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Microbial Production of 3-Oxobisnorchola-1,4-dien-22-oic Acid†

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Among examined microorganisms which have ability to decompose cholesterol, *Nocardia corallina* IFO 3338 converted cholesterol to 3-oxobisnorchola-1,4-dien-22-oic acid (BDA) at the conversion rate of 63%. The conversion product was identified as BDA by ^{1}H-NMR, ^{13}C-NMR, UV, IR, MS and elemental analysis.

The presence of chelating agents or some metal ions was necessary for the accumulation of BDA. The most effective agents for the accumulation were α,α′-dipyridyl and *o*-phenanthroline.

For the production of BDA, cholestanol, β-sitosterol, soy sterol (mixture), cholest-4-en-3-one and lithocholic acid could also be used as substrates other than cholesterol.

Various steroid hormones, such as sex hormones, corticosteroid hormones, protein anabolic hormones *etc.*, have been produced by chemical transformation starting from diosgenine, a sapogenine found in *Dioscorea*, however, several difficulties to obtain the plant will cause profound shortage of the starting material for hormone production in near future. We have recently established the microbial process to obtain steroid skeleton from cholesterol.[1] Incubation of cholesterol with a cholesterol-decomposing bacterium in the presence of some inhibitors caused selective cleavage of the cholesterol side chain to yield androsta-1,4-diene-3,17-dione (ADD). Although this process will possibly supply ADD as a starting material to derive large part of steroid hormones such as androgen and estrogen, chemical synthesis of several steroid hormones having a C_2-side chain at C–17 of the steroid skeleton, such as progestagen and adrenocortical hormones, from ADD is still difficult.

Present investigation was started intending to search for a microbial transformation process of cholesterol suitable for production of progestagen and adrenocortical hormones. Cholesterol-decomposing microorganisms selected from culture collection and natural sources were examined for their reaction products from cholesterol. Several strains were found to produce 3-oxobisnorchola-1,4-dien-22-oic acid (BDA) in a relatively good yield. This product will be able to be converted to C_{21}-steroid with a C_2-side chain by decarboxylation at C–22. Although an economical process for this decarboxylation has not yet been established, the present finding will produce a new starting material for several interesting steroid compounds. In this paper, we report on microorganisms and conditions to achieve effective transformation of cholesterol into BDA.

METHODS AND MATERIALS

UV spectra were measured with a Hitachi double beam spectrophotometer model 124. IR spectra were recorded with a JASCO IR–S spectrophotometer. The ^{1}H-NMR and ^{13}C-NMR spectra were measured with a Varian XL–100–15 spectrometer operating at 100 MHz and 25.2 MHz, respectively, in $CDCl_3$ solution with TMS as an internal standard. Mass spectra were recorded with a Hitachi RMU–6MG spectrometer.

Microorganisms. Following strains were selected according to their steroid decomposing ability and used in the present experiment. *Arthrobater simplex* IAM 1660, *Bacillus roseus* IAM 1250, *Brevibacterium*

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lypolyticum IAM 1390, *Corynebacterium equi* IAM 1038, *Corynebacterium sepedonicum* IFO 3306, *Microbacterium lacticum* IAM 1640, *Protaminobacter alboflavus* IAM 1040, *Serratia marcescens* IAM 1255, *Mycobacterium avium* IFO 3082, *Mycobacterium phlei* IFO 3158, *Mycobacterium smegmatis* IFO 3083, *Nocardia gardneri* IAM 0105, *Nocardia corallina* IFO 3338, *Nocardia minima* IFO 0374, *Nocardia restrictus* ATCC 14887, *Nocardia erythropolis* NI 9110, *Streptomyces coelicolar* IAM 1023, *Streptomyces rubescens* IAM 0074, *Streptomyces tanasiensis* IAM 0016 and unidentified 14 strains isolated from natural sources.

Fermentation. Cholesterol decomposing ability was examined after cultivation at 30°C for 48 hr aerobically in 20×200 mm test tubes containing 5 ml of the medium consisting of 0.3% meat extract, 1.0% peptone, 0.5% NaCl and 0.2% cholesterol (pH 7.0).

Microbial conversion of steroids was carried out according to the following procedure unless otherwise described. Microorganisms were cultured in the medium similar to the above except for omitting cholesterol. After 20 hr cultivation, steroids were added as fine powders to give a concentration of 0.2%. Inhibitors for steroid decomposition, such as α,α′-dipyridyl, were added 4 hr after the steroid addition and fermentation was continued for another 48 hr.

Determination of cholesterol. Cholesterol was assayed by the Liebermann-Burchard color reaction. Cholesterol in broth was extracted with ethylacetate and after complete removal of the solvent from the extract, colorimetrical determination was carried out by the procedure of I. Smith.[2]

Detection of degradation products of steroid. Degradation products of steroid were extracted from the cultured broth with ethylacetate under acidic condition (pH 1~2 with aqueous H_2SO_4 soln.), and an aliquot of the extract was subjected to thin-layer chromatography on silica gel G–60 plates (0.25 mm thick, 20×20 cm) with $CHCl_3$: Et_2O (9: 1) at room temperature. Spots on the chromatogram were detected by the following methods; spraying 10% H_2SO_4 and heating at 120°C for 5~10 min, UV light, or exposing to iodine vapor. *Rf* values were compared with those of authentic cholest-4-en-3-one, cholesta-1,4-dien-3-one, androsta-1,4-diene-3,17-dione (ADD), and 3-oxobisnorchola-1,4-dien-22-oic acid (BDA). Each spot was also quantitatively determined by extracting with ethanol followed by measuring their UV absorption at 243 nm.

RESULTS

Microbial conversion of cholesterol to 3-oxobisnorchola-1,4-dien-22-oic acid (BDA)

Although microbial conversion of cholesterol produced mainly ADD which lost the gross aliphatic side chain of cholesterol by cleavage between C–17 and C–20, slight accumulation of BDA having an intermediary degraded sidechain was observed. Therefore, we tried to obtain BDA in high yield.

More than 200 strains of various microorganisms were tested for their ability to decompose cholesterol, and 33 strains of them listed in METHODS AND MATERIALS were selected for this experiment. Almost all the selected strains accumulated only cholest-4-en-3-one from cholesterol in the absence of inhibitors, except for *Corynebacterium sepedonicum* IFO 3306 which accumulated no detectable degradation products.

Conversion of cholesterol by these microorganisms were examined in the presence of 10^{-3} M α,α′-dipyridyl, and 11 strains listed in Table I were found to accumulate significant

TABLE I. ACCUMULATION OF 3-OXOBISNORCHOLA-1,4-DIEN-22-OIC ACID BY VARIOUS MICROORGANISMS

Decomposition of cholesterol by various microorganisms was carried out in the presence of α,α′-dipyridyl. Cholesterol was added to 20 hr cultured broth at the concentration of 0.1%, and after an additional 4 hr, α,α′-dipyridyl was added at the level of 10^{-3} M. The other experimental methods were same as METHODS AND MATERIALS.

Microbial strains	BDA	
	Accumulation (mg/ml)	Yield (%)[a]
Nocardia corallina IFO 3338	0.38	43
Nocardia restrictus ATCC 14887	0.14	16
Nocardia erythropolis NI 9110	0.14	16
Mycobacterium avium IFO 3082	<0.10	
Arthrobacter simplex IAM 1660	<0.10	
Bacillus roseus IAM 1257	0.16	18
Brevibacterium lypolyticum IAM 1398	<0.10	
Corynebacterium equi IAM 1038	0.12	14
Microbacterium lacticum IAM 1640	<0.10	
Protaminobacter alboflavus IAM 1040	<0.10	
Serratia marcescens IAM 1255	0.32	36

[a] Conversion rate (%) from cholesterol.

TABLE II. ACCUMULATION OF INTERMEDIATES OF CHOLESTEROL DECOMPOSITION BY MICROORGANISMS

Decomposition of cholesterol was carried out in a similar way as Table I in the presence of α,α′-dipyridyl (10^{-3} M)[a] or *o*-phenanthroline (10^{-4} M).[b]

The table indicates accumulated intermediates in the digestion mixture, and their conversion rates from cholesterol.

Microbial strains	Cholest-4-en-3-one (%)	Cholesta-1,4-dien-3-one (%)	ADD (%)	BDA (%)	Remaining cholesterol (%)
Nocardia corallina IFO 3338[a]	4	2	5	43	28
Nocardia corallina IFO 3338[b]	4	3	5	63	22
Serratia marcescens IAM 1255[a]	10	1	4	32	16
Bacillus roseus IAM 1257[a]	14	—	7	19	7

amounts of BDA. Among them, the most excellent one was *Nocardia corallina* IFO 3338, which converted cholesterol to BDA at a conversion rate of 43% under the given condition. In addition to BDA, most strains in Table I accumulated considerable amounts of other cholesterol degradation products. Those were cholest-4-en-3-one, cholesta-1,4-dien-3-one, androsta-1,4-dien-3,17-dione and some minor unknown products. However, *N. corallina* IFO 3338 produced mainly BDA from cholesterol with small amounts of the other products as shown in Table II.

It was also observed that fairly large amounts of cholesterol remained even after the prolonged incubation (Table II). This problem was partially solved by changing the chelating agent α,α′-dipyridyl to *o*-phenanthroline, which stimulated both the accumulation of BDA and consumption of cholesterol. *o*-Phenanthroline seems to be a superior inhibitor, however, the critical range of the concentration to give a good conversion rate was relatively narrow as described below.

Isolation and identification of BDA

The main cholesterol degradation product by *N. corallina* IFO 3338 was purified and identified as follows. The culture broth was acidified with aqueous solution of H_2SO_4 to pH 1~2 and extracted twice with equal volume of ethylacetate. After concentration *in vacuo*, the extract was subjected to preparative thin-layer chromatography (silica gel G-60, 2 mm thickness) and developed three times with $CHCl_3$: ethylether (9: 1). The main zone, detected by iodine vapor, was scraped out and extracted with ethanol, followed by removal of the solvent *in vacuo* to dryness. The dried matter was crystallized from ethylacetate and recrystallized from acetone. Thus 90 mg of crystals were obtained from 500 ml culture broth which contained 1 g of cholesterol before the incubation.

The elemental analysis of this substance indicated $C_{22}H_{30}O_3$ as its molecular formula (found: C, 77.43; H, 8.65. calcd. for $C_{22}H_{30}O_3$: C, 77.19; H, 8.77). The mass spectrum of this substance revealed an ion peak of *m/e* 342 in agreement with the formula of $C_{22}H_{30}O_3$. This substance is acidic in nature and when methylated with diazomethane, an ion peak of *m/e* 356 was observed. This suggests the presence of one carboxy group in the molecule. The UV absorption spectrum showed λ_{max}^{EtOH} nm (ε); 243 (16214), due to a conjugated dienone system at $\Delta^{1,4}$-3-C=O. The IR absorption spectrum, ^{1}H-NMR spectrum (methyl ester) and ^{13}C-NMR spectrum of the substance are shown in Fig. 1, Fig. 2 and Fig. 3, respectively. The assignment of the spectra were made as follows referring to the assigned spectra of cholesterol degradation products[7] and related compounds.[8]

IR ν_{max}^{Nujol} cm^{-1}; 1720 (CO_2H), 1655, 1612 and 1600 ($\Delta^{1,4}$-3-C=O) (Fig. 1). ^{1}H-NMR $\delta_{Me_4Si}^{CDCl_3}$; 0.76 (3H, s, 19-Me), 1.24 (3H, s, 18-Me), 6.09 (1H, broad s, 4-H), 6.25 (1H, dd, J=10 Hz and 2 Hz, 2-H) and 7.05 (1H, d, J=10 Hz, 1-H), and the metylated substance showed one more signal at δ 3.66 (3H, s, CO_2Me) (Fig. 2). ^{13}C-NMR $\delta_{Me_4Si}^{CDCl_3}$; 12.2 (C-19, q), 17.0, C-21

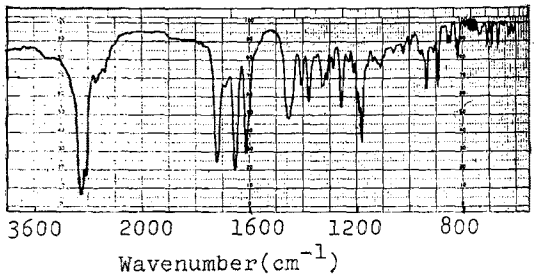


FIG. 1. IR Spectrum of BDA (in nujol).

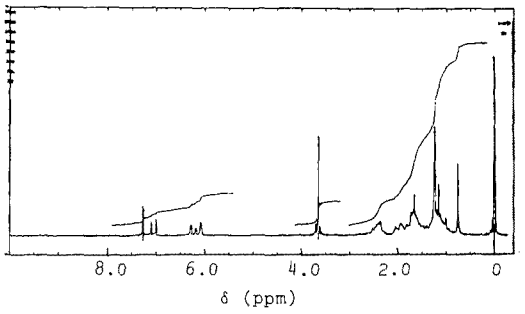


FIG. 2. ^{1}H-NMR Spectrum of BDA Methyl Ester (100 MHz).

Spectrum was recorded in $CDCl_3$ with TMS as internal standard.

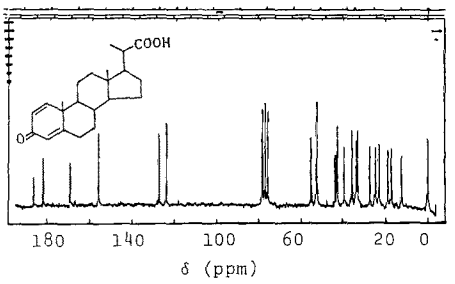
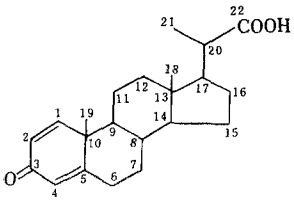


FIG. 3. Proton Noise Decoupled ^{13}C-NMR Spectrum of BDA (25 MHz).

Spectrum was recorded in $CDCl_3$ with TMS as internal standard. The number of hydrogen atoms was determined by using off-resonance decoupling technique.

q), 18.7 (C-18, q), 22.8 (C-11, t), 24.4 (C-15, t), 27.2 (C-12, t), 32.8 (C-7, t), 33.6 (C-6, t), 35.5 (C-8, d), 39.3 (C-16, t), 42.4 (C-13, s), 42.7 (C-20, d), 43.6 (C-10, s), 52.3 (C-9, C-17, d), 55.1 (C-14, d), 123.7 (C-4, d), 127.3 (C-2, d), 155.7 (C-1, d), 169.1 (C-5, s), 181.8 (C-22, s), 186.2 (C-3, s).

From the above results, it was concluded that the structure of the substance was 3-oxobisnorchola-1,4-dien-22-oic acid.



Effects of inhibitors on accumulation of BDA

Effects of various inhibitors on cholesterol degradation was examined for the production of BDA by *N. corallina* IFO 3338. As shown in Table III, obvious production of BDA was seen in the presence of chelating agents (α,α′-dipyridyl, *o*-phenanthroline and 8-oxyquinoline) and metal ions ($CoCl_2$). On the other hand, EDTA, a powerful chelating agent, did not show any effects on the production. These results were similar to the case of ADD production by *Arthrobacter simplex* IAM 1660.[3] In that report, the relationship between ADD production and the lipophilic structure of chelating agents was mentioned. In the present work, chelating agents having the lipophilic structures were also required for BDA production by *N. corallina* IFO 3338.

TABLE III. EFFECT OF VARIOUS INHIBITORS ON THE ACCUMULATION OF BDA BY *Nocardia corallina* IFO 3338

Inhibitors	Concn. (M)	BDA	
		Accumulation (mg/ml)	Yield (%)
α,α′-Dipyridyl	10^{-3}	0.38	43
o-Phenanthroline	10^{-4}	0.56	63
8-Oxyquinoline	10^{-3}	0.16	18
Diphenylthiocarbazone	10^{-3}	trace	
Isatin	10^{-3}	trace	
Methylene blue	10^{-3}	trace	
$CoCl_2$	2×10^{-3}	0.15	17
$NiCl_2$	2×10^{-3}	trace	
$CdSO_4$	10^{-3}	trace	
$NaAsO_2$	10^{-3}	trace	
LiCl	10^{-3}	trace	
$HgCl_2$	10^{-4}	trace	

TABLE IV. EFFECT OF *o*-PHENANTHROLINE AND α,α′-DIPYRIDYL CONCENTRATION ON THE ACCUMULATION OF BDA BY *Nocardia corallina* IFO 3338

Nocardia corallina IFO 3338 was inoculated to the medium containing 0.2% or 0.5% cholesterol and after 24 hr cultivation, various concentration of *o*-phenanthroline or α,α′-dipyridyl were added, and the cultivation was continued another 48 hr.

Inhibitors	Concn. (M)	BDA A[a] (mg/ml)	BDA B[b] (mg/ml)
o-Phenanthroline	10^{-3}	0.07	—
	5×10^{-4}	0.05	—
	2×10^{-4}	0.06	0.09
	10^{-4}	0.67	0.49
	5×10^{-5}	0.05	0.08
	2×10^{-5}	—	—
	10^{-5}	—	—
α,α′-Dipyridyl	2×10^{-3}	0.19	0.42
	10^{-3}	0.62	0.43
	5×10^{-4}	0.86	0.41
	2×10^{-4}	0.09	0.08
	10^{-4}	—	—
	5×10^{-5}	—	—

a) Cholesterol concentration was 0.5%.
b) Cholesterol concentration was 0.2%.

These results suggest that the degradative enzyme systems requiring certain metal ions for BDA or ADD production might function in lipid circumstances.

Optimum concentration of α,α′-dipyridyl and *o*-phenanthroline for the BDA production was examined and the results are shown in Table IV. Optimum concentration of *o*-phenanthroline was 10^{-4} M, and slight changes in the concentration resulted in marked decrease of the BDA production. On the other hand, α,α′-dipyridyl was effective in more wider concentration range, 2×10^{-3} M ~ 5×10^{-4} M.

Effects of substrates on BDA production

It is necessary for the effective production of BDA to increase cholesterol concentration. As shown in Table V, the maximum production of BDA was attained at the cholesterol concentration of 0.6%, with the conversion rate of 31%. The addition of cholesterol as a fine powder or as a solution in an organic solvent such as dimethylformamide gave the same results.

TABLE V. EFFECT OF CHOLESTEROL CONCENTRATION ON THE ACCUMULATION OF BDA BY *Nocardia corallina* IFO 3338

Cholesterol concentration (%)	BDA Accumulation (mg/ml)	BDA Yield (%)
0.1	0.44	50
0.2	0.56	32
0.4	1.20	34
0.6	1.60	31
1.0	0.54	6
2.0	0.78	5

N. corallina IFO 3338 also produced BDA from various steroids other than cholesterol (Table VI), such as cholestanol, β-sitosterol, soy sterol (mixture), cholest-4-en-3-one and lithocholic acid. But BDA was not produced from steroids which have a double bond or a hydroxy group in the side chain, or the B ring substituted by a hydroxy or carbonyl group or bromine.

TABLE VI. EFFECT OF VARIOUS SUBSTRATES ON THE ACCUMULATION OF BDA BY *Nocardia corallina* IFO 3338

Nocardia corallina IFO 3338 was cultured in media containing various steroids (0.2%) and after 24 hr culture, α,α′-dipyridyl was added at the concentration of 10^{-3} M. For other experimental conditions, see METHODS AND MATERIALS.

Substrates (0.2%)	BDA Accumulation (mg/ml)	BDA Yield (%)
Cholesterol	0.47	24
Cholestanol	0.11	6
β-Sitosterol	0.19	10
Stigmasterol	—[a]	
Cholest-4-en-3-one	0.07	4
Soy sterol (mixture)	0.10	6
Lithocholic acid	0.78	39
Cholic acid	—	
Deoxycholic acid	—	
20α-Hydroxycholesterol	—	
6-Ketocholestanol	—	
Dibromocholestanol	—	

a) undetectable

DISCUSSION

Although there were several observations detecting BDA or its related substances in the microbial degradation products of 19-hydroxycholest-4-en-3-one,[4,5] production of large amounts of BDA by microorganisms was not reported. It has been observed for the first time that addition of a chelator caused accumulation of large amounts of BDA from cholesterol by microorganisms.

As reported in previous papers,[1,3,6,7] addition of chelating agents to the cholesterol decomposing microorganisms caused marked accumulation of ADD, suggesting that the main inhibited site in the cholesterol metabolism was the 9α-hydroxylation reaction which precedes the cleavage of the steroid ring. However, the present finding that chelators induced also marked accumulation of BDA from cholesterol in several microorganisms including *Nocardia corallina* suggested the possibility that enzymatic reactions from BDA to ADD might be also sensitive to the chelators and that extent of the inhibition of the reactions might differ in various microorganisms. Another possible explanation is that accumulation of small amounts of ADD would suppress the conversion of BDA to ADD in several microorganisms, since ADD was also produced in smaller amounts besides BDA.

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