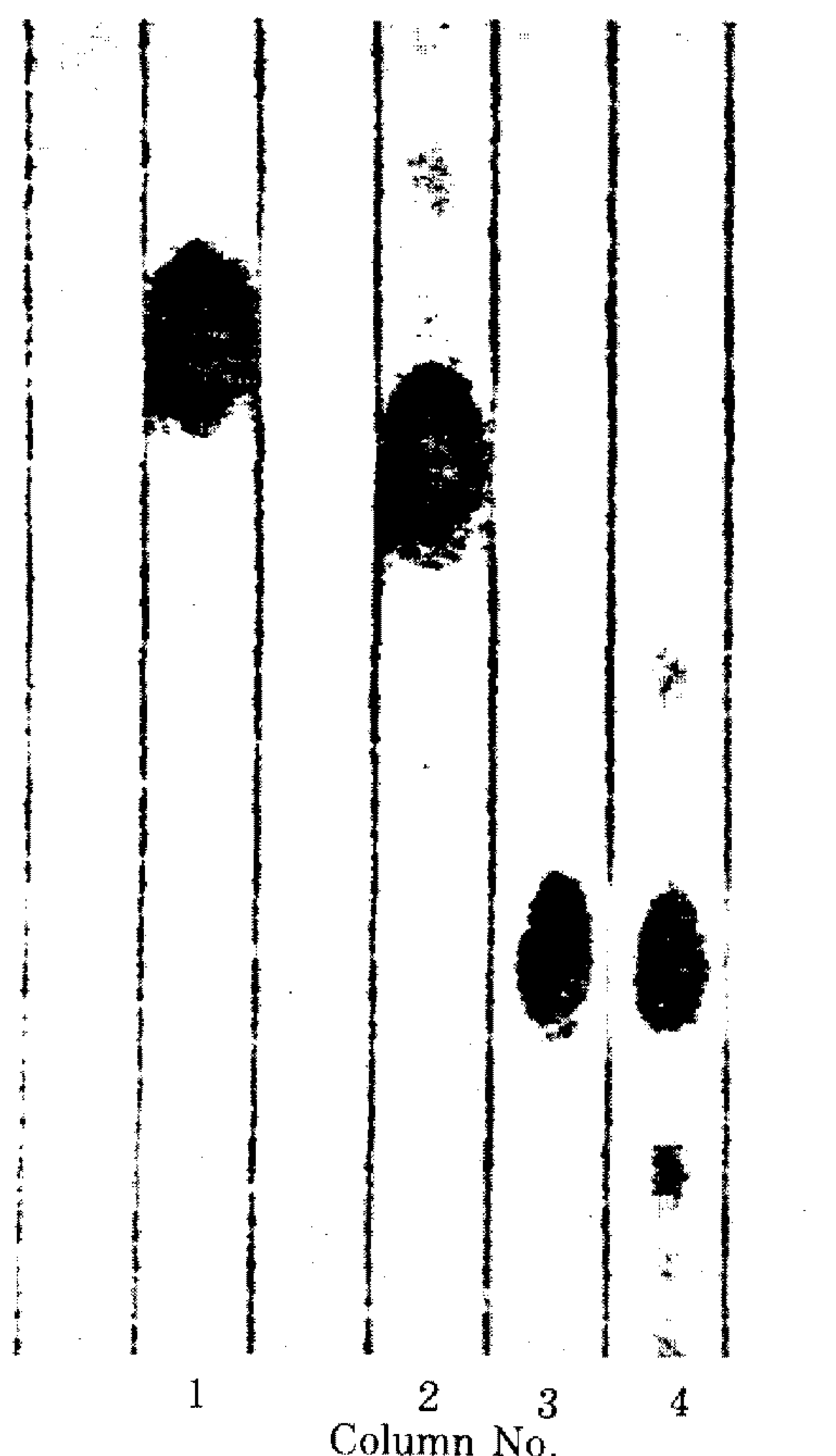

Fig. 1. Representative TLC of reference compounds and methylene chloride extracts of the bioconversion beers.
column 1 AD control, column 2 ADD control, column 3 9α-OHAD control, column 4 Bioconversion beers of the sitosterol

Shake culture growth and fermentation condition

500 m*l* Erlenmeyer flasks containing 100 m*l* of medium A were inoculated with 3 m*l* of a cell suspension from a 7 day old agar slant culture of *Mycobacterium* sp. The seed culture flask was incubated in a controlled environment on a rotary shaker (New Briuinswick Scientific Co) at 200 rec/min. After 72 hours at 31°C, 10 m*l* of the inoculum was taken from the seed culture flask and transferred to 100 m*l* of the inoculum was taken from the seed culture flask and transferred to 100 m*l* of media B in a 500 m*l* Erlenmeyer flask.

For the preparation of sterol substrate in medium B, 100 mg powdered sterol, prepared by pulverizing with a mortar and pestal, was added to each 500 m*l* Erlenmeyer flask containing 100 m*l* of media. For the preparation of steroids substrate in medium B, 2100 mg of powdered steroids were added to each 500 m*l* Erlenmeyer flask containing 100 m*l* medium

Table 1. The results of IR, NMR, and GC analysis of the products

Amounts of Substrate	Instrument used	% of Yield		
		Sitosterol	AD	9 OHAD
2.1g/100 m*l*	IR/NMR	--	--	24
1.5g/100 m*l*	GC	76	4	14

B. The inoculated flasks were incubated in a controlled environment on a rotary shaker at 200 rec/min for 168 hours at 31°C. The results of IR, NMR, and GC analysis were listed at Table 1.

Extraction procedure and preliminary analysis of the products

The bioconversion beers were extracted twice with one-half volumes of methylene chloride, and the extracts were combined. Preliminary analysis of the extracts were performed by thin-layer chromatography on silica gel G plates. The plates were developed using toluene-ethanol (85:15, v/v) as the developing solvents. The spots were visualized by spraying the plates with 50% sulphuric acid and heating at 160°C.

Conversion of sitosterol to 9α-hydroxy androst-4-ene-3,17-dione (9α OHAD)

The crude extract (from 2.1g sitosterol substrate) was dissolved in 20 m*l* of methanol. The solution was slurried with 10g of Woelm silica gel, evaporated, and loaded into a 15 mm I.D. × 1000 mm column already containing 80g of Woelm silica gel. The column was eluted succesively with:

35:65 ethyl acetate: methylene chloride (v/v)
50:50 ethyl acetate: methylene chloride
65:35 ethyl acetate: methylene chloride
80:20 ethyl acetate: methylene chloride
100 ethyl acetate

The initial flow rate was 3 m*l*/minute. Fraction of 18 m*l* volume were collected. The fractions were monitored by TLC. TLC plates of 20×20 cm (Woelm GF) were spotted with 10 μ*l* of each fraction. The plates were developed using a solvent system consisting of 80:20 ethyl acetate: methylene chloride (v/v). The developed plates were analyzed under short-wavelength UV. The plates were sprayed with a 50% sulfuric acid reagent, further analyzed under visible light and long-wave length UV. The plates were finally sprayed with a phosphomolybdic acid reagent.

The desired product, eluted with solvent systems 35:65 and 50:50 ethyl acetate: methylene chloride, was further purified to give 9α-hydroxy androst-4-ene-3,17-dione in 24% yield. The desired product, recrystalized from methanol, have a melting point of 221-224°C. Elemental analysis for $C_{19}H_2O\ O_3$: molecular weight 302.41; calculated C75.46 H 8.67.

Infrared absorption (chloroform) were observed at 3575, 3605 cm^{-1} for the hydroxy group; 1734 cm^{-1} for the C-17 keto group and 1613 and 1667 cm^{-1} for the 3-keto-4-ene system. NMR absorption were observed at δ0.93 (singlet) for the C-18 methyl, δ1.37 (singlet) for the C-19 proton. UV maxima was observed at 241 nm with an extinction coefficient of ξ=14,640 for the 3-keto-4-ene system. Mass spectrum exhibited peaks at m/e=302, 204, 166, 151, 137, 136, 134, and 124 (base peak).

Conversion of 21-hydroxy-20-methyl preg-4-ene-3-one to 9α, 21-dihydroxy-20-methyl pregn-4-ene-3-one.

The crude extract from 25g substrate was dissolved in 50 m*l* methylene chloride and was loaded into a 25×1000 mm column containing 500g of Woelm silica gel.

The column was eluted successively with:

20:80 ethyl acetate: toluene (v/v)
50:50 ethyl acetate: toluene
100 percent ethyl acetate

The initial flow rate was 5 m*l* per minute. Fractions of 16 m*l* volume were collected. The fractions were monitored using TLC. The plates were developed in a solvent system consisting of 50:50 ethyl acetate: toluene. The 9α, 21-dihydroxy-20-methyl-pregn-4-ene-3-one was eluted with 100% ethyl acetate. Recrystalization from acetone gave a crystallin material having a melting point of 181-183°C. Elemental analysis for $C_{22}H_{34}O_3$ with a molecular weight of 346.51 calculated C71.24, H 9.89, found C 76.35 H 10.03. Infrared absorption were observed at 3620, 3480, 1670, 1616, and 1088 cm^{-1}. NMR absorption were observed at δ 0.75, 1.04, 1.32, 2.40, 3.33, 3.67, and 5.85. Mass spectrum exhibited peaks at m/e 346, 328, 137, 135, and 124 (base peak).

Results and Discussion

The *Mycobacterium* sp. isolated during our screen-

Table 2. The effects of different media on the production of 9α OHAD from sitosterol (TLC)

Media	Conc. of Sitosterol	Production of	
		9α OHAD	AD
A	1 g/100 ml	+ + + +	+
B	1 g/100 ml	+ +	+
C	1 g/100 ml	+ + + + +	+ + + +
D	1 g/100 ml	+ + +	+
E	1 g/100 ml	+ + + +	+ + + +

Control of 9α OHAD + + + + + +, Control of AD + + + + + +

Table 3. The effects of substrate concentration on the production of 9α OHAD (TLC)

Sitosterol Conc. (%)	9α OHAD	AD
0.1	+ + + + +	+
0.25	+ + + + +	+
0.5	+ + + + +	+
1	+ + + +	+ +
1.5	+ + +	+ + +

Control of 9α OHAD + + + + + +, Control of AD + + + + + +

ing program was shown to degrade sitosterol and other sterols to predominantly 9 α OHAD, along with varing amounts of AD. G.C. and other physicochemical techniques were used to identify substrate.

The effect of media

Five different kinds of media were evaluated for the production of 9α OHAD from sitosterol (Table 3). Medium C gave the best yields of 9α OHAD. However it also produced a fair amounts of AD. The best yields of 9α OHAD with the least amounts of AD (ratio yield) was produced using medium A (Table 2).

The effects of substrate concentrations

Five different concentrations of substrate in medium C were evaluated for the production of 9α OHAD. Sitosterol concentrations in the range of 0.1% and 0.25% gave the best ratio yields (Table 3).

Various sterols and steroids were used as substrates. The degradation products of these substrates by *Mycobacterium* sp. B 1951-99 are listed in Table 4.

Table 4. The effects of different substrates on the production of steroidal intermediates (TLC)

Substrate	Products	
	9α OHAD	9α OH 30571
Sitosterol	+ + + + +	
AD	+ + + + +	
DHA	+	
SC-30571		+ + +
Cholesterol	+ + + + +	
Stigmasterol	+ + +	

Control of 9α OHAD + + + + + +, Control SC-30571 + + + + + +

Microscopic morphology

The color of the cell colony and culture condition of the microorganism was almost identical to *Mycobacterium* sp. NRRL B-3805. It was therefore designated to *Mycobacterium* sp. B-1951-99.

Several studies have shown that many microorganisms are capable of degrading sterols. This has entailed the decomposition of both the sterol side chain and the steroid nucleus (1).

If a microorganism could partially decompose the sterol by selectively cleaning the C-17 side chain without degrading the steroid nucleus, such process might have great commercial application. In principle, three different methods have been employed to inhibit one or both of the enzymes involved in the initial attack on the steroidal nucleus: (1) Structural modification of the substrate to prevent enzymatic attack on the steroid nucleus. (2) Conversion of unmodified substrates in the presence of inhibitor to prevent C-1 (2) dehydrogenation or 9α-hydroylation. (3) Mutation of the microorganism that decompose the sterols completely, in order to inactivate the C-1 (2)-dehydrogenase and/or 9α-hydroxylase.

Several investigators studied the procedures for the selective microbiol side chain cleavage of sterols in the presence of enzyme inhibitor.

The α-α' dipyridye is the good inhibitor of dehydrogenation at C-1 and 2 position, and inhibit the hydroxylation at C-9 of sitosterol by a *Nocardia* sp.

These inhibitory effects let to accumulation of 4-androstene-3,17-dione (AD) or 1,4-androstandiene-3,17-dione (ADD). Since the 9 α-hydroxylase of *Nocardia* sp. was not inhibited completely, further metabolism of AD and ADD occurred, and results in loss of substrate and low yields. Because of the partial inhibitory of the side chain hydroxylase of sitosterol, higher concentrations of inhibitors did not cause increased accumulation of C-17-ketosteroid. Therefore, the usage of the inhibitors appear to have limited practical usefulness due to their relative inefficiencis or costliness.

The generation and isolation of mutant is a well established process in microbiol genetics, and another approach is the isolation of the new mutant from natural habitats.

Recently, mutagenic treatment has been employed for the production of microorganisms capable of degrading the sterol side chain selectively. Such mutants were biochemically blocked from degrading the nucleus and can be used for the production of steroids from sterols without modifying the substrate or adding chemical inhibitors (4).

Wovcha *et al.* (6) described a methodology that allows for the production and selection of mutant organisms capable of specific side-chain cleavage of sterols. This methodology is based upon the mutation of wild-type strains that are capable of completely degrading cholesterol, and selection of mutants blocked at the desired conversion step.

Knight *et al.* (2) further mutated these organisms, and a mutant of *Mycobacterium fortuitum*, degraed β-sitosterol to 9 α -hydroxy-27-nor-4-cholestene-3,24-dione, whereas cholesterol is degraded mainly to 9 α -hydroxy-4-androstene-3,17-dione.

Marsheck *et al.* (3) earlier described the Mycobacterial degradation of sterol to 17-keto steroid by two newly isolated *Mycobacterium* sp. NRRL B-3683 and NRRL B-3805. *Mycobacterium* sp. NRRL B-3683 was produced by U.V. irradiation of a soil *Mycobacterium* sp. capable of conversion sitosterol to ADD. Further irradiation of *Mycobacterium* sp. NRRL B-3683 produced *Mycobacterium* sp. NRRLB-3805, which lacked the ability to C-1 (2) dehydrogenate steroids, resulting in the formation of Ad from sterols.

We found that B 1951-99 degraded sitosterol to AD and 9α OHAD, and cholesterol to AD and 9α OHAD. This microorganism also hydroxylates the steroidal nucleus at the 9α position. This isolated (B 1951-99) is very useful for the conversion of sterol or steroids to 9 α -hydroxylated steroids without the need of any substrate modification or added inhibitory agent.

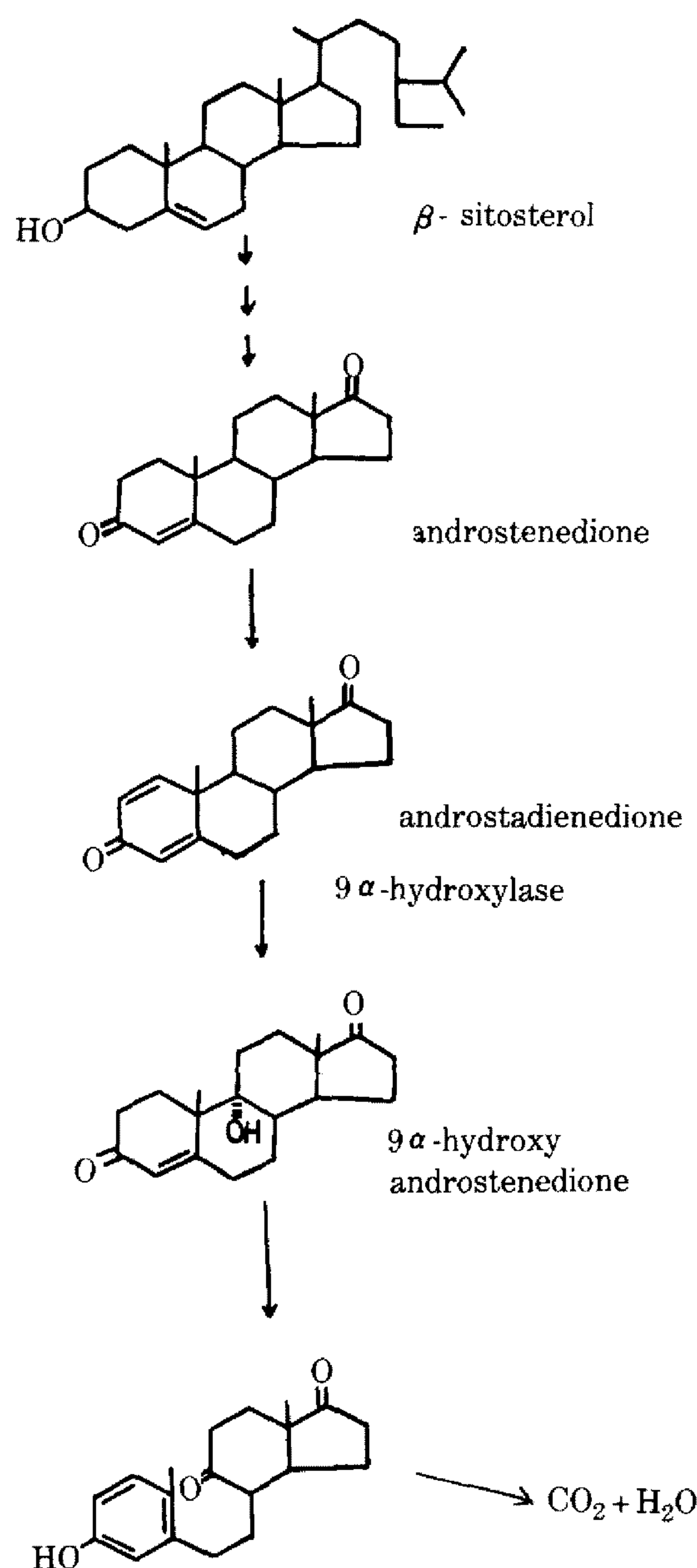

Fig. 2. Sitosterol degradation by *Mycobacterium* sp. B1951-99

The sitosterol degradation by this organism is illustrated in Fig. 2.

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요 약

콜레스테롤과 식물스테롤을 AD와 9-α-AD로 분해하는 Mycobacterium 속의 돌연변이주를 분리하였다. 또한, 이 변이주는 스테로이드성 핵의 9α 위치에 Hydroxylation을 시켰다. 이러한 반응은 고리파괴저해제 없이도 가능하였다.

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