

· 工业生物技术 ·

分枝杆菌 HGMS2 甾醇降解中新 C-23 代谢产物的鉴定及其代谢途径分析

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摘要: 新金分枝杆菌(*Mycobacterium neoaurum*)通过敲除羟酰基辅酶 A 脱氢酶(hydroxyacyl-CoA dehydrogenase, Hsd4A)或酰基辅酶 A 硫解酶(acyl-CoA thiolase, FadA5)基因来生产 22-羟基-23,24-双降甾-4-烯-3-酮(22-hydroxy-23,24-bisnorchol-4-en-3-one, BA)甾体类药物中间体来受到广泛关注。本实验室前期发现, 敲除 *fadA5* 基因后, 发酵产物中出现了一种新的代谢产物。本研究通过对该物质的结构鉴定、*fadA5* 基因的蛋白同源序列比对、*M. neoaurum* HGMS2 的系统发育树分析和基因敲除等手段探究该物质的代谢途径。结果表明该物质为 C23 类代谢中间体, 即 24-norchol-4-ene-3,22-dione (将其命名为 3-OPD)。该物质是通过硫酯酶(thioesterase, TE)催化 3,22-dioxo-25,26-bisnorchol-4-ene-24-oyl CoA (22-O-BNC-CoA)生成侧链为 β -酮酸结构的物质后通过生物体的自动脱羧反应生成的。这些结果对开发新的甾体类中间体有重要价值。

关键词: 甾体药物; *fadA5*; 基因敲除; 新金分枝杆菌; C-23 代谢中间体

Identification of a new C-23 metabolite in sterol degradation of *Mycobacterium neoaurum* HGMS2 and analysis of its metabolic pathways

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Abstract: *Mycobacterium neoaurum* has the ability to produce steroidal intermediates known as 22-hydroxy-23,24-bisnorchol-4-en-3-one (BA) upon the knockout of the genes for either the hydroxyacyl-CoA dehydrogenase (Hsd4A) or acyl-CoA thiolase (FadA5). In a previous study, we discovered a novel metabolite in the fermentation products when the *fadA5* gene was deleted. This research aims to elucidate the metabolic pathway of this metabolite through structural identification, homologous sequence analysis of the *fadA5* gene, phylogenetic tree analysis of *M. neoaurum* HGMS2, and gene knockout. Our findings revealed that the metabolite is a C23 metabolic intermediate, named 24-norchol-4-ene-3,22-dione (designated as 3-OPD). It is formed when a thioesterase (TE) catalyzes the formation of a β -ketonic acid by removing CoA from the side chain of 3,22-dioxo-25,26-bisnorchol-4-ene-24-oyl CoA (22-O-BNC-CoA), followed by spontaneously undergoing decarboxylation. These results have the potential to contribute to the development of novel steroid intermediates.

Keywords: steroid drugs; *fadA5*; gene knockout; *Mycobacterium neoaurum*; C-23 steroid intermediate

甾体药物在制药行业占据着重要的地位, 每年全球市场销售额超过 100 亿美元, 其中雄烯二酮(4-androstenedione, 4-AD)作为甾体药物核心原料可合成绝大部分甾体激素类药物^[1]。目前, 工业生产中常利用微生物转化法生产甾体药物中间体, 在经济、生态环保等方面展现出巨大的优势^[2]。分枝杆菌(*Mycobacterium*)作为主要的工业微生物菌株, 能够对植物甾醇进行选择性功能修饰, 已成为甾体药物中间体生物制造的主流生成方式^[3]。目前, 只有少数从植物甾醇合成的甾体药物中间体可以规模化生产^[4], 例如 4-雄甾-3,17-二酮(4-androstene-3,17-dione, 4-AD)^[2]、1,4-雄甾二烯-3,17-二酮

(1,4-androstadiene-3,17-dione, ADD)^[5]、9 α -羟基-4-雄烯二酮(9 α -hydroxy-4-androstenediol, 9-OH-AD)等^[6-7], 因此仍然需要高效的分枝杆菌细胞工厂以工业化规模生产新的甾药中间体。相比于传统的菌种诱变, 基因和代谢工程改造可以更高效地实现对分枝杆菌甾醇降解途径的人工改造, 以改善植物甾醇的生物转化和构建高产目标甾药中间体的新菌株^[8-9]。

前期通过对包皮分枝杆菌(*Mycobacterium smegmatis*) str. mc² 155、*Rhodococcus jostii* RHA1、结核分枝杆菌(*Mycobacterium tuberculosis*) H37Rv 等放线菌甾醇降解途径的大量研究^[10-15], 植物甾醇的代谢途径中的关键酶已逐渐被揭示

出来, 其中甾醇侧链降解途径(图 1A)主要是植物甾醇首先在胆固醇氧化酶(cholesterol oxidase, ChO)^[16]和 3 β -羟基类固醇脱氢酶/异构酶(3 β -hydroxysteroid dehydrogenase/isomerase, HSD)^[17]催化作用下, 被降解为 4-cholesten-3-one, 其次细胞色素 P450 蛋白(cytochrome P450 protein, CYP125)^[18]在 C27 位进行羟基化反应生成 27-hydroxy-4-en-3-one, 并进一步被氧化生成甾醇 C-27 羧酸。然后, 甾醇 C27 羧酸在酰基辅酶 A 合成酶(acyl-CoA synthetase, FaD19)^[19]作用下进行酯化反应得到甾醇 C-27 羧酰 CoA, 接着在长链酰基辅酶 A 脱氢酶(long-chain acyl-CoA dehydrogenase, ChsEs)^[20-22]、羟酰基辅酶 A 脱氢酶(hydroxyl-CoA dehydrogenase, Hsd4A)^[23]、酰基辅酶 A 硫解酶(acyl-CoA thiolase, FadA5)^[24]、酰基辅酶 A 水合酶(acyl-CoA hydratase, ChsHs)^[25-27]和酰基辅酶 A 水合酶-醛缩酶复合体(acyl-CoA hydratase-aldolase complex, ChsHs-Ltp2)^[28]作用下进入 3 轮类 β -氧化过程, 最终转化成为 4-AD。而在部分积累 22-羟基-23,24-双降甾-4-烯-3-酮(22-hydroxy-23,24-bisnorchol-4-ene-3-one, BA)中间体的菌株中, 侧链降解过程表现出不完全氧化状态, 类 β -氧化过程仅完成前 2 轮, 然后在醛缩酶(aldolase, Sal)^[29]、醛还原酶(aldehyde reductase, OpccR)^[30]作用下生成了 BA。母核降解途径(图 1B)主要是在 3-甾酮- Δ 1,2-脱氢酶(3-ketosteroid- Δ 1,2- dehydrogenase, KstD)^[31]和类固醇-9 α -羟化酶(3-ketosteroid-9 α -hydroxylase, KshA/B)^[6,32]共同作用下致使甾体母核中 B 环裂解, 然后进一步降解为 CO₂ 和 H₂O。随着基因编辑技术的不断成熟, 越来越多的研究人员通过基因敲除和异源基因过表达等手段, 以分枝杆菌为底盘设计改造获得多种类目标甾药中间体, 例如 BA、22-羟基-23,24-双降

甾-1,4-二烯-3-酮(22-hydroxy-23,24-bisnorchol-1,4-diene-3-one, DBA)、9 α ,21-二羟基-20 α -甲基-孕甾-4-烯-3-酮(9,21-dihydroxy-20-methylpregna-4-en-3-one, 9-OH-BA)、9 α -羟基-3-酮-4,17(20)-二烯-20-羧基孕甾(9 α -hydroxy-3-oxo-4,17(20)-pregnadiene-20-carboxylic acid, 9-OH-PDC)和 9 α -羟基-3-酮-4,17(20)-二烯孕甾-20-羧酸甲酯(9 α -hydroxy-3-oxo-4,17(20)-pregnadiene-20-carboxylic acid methyl ester, 9-OH-PDCM)等甾体类药物中间体^[23,33-36](表 1)。然而, 通过基因工程改造分枝杆菌生产甾体药物前体的类型非常有限, 需要更进一步探究分枝杆菌甾醇降解分子机制, 挖掘新的代谢途径或新的代谢机制^[37], 故开发新型甾药中间体显得十分有必要。

本研究团队, 对 *hsd4A* 和 *fadA5* 等基因在甾醇降解中的功能作用进行了鉴定, 通过人工改造新金分枝杆菌(*Mycobacterium neoaurum*) HGMS2 的代谢途径, 以获得可高产 BA 的基因工程菌株, 该产物可用于进一步合成孕酮^[33]。然而, 当 *fadA5* 基因缺失后, 发酵产物中检测到除 4AD、BA 类固醇中间体以外的一种新代谢产物, 该发现和其他文献报道的结果不同^[23,35]。该结果说明分枝杆菌中可能存在一条新的代谢途径, 以此为出发点, 本文进一步研究 *M. neoaurum* HGMS2 的甾醇代谢途径。首先对 *fadA5* 基因敲除型菌株的发酵产物中的新物质进行了分离纯化, 然后通过质谱和核磁共振等手段对其分子结构进行了鉴定, 进一步, 通过产物分析、基因敲除、序列分析及系统发育树分析等手段阐明了新物质产生的代谢途径, 对改造 BA 生产菌株, 研究分枝杆菌甾醇的降解机制, 挖掘新的代谢途径或新的代谢机制, 开发新的甾类中间体生产菌株具有重要价值。

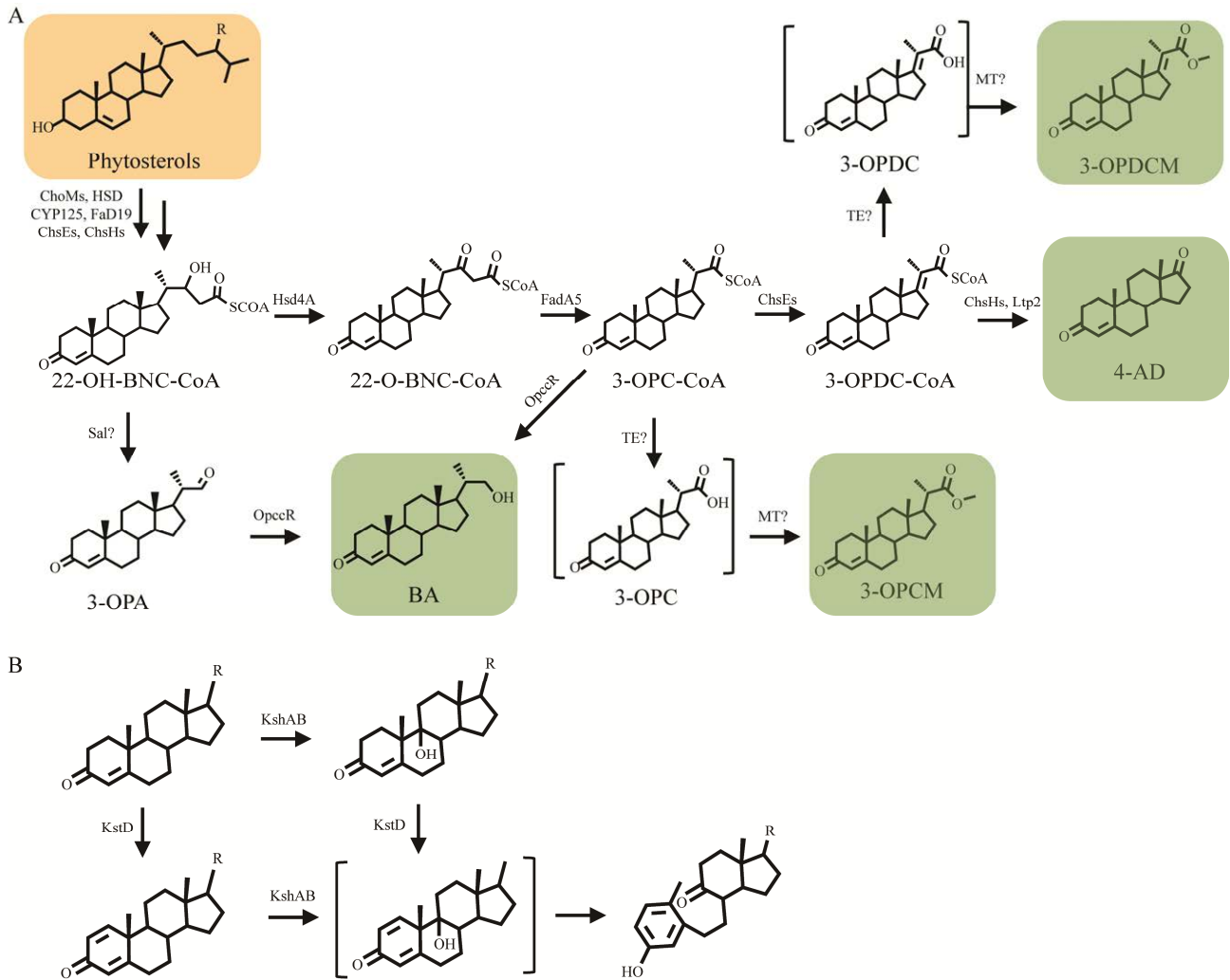


图 1 植物甾醇在新金分枝杆菌中的生物降解途径 A: 侧链降解途径. 植物甾醇(淡黄色)、植物甾醇降解产生的类固醇化合物(淡绿色). 带问号的酶和带括号的中间体是假定的, 还没有在分枝杆菌中得到验证. CHOMS: 胆固醇氧化酶; HSD: 3 β -羟基类固醇脱氢酶/异构酶; CYP125: 细胞色素 P450 蛋白; FAD19: 酰基辅酶 A 合成酶; CHSES: 长链酰基辅酶 A 脱氢酶; HSD4A: 羟酰基辅酶 A 脱氢酶; FADA5: 酰基辅酶 A 硫解酶; CHSHS: 酰基辅酶 A 水合酶; SAL, LTP2: 醛缩酶; TE: 硫酯酶; OPCCR: 醛还原酶; MT: 甲基转移酶. B: 母核降解途径. KSTD: 3-酮类固醇- $\Delta^{1,2}$ -脱氢酶; KSHAB: 3-酮类固醇-9 α -羟化酶

Figure 1 Biodegradation pathway of phytosterols in *Mycobacterium neoaurum*. A: Side chain degradation pathway. Phytosterols (light yellow), steroid compounds produced by phytosterol degradation (light green). The enzyme with question marks and the intermediate with brackets are hypothesized and have not been tested in *Mycobacterium*. ChoMs: Cholesterol oxidase; HSD: 3 β -hydroxysteroid dehydrogenase/isomerase; CYP125: Cytochrome P450 protein; FaD19: Acyl-CoA synthetase; ChsEs: Long-chain acyl CoA dehydrogenase; Hsd4A: Hydroxyl-CoA dehydrogenase; FadA5: Acyl-CoA thiolase; ChsHs: Acyl CoA hydratase; Sal, Ltp2: Aldolase; TE: Thioesterase; OpccR: Aldehyde reductase; MT: Methyltransferase. B: Parent nucleus degradation pathway. KstD: 3-ketosteroid- $\Delta^{1,2}$ -dehydrogenase; KshAB: 3-ketosteroid-9 α -hydroxylase.

1 材料与amp;方法

1.1 材料

1.1.1 质粒、菌株和引物

M. neoaurum HGMS2 由本实验室保存，其基因组序列可在 GenBank (登录号：CP031414.1) 中获得。HG-I、HG-IΔ*hsd4A1*、HG-IΔ*fadA5* 菌株以及基因敲除所需的工具质粒 p2NIL-*sacB* 和 p2NIL-*sacB*-*hsd4A1* 由实验室前期通过基因工程手段构建^[33]。本研究以 HG-IΔ*fadA5* 为出发菌

株，构建了 *hsd4A* 和 *fadA5* 双基因敲除菌。本研究中所使用的菌株和质粒见表 2。

1.1.2 主要试剂

Taq 和 *Pfu* DNA 聚合酶、限制性内切酶、dNTPs 购自 TaKaRa (大连)。质粒纯化试剂盒、PCR 产物纯化试剂盒、DNA 提取试剂盒购自天根生化科技(北京)有限公司。4-androstene-3,17-dione (4-AD)、22-hydroxy-23,24-bisnorcholesterol-4-ene-3-one (BA) 标准样品由 Amersino (武汉)制备。植物甾醇(98%, 410.40 Da)购自湖北共同药业股份有限公司(襄阳)。

表 1 通过基因编辑菌株产生的甾药中间体

Table 1 Steroid intermediates produced by gene edited *Mycobacterium neoaurum* strains

Parental strain	Genetic manipulations	Main product
<i>M. neoaurum</i> HGMS2	(1) Deletion of multiple <i>kshAB</i> ($\Delta kshAB12$), <i>kstD</i> and <i>hsd4A</i> genes (2) Deletion of multiple <i>kshAB</i> ($\Delta kshAB12$) and <i>hsd4A</i> genes, overexpressing <i>kstD</i> gene (3) Deletion of <i>kstD</i> and <i>hsd4A</i> genes, overexpressing <i>kshA1/B1</i> gene ^[33]	(1) BA (2) DBA (3) 9-OH-BA
<i>M. neoaurum</i> DSM 44074	Deletion of multiple <i>kstD</i> ($\Delta kstD123$), <i>hsd4A</i> and <i>fadA5</i> genes, overexpressing <i>katE</i> and <i>NADH</i> genes ^[35]	9-OH-BA
<i>M. neoaurum</i> ATCC 25795	(1) Deletion of multiple <i>kshAB</i> ($\Delta kshB12$), multiple <i>kstD</i> ($\Delta kstD123$) and <i>hsd4A/fadA5</i> genes (2) Deletion of multiple <i>kshAB</i> ($\Delta kshAB12$) and <i>hsd4A</i> genes, overexpressing <i>kstD1</i> gene (3) Deletion of multiple <i>kstD</i> ($\Delta kstD123$) and <i>hsd4A</i> genes ^[23]	(1) BA (2) DBA (3) 9-OH-BA
<i>M. neoaurum</i> ATCC 25795	Deletion of multiple <i>kstD</i> ($\Delta kstD123$) and <i>ltp2</i> genes, overexpressing <i>chsE1-E2</i> and <i>hsd4A</i> genes ^[36]	9-OH-PDC, 9-OH-PDCM
<i>M. neoaurum</i> DSM 44074	Deletion of multiple <i>kstD</i> ($\Delta kstD123$) and <i>chsH2</i> genes, overexpressing <i>hsd4A</i> and <i>chsE1-E2</i> genes ^[34]	9-OH-PDCM

表 2 本研究使用的菌株、质粒和引物

Table 2 Strains, plasmids and primers used in this study

Name	Description	Source
Strains		
<i>M. neoaurum</i> HGMS2	<i>M. neoaurum</i> HGMS2 was maintained in our laboratory, and its genome sequence is available in GenBank (CP031414.1)	[38]
HG-I	<i>kstD211</i> deleted in <i>M. neoaurum</i> HGMS2	[39]
HG-IΔ <i>fadA5</i>	<i>fadA5</i> deleted in HG-I	[33]
HG-IΔ <i>hsd4A1</i>	<i>hsd4A1</i> deleted in HG-I	[33]
HG-IΔ <i>fadA5</i> Δ <i>hsd4A1</i>	Combined deletion of <i>fadA5</i> and <i>hsd4A1</i> in HG-I	This study
Plasmids		
P2NIL	Nonreplicating plasmid for allelic recombination in <i>Mycobacterium</i> , KanR	[39]
p2NIL- <i>sacB</i>	Derived from P2NIL and contains <i>sacB</i> <i>Bacillus subtilis</i> 168, KanR	[39]
p2NIL- <i>sacB</i> - <i>hsd4A1</i>	Suicide plasmid derived from p2NIL carrying two homologous arms of <i>hsd4A1</i> and selective marker gene cassette from p2NIL- <i>sacB</i>	[33]
Primer		
<i>hsd4A1</i> -D-R	CCCAAGCTTGATGCCAGCACACGCGGC	[33]
<i>hsd4A1</i> -U-F	GAAGATCTTGCCGAAATGCATGATCG	[33]

1.2 方法

1.2.1 生物信息学分析

在 GenBank (<http://www.ncbi.nlm.nih.gov/>) 数据库中下载其他物种 *fadA5* 的基因。在网站 <https://www.genome.jp/tools-bin/clustalw> 及 <https://esprict.ibcp.fr/ESPript/cgi-bin/ESPript.cgi> 进行序列比对。在网站 <https://www.ncbi.nlm.nih.gov/genome/?term=HGMS2> 进行系统发育树构建。

1.2.2 目的菌株构建

根据 Li 等^[39]先前报道的程序,运用同源重组的方法进行基因敲除。使用来自 ScienTZ (宁波)的 ScienTZ-2C 电穿孔系统将重组质粒电穿孔到感受态分枝杆菌细胞中(电击参数:电压 2 500 V,电阻 1 000 Ω ,电容 25 μ F)。通过菌落 PCR 选出敲除型菌株,将阳性重组菌落转入含有 0.05% Tween-80 的 5 mL LB 培养基,30 $^{\circ}$ C、200 r/min 培养 2 d,抽提基因组进行 PCR 鉴定,经验证的突变菌株储存在-80 $^{\circ}$ C。

1.2.3 微生物培养和生物转化

首先将分枝杆菌菌株放在含有 0.05% Tween-80 的 LB 培养基中于 30 $^{\circ}$ C 培养 48 h,待 OD_{600} 值达到 13-15 时,将培养物接种到 50 mL 由酵母粉(15 g/L)、葡萄糖(1 g/L)、 NaNO_3 (5.4 g/L)、 $(\text{NH}_4)_2\text{HPO}_4$ (0.6 g/L)、 β -环糊精(3 g/L)、TW-80 (0.05%,质量体积分数)和植物甾醇(10 g/L)组成的发酵培养基中,并在 30 $^{\circ}$ C 和 200 r/min 下摇动 7 d。然后,每天收集 1 mL 培养液提取代谢物,用于监测植物甾醇的降解过程。为了进行比较,仅显示第 7 天发酵液样品的高效液相(high-performance liquid chromatography, HPLC)图谱。

1.2.4 新产物的分离纯化

将发酵液与乙酸乙酯以 1:3 的比例充分混合。混合物以 12 000 r/min 离心 5 min,收集上清液,然后将上清液进行减压干燥得到固体粉末,将固体产物倒入硅胶柱中,用石油醚和乙酸

乙酯(5:1,体积比)进行洗脱来分离产物。

1.2.5 高效液相色谱分析(HPLC)

将发酵液与乙酸乙酯以 1:1 的比例充分混合。将混合物以 12 000 r/min 离心 5 min,收集上清液,使用金属浴加热干燥。将干燥的样品溶解在 40%的甲醇溶液中然后通过 0.22 μ m 微孔膜过滤用于 HPLC 测定。通过与 4-AD、BA 的标准品比较,确认了 HPLC 峰的身份。每个峰面积用 Waters 提供的软件集成在一起,用于评估各个化合物浓度。

1.2.6 质谱分析

在选择最佳条件后,即乙腈:水=60:40,0.2%甲酸,根据 Li 等^[39]的方法进行液相色谱-质谱联用(liquid chromatography/mass spectrometry, LC/MS)检测。

1.2.7 核磁分析

小分子核磁(nuclear magnetic resonance, NMR)鉴定中,将小分子溶于 CDCl_3 后,用 Bruker 核磁共振仪扫描 ^1H 和 ^{13}C NMR 谱图,采用 Mestrenova 14.0 对核磁数据进行处理。

2 结果与分析

2.1 HG-I Δ fadA5 发酵产物分析

如图 2 所示,菌株 HG-I 中主要代谢产物是 4AD,含有少量的 BA,敲除 *fadA5* 基因以后,

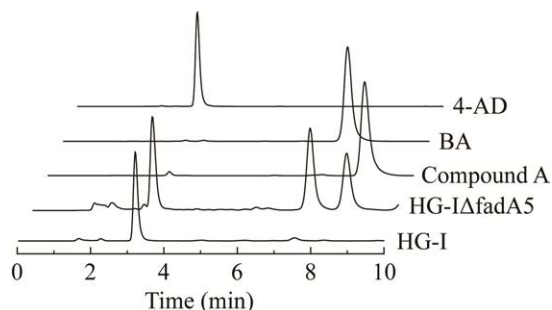


图 2 HG-I Δ fadA5 发酵产物的 HPLC 分析
Figure 2 HPLC analysis of HG-I Δ fadA5 fermentation products.

在液相图上,除了 4AD、BA 以外,出现了新的代谢产物,将其命名为化合物 A。

2.2 酰基辅酶 A 硫解酶(FadA5)蛋白序列分析

在 *M. neoaurum* ATCC 25795 和 *M. neoaurum* DSM 44074 中敲除 *fadA5* 并未发现该化合物 A^[23,35]。本研究首先猜测是 FadA5 的功能不同影响了代谢途径。因此比较模式生物 *M. neoaurum* ATCC 25795 和 *M. neoaurum* DSM 44074 与本菌株的 FadA5 蛋白的序列差异关系。从 NCBI 数据库下载 *M. tuberculosis* H37Rv (登录号:AXK73888.1)、*M. smegmatis* mc² 155 (登录号:ABK75580.1)、*R. jostii* RHA1 (登录号:ABG96464.1)、*M. neoaurum* ATCC 25795 (登录号:AKK31743.1)、*M. neoaurum* DSM 44074 (登录号:CDQ47076.1)、*M. neoaurum* HGMS2 (登录号:AXK73888.1)的 FadA5 蛋白序

列。如图 3 中本菌株与 *M. tuberculosis* H37Rv、*M. smegmatis* mc² 155、*R. jostii* RHA1 差异较大,序列相似性分别为 85%、92%、74%,与 *M. neoaurum* ATCC 25795、*M. neoaurum* DSM 44074 较为接近,序列相似性都达到了 99%,说明本菌株的 FadA5 与它们的功能相似,可能不是 FadA5 的差异引起代谢途径的变化。

2.3 化合物 A 的结构鉴定

对该化合物的 HR-MS 分析表明(图 4A),与 BA (*m/z* 330.256)相比,含有 *m/z* 343.261 的未知产物的结构中可能有一个额外的甲基。通过和 BA 的氢谱图对比表明(图 4B),它与 BA 具有相同的主碳骨架,该化合物 A 与化合物 BA 有两个主要区别,其一是 C22 上质子的化学位移发生了变化,未知产物的 C22 位没有氢,BA



图 3 来自不同菌株 FadA5 的序列比对

Figure 3 Sequences alignment of FadA5 from different strains.

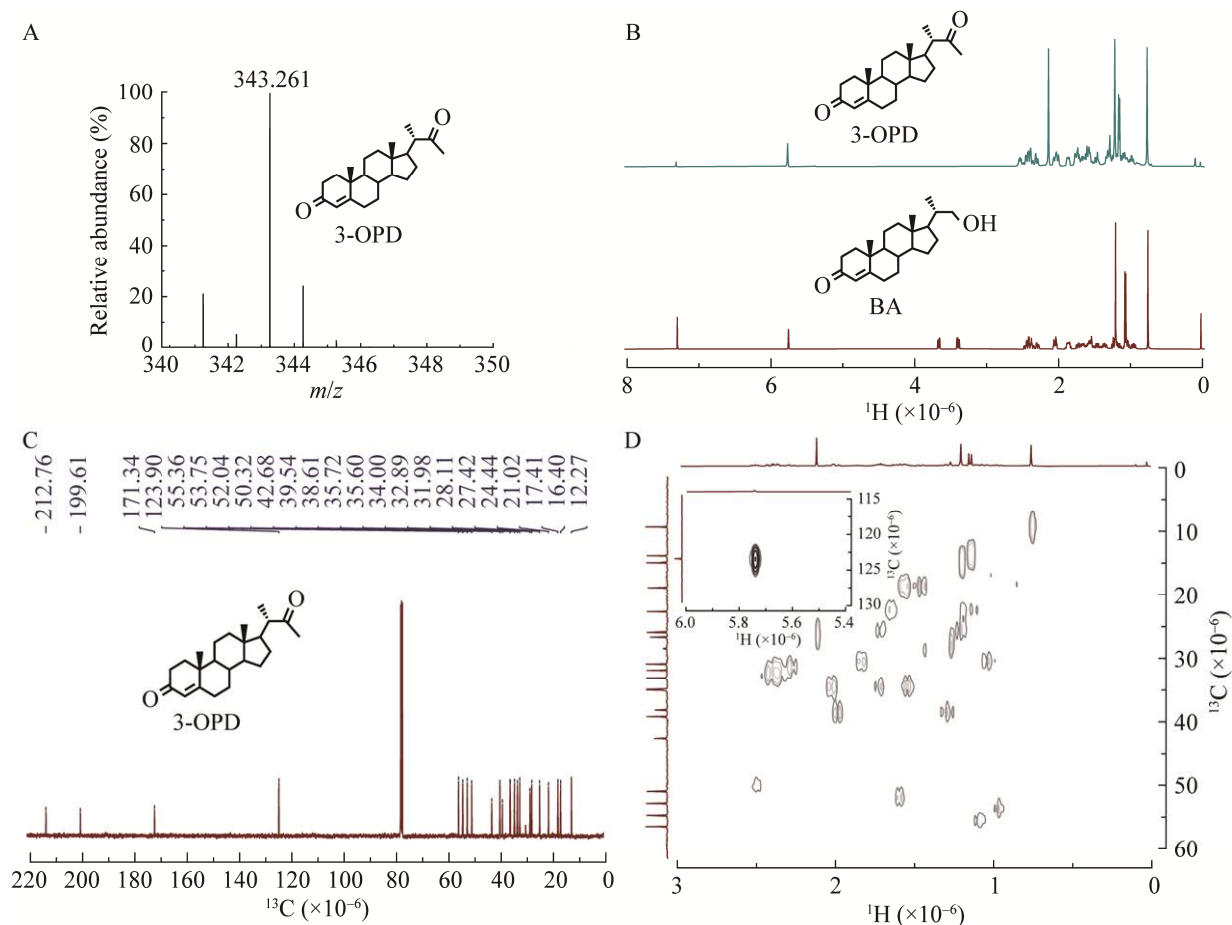


图4 化合物A的结构鉴定 A: LC/MS分析. B: ¹H分析. C: ¹³C分析. D: HSQC分析
Figure 4 Structure identification of compound A. A: LC/MS analysis. B: ¹H analysis. C: ¹³C analysis. D: HSQC analysis.

为(δ3.64, 3.37), 其二是多了个新的单峰(δ2.13)。该化合物A的碳谱(图4C)显示该物质有23个碳, 比BA多了一个碳, C22的 $\delta > 200 \times 10^{-6}$, 所以判断该化合物C22有一个酮基。通过异核单量子关系(heteronuclear singular quantum correlation, HSQC)分析(图4D), $\delta 2.13$ 附着在27.42上(图5, 表3)。因此, 该化合物A被鉴定为24-norchol-4-ene-3,22-dione, 命名为3-OPD。

2.4 化合物A的代谢途径分析

2.4.1 HG- Δ fadA5/ Δ hsd4A1 敲除型菌株构建

为进一步明确24-norchol-4-ene-3,22-dione(3-OPD)的代谢产生途径, 随即构建了基因缺陷

株HG- Δ fadA5/ Δ hsd4A1。根据Li等^[39]先前报道的程序, 运用同源重组的方法对该基因进行敲除, 采用Song等^[33] hsd4A1-U-F、hsd4A1-D-R的同源臂两端的引物做菌落PCR鉴定阳性重组菌落。如果成功从新金分枝杆菌HG- Δ fadA5基因组中敲除 hsd4A1 基因, 那么与野生型菌株相比, 野生型菌株约2 100 bp, 敲除型PCR产物大小约1 400 bp, 相比之下更短。如图6所示, 泳道6、7号为未敲除型菌株, 2-5号和8、9号为敲除型菌株。挑选3-4个敲除型菌株, 抽提基因组, 进行DNA测序, 结果证实, 从HG- Δ fadA5突变体中敲除了 hsd4A1 基因。

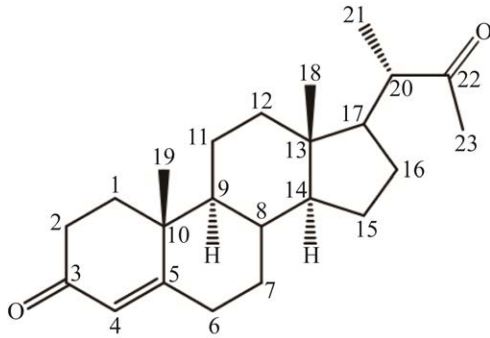


图 5 化合物 A 结构

Figure 5 Structure of compound A.

表 3 3-OPD 的核磁数据

Table 3 ^1H and ^{13}C data of 3-OPD

Carbon No.	^{13}C (δ , $\times 10^{-6}$)	^1H (δ , $\times 10^{-6}$)
1	34.00	2.30, 2.26
2	32.89	2.47, 2.38
3	199.61	—
4	123.90	5.74
5	171.34	—
6	31.98	1.83, 1.03
7	28.11	2.11, 1.73
8	35.60	1.54
9	52.04	1.60
10	35.72	—
11	21.02	1.57, 1.41
12	38.61	1.32, 1.28
13	42.68	—
14	55.36	1.08
15	24.44	1.66, 1.19
16	39.54	2.01, 1.97
17	53.75	0.96
18	12.27	0.76
19	17.41	1.20
20	50.32	2.52
21	16.40	1.14
22	212.76	—
23	27.42	2.13

—: That means there are no hydrogens on this carbon.

2.4.2 植物甾醇发酵小试产物分析

对菌株 HG-I Δ fadA5/ Δ hsd4A1、HG-I Δ hsd4A1 与 HG-I Δ fadA5 进行发酵小试。如图 7 所示，在 HG-I Δ fadA5 菌株的基础上敲除 *hsd4A1*，发

现 3-OPD 消失了，该菌株与菌株 HG-I Δ hsd4A1 产物相同，都是以 BA 为主要产物。因此推测 3-OPD 的产生可能在 *hsd4A* 和 *fadA5* 代谢途径之间。据报道，分枝杆菌代谢过程中的一些类固醇中间体会被硫酯酶(thioesterase, TE)水解变成侧链末端为羧酸结构的物质^[30,36-37]。因此推测(图 8)产生 3-OPD 的原因可能是 *fadA5* 的敲除导致 3,22-dioxo-25,26-bisnorchol-4-ene-24-oyl CoA (22-O-BNC-CoA)的积累，被 TE 水解成 3,22-dioxo-25,26-bisnorchol-4-ene-24-formic acid (22-O-BNC)，由于该物质侧链有 β -酮酸结构，在生物体内不稳定^[40-41]，可以在温和条件下自发脱羧产生 3-OPD。

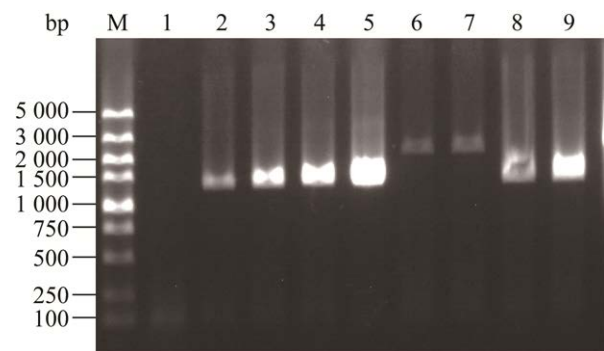
图 6 *hsd4A1* 基因敲除菌二次重组菌落 PCR 鉴定

Figure 6 PCR Identification of *hsd4A1* knockout strain. M: DNA marker. 2–5, 8–9: Knockout strains. 6–7: Unknockout strains.

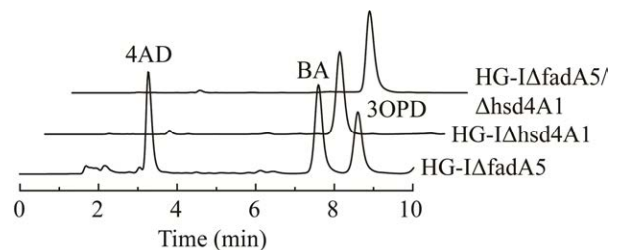
图 7 菌株 HG-I Δ fadA5、HG-I Δ hsd4A1、HG-I Δ fadA5/ Δ hsd4A1 发酵产物对比图

Figure 7 Comparison of fermentation products of strains HG-I Δ fadA5, HG-I Δ hsd4A1, HG-I Δ fadA5/ Δ hsd4A1.

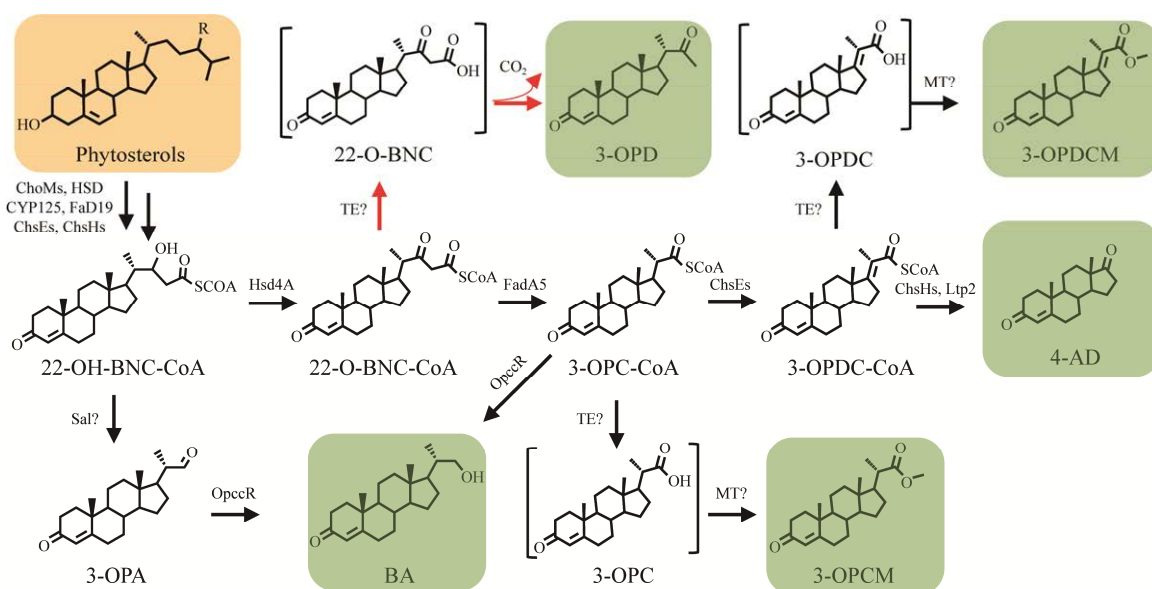


图 8 植物甾醇在新金分枝杆菌中的生物降解途径 植物甾醇(淡黄色)、植物甾醇降解产生的类固醇化合物(淡绿色)。带问号的酶和带括号的中间是假定的,还没有在分枝杆菌中得到验证。黑线指的是已知代谢途径。红线表示推测积累 3-OPD 的新代谢途径

Figure 8 Biodegradation pathways of phytosterols in *Mycobacterium neoaurum*. Phytosterols (light yellow), steroid compounds from the degradation of phytosterols (light green). The enzyme with question marks and the intermediate with brackets are hypothesized and have not been tested in *Mycobacterium*. The black line refers to known metabolic pathways. The red line indicates a new metabolic pathway for hypothesized accumulation of 3-OPD.

2.5 *M. neoaurum* HGMS2 系统发育树分析

如图 9 所示, *M. neoaurum* HGMS2 更接近 *M. neoaurum* VKM Ac-1815D, 两种菌株可能具有相类似的代谢途径, 与 *M. neoaurum* ATCC 25795 和 *M. neoaurum* DSM 44074 相差较远, 说明本菌株可能含有某种特殊的 TE, 能催化 22-O-BNC-CoA 水解, 导致了代谢途径的不一致。 *M. neoaurum* ATCC 25795 和 *M. neoaurum* DSM 44074 较为接近, 说明两种菌株的代谢途径可能相似, 这与 Yuan 等^[35]和 Xu 等^[23]敲除这两株菌株的 *fadA5* 结果一致。

3 讨论与结论

为了确定 *fadA5* 基因在分枝杆菌甾醇降解过程中的功能作用, 以 HG-I 作为初始菌株, 对

其进行了敲除鉴定分析, 结果发现 HG- Δ *fadA5* 的发酵产物中主要积累 3 种代谢产物, 分别为 4-AD、BA 和 3-OPD, 并且它们在代谢产物中的占比分别为 27.8%、41.74%和 17.47%。其中, BA 为主优势产物, 4-AD 的存在表明了分枝杆菌中必然存在其他 *FadA5* 同工酶, 当 *FadA5* 功能活性缺失后对其进行了功能补偿。与他人研究结果不同的是^[23,35], 在 HG- Δ *fadA5* 的发酵产物中还鉴定出一种 C23 类代谢产物 3-OPD, 根据相关报道, C23 类代谢中间体是目前甾醇代谢过程中首次发现的新代谢支路途径, 是对 *FadA5* 缺失后所诱发的补偿途径。

据相关文献报道, 具有 β -酮酸结构的化合物会自动发生脱酸反应^[40-41]。因此, 推测当 *FadA5* 活性缺失后, 甾醇代谢途径中的 22-O-BNC-CoA

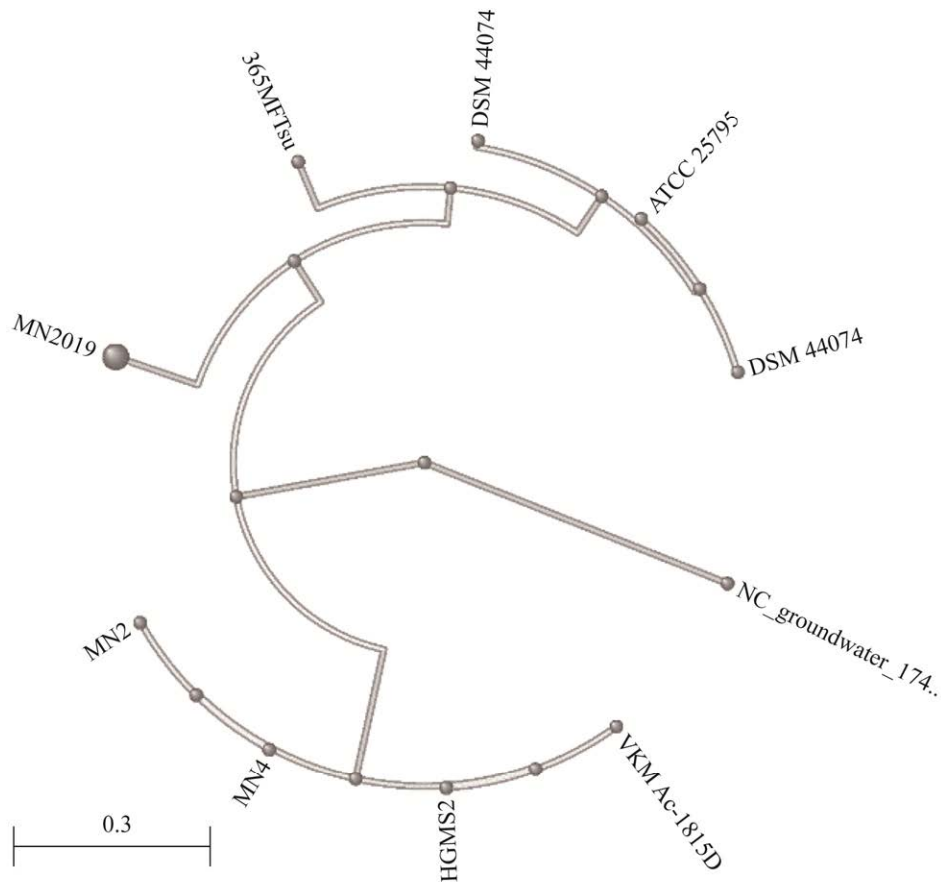


图9 *Mycobacterium neoaurum* HGMS2 系统发育树分析

Figure 9 Phylogenetic tree analysis of *Mycobacterium neoaurum* HGMS2.

会在 TE 的催化作用下生成 22-O-BNC，然后自动脱羧，最终产生了 3-OPD。

本文在分枝杆菌中揭示了一种新的甾醇代谢支路途径，通过核磁共振对 3-OPD 的分子结构进行了解析，并从产物出发，对 3-OPD 合成途径的代谢机理进行了初步探讨，不仅为新型甾药中间体的开发提供了理论基础，而且为结核杆菌新靶点的发现提供了新的线索。

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