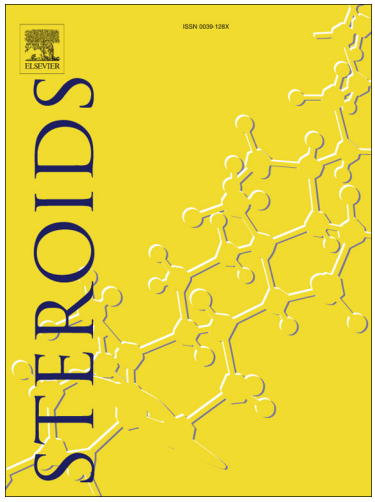

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New product identification in the sterol metabolism by an industrial strain *Mycobacterium neoaurum* NRRL B-3805

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Running title: New product of *M. neoaurum* NRRL B-3805

Abstract

Mycobacterium neoaurum NRRL B-3805 metabolizes sterols to produce androst-4-en-3,17-dione (AD) as the main product, and androsta-1,4-dien-3,17-dione, 9α-hydroxyandrost-4-en-3,17-dione and 22-hydroxy-23,24-bisnorchol-4-en-3-one have been identified as by-products. In this study, a new by-product was isolated from the metabolites of sterols and identified as methyl 3-oxo-23,24-bisnorchol-4-en-22-oate (BNC methyl ester), which was proposed to be produced via the esterification of BNC catalyzed by an *O*-methyltransferase using *S*-adenosyl-L-methionine as the methyl group donor. These results might open a new dimension for improvement of the efficiency of microbial AD production by eliminating this by-product via genetic manipulation of the strain.

Key words: sterol degradation pathway, *Mycobacterial neoaurum*, androst-4-en-3,17-dione, methyl 3-oxo-23,24-bisnorchol-4-en-22-oate

1. Introduction

Steroidal pharmaceuticals are widely used in clinical applications. Androst-4-en-3,17-dione (AD), androsta-1,4-dien-3,17-dione (ADD) and 9α-hydroxy androst-4-en-3,17-dione (9-OH-AD) are the most marketed precursors for the production of steroidal drugs [1, 2]. Many actinobacteria can use sterols including cholesterol or phytosterols as carbon and energy sources and convert sterols to these steroidal precursors in a biotechnological step.

Mycobacterium neoaurum NRRL B-3805 (hereafter B-3805), which was isolated after two rounds of ultraviolet irradiation, has been used at industrial level to produce AD from various sterols with up to 39% yield [3-6]. However, ADD, 22-hydroxy-23,24-bisnorchol-4-en-3-one (4-HBC) and 22-hydroxy-23,24-biosnorchol-1,4-dien-3-one (1,4-HBC) were produced in small amounts, limiting the product yield and increasing the difficulty in the product purification. The cholesterol metabolic pathway was proposed as Figure 1 [7-11]. Recently, the genome of B-3805 was sequenced (GenBank accession number CP011022), providing essential genetic information for molecular engineering of this industrial strain [12]. Identification of the products in the microbial transformation of sterols provides useful information for further understanding of the microbial sterol degradation pathway, thus offering guidance for molecular engineering of the industrial strains to improve the process efficiency for the production of the useful steroidal intermediates. Therefore, we attempted to characterize the products in the transformation of cholesterol with B-3805. This would lead to further understand

1 the metabolic pathway of sterols by this strain and provide more information for its
2 engineering to eliminate the by-products, thus enhancing the AD production
3 efficiency of this strain.

2. Materials and methods

2.1 Materials

Cholesterol (>95%), β-sitosterol (75%), campesterol (95%), AD, ADD, 4-HBC, 1,4-HBC, testosterone (TS) and other chemical reagents and solvents were supplied by chemical companies with reagent grade or the highest purity available. Silica gel and thin layer chromatography (TLC) plate were both obtained from Qingdao Haiyang Chemical Company. The 3-oxo-23,24-bisnorchol-4-en-22-oate (BNC, V) was prepared by the oxidation of 4-HBC in our laboratory according to the reference [13, 14]. The ^{1}H nuclear magnetic resonance (NMR) spectra of BNC was recorded on Bruker AVANCE III at 400 MHz and shown in Figure S1.

2.2 Microorganism cultivation and biotransformation

Strain B-3805 was grown on Luria-Bertani (LB) medium at 30°C in a rotary shaker at 200 rpm for 3 days. The LB pre-grown culture was inoculated into 18 mL of medium SCN (inoculum volume 1:9, v/v), which contained defatted soy flour (10 g/L), corn steep powder (5 g/L), $(NH_4)_2HPO_4$ (2 g/L), cholesterol (20 g/L) or BNC (10 g/L), and soy oil (200 g/L), and the pH was adjusted to pH 7.5 by NaOH. The resulting culture was shaken at 30°C in a rotary shaker at 200 rpm for 4-7 days. The biotransformation products were worked-up and analyzed as described below.

2.3 Analytical methods

The bioconversion mixture was extracted with 3 volume of ethyl acetate, and the

solvent was removed to give a residue, which was re-dissolved in methanol. The resulting solution was used for TLC and HPLC analysis. When the BNC served as a substrate, the products were extracted with 4 volume of CH_2Cl_2. TLC was developed with petroleum ether and acetone (7:3, v/v) and visualized by UV light (254 nm) or by phosphmolybdic acid. HPLC was performed on an Agilent 1200 system equipped with a C18-column (250 mm × 4.6 mm × 5 μm) and a diode array detector (DAD) at the wavelength of 210 nm, 230 nm and 254 nm. A mixture of methanol and water (80:20, v/v) was used as mobile phase at flow rate of 0.8 mL/min. The retention times of AD, 9-OH-AD, BNC, ADD, 4-HBC, 1,4-HBC and TS were 5.6 min, 4.1 min, 4.5 min, 4.7 min, 12.1 min, 8.9 min and 6.2 min, respectively. The products were also analyzed by LC-MS with an Agilent 1200 HPLC and a Bruker micro TOF_QII high resolution mass spectrometer (HRMS) equipped with an ESI ionization source. Masses were recorded over a 50-1000 *m/z* range in positive mode.

In order to examine the potential effect of methanol on the formation of methyl ester, acetonitrile was also used to instead of methanol in the analytical procedure. HPLC was performed with a mixture of acetonitrile and water (80:20, v/v) as mobile phase. Some of the samples were re-dissolved in acetonitrile. The retention time of BNC methyl ester was 16.1 min.

2.4 Isolation of the new product

To isolate the products, the biotransformation mixture was extracted with 3 volumes of ethyl acetate. After being centrifuged at 4000 × *g*, the supernatant was

separated and dried under reduced pressure. Then suitable volumes of methanol were added to extract the products and the extract was dried again under the same conditions. After that, the product was dissolved in CH_2Cl_2 and applied to a silica gel column. The column was eluted by petroleum ether and ethyl acetate (6:1, v/v) to separate the products. Preparative reverse phase recycling high performance liquid chromatography (HPLC, Agilent 1260) with C18-column was used for the final purification, and was performed with the mixture of water and methanol (30:70, v/v) as eluent at flow rate of 11 mL/min. The detecting wavelengths were set as 254 nm and 210 nm. The ^{1}H and ^{13}C NMR spectra were recorded at 290.15 K on a Bruker AVANCE III at 400 MHz. Tetramethylsilane (TMS) served as the internal standard in $CDCl_3$ and chemical shifts (δ) are shown as parts per million (ppm) relative to TMS (Table 1).

3. Results and discussion

3.1 Products of strain B-3805

Because of the poor water-solubility of sterols, the biocompatible organic-aqueous two-liquid phase systems were used in the biotransformation by *Mycobacterium* strains [15, 16]. In this study, soy oil (200 g/L) was used as the organic phase and the incubated medium SCN without any sterols served as the control. As shown in Figure 2A, only a very small amount of AD, which was formed from the phytosterols in the soy oil, was detected in the control experiment. Strain B-3805 metabolized cholesterol to produce AD as main product (peak 3), and 9-OH-AD (peak 1), ADD (peak 2) and 4-HBC (peak 4) as by-products (Figure 2B, C, D, and E, and Table 2). When campesterol or β-sitosterol served as substrate, same products were found (Figure S2, Table S1 and Table S2). These results are in accordance with earlier report [3]. In this study, trace amount of 9-OH-AD was detected (peak 1). It is reasonable because two *kshA* genes and one *kshB* gene is annotated as the 3-ketosteroid-9α-hydroxylase gene in the genome [12]. In addition to these previously identified by-products, a new by-product (named compound A, peak 5) was detected with larger peak area than 9-OH-AD. Xu et al. found a trace amounts of TS and boldenone from the metabolism of cholesterol by derivative *M. neoaurum* strain [17].

The putative sterol degradation pathway was proposed according to the identified intermediates and the catabolic gene cluster in *Mycobacterium*, *Nocardia*, and

Rhodococcus [1, 7-10, 18, 19]. As shown in Figure 1, the metabolic pathway is initiated by the cholesterol oxidase (Cho) and/or 3β-hydroxysteroid dehydrogenase (3β-HSD), which transform 3β-ol-5-en- to 3-keto-4-en- moiety [20, 21]. This is followed by the sequential terminal oxidations catalyzed by cytochromes P450 (CYP125 and/or CYP142), to produce the terminal alcohol and the carboxylic acid (III) [22, 23]. Further degradation of the side-chain is proposed to employ a β-oxidation-like mechanism, which is catalyzed by sterol-CoA ligases, dehydrogenases, hydratases, and acyl-CoA thiolases. As such, cholesterol or phytosterols are shortened to a C24 carboxylic acid compound (IV). Another two β-oxidation-like cycles are proceeded to produce AD (VI) or ADD (VIII) [10]. The transformations of AD to ADD or 9-OH-AD (VII) are catalyzed by 3-ketosteroid-Δ^1-dehydrogenases (KstDs) or 3-ketosteroid-9α-hydroxylase (KshAB), respectively [24, 25]. The 9α-hydroxy-1,4-androstadiene-3,17-dione (9-OH-ADD) can also be produced, which is unstable and the B ring will be destructed spontaneously to afford 3-hydroxy-9,10-seco-androst-1,3,5(10)-triene-9,17-dione (3-HSA, IX) [26]. The steroidal polycyclic ring opening can occur at the earlier steps of the metabolic pathway by following similar mechanisms with both the 3-keto-1,4-dien moiety and 9α-hydroxy group in the molecule [1]. The compound IX is further degraded via oxygenolytic cleavage of ring A to produce 9,17-dioxo-1,2,3,4,10,19-hexanorandrostan-5-oic acid (DOHNAA, X) and 2-hydroxyhexa-2,4-dienoic acid [27, 28].

Some studies have been carried out to improve the yield and purity of the main

product by engineering the production strains. The *kstD*s were inactivated for the production of AD and 9-OH-AD [25, 29]. On the contrast, the enhanced *kstD* improved the yield of ADD [25]. The genes of *kshB* and *kstD* were also knocked out in the strain *M. smegmatis* mc^2155 for the production of ADD (Δ*MSMEG_6039*, Δ*kshB1*) and AD (Δ*MSMEG_6039*-Δ*MSMEG_5941*, Δ*kshB1*-Δ*kstD1*) [30]. The characterizations of KstDs and KshABs were studied in detail [24, 26, 31, 32]. There were some studies to produce other products, such as TS and 4-HBC/1,4-HBC, by engineering strains. Fernández-Cabezón et al. cloned and overexpressed the 17-ketosteroid reductase in the strain *M. smegmatis* mc^2155 to produce TS from phytosterols or AD [33]. Xu et al. knocked out *hsd4A* in the strains of *M. neoaurum* to produce 4-HBC or 1,4-HBC [17].

In this context, genetic manipulation would reduce or eliminate the by-products to improve the yield and purity of the main product. Identification of by-products is essential for understanding their formation mechanisms, and thus providing useful information for the genetic manipulation. As such, we were interested in the isolation and characterization of the new by-product (compound A) in the metabolism of sterols by strain B-3805.

3.2 Isolation and identification of the new by-product

To isolate the compound A, 480 mL culture of strain B-3805 with cholesterol as the substrate were extracted with ethyl acetate. After the solvent was removed, the products were separated on a silica column. The fractions with compound A were

combined and the solvents were removed. The residue was re-dissolved in methanol, and the compound A was purified by a preparative HPLC to give a white powder (11 mg). HR-MS analysis indicated that the compound A with *m/z* 358.2509 possibly had an additional methyl group in its structure compared to BNC. The ^{1}H NMR spectra of the compound A clearly showed that it had the same main carbon skeleton with BNC. There were two main differences of BNC with the compound A. One is the chemical shift of proton on C21 was changed (δ 1.19, 1.18 for compound A, δ 1.25, 1.23 for BNC), but still as a duplet peak; the other was a new single peak appeared (δ 3.67). The ^{13}C NMR spectra of compound A showed the compound bearing 23 carbon, which is one more carbon than BNC, but the C22 (δ 177.2) still the carbonyl group. By HSQC, HMBC and ^{1}H NMR analyses, δ 3.64 was attached to 51.4, and showed no interaction with the main skeleton (Table 1). Therefore, the compound A was identified as the methyl ester of BNC, and named as BNC methyl ester.

Owen et al. have identified some intermediates in the cholesterol degradation pathway of strain *Pseudomonas* sp. NCIB 10590 by infrared spectra, NMR and MS. These intermediates include chol-4-ene-3-one-24-oic acid (IV), chola-1,4-dien-3-one-24-oic acid, pregn-4-ene-3-one-20-carboxylic acid (3-oxo-23,24-bisnorchol-4-ene-22-oate, V), and pregna-1,4-dien-3-one-20-carboxylic acid [11]. The last compound was also found in the sterol degradation by *Rhodococcus rhodochrous* strain RG32, a *kshA* deletion mutant [34]. These results are consistent with the proposed β-oxidation-like mechanism of the side chain

degradation. On the other hand, in the sterol metabolism by the engineering strains of *M. neoaurum*, 4-HBC, 1,4-HBC, and 9,22-dihydroxy-23,24-bisnorchol-4-en-3-one (9-OH-HBC) were observed. These compounds may be produced via reduction of 3-oxo-23,24-bisnorchol-4-en-20-carbaldehyde which formed by an retro-aldol reaction [17]. The same reaction also ultimately produced 17-keto-steroids including AD and ADD [7-9].

In theory, the C22 terminal aldehyde compound can be oxidized to BNC *in vivo*. However, the authors did not detect the carboxylic acid (V) [17]. Thomas et al. found the accumulation of methyl 1*β*-(2'-propanoate)-3a*α*-H-4*α*-(3'-propanoic acid)-7a*β*-methylhexahydro-5-indanone (methyl ester of DOHNAA), which was identified by HRMS and NMR. However, no more suggestion was given for the esterification reaction [35]. In this study, the possibility for the formation of methyl ester during the reaction work-up and analysis was excluded, because similar amounts of BNC methyl ester was formed when acetonitrile was used instead of methanol for the reaction work-up and analysis (Figure S3). Therefore, BNC methyl ester should be produced during the degradation of sterols by strain B-3805.

3.3 Biotransformation of BNC

BNC, which could be oxidatively converted from 4-HBC, was tested as the substrate for the biotransformation by strain B-3805 with 100 g/L soy oil, and the products were analyzed. Only trace amount of AD was detected from the control experiment without BNC (Figure 3A). As shown in Figure 3B, 2 g/L BNC was

converted to 0.46 g/L AD and 0.36 g/L BNC methyl ester after 168 h, and 1.21 g/L BNC was recovered. Similar results were obtained when the concentration of BNC was increased to 10 g/L, after 168 h, the concentrations of AD, BNC methyl ester and remaining BNC were 0.75 g/L, 0.80 g/L and 8.17 g/L, respectively (Figure 3C). These results suggested that BNC was converted to AD and BNC methyl ester by strain B-3805, and this may be the reason that the carboxylic acid compounds (BNC and pregna-1,4-dien-3-one-20-carboxylic acid) did not detected for strain B-3805.

It has been reported that *O*-methyltransferases can act on carboxyl moiety of molecules using *S*-adenosyl-L-methionine (SAM) as the methyl group donor [36]. It is possible that the esterification reaction to yield BNC methyl ester might be a methyl transfer reaction catalyzed by an *O*-methyltransferase in the strain B-3805, although the gene encoding the methyltransferase is still unknown.

4. Conclusion

In summary, in the sterols metabolism by *M. neoaurum* NRRL B-3805, AD was produced as the main product. In addition to the by-products 9-OH-AD, ADD, and 4-HBC, BNC methyl ester was isolated and identified. This new by-product might be produced by the esterification of BNC catalyzed by an *O*-methyltransferase using SAM as the methyl group donor. These results would provide new opportunity for the genetic manipulation of strain B-3805 to improve the efficiency of AD production. In this context, further studies on the gene encoding *O*-methyltransferase and characterization of the enzyme are warranted.

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2

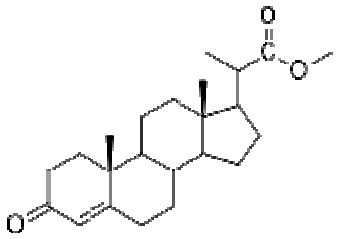
Figure legends

Figure 1 Overview of the cholesterol degradation pathway [7-11]. Cholesterol degradation is initiated by the oxidation of 3β-hydroxyl group, and the side-chain was degraded by sequential terminal oxidations and then by 3 rounds of β-oxidation like reactions to yield AD or ADD. Retro-aldol reaction maybe participate in the degradation pathway. The ring B is destructed spontaneously via 9-OH-ADD to form 3-HSA (IX), and then was metabolized further to produce DOHNAA (X) and 2-hydroxyhexa-2,4-dienoic acid.

Figure 2 Phenotypic analyses of cholesterol metabolism of *M. neoaurum* NRRL B-3805. A, HPLC chromatogram of the products from the degradation of cholesterol by strain B-3805. Black line, cholesterol served as substrate; red line, control experiment. B, MS of metabolite 9-OH-AD (peak 1). C, MS of metabolite ADD (peak 2). D, MS of metabolite AD (peak 3). E, MS of metabolite 4-HBC (peak 4). Strain B-3805 can use cholesterol and phytosterol as substrate to accumulate AD as main product, and 9-OH-AD, ADD, and 4-HBC, et al. as by-products.

Figure 3 The biotransformation of BNC by strain B-3805. A, only minimum of AD was detected from the culture of control. B, the transformation of 2 g/L BNC. C, the transformation of 10 g/L BNC. Both AD and BNC methyl ester were detected from the culture of strain B-3805 when BNC served as substrate.

Table 1. ^{1}H and ^{13}C Data of compound A.



Compound A

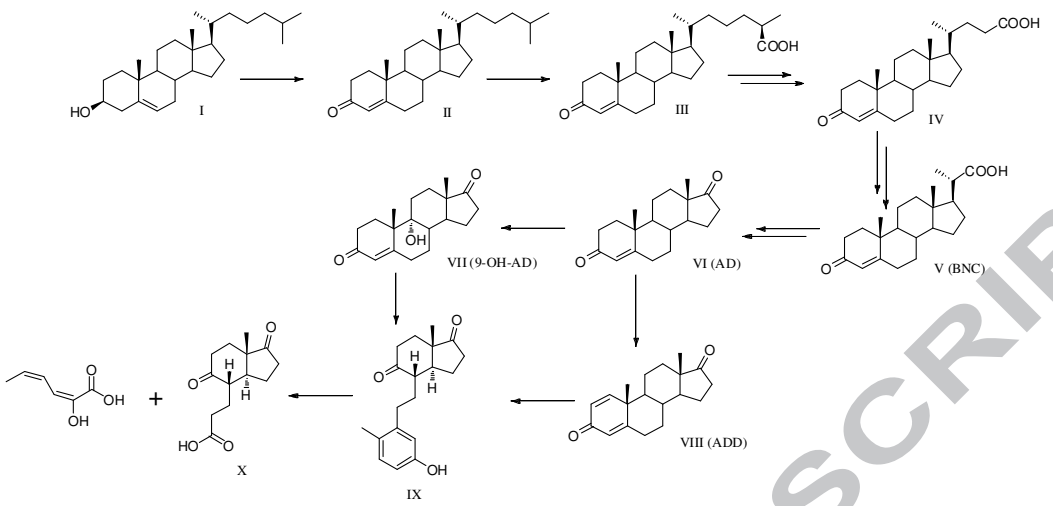
Carbon No.	^{13}C (δ, ppm)	^{1}H (δ, ppm)
1	35.7	2.02, 1.72
2	34.0	2.42, 2.35
3	199.6	-
4	123.8	5.78
5	171.3	-
6	32.9	2.39, 2.29
7	32.0	1.85, 1.04
8	35.6	1.54
9	53.7	0.98
10	38.6	
11	20.9	1.55, 1.45
12	39.4	1.98, 1.27
13	42.5	-
14	52.8	1.64
15	24.2	1.65
16	27.0	1.73, 1.36
17	55.4	1.08
18	12.1	0.77
19	17.3	1.18
20	42.4	2.45
21	17.0	1.18, 1.19
22	177.2	-
23	51.3	3.67

1 **Table 2.** HPLC-HRMS Data of the products in the cholesterol degradation by *M. neoaurum* NRRL B-3805.

Compound ID	HPLC (RT[a], min)	Molecular formula	Predicted molecular mass[b]	Experimental ionic mass	Identification of product ions
9α-hydroxy androst-4-en-3,17-dione (9-OH-AD)	4.6	$C_{19}H_{26}O_3$	302.1882	303.1921 325.1756	$[M+H]^+$ $[M+Na]^+$
androsta-1,4-dien-3,17-dione (ADD)	5.2	$C_{19}H_{24}O_2$	284.1776	285.1833 307.1720	$[M+H]^+$ $[M+Na]^+$
androst-4-en-3,17-dione (AD)	6.2	$C_{19}H_{26}O_2$	286.1933	287.2004 309.1822	$[M+H]^+$ $[M+Na]^+$
22-hydroxy-23,24-bisnorchol-4-en-3-one (4-HBC)	12.7	$C_{22}H_{34}O_2$	330.2559	331.2610 353.2431	$[M+H]^+$ $[M+Na]^+$

2 [a] RT, retention time.

3 [b] The predicated molecular mass was calculated using the atom mass of ^{12}C (12.0000), ^{16}O (15.9949), and ^{1}H (1.0078).



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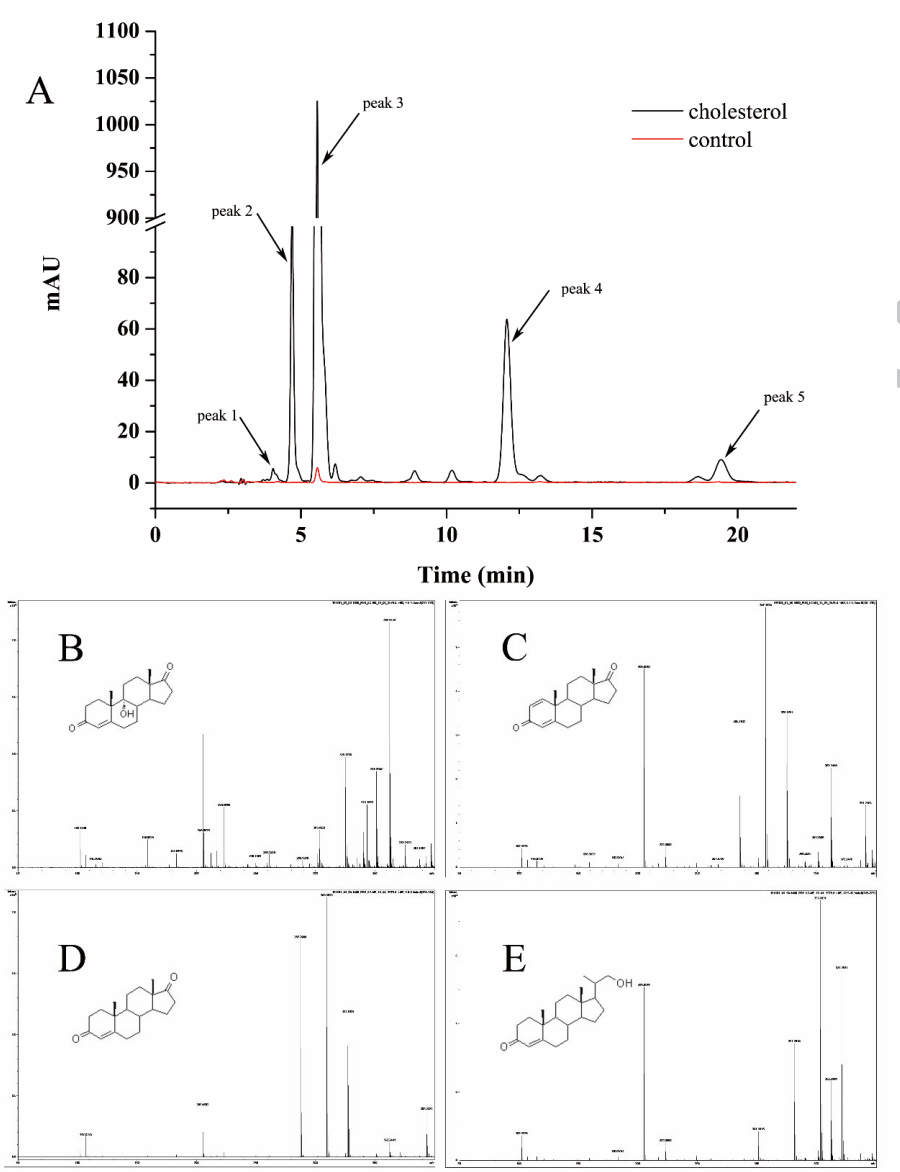

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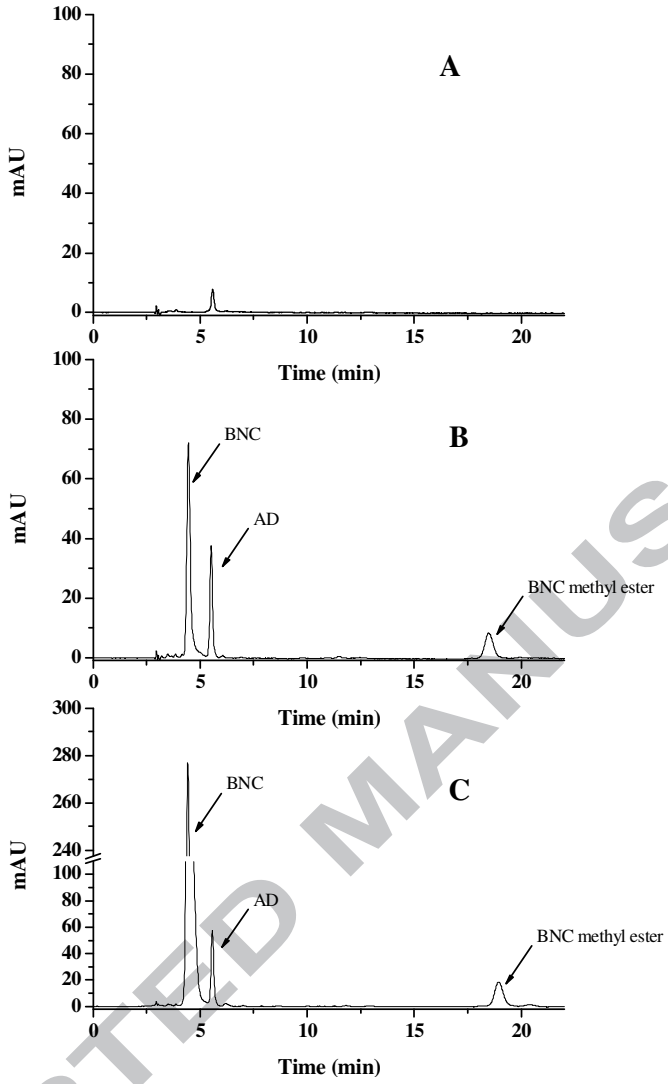

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Highlights:

1. A new by-product was isolated from the degradation of sterol by the strain *Mycobacterium neoaurum* NRRL B-3805.
2. It was identified as methyl 3-oxo-23,24-bisnorchol-4-en-22-oate by NMR and HRMS.
3. The methyl ester was formed from the corresponding acid catalyzed by an *O*-methyltransferase.
4. This provides new information on the microbial metabolism pathway of sterols.