Improving the Production of 9,21-dihydroxy-20-methyl-pregna-4-en-3-one from Phytosterols in Mycobacterium Neoaurum by Modifying Multiple Genes and Improving the Intracellular Environment

Chen-Yang Yuan  
SARI: Chinese Academy of Sciences Shanghai Advanced Research Institute

Zhi-Guo Ma  
SARI: Chinese Academy of Sciences Shanghai Advanced Research Institute

Jing-Xian Zhang  
SARI: Chinese Academy of Sciences Shanghai Advanced Research Institute

Xiang-Cen Liu  
SARI: Chinese Academy of Sciences Shanghai Advanced Research Institute

Gui-Lin Du  
SARI: Chinese Academy of Sciences Shanghai Advanced Research Institute

Jun-Song Sun  
SARI: Chinese Academy of Sciences Shanghai Advanced Research Institute

Ji-Ping Shi  
SARI: Chinese Academy of Sciences Shanghai Advanced Research Institute

Bao-Guo Zhang (✉ zhangbg@sari.ac.cn)  
Shanghai Advanced Research Institute  https://orcid.org/0000-0002-0800-0342

Research

Keywords: 9,21-dihydroxy-20-methyl-pregna-4-en-3-one (9-OH-4-HP), kstd, hsd4A, fadA5, intracellular environment

Posted Date: August 10th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-783513/v1

License: © This work is licensed under a Creative Commons Attribution 4.0 International License.  
Read Full License
**Abstract**

**Background**

Steroid drugs are particularly important for disease prevention and clinical treatment. However, traditional chemical methods are rarely implemented during the whole synthetic process to generate steroid intermediates due to the intricate steroid structure. Novel steroid drug precursors and their ideal bacterial strains for industrial production have yet to be developed. Among these, 9-OH-4-HP is a potential steroid drug precursor for the synthesis of corticosteroids. In this study, a combined strategy of blocking Δ<sup>1</sup>-dehydrogenation and the C19 pathway as well as improving the intracellular environment was investigated to construct an effective 9-OH-4-HP-producing strain.

**Results**

A Δ<sup>1</sup>-dehydrogenation-deficient strain of wild-type *Mycobacterium neoaurum* DSM 44074 produces 9-OH-4-HP with a molar yield of 4.8%. *hsd4A*, encoding a β-hydroxyacyl-CoA dehydrogenase, and *fadA5* encoding an acyl-CoA thiolase, were separately knocked out to block the C19 pathway in the Δ<sup>1</sup>-dehydrogenation-deficient strain. The two engineered strains could accumulate 0.59 g L<sup>-1</sup> and 0.47 g L<sup>-1</sup> 9-OH-4-HP from 1 g L<sup>-1</sup> phytosterols. Furthermore, *hsd4A* and *fadA5* were knocked out simultaneously in the Δ<sup>1</sup>-dehydrogenation-deficient strain. The 9-OH-4-HP production from the Hsd4A and FadA5 double-deficient strain was 11.9% higher than that of the Hsd4A -deficient strain and 40.4% higher than that of the strain with FadA5 deficiency, and its selectivity reached 94.9%. Subsequently, the catalase *katE* from *Mycobacterium* and an NADH oxidase, *nox*, from *Bacillus subtilis* were overexpressed to improve the intracellular environment. Ultimately, 9-OH-4-HP production reached 3.58 g L<sup>-1</sup> from 5 g L<sup>-1</sup> phytosterols, and the selectivity of 9-OH-4-HP improved to 97%.

**Conclusion**

*hsd4A* and *fadA5* are key enzymes in the C19 pathway for phytosterol side chain degradation. Deletion of *hsd4A* and *fadA5* could almost entirely block the C19 pathway. Improving the intracellular environment of *Mycobacterium* during phytosterol bioconversion could accelerate the conversion process and enhance the productivity of target sterol derivatives.

**Background**

Steroid drugs, including mineralocorticoids, glucocorticoids, and sex hormones, are crucial in the prevention and clinical treatment of various diseases, including diabetes, asthma, neurodegenerative disorders, cancer, hormone metabolic syndrome, and many others [1, 2]. Steroid drugs represent one of the best-selling classes of drugs and are the second-largest pharmaceutical on the market. Global pharmaceutical industry predictions indicate that many drugs based on steroid compounds will retain market leadership in the future.
In industrial manufacturing, two major valuable intermediates of sterols, C19 steroids and C22 steroids, can be used to synthesize sex and adrenocortical hormones. However, traditional chemical methods are rarely implemented in the whole synthetic processes of modifying steroid intermediates due to the intricate steroid structure. Thus, the pursuit of novel steroid drug precursors has intrigued researchers. Certain C22 steroids, including 21-hydroxy-20-methyl-pregna-4-en-3-one (4-HP), 21-hydroxy-20-methyl-pregna-1,4-dien-3-one (1,4-HP), and 9,21-dihydroxy-20-methyl-pregna-4-en-3-one (9-OH-4-HP), are ideal precursors for steroid drug synthesis [3]. Among these steroids, 9-OH-4-HP is a highly valuable steroid derivative for the synthesis of corticosteroids because of its substituents at positions 9 and 21. Early studies identified 9-OH-4-HP as a by-product of sterol bioconversion to generate 9-hydroxy steroid derivatives in several Mycobacterium species, such as Mycobacterium sp. 2-4M, which produces a 1.5%-1.6% molar yield of 9-OH-4-HP [4]. Mycobacterium sp. VKM Ac-1815D, Mycobacterium sp. VKM Ac-1817D, and Mycobacterium fortuitum ATCC-6842 have also been reported to accumulate a small amount of 9-OH-4-HP during the 9-OH-AD production process [4–6]. However, ideal industrial strains have yet to be developed.

Actinomycetes, such as Mycobacterium, share similar sterol metabolic pathways. The initial step of sterol metabolism is the conversion of sterols to 4-en-3-sterone, followed by degradation of the sterol side chain and cleavage of the steroid skeleton[3, 7, 8]. The cleavage of the steroid B ring is performed by Δ^1-dehydrogenation catalysed by 3-ketosteroid-1(2)-dehydrogenase (KstD) and C9-hydroxylation catalysed by 9α-hydroxylase (KSH) [9, 10]. Consequently, blocking C9 hydroxylation resulted in various C19 steroids. 9α-Hydroxy derivatives, such as 9-hydroxy-androst-4-ene-3,17-dione (9-OH-AD), are important precursors in the manufacture of several modern glucocorticoid drugs bearing a halogen at the 9α position [11]. An engineered strain of Mycobacterium neoaurum ATCC 25795 in which kstDs are knocked out can accumulate 6.02 g L^-1 9-OH-AD as the main product from 15 g L^-1 phytosterols [12]. kstd1 and kstd2 have also been reported to be effective for 9-OH-AD degradation in Rhodococcus rhodochrous DSM43269 [13]. By overexpression of certain related genes and knockout of kstDs, the production of 9-OH-AD increased by 45% in M. neoaurum MS136 [14]. A few wild-type strains of Mycobacterium have been reported to be able to produce 9-OH-AD from plant or animal sterols via a single-step microbial conversion [4]. Normally, 9α-hydroxy derivatives can be obtained from industrial strains in which kstDs are deleted.

In contrast, side chain degradation is rather complicated. Some key enzymes involved in the side chain metabolic pathway remain uncertain, limiting the comprehensive understanding of the process. Dual competing pathways, the overwhelming C19 steroid pathway and the C22 steroid pathway, are involved in phytosterol side chain degradation. Recently, the 17-hydroxysteroid/22-OH-BNC-CoA dehydrogenase Hsd4A was found to be relevant during C22 steroid formation [3]. Inactivation of Hsd4A enabled the production of C22 steroids from sterols. For example, M. neoaurum NwIB-XII accumulates androst-4-ene-3,17-dione (AD) and androst-1,4-diene-3,17-dione (ADD), which are both C19 steroids, as the main products after culture with cholesterol. The hsd4A knockout strain accumulates 4-HP and 1,4-HP as the main products. Nevertheless, C19 steroids still accumulated in the hsd4A knockout strain from
phytosterols, indicating incomplete blockage of the C19 steroid pathway. An Hsd4A- and KstDs-deficient strain of *M. neoaurum* ATCC 25795 gives a 32% molar yield of 9-OH-4-HP and a 15% molar yield of 9-OH-AD from 40 g L\(^{-1}\) phytosterols [3]. FadA5, a thiolase, was reported to be essential for the production of AD/ADD from cholesterol by *M. tuberculosis* H37Rv [15]. A FadA5-deficient strain of *M. neoaurum* NwLB-XII also accumulates 4-HP and 1,4-HP as the main products. Thus, the deletion of *fadA5* may contribute to further blockage of the C19 pathway. Therefore, *hsd4A* and *fadA5* are important targets for modification by genetic engineering to develop microorganisms that can transform sterols into the valuable steroidal intermediate 9-OH-4-HP.

On the other hand, phytosterols and their metabolites are toxic to cells, as they could inhibit cell growth and biocatalytic activity [16]. A steady-state intracellular environment could be beneficial for phytosterol degradation by *Mycobacterium*. Toxic steroid intermediates cause cells to produce reactive oxygen species (ROS), including hydrogen peroxide (H\(_2\)O\(_2\)), during aerobic metabolism. A high level of H\(_2\)O\(_2\) might harm cell growth, hence slowing the rate of phytosterol degradation and decreasing the yield of metabolites [17], and vice versa. For example, elimination of H\(_2\)O\(_2\) from *M. neoaurum* JC-12 increased the 4-HP yield by 24%. In addition, during the phytosterol degradation process, intracellular nicotinamide adenine dinucleotides (NAD\(^+\) and NADH) are consumed, which participate in multistep reactions during phytosterol degradation, such as when dehydrogenation occurs. NAD\(^+\)/NADH regeneration and maintenance of the redox balance are considered the rate-limiting factors in the steroid degradation pathway [17, 18]. Manipulation of NAD\(^+\)/NADH contents could enhance the production of AD and ADD to various degrees [17–19]. Overexpression of NADH oxidase in *M. neoaurum* JC-12 increased ADD production by 43% [17], and an increase in the amounts of the ratio of NAD\(^+\)/NADH in *M. neoaurum* TCCC 11978 enhanced the productivity of ADD by 93%. Thus, the elimination of H\(_2\)O\(_2\) and regeneration of NAD\(^+\) could contribute to higher concentrations of phytosterol metabolites.

Herein, an engineered strain of *M. neoaurum* DSM 44074, which is a sterol consumer, was constructed for the bioconversion of phytosterols to 9-OH-4-HP. A kstDs knockout strain was constructed based on *M. neoaurum* DSM 44074, and the C19 steroid pathway was further blocked by knocking out both *hsd4A* and *fadA5*. By improving the intracellular environment, an efficient 9-OH-4-HP-producing strain was generated. This strain may contribute to the development of steroid drug precursors.

### Results

#### Accumulation of 9α-hydroxy derivatives

To eliminate Δ\(^1\)-dehydrogenation and accumulate 9α-hydroxy derivatives from phytosterols, *kstDs* were identified and knocked out from the genome of the wild-type strain *M. neoaurum* DSM 44074, a steroid-degrading *Mycobacterium* that can completely degrade phytosterols to produce CO\(_2\) and H\(_2\)O [7]. The genome of *M. neoaurum* DSM 44074 was sequenced as described in the Methods section. Three putative *kstD* genes (gene 5102 for *kstd1*, gene 5236 for *kstd2*, and gene 5233 for *kstd3*) were identified in
M. neoaurum DSM 44704. kstDs was successfully knocked out from the genome of M. neoaurum DSM 44704 as described in the Methods section, and the mutant strain ΔKstD was obtained. The cell growth of the ΔkstD strain showed no significant difference from that of the wild-type strain (Fig. S1). The wild-type M. neoaurum DSM 44074 strain and the genetically modified strain ΔkstD were incubated with phytosterols for 168 h. The resulting metabolites were extracted from the culture supernatants and analysed by HPLC. Compared with the wild-type strain M. neoaurum DSM 44074, which showed no detectable product by HPLC analysis (Fig. 2a), the ΔkstD strain produced 9-OH-AD as the main product with a retention time of 4.2 min (Fig. 2a, peak A), along with 9-OH-4-HP as a by-product with a retention time of 7.1 min (Fig. 2a, peak B). No ADD was detected during phytosterol bioconversion by ΔkstD, which indicated that the phytosterol degradation pathway was interrupted because of the elimination of Δ1-dehydrogenation by kstDs knockout. When MP01 medium plus 1 g L⁻¹ phytosterols was used for incubation of the ΔkstD strain, 0.62 g L⁻¹ 9-OH-AD and 0.04 g L⁻¹ 9-OH-4-HP were produced within 60 h (Fig. 3a and 3b). The molar yield of 9-OH-AD reached 84.9%. 9-OH-4-HP was the major by-product during phytosterol bioconversion by ΔkstD, but the molar yield of 9-OH-4-HP was only 4.8%, and the selectivity of 9-OH-4-HP was 5.6% (Table 2). Thus, 9α-hydroxy derivatives successfully accumulated during phytosterol bioconversion by Mycobacterium, but the purity and yield of 9-OH-4-HP remained unsatisfactory.

Construction of a 9-OH-4-HP-producing strain

Dual pathways, the C19 steroid pathway and the C22 steroid pathway, competes during phytosterol side chain degradation. The C19 steroid pathway is the dominant pathway in M. neoaurum DSM 44074, which could be confirmed that ΔkstD produces 9-OH-AD as the main product along with 9-OH-4-HP as a by-product after culture with phytosterols. The two pathways diverge at 22-hydroxy-3,24-dioxo-4-ene-cholest-COA (22-OH-24-CDOE-COA), which could be Δ22-dehydrogenated by the β-hydroxyacyl-CoA dehydrogenase Hsd4A to generate 3,22,24-trioxo-4-ene-cholest-COA (24-CTOE-COA). 24-CTOE-COA could subsequently be catalysed by the thiolase FadA5, leading the phytosterol degradation pathway to the C19 pathway.

Thus, to construct a 9-OH-4-HP-producing strain, hsd4A and fadA5 were identified in the genome of M. neoaurum DSM 44074 and separately knocked out in ΔkstD, resulting in the strains ΔkstDΔhsd4A and ΔkstDΔfadA5.

The cell growth of ΔkstDΔhsd4A and ΔkstDΔfadA5 showed no significant difference from that of the wild-type strain M. neoaurum DSM 44074 (Fig. S1). The strains ΔkstDΔhsd4A and ΔkstDΔfadA5 were cultured with phytosterols for 168 h, and the metabolites were analysed by HPLC (Fig. 2b). As shown in Fig. 3b, 9-OH-4-HP successfully accumulated in both strains ΔkstDΔhsd4A and ΔkstDΔfadA5. The selectivity of 9-OH-4-HP from the ΔkstDΔhsd4A and ΔkstDΔfadA5 strains were 88.6% and 86.0%, respectively. Nevertheless, both strains still showed only a small amount of 9-OH-AD accumulation (Fig. 3a). The selectivity of 9-OH-AD from strains ΔkstDΔhsd4A and ΔkstDΔfadA5 were 7.2% and 8.0%,
respectively. After culture with 1 g L\(^{-1}\) phytosterols in MP01 medium, the strain \(\Delta kstD\Delta hsd4A\) accumulated 0.59 g L\(^{-1}\) 9-OH-4-HP and 0.13 g L\(^{-1}\) 9-OH-AD, while 0.47 g L\(^{-1}\) 9-OH-4-HP and 0.08 g L\(^{-1}\) 9-OH-AD were obtained from strain \(\Delta kstD\Delta fadA5\). The molar yields of 9-OH-4-HP and 9-OH-AD from strain \(\Delta kstD\Delta fadA5\) were both lower than those from strain \(\Delta kstD\Delta hsd4A\). The molar yield of 9-OH-4-HP from strain \(\Delta kstD\Delta fadA5\) was 20.3% lower than that from strain \(\Delta kstD\Delta hsd4A\), and the molar yield of 9-OH-AD from strain \(\Delta kstD\Delta fadA5\) which was 38.5% lower than that from strain \(\Delta kstD\Delta hsd4A\).

Considering that 9-OH-AD still accumulated in both the \(\Delta kstD\Delta hsd4A\) and \(\Delta kstD\Delta fadA5\) strains, the C19 steroid pathway of the phytosterol degradation pathway was not completely blocked in either strain. Thus, to enhance the purity and productivity of 9-OH-4-HP and obstruct the yield of 9-OH-AD, \(hsd4A\) and \(fadA5\) were both knocked out in strain \(\Delta kstD\), and strain \(\Delta kstD\Delta hsd4A\Delta fadA5\) was obtained. The cell growth of the \(\Delta kstD\Delta hsd4A\Delta fadA5\) strain showed a trend similar to that of the wild-type strain \(M. neoaurum\) DSM 44074 (Fig. S1). As shown in Fig. 2b, after culture with phytosterols and metabolites being analysed by HPLC, the strain \(\Delta kstD\Delta hsd4A\Delta fadA5\) accumulated 9-OH-4-HP as the main product, while the accumulation of 9-OH-AD was significantly decreased compared with the strains \(\Delta kstD\Delta hsd4A\) and \(\Delta kstD\Delta fadA5\). The selectivity of 9-OH-4-HP and 9-OH-AD from strain \(\Delta kstD\Delta hsd4A\Delta fadA5\) were 94.9% and 2.0%, respectively. The purity of 9-OH-4-HP from strain \(\Delta kstD\Delta hsd4A\Delta fadA5\) was higher than those from strains \(\Delta kstd\Delta hsd4A\) and \(\Delta kstD\Delta fadA5\). After culture with 1 g L\(^{-1}\) phytosterols, 0.66 g L\(^{-1}\) 9-OH-4-HP was obtained from strain \(\Delta kstD\Delta hsd4A\Delta fadA5\), which is 11.9% more than that from strain \(\Delta kstD\Delta hsd4A\) and 40.4% more than that from strain \(\Delta kstD\Delta fadA5\) (Fig. 3b). The selectivity and production of 9-OH-4-HP from strain \(\Delta kstD\Delta hsd4A\Delta fadA5\) were both higher than those from strains \(\Delta kstD\Delta hsd4A\) and \(\Delta kstD\Delta fadA5\), indicating that double knockout of \(hsd4A\) and \(fadA5\) could effectively block the accumulation of AD homologues.

Moreover, to verify the functions of \(hsd4A\) and \(fadA5\) during phytosterol degradation, \(\Delta kstD\Delta hsd4A\Delta hsd4A\), the \(hsd4A\) complementation strain, and \(\Delta kstD\Delta fadA5\Delta fadA5\), the \(fadA5\) complementation strain, were also constructed. As shown in Figure 2c, when the two complementation strains were cultured with phytosterols and metabolites being analysed by HPLC, the accumulation of 9-OH-AD was recovered. The purities of 9-OH-AD from \(\Delta kstD\Delta hsd4A\Delta hsd4A\) and \(\Delta kstD\Delta fadA5\Delta fadA5\) were 90.0% and 88.5%, respectively, which is nearly consistent with those of strain \(\Delta kstD\). These results indicated that Hsd4A and FadA5 were key enzymes in the C19 steroid pathway during phytosterol degradation. Phylogenetic trees of Hsd4A and FadA5 were constructed to elucidate evolutionary relationship of the two enzymes (Fig. S2).

**Evaluation of the 9-OH-4-HP producer**

After culture with 1 g L\(^{-1}\) phytosterols, the molar yield of 9-OH-4-HP from \(\Delta kstD\Delta hsd4A\Delta fadA5\) was 78.9%. To evaluate the ability of \(\Delta kstD\Delta hsd4A\Delta fadA5\) to transform phytosterols into 9-OH-4-HP, different concentrations of phytosterols were incubated with \(\Delta kstD\Delta hsd4A\Delta fadA5\).
As shown in Fig. 4b, the yields of 9-OH-4-HP from the bioconversion of 2 g L\(^{-1}\), 5 g L\(^{-1}\), 8 g L\(^{-1}\), and 10 g L\(^{-1}\) phytosterols by \(\Delta kstD\Delta hsd4A\Delta fadA5\) were 1.43 g L\(^{-1}\), 2.78 g L\(^{-1}\), 1.98 g L\(^{-1}\), and 1.73 g L\(^{-1}\), respectively. 9-OH-AD was also obtained during the incubation, showing yields of 0.06 g L\(^{-1}\), 0.10 g L\(^{-1}\), 0.03 g L\(^{-1}\), and 0.04 g L\(^{-1}\), respectively (Fig. 4a). The productivity of 9-OH-4-HP was enhanced as the concentration of phytosterols increased up to 5 g L\(^{-1}\). However, at phytosterol concentrations of 8 g L\(^{-1}\) and 10 g L\(^{-1}\), the productivity of 9-OH-4-HP decreased, showing results only slightly higher than that with 2 g L\(^{-1}\) phytosterols, and was obviously lower than that with 5 g L\(^{-1}\) phytosterols. The molar yields of 9-OH-4-HP from different concentrations of phytosterols are listed in Table 3. A downward trend in the molar yield of 9-OH-4-HP appeared as the concentration of phytosterols increased from 2 g L\(^{-1}\) to 10 g L\(^{-1}\). However, the purity of 9-OH-4-HP remained stable. Previous research has reported that phytosterols and their metabolites could be noxious to cells during bioconversion [16, 20-22], which might account for the poor performance of \(\Delta kstD\Delta hsd4A\Delta fadA5\) during phytosterol bioconversion as the concentration of phytosterols increased.

**Intracellular environmental balance contributes to higher 9-OH-4-HP production**

The 9-OH-4-HP-producing strain \(\Delta kstD\Delta hsd4A\Delta fadA5\) did not perform well during the bioconversion of phytosterols when the concentration of phytosterols was higher than 2 g L\(^{-1}\). This might be due to multiple factors that influence the bioconversion of phytosterols.

A series of redox reactions occur during phytosterol degradation, which use oxygen as an electron acceptor, and cholesterol dehydrogenases/isomerases require intracellular nicotinamide adenine dinucleotides (NAD\(^{+}\) and NADH) as cofactors [23, 24]. NAD\(^{+}\) and NADH play important roles during phytosterol transformation. They act in many oxidation-reduction reactions and regulate various enzymatic activities and genetic processes. The intracellular NAD\(^{+}\) concentration decreased due to its consumption. Therefore, NAD\(^{+}\) and NADH have critical effects on the maintenance of the intracellular redox balance. Regeneration of NAD\(^{+}\) and enhancement of the NAD\(^{+}\)/NADH ratio may be of great assistance during phytosterol transformation.

In addition, hydrogen peroxide (H2O2) is produced due to incomplete oxidation during aerobic metabolism and the regeneration of flavin adenine dinucleotide (FAD) during the phytosterol transformation process [17]. A high level of H2O2 can damage proteins, DNA, and lipids in cells, resulting in inhibition of cell growth and metabolite yield [25].

To enhance the ability of strain \(\Delta kstD\Delta hsd4A\Delta fadA5\) to transform phytosterols into 9-OH-4-HP, the catalase katE from DSM 44074 and the NADH oxidase nox from Bacillus subtilis [17] were co-expressed in strain \(\Delta kstD\Delta hsd4A\Delta fadA5\) to construct strain \(\Delta kstD\Delta hsd4A\Delta fadA5\)-NK.
The extracellular H$_2$O$_2$ concentrations of the two strains ΔkstΔhsd4ΔfadA5 and ΔkstΔhsd4ΔfadA5-NK were measured when they were cultured with 5 g L$^{-1}$ phytosterols for 168 h. As shown in Figure 5b, the extracellular H$_2$O$_2$ concentration of strain ΔkstΔhsd4ΔfadA5 showed an upward trend during the bioconversion process. The extracellular H$_2$O$_2$ concentration increased from 0.59 µmol L$^{-1}$ at the beginning to 1.05 µmol L$^{-1}$ after 168 h and reached a peak of 1.10 µmol L$^{-1}$ at 120 h. In contrast, the extracellular H$_2$O$_2$ concentration of strain ΔkstΔhsd4ΔfadA5-NK remained nearly stable during the bioconversion process at approximately 0.51 µmol L$^{-1}$. Therefore, the overexpression of katE eliminated excessive extracellular H$_2$O$_2$. Moreover, to verify the toxicity of H$_2$O$_2$, cell growth of the strains ΔkstΔhsd4ΔfadA5 and ΔkstΔhsd4ΔfadA5-NK was also measured. As shown in Figure 5a, the biomass of strain ΔkstΔhsd4ΔfadA5-NK was higher than that of strain ΔkstΔhsd4ΔfadA5, indicating that the elimination of extracellular H$_2$O$_2$ could help with cell growth.

Likewise, the NAD$^+/$/NADH ratios of the strains ΔkstΔhsd4ΔfadA and ΔkstΔhsd4ΔfadA-NK were also measured after they were cultured with 5 g L$^{-1}$ phytosterols for 168 h. As shown in Figure 5c, the NAD$^+/$/NADH ratio of strain ΔkstΔhsd4ΔfadA-NK was consistently higher than that of strain ΔkstΔhsd4ΔfadA by at least 10.9%. At 96 h, the NAD$^+/$/NADH ratio was enhanced by 25.4% in strain ΔkstΔhsd4ΔfadA-NK compared with strain ΔkstΔhsd4ΔfadA. The overexpression of nox could significantly influence the NAD$^+/$/NADH ratio during phytosterol bioconversion.

9-OH-4-HP productivity was also measured to test whether overexpression of katE and nox could enhance the ability of ΔkstΔhsd4ΔfadA to transform phytosterols into 9-OH-4-HP. The recombinant strain ΔkstΔhsd4ΔfadA-NK was cultured with 1 g L$^{-1}$, 2 g L$^{-1}$, 5 g L$^{-1}$, 8 g L$^{-1}$, and 10 g L$^{-1}$ phytosterols for 168 h, and the productivity of 9-OH-4-HP was measured every 24 h. As shown in Figure 5d, the final productivities of 9-OH-4-HP from 1 g L$^{-1}$, 2 g L$^{-1}$, 5 g L$^{-1}$, 8 g L$^{-1}$, and 10 g L$^{-1}$ phytosterols were 0.68 g L$^{-1}$, 1.53 g L$^{-1}$, 3.58 g L$^{-1}$, 2.51 g L$^{-1}$, and 2.73 g L$^{-1}$, respectively. Compared with ΔkstΔhsd4ΔfadA cultured with the same concentrations of phytosterols, the productivities of 9-OH-4-HP were enhanced by 3.03%, 6.99%, 28.7%, 26.8%, and 57.8%. The highest yield of 9-OH-4-HP was obtained when strain ΔkstΔhsd4ΔfadA-NK was cultured with 5 g L$^{-1}$ phytosterols, with a molar yield that reached 85.5%, which was 28.8% higher than that of ΔkstΔhsd4ΔfadA. Moreover, no significant difference in the productivity of 9-OH-AD was observed between the two strains ΔkstΔhsd4ΔfadA5 and ΔkstΔhsd4ΔfadA5-NK (Fig. 5e), indicating that the purity of 9-OH-4-HP was also enhanced during phytosterol bioconversion by the strain ΔkstΔhsd4ΔfadA5-NK. All of the results above confirm that regulation of the intracellular NAD$^+/$/NADH ratio and H$_2$O$_2$ levels could be an effective way to improve sterol transformation efficiency and the production of steroid intermediates.

**Discussion**

By genome sequencing, three kstDs were found in *M. neoaurum* DSM 44074. kstD2 and kstD3 in *M. neoaurum* DSM 44074 showed 100% similarity with those in *M. neoaurum* ATCC 25795, a strain that was
deemed to be the same strain as *M. neoaurum* DSM 44074. However, *kstD1* in *M. neoaurum* DSM 44074 showed 5 mismatches with that in *M. neoaurum* DSM 44074, causing 3 amino acid changes. When the *kstds* knockout strain of *M. neoaurum* DSM 44074 was cultured with phytosterols, AD and 4-HP were nearly undetectable in the final products. The *kstd* knockout strain accumulated 9-OH-AD as the main product and 9-OH-4-HP as a by-product. Certain other strains have also been reported to accumulate 9-OH-AD from phytosterols. *Mycobacterium* sp. 2-4M [26] showed a 50% molar yield of 9-OH-AD, a 22% molar yield of AD and a 2% molar yield of 4-HP from 5 g L\(^{-1}\) sitosterol [27]. In a *kstds* knockout strain of *M. neoaurum* ATCC 25795, a 55% molar yield of 9-OH-AD and a 15% molar yield of AD were obtained from 15 g L\(^{-1}\) phytosterols. Compared with other 9-OH-AD-producing strains, the purity and molar yield of 9-OH-AD from \(\Delta kstD\) after culture with phytosterols were notably higher. The accumulation of AD from *kstds* knockout strains might be due to residual \(\Delta 1\)-dehydrogenation activity. The genome of *R. ruber* contains at least two other possible ORFs other than *kstD1*, *kstD2*, and *kstD3* with certain identity to *kstds* (approximately 38%) [28]. The existence of more than 3 KstDs has also been reported for other *Rhodococcus* species, such as *R. jostii* Rha1 [29]. Thus, inactivation of all KstD activities ought to be the fundamental premise to develop promising 9\(\alpha\)-hydroxy derivatives.

Due to dual competing pathways, the dominant C19 steroid pathway and the C22 steroid pathway exist in the phytosterol degradation pathway. 9-OH-4-HP is usually produced as a by-product in 9-OH-AD-producing strains. M. V. Donova reported a wild-type strain *Mycobacterium* sp. 2-4M, which produces 9-OH-AD as the major product with a 1.5%-1.6% molar yield of 9-OH-4-HP [26]. *Mycobacterium* sp. VKM Ac-1815D, *Mycobacterium* sp. VKM Ac-1817D, and *Mycobacterium fortuitum* ATCC-6842 have also been reported to accumulate a small amount of 9-OH-4-HP during the 9-OH-AD production process [4–6]. The enzymes that catalyse 22-hydroxy-3,24-dioxo-4-ene-cholest-COA into 4-HP homologues remain unidentified. Thus, Hsd4A is normally chosen to manipulate the metabolic flux to generate AD homologues or 4-HP homologues. Xu reported the characterization of Hsd4A *in vivo* and *in vitro*, testifying that deletion of *hsd4A* resulted in blockage of the C19 steroid pathway and enhanced the accumulation of 4-HP homologues. During the Hsd4A investigation, Xu constructed a 9-OH-4-HP-producing strain by knocking out *hsd4A* in the *kstD*-deficient strain of *M. neoaurum* ATCC 25795. This mutant strain displayed 32% molar yield of 9-OH-4-HP and 15% molar yield of 9-OH-AD from 40 g L\(^{-1}\) phytosterols [3]. Here, in this research, it was confirmed that double knockout of *hsd4A* and *fadA5* could further block the C19 steroid pathway. The purity and molar yield of 9-OH-4-HP of strain \(\Delta kstds\Delta hsd4A\Delta fadA5\) were notably higher than those of Xu’s strain. Although strain \(\Delta kstds\Delta hsd4A\Delta fadA5\) did not perform well when cultured with higher concentrations of phytosterols, the purity of 9-OH-4-HP was not influenced, indicating its potency as a promising 9-OH-4-HP producer.

AD homologues accumulated in the 9-OH-4-HP producer strains \(\Delta kstds\Delta hsd4A\), \(\Delta kstds\Delta fadA5\), and \(\Delta kstds\Delta hsd4A\Delta fadA5\), indicating incomplete blockage of the C19 steroid pathway. Similar results have been previously reported. Analysis of the *M. neoaurum* CCTCC AB2019054 genome revealed that there were 6 proteins with \(>38\%\) identity and 10 proteins with \(31–38\%\) identity to Hsd4A, which may compensate for its function [30]. The most identical gene, *hsd4A2* (45% identity to *hsd4A*), was deleted,
and fermentation analysis revealed that it can indeed produce 4-HBC at a significantly increased ratio. A similar result was found in *M. neoaurum* DSM 44074. Eight proteins showed certain identities to Hsd4A, indicating the presence of isoenzymes of Hsd4A in the genome of *M. neoaurum* DSM 44074.

Some attempts have been made to enhance the ability of microorganisms to transform phytosterols. Considering the toxicity of phytosterols and their derivatives to cells, the balance of the intracellular environment could improve the ability of microorganisms to transform phytosterols. Intracellular factors such as NAD$^+$ and NADH have drawn increasing attention in recent years. NAD$^+$ and NADH participate in multiple steps during steroid bioconversion, and the intracellular NAD$^+$ concentration decreases as it is consumed. Regeneration of NAD$^+$ and enhancement of the NAD$^+$/NADH ratio have been proven to be able to enhance the ability of microorganisms to transform phytosterols. Overexpression of NADH oxidase in *M. neoaurum* JC-12 increased ADD production by 43% [17]. Overexpression of flavin oxidoreductase and NADH oxidase from *Lactobacillus brevis* in *M. neoaurum* TCCC 11978 increased the NAD$^+$/NADH ratio by 113% and 192%, respectively, and significantly enhanced the conversion ratio of AD(D) [18]. The type II NADH dehydrogenases ndhN and ndhF were overexpressed in *M. neoaurum* MNR, resulting in an increase in the NAD$^+$/NADH ratio from 3.93 to 5.91 and 10.96, respectively. The highest molar biotransformation rates of AD with 5 g L$^{-1}$ phytosterol feed were 5.32% and 12.38% higher than those of the original strain, respectively.

In addition, reactive oxygen species are generated during the conversion process of sterols, which impair cell viability and hinder the conversion of sterols to steroid synthons. Elimination of ROS and H$_2$O$_2$ have been reported to be an effective method to improve cell growth under phytosterol feed and enhance phytosterol bioconversion. Combinatorial augmentation with catalase, mycothiol, and ergothioneine increased 4-HP productivity by 47.5% in *M. neoaurum* WIII-egt&msh&cat [31]. Elimination of H$_2$O$_2$ in *M. neoaurum* JC-12 increased the 4-HP yield by 24%. In this study, when combining the abilities of NAD$^+$ regeneration and H$_2$O$_2$ elimination, the performance of the new mutant strain ΔkstDsΔhsd4ΔfadA5-NK to transform phytosterols into 9-OH-4-HP improved. The extracellular H$_2$O$_2$ concentration of ΔkstDsΔhsd4ΔfadA5-NK after culture with 5 g L$^{-1}$ phytosterols remained at a low level, which was only 49.4% that of the extracellular H$_2$O$_2$ concentration of ΔkstDsΔhsd4ΔfadA5 culture under the same conditions. The NAD$^+$/NADH ratio was also enhanced by 25.4% after 96 h. Although the molar yield of 9-OH-4-HP decreased as the phytosterol concentration increased, the molar yield of 9-OH-4-HP from strain ΔkstDsΔhsd4ΔfadA5-NK was significantly higher than that from strain ΔkstDsΔhsd4ΔfadA5 at the same phytosterol concentration. The highest yield of 9-OH-4-HP was 3.58 g L$^{-1}$, which was achieved when ΔkstDsΔhsd4ΔfadA5-NK was fed 5 g L$^{-1}$ phytosterols. The molar yield of 9-OH-4-HP from strain ΔkstDsΔhsd4ΔfadA5-NK was 28.7% higher than that from strain ΔkstDsΔhsd4ΔfadA5 with 5 g L$^{-1}$ phytosterol feeding. All of these results proved that the elimination of H$_2$O$_2$ and regeneration of NAD$^+$ could be an effective method to improve phytosterol bioconversion.
When 10 g L\(^{-1}\) phytosterols were cultured with \(\Delta kstDs\Delta hsd4A\Delta fadA5\)-NK, the molar yield of 9-OH-4-HP was 32.6%, indicating further manipulation to enhance its molar yield. A number of different aqueous-organic two-phase systems have been studied, such as water-vegetable oils and water-ionic liquids \[32\]. The use of cyclodextrins could enhance the uptake of phytosterols by microorganisms \[16, 33\]. Recently, some novel methods have been developed to improve phytosterol bioconversion. Increasing cell permeability could improve the production of phytosterol metabolites \[34, 35\]. Deletion of the transmembrane transporter trehalose monomycolate \(mmpL3\) in a 4-HP-producing strain derived from \(M. neoaurum\) ATCC 25795 increased 4-HP production by 24.7%. A “resting cell-cyclodextrin” system has also been widely used in industry and research to improve bioconversion ability.

**Methods**

**Bacterial strains, plasmids, medium, and reagents**

The strains and plasmids used in this study are described in Table 1. \(E. coli\) DH5\(\alpha\) stored in the laboratory was used for plasmid amplification. Wild-type \(M. neoaurum\) DSM 44074 (DSM 44704) was purchased from Deutsche Sammlung von Mikroorganismenund Zellkulturen (DSMZ, GERMANY). All other strains were derived from \(M. neoaurum\) DSM 44704. Common plasmids and primers (Table S1) were used to construct the mutants. \(E. coli\) DH5\(\alpha\) was cultured at 37°C and 200 rpm in 50 mL of Luria-Bertani (LB) medium (10 g L\(^{-1}\) tryptone, 10 g L\(^{-1}\) NaCl, 5 g L\(^{-1}\) yeast extracts, pH 7.0). \(Mycobacterium\) cells were cultured in MYD medium (0.6 g L\(^{-1}\) \(K_2\)HPO\(_4\)·3H\(_2\)O, 5.4 g L\(^{-1}\) NaNO\(_3\), 6 g L\(^{-1}\) glucose, 15 g L\(^{-1}\) yeast extract and an initial pH value 7.5) and fermented with MP01 medium (10 g L\(^{-1}\) corn steep powder, 20 g L\(^{-1}\) glucose, 2 g L\(^{-1}\) \(K_2\)HPO\(_4\)·3H\(_2\)O, 1.0 g L\(^{-1}\) MgSO\(_4\)·7H\(_2\)O, 2.0 g L\(^{-1}\) NaNO\(_3\), 2‰ Tween 80 (v/v), and an initial pH value 7.5) at 30°C and 200 rpm.

The phytosterols consisted of 45% \(\beta\)-sitosterol, 37% campesterol, and 18% stigmasterol, which were purchased from Yunnan Biological Products Co., Ltd. (Yunnan, China). AD and 9-OH-AD were obtained from Shanghai Macklin Biochemical Co., Ltd. (China). \((2\text{-Hydroxypropyl})\text{-}\beta\text{-cyclodextrin (HP-}\beta\text{-CD)}\) was purchased from Zhiyuan Biotechnology Co., Ltd. (Shandong, China). The ClonExpress\textsuperscript{\textregistered} One Step Cloning Kit was purchased from Vazyme Biotech Co., Ltd. (Nanjing, China). The Hydrogen Peroxide (\(H_2O_2\)) Content Assay Kit and Nicotinamide Adenine Dinucleotide NAD(H) Content Assay Kit were obtained from Sangon Biotech Co., Ltd. (Shanghai, China).

**Bioinformatic analysis**

The genome of \(M. neoaurum\) DSM 44074 was sequenced by Shanghai Majorbio Co., Ltd. The DNA sample was extracted and sheared into 400-500 bp fragments using a Covaris M220 Focused Acoustic Shearer (Covaris, USA). Illumina sequencing libraries were prepared from the sheared fragments using a NEXTflex\textsuperscript{\textregistered} Rapid DNS-Seq Kit (Bioo Scientific, USA). The sequencing data were assembled using SOAPdenovo2\textsuperscript{(GitHub - aquaskyline/SOAPdenovo2: Next generation sequencing reads de novo)}
assembler). Further prediction and annotation were produced by Glimmer (Glimmer (jhu.edu)) and BLAST (blast.ncbi.nlm.nih.gov). The putative genes for kstD, hsd4A, and fadA5 were identified by comparison with known gene sequences taken from the NCBI database. MEGA-X software (Home (megasoftware.net)) was used to construct a phylogenetic tree of hsd4A and fadA5 with the known amino acid sequences taken from the NCBI.

**Mutant strain construction**

A CRISPR-assisted nonhomologous end-joining strategy was used to delete the target gene in *M. neoaurum* DSM 44074 based on previous reports. The PSBY1 plasmid harbouring cpf1 was obtained from Jiang [36], and the PCR-Hyg plasmid harbouring sgRNA was obtained from Sun [37]. ClonExpress® One Step Cloning Kit mutated spacers were used to construct different plasmids harbouring target sgRNA. The plasmid harbouring target sgRNA was transfected into *M. neoaurum*, and the PSBY1 plasmid was transfected beforehand by electroporation. The recombinant clones were sequenced using specific primers to determine the deletion.

The vector P38Mu (pMV306 with the Psmyc promoter) with kanamycin resistance was used to overexpress the target gene. The genes hsd4A, fadA5, katE from *M.neoaurum* DSM 44074, and nox from *Bacillus subtilis* were recombined on P38Mu. Specific primers were used to amplify the corresponding gene, and the PCR product was inserted into the NdeI site (and HindIII site, if two genes were inserted) of P38Mu using the ClonExpress® One Step Cloning Kit.

**Bioconversion and analysis**

The transformation capability of the mutant strains was identified in MP01 medium with an initial phytosterol concentration of 1 g L\(^{-1}\). A concentration gradient was later tested to further determine the capability of phytosterol bioconversion. Phytosterols were prepared in (2-hydroxypropyl)-β-cyclodextrin (HP-β-CD) at a ratio of 1:1.5. The recombinant cells were inoculated into 30 mL of MYD medium in a 250 mL shaker flask and cultured at 30°C and 200 rpm. Three millilitres of seed medium was transferred to 30 mL of MP01 medium in a 250 mL shaker flask with a baffle when the optical density reached the mid-log exponential phase. The fermentation of *M. neoaurum* DSM 44074 and recombinant strains was sampled every 12 or 24 h, and three replicates were used to measure the steroids. The bioconversion mixture was extracted with 3 volumes of ethyl acetate, and the solvent was removed to give a residue that was redissolved in methanol. The resulting solution was used for HPLC analysis. HPLC was performed on a Shimadzu Separations module connected to a Shimadzu SPD-M20A detector equipped with a C18 column (250 mm × 4.6 mm, 5 µm) and detected at a wavelength of 254 nm. A mixture of methanol and water (80:20, v/v) was used as the mobile phase at a flow rate of 0.8 mL min\(^{-1}\).

Extracellular H\(_2\)O\(_2\) concentrations were measured according to the operating manual of the Hydrogen Peroxide (H\(_2\)O\(_2\)) Content Assay Kit. NADH and NAD\(^+\) intracellular concentrations were measured
according to the operating manual of the Nicotinamide Adenine Dinucleotide, NAD(H) Content Assay Kit.

The accumulation of 9-OH-4-HP was achieved by blocking the C19 steroid pathway and 3-ketosteroid-Δ¹-dehydrogenation. Compared with single deletion of *hsd4A* and single deletion of *fadA5*, double deletion of *hsd4A* and *fadA5* could further block the C19 steroid pathway. By eliminating H₂O₂ and regenerating NAD⁺ in the triple *hsd4A*, *fadA5*, and *kstDs* knockout strain, the highest 9-OH-4-HP productivity was 3.58 g L⁻¹ with 5 g L⁻¹ phytosterol feed.

**Abbreviations**

AD: androst-4-ene-3,17-dione; ADD: androst-1,4-diene-3,17-dione; 9-OH-AD: 9-hydroxy-androst-4-ene-3,17-dione; 4-HP: 21-hydroxy-20-methyl-pregna-4-en-3-one; 1,4-HP: 21-hydroxy-20-methyl-pregna-1,4-dien-3-one; 9-OH-4-HP: 9,21-dihydroxy-20-methyl-pregna-4-en-3-one; KstDs: 3-ketosteroid-Δ¹-dehydrogenases; KSHs: 9α-hydroxylase; HPs: C22 steroids; Hsd4A: β-hydroxyacyl-CoA dehydrogenase; FadA5: acyl-COA thiolase; ROS: Reactive oxygen species; H₂O₂: hydrogen peroxide; NAD⁺ / NADH: nicotinamide adenine dinucleotides; NOX: NADH oxidase; KatE: catalase; HPLC: high performance liquid chromatography

**Declarations**

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Availability of data and materials**

All data generated and analyzed during this study are included in this published article and its additional files.

**Author’s contributions**

BGZ and YCY designed the study. YCY carried out the gene knockout and overexpression. YCY and ZGM performed the phytosterol bioconversion. YCY, XCL, GLD and JXZ analyzed the data. YCY wrote the manuscript. BGZ, JSS and JPS reviewed the manuscript.

All authors read and approved the final manuscript.
Competing interests

The authors declare no conflict of interest

Funding

This research was funded by National Key R&D Program of China, grant number 2017YFE0112700.

Acknowledgement

We sincerely thank Yu Jiang (CAS Center for Excellence in Molecular Plant Sciences Institute of Plant Physiology and Ecology, Chinese Academy of Sciences, Shanghai, China) for providing the plasmid PSBY1 and Yicheng Sun (Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China) for providing the plasmid Pcr-Hyg. We also thank W.R. Jacobs Jr. (Howard Hughes Medical Institute) for providing the plasmids pMV306

References

1. Finocchi C, Ferrari M. Female reproductive steroids and neuronal excitability. Neurol Sci. 2011;32(Suppl 1):S31-5.
2. Rugutt JK, Rugutt KJ. Antimycobacterial activity of steroids, long-chain alcohols and lytic peptides. Nat Prod Res. 2012;26(11):1004–11.
3. Xu LQ, et al. Unraveling and engineering the production of 23,24-bisnorcholesterol in sterol metabolism. Rep. 2016;6:21928.
4. Donova MV, et al. Mycobacterium sp. mutant strain producing 9alpha-hydroxyandrostenedione from sitosterol. Appl Microbiol Biotechnol. 2005;67(5):671–8.
5. Shtratnikova VY, et al. Effect of methyl-beta-cyclodextrin on gene expression in microbial conversion of phytosterol. Appl Microbiol Biotechnol. 2017;101(11):4659–67.
6. Wovcha MG, et al. Bioconversion of sitosterol to useful steroidal intermediates by mutants of Mycobacterium fortuitum. Biochim Biophys Acta. 1978;531(3):308–21.
7. Yao K, et al. Identification and engineering of cholesterol oxidases involved in the initial step of sterols catabolism in Mycobacterium neoaurum. Metab Eng. 2013;15:75–87.
8. Brzostek A, et al. Cholesterol oxidase is required for virulence of Mycobacterium tuberculosis. FEMS Microbiol Lett. 2007;275(1):106–12.
9. Petrusma M, et al. Multiplicity of 3-Ketosteroid-9alpha-Hydroxylase enzymes in Rhodococcus rhodochrous DSM43269 for specific degradation of different classes of steroids. J Bacteriol. 2011;193(15):3931–40.
10. Van, d.G., R., et al., Targeted disruption of the kstD gene encoding a 3-ketosteroid delta(1)-dehydrogenase isoenzyme of Rhodococcus erythropolis strain SQ1. Applied & Environmental Microbiology, 2000. 66(5): p. 2029–36.

11. Sedlaczek L, Smith LL. Biotransformations of steroids. Crit Rev Biotechnol. 1988;7(3):187–236.

12. Yao K, et al. Characterization and engineering of 3-ketosteroid-Δ1-dehydrogenase and 3-ketosteroid-9α-hydroxylase in Mycobacterium neoaurum ATCC 25795 to produce 9α-hydroxy-4-androstene-3,17-dione through the catabolism of sterols. Metab Eng. 2014;24:181–91.

13. Yang, et al. The effect of 3-ketosteroid-Δ1-dehydrogenase isoenzymes on the transformation of AD to 9α-OH-AD by Rhodococcus rhodochrous DSM43269. Journal of Industrial Microbiology Biotechnology. 2016;43(9):1303–11.

14. Sun H, et al. Enhancing production of 9α-hydroxy-androst-4-ene-3,17-dione (9-OHAD) from phytosterols by metabolic pathway engineering of mycobacteria. Chem Eng Sci. 2021;230(47):116195.

15. Nesbitt NM, et al. A thiolase of Mycobacterium tuberculosis is required for virulence and production of androstenedione and androstadienedione from cholesterol. Infect Immun. 2010;78(1):275–82.

16. Su L, et al., The Sterol Carrier Hydroxypropyl-beta-Cyclodextrin Enhances the Metabolism of Phytosterols by Mycobacterium neoaurum. Appl Environ Microbiol, 2020. 86(15).

17. Shao M, et al., Intracellular Environment Improvement of Mycobacterium neoaurum for Enhancing Androst-1,4-Diene-3,17-Dione Production by Manipulating NADH and Reactive Oxygen Species Levels. Molecules, 2019. 24(21).

18. Su LQ, et al., Cofactor engineering to regulate NAD(+) /NADH ratio with its application to phytosterols biotransformation. Microbial Cell Factories, 2017. 16.

19. Zhou XL, et al. Efficient production of androstenedione by repeated batch fermentation in waste cooking oil media through regulating NAD(+) /NADH ratio and strengthening cell vitality of Mycobacterium neoaurum. Biorec Technol. 2019;279:209–17.

20. Orrego R, et al. Pulp and Paper Mill Effluent Treatments Have Differential Endocrine-Disrupting Effects on Rainbow Trout. Environ Toxicol Chem. 2009;28(1):181–8.

21. Nieminen P, et al. Phytosterols act as endocrine and metabolic disruptors in the European polecat (Mustela putorius). Toxicol Appl Pharmacol. 2002;178(1):22–8.

22. Denton TE, et al. Masculinization of female mosquitofish by exposure to plant sterols and Mycobacterium smegmatis. Bull Environ Contam Toxicol. 1985;35(5):627–32.

23. Li JY, et al. Crystal-Structure of Cholesterol Oxidase Complexed with a Steroid Substrate - Implications for Flavin Adenine-Dinucleotide Dependent Alcohol Oxidases. Biochemistry. 1993;32(43):11507–15.

24. Uhia I, et al. Initial step in the catabolism of cholesterol by Mycobacterium smegmatis mc2155. Environ Microbiol. 2011;13(4):943–59.
25. Ezraty B, et al. Oxidative stress, protein damage and repair in bacteria. Nat Rev Microbiol. 2017;15(7):385–96.
26. Donova MV, et al. Mycobacterium sp. mutant strain producing 9α-hydroxyandrostenedione from sitosterol. Applied Microbiology Biotechnology. 2005;67(5):671–8.
27. Engineered 3-Ketosteroid 9α-Hydroxylases in Mycobacterium neoaurum: an Efficient Platform for Production of Steroid Drugs. Applied and Environmental Microbiology, 2018. 84(14): p. e02777-17.
28. Heras LFDL, et al. Molecular characterization of three 3-ketosteroid-Δ(1)-dehydrogenase isoenzymes of Rhodococcus ruber strain Chol-4. J Steroid Biochem Mol Biol. 2012;132(3–5):271–81.
29. Mathieu JM, et al. 7-Ketocholesterol Catabolism by Rhodococcus jostii RHA1. Applied Environmental Microbiology. 2010;76(1):352–5.
30. Peng H, et al. A Dual Role Reductase from Phytosterols Catabolism Enables the Efficient Production of Valuable Steroid Precursors. Angew Chem Int Ed Engl. 2021;60(10):5414–20.
31. Sun WJ, et al. Characterization and engineering control of the effects of reactive oxygen species on the conversion of sterols to steroid synthons in Mycobacterium neoaurum. Metab Eng. 2019;56:97–110.
32. Yuan JJ, et al. Side-chain cleavage of phytosterols by Mycobacterium sp MB 3683 in a biphasic ionic liquid/aqueous system. J Chem Technol Biotechnol. 2016;91(10):2631–7.
33. Shen Y, et al. Cyclic utilization of HP-β-CD in the bioconversion of cortisone acetate by Arthrobacter simplex. Biotech Lett. 2016;38(4):597–602.
34. Xiong LB, et al., Improving the production of 22-hydroxy-23,24-bisnorchol-4-ene-3-one from sterols in Mycobacterium neoaurum by increasing cell permeability and modifying multiple genes. Microbial Cell Factories, 2017. 16.
35. Xiong LB, et al., Enhancing the bioconversion of phytosterols to steroidal intermediates by the deficiency of kasB in the cell wall synthesis of Mycobacterium neoaurum. Microbial Cell Factories, 2020. 19(1).
36. Sun BB, et al., A CRISPR-Cpf1-Assisted Non-Homologous End Joining Genome Editing System of Mycobacterium smegmatis. Biotechnology Journal, 2018. 13(9).
37. Yan MY, et al., CRISPR-Cas12a-Assisted Recombineering in Bacteria. Applied and Environmental Microbiology, 2017. 83(17).
38. Stover CK, et al. New Use of Bcg for Recombinant Vaccines. Nature. 1991;351(6326):456–60.
39. Liu XC, et al., Biotransformation of Phytosterols to Androst-1,4-Diene-3,17-Dione by Mycobacterium sp. ZFZ Expressing 3-Ketosteroid-Delta(1)-Dehydrogenase. Catalysts, 2020. 10(6).

Tables

| Table 1 |

| Strains and plasmids used in this study |
| Name                        | Description                                                                                       | Source       |
|-----------------------------|----------------------------------------------------------------------------------------------------|--------------|
| **Strains**                 |                                                                                                    |              |
| *M. neoaurum DSM 44704*     | Wild type strain, Sterol consumer with no detectable intermediates                                |              |
| ΔkstD                       | *kstD1, kstD2, and kstD3 deleted in *M. neoaurum DSM 44704*                                        | This study   |
| ΔkstDΔhsd4A                 | *hsd4A deleted in ΔkstD strain*                                                                    | This study   |
| ΔkstDΔfadA5                 | *fadA5 deleted in ΔKstD strain*                                                                    | This study   |
| ΔkstDΔhsd4A-hsd4A           | ΔkstDΔhsd4A strain harboring P38Mu-Hsd4A                                                          | This study   |
| ΔkstDΔfadA5-fadA5           | ΔkstDΔfadA5 strain harboring P38Mu-FadA5                                                           | This study   |
| ΔkstDΔhsd4AΔfadA5           | *hsd4A and fadA5 deleted in ΔkstD strain*                                                          | This study   |
| ΔkstDΔhsd4AΔfadA5-NK        | ΔkstDΔhsd4AΔfadA5 strain harboring P38Mu-NK                                                       | This study   |
| **Plasmids**                |                                                                                                    |              |
| PSBY1                       | Derived from pMV261 and contains FnCpf1 *C. glutamicum* codon-optimized; KanR                      | [36]         |
| PCR-Hyg                     | Plasmid for sgRNA production                                                                         | [37]         |
| Pam-KstD1                   | PCR-Hyg containing *kstD1* spacer                                                                    | This study   |
| Pam-KstD2                   | PCR-Hyg containing *kstD2* spacer                                                                    | This study   |
| Pam-KstD3                   | PCR-Hyg containing *kstD3* spacer                                                                    | This study   |
| Pam-Hsd4A                   | PCR-Hyg containing *hsd4A* spacer                                                                    | This study   |
| Pam-FadA5                   | PCR-Hyg containing *fadA5* spacer                                                                    | This study   |
| pMV306                      | *Mycobacterium* integrative vector without promoter, kanR                                            | [38]         |
| P38Mu                       | pMV306 with Psmyc promoter, KanR                                                                     | [39]         |
| P38Mu-Hsd4A                 | Recombinant P38Mu for expression of *hsd4A*                                                        | This study   |
| P38Mu-FadA5                 | Recombinant P38Mu for expression of *fadA5*                                                        | This study   |
P38Mu-NK  Recombinant P38Mu for expression of *nox* and *katE*  This study

Table. 2

Relative production purity of *M. neoaurum* DSM 44074 and its derivative strains

| strain                  | Relative Purity (%) | 9-OH-AD  | 9-OH-4-HP | AD    | Others |
|-------------------------|---------------------|----------|-----------|-------|--------|
|                         |                     | 0        | 0         | 0     | 0      |
| DSM 44704               | 0.0±0.0             | 90.4±3.1 | 5.6±2.5   | 1.9±0.2 | 2.1±0.8 |
| ΔkstD                   | 7.2±2.2             | 88.6±1.3 | 2.4±0.5   | 1.8±1.3 |
| ΔkstDΔhsd4A             | 8.0±1.9             | 86.0±3.5 | 3.3±0.4   | 2.7±0.5 |
| ΔkstDΔfadA5             | 90.0±2.2            | 5.2±1.7  | 1.8±0.2   | 3.0±0.3 |
| ΔkstDΔhsd4A-hsd4A       | 88.5±3.6            | 4.4±2.3  | 1.3±0.1   | 5.8±1.2 |
| ΔkstDΔfadA5-fadA5       | 2.0±1.1             | 94.9±1.2 | 0.3±0.1   | 2.8±0.3 |
| ΔkstDΔhsd4AΔfadA5       | 2.0±1.1             | 97.0±1.1 | 0.3±0.1   | 0.7±0.3 |
| ΔkstDΔhsd4AΔfadA5-NK    | 0.0±0.0             | 90.4±3.1 | 5.6±2.5   | 1.9±0.2 | 2.1±0.8 |
| ΔkstDΔhsd4AΔfadA5-NK    | 7.2±2.2             | 88.6±1.3 | 2.4±0.5   | 1.8±1.3 |
| ΔkstDΔhsd4AΔfadA5-NK    | 8.0±1.9             | 86.0±3.5 | 3.3±0.4   | 2.7±0.5 |
| ΔkstDΔhsd4AΔfadA5-NK    | 90.0±2.2            | 5.2±1.7  | 1.8±0.2   | 3.0±0.3 |
| ΔkstDΔhsd4AΔfadA5-NK    | 88.5±3.6            | 4.4±2.3  | 1.3±0.1   | 5.8±1.2 |
| ΔkstDΔhsd4AΔfadA5-NK    | 2.0±1.1             | 94.9±1.2 | 0.3±0.1   | 2.8±0.3 |
| ΔkstDΔhsd4AΔfadA5-NK    | 2.0±1.1             | 97.0±1.1 | 0.3±0.1   | 0.7±0.3 |

Table. 3

Maximum yield and molar yield of 9-OH-4-HP from ΔkstDΔhsd4AΔfadA5 and ΔkstDΔhsd4AΔfadA5-NK

| phytosterols concentration (g L⁻¹) | ΔkstDΔhsd4AΔfadA5 | ΔkstDΔhsd4AΔfadA5-NK |
|------------------------------------|-------------------|----------------------|
| Maximum yield (g L⁻¹)              | Maximum molar yield (%) | Maximum yield (g L⁻¹) | Maximum molar yield (%) |
| 1                                  | 0.66±0.02         | 78.9±2.4             | 0.68±0.04         | 81.2±4.8         |
| 2                                  | 1.43±0.06         | 84.8±3.6             | 1.53±0.06         | 90.8±3.6         |
| 5                                  | 2.78±0.11         | 66.4±2.6             | 3.58±0.15         | 85.5±3.6         |
| 8                                  | 1.98±0.08         | 31.5±1.2             | 2.51±0.14         | 38.5±2.1         |
| 10                                 | 1.73±0.09         | 20.7±1.1             | 2.73±0.19         | 32.6±1.1         |

Figures
A schematic diagram of physterols side chain degradation in Mycobacterium. ChoM, Cholesterol oxidase; CYP125, cytochrome P450 125; ChsEs, acyl-CoA dehydrogenases; ChsHs, 3-oxo-23,24-bisnorcholest-4,17(20)-dien-22-yl-CoA-hydrolase; Ltp2, lipid transfer protein 2; Hsd4A, 17β-hydroxysteroid dehydrogenase / β-hydroxyacyl-CoA dehydrogenase; FadA5, acetyl-CoA acetyltransferase/thiolase; KstD, 3-ketosteroid-Δ1-dehydrogenase; KSH, 3-ketosteroid-9α-hydroxylase; 22-OH-24-CDOE-COA, 22-hydroxy-3,24-dioxo-4-ene-cholest-COA; 24-CTOE-COA, 3,22,24-trioxo-4-ene-cholest-COA; 22-PDOE-COA, 3,22-dioxo-4-ene-pregna-COA; 20-POECA, 3-oxo-4-ene-pregna-20-carboxylic acid; 20-POECAH, 3-oxo-4-ene-pregna-20-carboxyaldehyde.
Figure 2

Phenotypic analyses of the metabolites of phytosterol by M. neoaurum DSM 44074 and its derivative strains. Peak A, 9-OH-AD; Peak B, 9-OH-4-HP. (a) HPLC chromatogram comparison of the products of M. neoaurum DSM 44704 and ΔkstD with 1 g L-1 phytosterols feed. (b) HPLC chromatogram comparison of the products of ΔkstD, ΔkstDΔhsd4A, ΔkstDΔfadA5 and ΔkstDΔhsd4AΔfadA5 with 1 g L-1 phytosterols feed. (c) HPLC chromatogram comparison of the products of ΔkstD, hsd4A complement strain ΔkstDΔhsd4A-hsd4A and fadA5 complement strain ΔkstDΔfadA5-fadA5, with 1 g L-1 phytosterols feed. (d) structure of peak A, 9-OH-AD, and peak B, 9-OH-4-HP.
Figure 3

9-hydroxy steroids accumulation from 1 g L-1 phytosterols. (a) Time course of 9-OH-AD accumulation; (b) Time course of 9-OH-4-HP accumulation; single deletion of hsd4A or fadA5 caused increase of 9-OH-4-HP, and double deletion of hsd4A and fadA5 could obviously increase the productivity and selectivity of 9-OH-4-HP.

Figure 4

9-hydroxy steroids accumulation of ΔkstDΔhsd4AΔfadA5 from different concentrations of phytosterols. (a) Time course of 9-OH-AD accumulation; (b) Time course of 9-OH-4-HP accumulation; As the phytosterols concentration increased, the ability of the strain ΔkstDΔhsd4AΔfadA5 to transform phytosterols was inhibited.
Figure 5

Time profiles of 9-hydroxy steroids accumulation, extracellular H2O2 concentration, and the NAD+/NADH ratio of the strain ΔkstDΔhsd4AΔfadA5 and the strain ΔkstDΔhsd4AΔfadA5-NK. (a) the cell growth; (b) intracellular NAD+/NADH ratio; (c) extracellular H2O2 concentration; (d) time course of 9-OH-4-HP accumulation of ΔkstDΔhsd4AΔfadA5-NK from different concentrations of phytosterols; (d) time course of 9-OH-AD accumulation of ΔkstDΔhsd4AΔfadA5-NK from different concentrations of phytosterols.
Expression of Nox and KatE is beneficial for cell growth, increases the ratio of intracellular NAD+/NADH, decreases the extracellular H2O2 concentration, and enhance the yield of 9-OH-4-HP under a high concentration of phytosterol.

**Supplementary Files**

This is a list of supplementary files associated with this preprint. Click to download.

- [AddtionalFiles.docx](#)