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 Yuan1,2,3, Zhi-Guo Ma1, Jing-Xian Zhang1,3, Xiang-Cen Liu1,3, Gui-Lin Du1,2,3, Jun-Song Sun1,2,3*, Ji-Ping Shi1,2,3 and Bao-Guo Zhang1,3*

**Abstract**

**Background:** Steroid drugs are essential for disease prevention and clinical treatment. However, due to intricate steroid structure, traditional chemical methods are rarely implemented into the whole synthetic process for generating steroid intermediates. Novel steroid drug precursors and their ideal bacterial strains for industrial production have yet to be developed. Among these, 9,21-dihydroxy-20-methyl-pregna-4-en-3-one (9-OH-4-HP) is a novel steroid drug precursor, suitable for the synthesis of corticosteroids. In this study, a combined strategy of blocking Δ1-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:** The Δ1-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 Δ1-dehydrogenation-deficient strain. The two engineered strains were able to accumulate 0.59 g L⁻¹ and 0.47 g L⁻¹ 9-OH-4-HP from 1 g L⁻¹ phytosterols, respectively. Furthermore, *Hsd4A* and *fadA5* were knocked out simultaneously in the Δ1-dehydrogenation-deficient strain. The 9-OH-4-HP production from the Hsd4A and FadA5 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 strain, respectively. The purity of 9-OH-4-HP obtained from the Hsd4A and FadA5 deficient strain has reached 94.9%. Subsequently, the catalase *katE* from *Mycobacterium neoaurum* and an NADH oxidase, *nox*, from *Bacillus subtilis* were overexpressed to improve the intracellular environment, leading to a higher 9-OH-4-HP production. Ultimately, 9-OH-4-HP production reached 3.58 g L⁻¹ from 5 g L⁻¹ phytosterols, and the purity of 9-OH-4-HP improved to 97%. The final 9-OH-4-HP production strain showed the best molar yield of 85.5%, compared with the previous reported strain with 30% molar yield of 9-OH-4-HP.

© The Author(s) 2021. Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/. The Creative Commons Public Domain Dedication waiver (http://creativecommons.org/publicdomain/zero/1.0/) applies to the data made available in this article, unless otherwise stated in a credit line to the data.
Conclusion: KstD, Hsd4A, and FadA5 are key enzymes for phytosterol side-chain degradation in the C19 pathway. Double deletion of hsd4A and fadA5 contributes to the blockage of the C19 pathway. Improving the intracellular environment of Mycobacterium neoaurum during phytosterol bioconversion could accelerate the conversion process and enhance the productivity of target steroid derivatives.

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

Background
Steroid drugs, including mineralocorticoids, glucocorticoids, and sex hormones, are crucial in the prevention and clinical treatment of various diseases [1, 2]. 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 some researchers. Certain C22 steroids are ideal precursors for steroid drug synthesis [3]. Among these steroids, 9,21-dihydroxy-20-methyl-pregna-4-en-3-one (9-OH-4-HP) is a valuable and novel steroid derivative for the synthesis of corticosteroids because of its substituents at positions C-9 and C-21. 9-OH-4-HP was commonly identified as a by-product during bioconversion of sterol to 9-hydroxy steroid derivatives in several Mycobacterium species, such as Mycobacterium sp. 2–4M that produces a 1.5–1.6% molar yield of 9-OH-4-HP [4]. However, ideal industrial strains for 9-OH-4-HP production have not yet to be developed.

Due to its mild reaction conditions in the process of steroid synthesis, the microbial transformation has caught increasing attention for medicinal chemists [5]. Among these reactions, Δ1-dehydrogenation was one of the most thoroughly investigated the Δ1-dehydrogenation of the sterol skeleton was catalyzed by 3-ketosteroid-1(2)-dehydrogenase (KstD) [6]. Strains with inactivation of KstD generally leads to various 9α-hydroxy derivatives after culture with steroids, such as 9-hydroxy-androst-4-ene-3,17-dione (9-OH-AD) and 9-OH-4-HP. 9α-hydroxy derivatives are important precursors in the production of several modern glucocorticoid drugs with a halogen at the 9α position [7]. Other than industrial strains with KstD deficiency that accumulate 9α-hydroxy derivatives after culture with sterol [6], a few wild-type strains of Mycobacterium have been reported to be able to produce 9-OH-AD [4].

Dual competing pathways, the overwhelming C19 steroid pathway, and the C22 steroid pathway are involved into phytosterol side-chain degradation (Fig. 1). Recently, the 17-hydroxysteroid/22-OH-BNC-CoA dehydrogenase Hsd4A was found relevant to C22 steroid formation [3].
oxidase in *M. neoaurum* JC-12 increased ADD production by 43% [10]. 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 44,074, as a sterol consumer with no steroid final products, was constructed for the bioconversion of phytosterols to 9-OH-4-HP. A kstD 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 (Fig. 1), kstDs were beforehand identified and subsequently knocked out from the genome of the wild-type strain *M. neoaurum* DSM 44074, a steroid-degrading *Mycobacterium* that can completely degrade phytosterols into CO$_2$ and H$_2$O [13]. 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 with
a CRISPR-assisted nonhomologous end-joining strategy as described in the Methods section, resulting in a mutant strain ΔKstD. The cell growth of the ΔkstD strain showed no significant difference from that of the wild-type strain (Additional file 1: Fig. S1). The wild-type M. neoaurum DSM 44074 strain and the genetically modified strain ΔkstD were incubated with phytosterols for 168 h. 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, proving the elimination of Δ1-dehydrogenation by kstDs knockout. When MP01 medium plus 1 g L\(^{-1}\) phytosterols was used for the 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 b). The molar yield of 9-OH-AD reached 84.9%. 9-OH-4-HP was the by-product during phytosterol bioconversion by ΔkstD, with a molar yield of 4.8%, and 5.6% purity (Table 1). 9α-hydroxy derivatives successfully accumulated during phytosterol bioconversion by engineered Mycobacterium, but the purity and yield of 9-OH-4-HP didn't show satisfactory.

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

Dual pathways, the C19 steroid pathway, and the C22 steroid pathway compete during phytosterol side-chain degradation (Fig. 1). The C19 steroid pathway is considered as the dominant pathway in M. neoaurum DSM 44074 because ΔkstD produces 9-OH-AD as the main

![Fig. 2 Phenotypic analyses of the metabolites of phytosterol by M. neoaurum DSM 44074 and its derivative strains. a HPLC chromatogram comparison of the products of M. neoaurum DSM 44074 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](image-url)
product along with little 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-3,24-dioxo-4-ene-cholest-CoA), which could be Δ^{22}-dehydrogenated by the β-hydroxyacyl-CoA dehydrogenase Hsd4A and generate 3,22,24-trioxo-4-ene-cholest-CoA (24-CTOE-CoA). 24-CTOE-CoA could subsequently be catalyzed by the thiolase FadA5, leading the phytosterol degradation flux to the C19 pathway (Fig. 1).

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 Δ*kstD*, resulting in the strains Δ*kstD*Δ*hsd4A* and Δ*kstD*Δ*fadA5*.

The cell growth of Δ*kstD*Δ*hsd4A* and Δ*kstD*Δ*fadA5* showed no significant difference from that of the wild-type strain *M. neoaurum* DSM 44074 (Additional file 1: Fig. S1). The strains Δ*kstD*Δ*hsd4A* and Δ*kstD*Δ*fadA5* were cultured with phytosterols for 168 h, and the metabolites were analyzed by HPLC (Fig. 2b). As shown in Fig. 3b, 9-OH-4-HP, successfully accumulated in both strains Δ*kstD*Δ*hsd4A* and Δ*kstD*Δ*fadA5* as a major production. The purity of 9-OH-4-HP from the Δ*kstD*Δ*hsd4A* and Δ*kstD*Δ*fadA5* strains was 88.6% and 86.0%, respectively. Still, both strains showed a small amount of 9-OH-AD accumulation (Fig. 3a). The purity of 9-OH-AD from strains Δ*kstD*Δ*hsd4A* and Δ*kstD*Δ*fadA5* were 7.2% and 8.0%, respectively. After culture with 1 g L⁻¹ phytosterols in MP01 medium, the strain Δ*kstD*Δ*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 Δ*kstD*Δ*fadA5*. Interestingly, the molar yields of 9-OH-4-HP and 9-OH-AD from strain Δ*kstD*Δ*fadA5* were both lower than that of from strain Δ*kstD*Δ*hsd4A*. The molar yield of 9-OH-4-HP from strain Δ*kstD*Δ*fadA5* was 20.3% lower than that from strain Δ*kstD*Δ*hsd4A*, and the molar yield of 9-OH-AD from strain Δ*kstD*Δ*fadA5* which was 38.5% lower than that from strain Δ*kstD*Δ*hsd4A*.

Considering that 9-OH-AD still accumulated in both the Δ*kstD*Δ*hsd4A* and Δ*kstD*Δ*fadA5* strains, the C19 steroidal pathway of the phytosterol degradation pathway was not probably completely blocked in either strain. To enhance the purity and production of 9-OH-4-HP and obstruct the yield of 9-OH-AD, *hsd4A* and *fadA5* were simultaneously knocked out in strain Δ*kstD*, resulting in the strain Δ*kstD*Δ*hsd4AΔ*fadA5*. The cell growth of the Δ*kstD*Δ*hsd4AΔ*fadA5* strain showed a similar trend with the wild-type strain *M. neoaurum* DSM 44,074 (Fig. S1). As shown in Fig. 2b, after culture with phytosterols, the strain Δ*kstD*Δ*hsd4AΔ*fadA5* accumulated 9-OH-4-HP as the main product, while the accumulation of 9-OH-AD was significantly decreased, compared with the strains

### Table 1 Relative production purity of *M. neoaurum* DSM 44074 and its derivative strains

| Strain           | Relative purity (%) | 9-OH-AD | 9-OH-4-HP | AD | Others |
|------------------|---------------------|--------|-----------|----|--------|
| DSM 44074        | 90.4 ± 3.1          | 5.6 ± 2.5 | 19 ± 0.2   | 21 ± 0.8 |
| Δ*kstD*          | 7.2 ± 2.2           | 88.6 ± 1.3 | 2.4 ± 0.5   | 18 ± 1.3 |
| Δ*kstDΔ*hsd4A*   | 8.0 ± 1.9           | 860 ± 3.5 | 3.3 ± 0.4   | 27 ± 0.5 |
| Δ*kstDΔ*fadA5*   | 900 ± 2.2           | 52 ± 1.7   | 18 ± 0.2   | 30 ± 0.3 |
| Δ*kstDΔ*hsd4AΔ*fadA5* | 885 ± 3.6    | 44 ± 2.3   | 13 ± 0.1   | 58 ± 1.2 |
| Δ*kstDΔ*hsd4AΔ*fadA5-NK | 2.0 ± 1.1   | 94.9 ± 1.2 | 0.3 ± 0.1   | 28 ± 0.3 |

*Fig. 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 increment of 9-OH-4-HP, and double deletion of *hsd4A* and *fadA5* could obviously increase the productivity and purity of 9-OH-4-HP.
ΔkstDΔhsd4A and ΔkstDΔfadA5. The purity of 9-OH-4-HP from strain ΔkstDΔhsd4AΔfadA5 was 94.9%, obviously higher than those from strains ΔkstDΔhsd4A and ΔkstDΔfadA5. Meanwhile, the accumulation of 9-OH-AD from strain ΔkstDΔhsd4AΔfadA5 was significantly decreased at a purity of 2.0%. After culture with 1 g L⁻¹ phytosterols, 0.66 g L⁻¹ 9-OH-4-HP was obtained from strain ΔkstDΔhsd4AΔfadA5, which is 11.9% more than that from strain ΔkstDΔhsd4A and 40.4% more than that from strain ΔkstDΔfadA5 (Fig. 3b). The purity and production of 9-OH-4-HP from strain ΔkstDΔhsd4AΔfadA5 were both higher than those from strains ΔkstDΔhsd4A and ΔkstDΔfadA5, indicating that the double knockout of hsd4A and fadA5 could effectively block the accumulation of AD homologues.

To verify the functions of hsd4A and fadA5 during phytosterol degradation, ΔkstDΔhsd4A-hsd4A, the hsd4A complementation strain of strain ΔkstDΔhsd4A, and ΔkstDΔfadA5-fadA5, the fadA5 complementation strain of ΔkstDΔfadA5 were also constructed. As shown in Fig. 2c, the accumulation of 9-OH-AD was recovered when the two complementation strains were cultured with phytosterols. The purities of 9-OH-AD from ΔkstDΔhsd4A-hsd4A and ΔkstDΔfadA5-fadA5 were 90.0% and 88.5%, respectively, which are consistent with those of strain ΔkstD. These results indicate that Hsd4A and FadA5 are key enzymes in the C19 steroid pathway during phytosterol side-chain degradation. Phylogenetic trees of Hsd4A and FadA5 were constructed to elucidate the evolutionary relationship of the two enzymes (Additional file 1: 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 ΔkstDΔhsd4AΔfadA5 was 78.9%. To evaluate the ability of ΔkstDΔhsd4AΔfadA5 of transforming phytosterols into 9-OH-4-HP, higher concentrations of phytosterols were incubated with ΔkstDΔhsd4AΔfadA5.

As shown in Fig. 4a, 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 ΔkstDΔhsd4AΔfadA5 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. 4b). The molar yields of 9-OH-4-HP from different concentrations of phytosterols are listed in Table 2. The highest molar yield, 84.8% of 9-OH-4-HP, was obtained when strain ΔkstDΔhsd4AΔfadA5 was cultured with 2 g L⁻¹ phytosterols. Thence 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 relatively higher than those from strains ΔkstDΔhsd4A and ΔkstDΔfadA5.

Table 2 Maximum yield and molar yield of 9-OH-4-HP from ΔkstDΔhsd4AΔfadA5 and ΔkstDΔhsd4AΔfadA5-NK

| Phytosterols concentration (g L⁻¹) | 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              |

Fig. 4 9-hydroxy steroids accumulation of ΔkstDΔhsd4AΔfadA5 from different concentrations of phytosterols. a Time course of 9-OH-4-HP accumulation; b time course of 9-OH-AD accumulation; As the phytosterols concentration increased, the ability of the strain ΔkstDΔhsd4AΔfadA5 to transform phytosterols was inhibited.
stable. Previous research has reported that phytosterols and their metabolites could be noxious to cells during bioconversion [9, 14–16], for the poor performance of ΔkstDΔhsd4AΔfadA5 under high concentration phytosterols bioconversion.

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

The 9-OH-4-HP-producing strain ΔkstDΔhsd4AΔfadA5 did not perform well when fed with high concentration phytosterols. This might be due to multiple factors that influence the bioconversion of phytosterols.

A series of redox reactions occur by oxygen as an electron acceptor, during phytosterols degradation, cholesterol dehydrogenases/isomerases require intracellular nicotinamide adenine dinucleotides (NAD⁺ and NADH) as cofactors [17, 18]. NAD⁺ and NADH play crucial roles during phytosterols transformation. They act in many oxidation–reduction reactions and regulate various enzymatic activities and genetic processes. One molecule of NAD⁺ accepts an H⁺ and two electrons then generates one molecule of NADH with oxidation occurrence, while the regeneration of NAD⁺ from NADH is insufficient during phytosterols side-chain degradation. 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 a great assistance for phytosterol transformation.

In addition, hydrogen peroxide (H₂O₂) is produced with incomplete oxidations during aerobic metabolism and the regeneration of flavin adenine dinucleotide (FAD) in the phytosterol transformation process [10]. A high level of H₂O₂ can damage proteins, DNA, and lipids in cells, resulting in an inhibition of cell growth and a low metabolite yield [19].

To enhance the ability of strain ΔkstDΔhsd4AΔfadA5 to transform phytosterols into 9-OH-4-HP, the catalase KATE from M. neoaurum DSM 44,074 and the NADH oxidase NOX from Bacillus subtilis [10], were co-expressed in strain ΔkstDΔhsd4AΔfadA5, resulting in the strain ΔkstDΔhsd4AΔfadA5-NK.

The extracellular H₂O₂ concentrations of the two strains ΔkstDΔhsd4AΔfadA5 and ΔkstDΔhsd4AΔfadA5-NK were measured when they were cultured with 5 g L⁻¹ phytosterols for 168 h. As shown in Fig. 5a, the extracellular H₂O₂ concentration of strain ΔkstDΔhsd4AΔfadA5 showed an upward trend during the bioconversion process. The extracellular H₂O₂ concentration increased from an initial concentration of 0.59 µmol L⁻¹ to a final concentration of 1.05 µmol L⁻¹ after 168 h. In contrast, the extracellular H₂O₂ concentration of strain ΔkstDΔhsd4AΔfadA5-NK remained low and stable during the bioconversion process at approximately 0.51 µmol L⁻¹, which could convince that the overexpression of katE eliminated excessive extracellular H₂O₂. Moreover, to verify the toxicity of H₂O₂, cell growth of the strains ΔkstDΔhsd4AΔfadA5 and ΔkstDΔhsd4AΔfadA5-NK were also measured. As shown in Fig. 5b, the biomass of strain ΔkstDΔhsd4AΔfadA5-NK was higher than that of strain ΔkstDΔhsd4AΔfadA5, indicating that the elimination of extracellular H₂O₂ could help with cell growth.

Likewise, the NAD⁺/NADH ratios of the strains ΔkstDΔhsd4AΔfadA5 and ΔkstDΔhsd4AΔfadA5-NK were also measured after they were cultured with 5 g L⁻¹ phytosterols for 168 h. As shown in Fig. 5c, the NAD⁺/NADH ratio of strain ΔkstDΔhsd4AΔfadA5-NK was consistently higher than that of strain ΔkstDΔhsd4AΔfadA5. At 96 h, the NAD⁺/NADH ratio of strain ΔkstDΔhsd4AΔfadA5-NK was enhanced by 25.4%, compared with that of strain ΔkstDΔhsd4AΔfadA5. 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 ΔkstDΔhsd4AΔfadA5 to transform phytosterols into 9-OH-4-HP. The recombinant strain ΔkstDΔhsd4AΔfadA5-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 production of 9-OH-4-HP was measured by every 12 h. As shown in Fig. 5d, the final productions 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 ΔkstDΔhsd4AΔfadA5, the productions of 9-OH-4-HP were enhanced by 3.03%, 6.99%, 28.7%, 26.8%, and 57.8% under the same conditions. The highest yield of 9-OH-4-HP was obtained when strain ΔkstDΔhsd4AΔfadA5-NK was cultured with 5 g L⁻¹ phytosterols, with a molar yield of 85.5%,28.8% higher than that of ΔkstDΔhsd4AΔfadA5. Moreover, no significant difference in the production of 9-OH-AD was observed between ΔkstDΔhsd4AΔfadA5 and ΔkstDΔhsd4AΔfadA5-NK (Fig. 5e), indicating that the purity of 9-OH-4-HP was also enhanced during phytosterol bioconversion by the strain ΔkstDΔhsd4AΔfadA5-NK. All of the results above confirm controlling the intracellular NAD⁺/NADH ratio and H₂O₂ levels are effective 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* ATCC 25795, 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 many 9-OH-AD as the main product and little 9-OH-4-HP as a by-product. Compared with other 9-OH-AD-producing strains, the purity and molar yield of 9-OH-AD from ΔkstD after culture with phytoesters were notably higher. For example, Mycobacterium sp. 2-4 M [4] 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 [20]. In a kstDs knockout strain of M. neoaurum ATCC 25795, both the 55% molar yield of 9-OH-AD, and the 15% molar yield of AD were obtained from 15 g L\(^{-1}\) phytoesters. 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 identities to kstDs (approximately 38%) [21]. The existence of more than 3 kstDs has also been reported for other Rhodococcus species, such as R. jostii Rha1 [22]. Thus, inactivation of all KstD may provide the fundamental premise to develop promising 9α-hydroxy derivatives producing strains.

Theoretically, lipid transfer protein Ltps catalyzes the transformation from 22-OH-24-CDOE-CoA to 4-HP (Fig. 1), but so far, no specific Ltps have been identified. Thus, manipulation of Hsd4A is usually chosen to control the metabolic flux to generate C19 steroids or C22 steroids. Xu reported the characterization of Hsd4A in vivo and in vitro, testing 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 from M. neoaurum ATCC 25,795. This mutant strain displayed a 32% molar yield of 9-OH-4-HP and a 15% molar yield of 9-OH-AD from 40 g L\(^{-1}\) phytoesters [3]. Here, in this research, it was confirmed that double knockout of hsd4A and fadA5 could further block the C19 steroid pathway. The molar yield of 9-OH-4-HP of strain ΔkstDsΔhsd4AΔfadA5 was notably higher than those of Xu’s strain, which is 90.8% versus 32%.

As shown in Table 2, the molar yield of 9-OH-4-HP decreased with the increase of phytoesters concentration. This might be due to the hindrance of phytoesters. On one hand, the aqueous solubility of phytoesters is too low to form a dispersive state [23], which impedes the contact within cells and further retard the phytoesters bioconversion. Therefore, with the phytosterol concentration increasing, phytosterol is hard to form dispersive solution, and further resulting in low molar yields of sterol derivatives productions. The major limitation on microbial transformation of phytoesters is due to its low solubility in aqueous media. The conversion yields can be improved by adding surface active agents to the transformation media, such as Tween 80 or Triton X-100, and other sterol-solubilizing agents like cyclodextrins [24]. Some surfactants like Tween 80 can reduce the tendency of Mycobacterium to aggregate [25] and thus promote the formation of stable suspensions of phytosterol by increasing phytosterol solubility and decreasing dynamic interfacial tension [23]. These above cause the improvement of phytosterol bioconversion. Sterol-solubilizing agents like cyclodextrins could form inclusion complexes with steroids and make them as the effective carriers to deliver of hydrophobic steroids to cells, which enhances the biotransformation of steroid compounds [26]. Phytoesters and their derivatives are also reported to be toxic and inhibitory to cell growth [26, 27], which also hinder the bioconversion and result in low molar yields. Balancing the intracellular environment is another promising method to improve phytoesters bioconversion. On one hand, NAD\(^+\) is greatly consumed during phytosterol bioconversion, which participates in many reactions of phytosterol side-chain degradation. On the other hand, phytoesters’ toxicity to cells impedes the bioconversion. In this study, when combining the abilities of NAD\(^+\) regeneration and H\(_2\)O\(_2\) elimination, the performance of the new mutant strain ΔkstDsΔhsd4AΔfadA5-NK was significantly higher than that from strain ΔkstDsΔhsd4AΔfadA5 at the same concentration phytoesters. The highest yield of 9-OH-4-HP was 3.58 g L\(^{-1}\) from ΔkstDsΔhsd4AΔfadA5-NK with 5 g L\(^{-1}\) phytoesters fed, which is 28.7% higher than that from strain ΔkstDsΔhsd4AΔfadA5. Again, these results proved that the elimination of H\(_2\)O\(_2\) and regeneration of NAD\(^+\) are effective methods to improve phytosterol bioconversion.

The accumulation of C19 steroids in the Hsd4A and/or FadA5 deficiency strains indicates incomplete blockage of the C19 steroid pathway. Similar results have been previously reported [3, 28]. An Hsd4A2 was testified to be dominant in M. neoaurum CCTCC AB2019054 rather than Hsd4A [28]. A protein in M. neoaurum DSM 44074 of 98.67% identity with Hsd4A2 in M. neoaurum CCTCC AB2019054 was found by blastp, indicating Hsd4A2 existing in M. neoaurum DSM 44074. Analysis of the M. neoaurum DSM 44074 genome implied more potential Hsd4A isoenzymes. Thus, the downstream gene of hsd4A, fadA5, was
chosen to be knocked out to reduce the accumulation of 9-OH-AD ulteriorly. However, 9-OH-AD still took up 2% of the products when Hsd4A and FadA5 deficiency strain was cultured with phytosterols. Similarly, analysis of the *M. neoaurum* DSM 44074 genome implied FadA5 isoenzymes existing, which could also explain why the strain with FadA5 deficiency also accumulated 9-OH-AD.

**Conclusion**

This study aimed to construct an efficient 9-OH-4-HP production strain, which is a novel and valuable sterol drugs precursor. The higher accumulation of 9-OH-4-HP was achieved by *hsd4A* and *fadA5* knockout to block the C19 steroid pathway and by 3-ketosteroid-Δ1-dehydrogenation deficiency with *kstDs* knockout. Compared with the Hsd4A deficiency or the FadA5 deficiency, the double deletion of *hsd4A* and *fadA5* could further block the C19 steroid pathway in phytosterol side-chain degradation. By eliminating H2O2 and regenerating NAD+ in the KstD, Hsd4A, and FadA5 deficiency strain, the 9-OH-4-HP yield was significantly improved. These findings provided some new insights into the accumulation of C22 steroids and methods by improving phytosterols bioconversion.

**Methods**

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

The strains and plasmids used in this study are described in Table 3. *E. coli* DH5α stored in the laboratory was used for plasmid amplification. Wild-type *M. neoaurum* DSM 44074 (DSM 44704) was purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, GERMANY). All other strains were derived from *M. neoaurum* DSM 44704. Common plasmids and primers (Additional file 1: Table S1) were used to construct the mutants. *E. coli* DH5α 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 K₂HPO₄·3H₂O, 5.4 g L⁻¹ NaNO₃, 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 K₂HPO₄·3H₂O, 1.0 g L⁻¹ MgSO₄·7H₂O, 2.0 g L⁻¹ NaNO₃, 2‰ Tween 80 (v/v), and an initial pH value 7.5) at 30 °C and 200 rpm.

The phytosterols consisted of 45% β-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-Hydroxypropyl)-β-cyclodextrin (HP-β-CD) was used as a sequestering agent.
Bioinformatic analysis
The genome of _M. neoaurum_ DSM 44,074 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™ Rapid DNA-Seq Kit (Bioo Scientific, USA). The sequencing data were assembled using SOAPdenovo2 (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 44,074 based on previous reports. The PSBY1 plasmid harbouring cpf1 was obtained from Jiang [29], and the PCR-Hyg plasmid harbouring sgRNA was obtained from Sun [30]. ClonExpressIIOne 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 Pmyc 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 Ndel site (and HindIII site, if two genes were inserted) of P38Mu using the ClonExpressIIOne 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 mL 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 44,074 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 H₂O₂ concentrations were measured according to the operating manual of the Hydrogen Peroxide (H₂O₂) 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.

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-Δ^1-dehydrogenases; KShs: 9α-Hydroxylase; Hps: CP2 steroids; HsdA: β-Hydroxysteracyl-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.

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s12934-021-01717-w.

Additional file 1: Table S1. Primers used in this work. Fig. S1. Cell growth of _M. neoaurum_ DSM 44074 and its mutant strains. Fig. S2. Phylogenetic trees of _hsd4A_ and _fadA5_.

Acknowledgements
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.
Authors' 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.

Funding
This research was funded by National Key R&D Program of Chin (No. 2017YFE0112700) and the Natural Science Foundation of China (No. 21906139).

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

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no conflict of interests.

Author details
1 Lab of Biofenery, Shanghai Advanced Research Institute, Chinese Academy of Sciences, No. 99 Haikai Road, Pudong, Shanghai 201210, China. 2 School of Life Science and Technology, ShanghaiTech University, Shanghai 201210, China. 3 University of Chinese Academy of Sciences, Beijing 100049, China.

Received: 5 August 2021 Accepted: 5 December 2021 Published online: 23 December 2021

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 lyric peptides. Nat Prod Res. 2012;26(11):1004–11.
3. Xu LQ, et al. Unraveling and engineering the production of 23,24-bisnorcholesterol by Rhodococcus jostii RHA1. Appl Microbiol Biotechnol. 2005;67(5):671–8.
4. Freydes P, et al. Microbial conversion of steroid compounds: recent developments. Enzyme Microb Technol. 2003;32(6):688–705.
5. Vender Geize R, et al. Targeted disruption of the kstD gene encoding a 3-ketosteroid delta-(1)-dehydrogenase isozyme of Rhodococcus erythropolis strain SQ1. Appl Environ Microbiol. 2000;66(5):2029–36.
6. Sedlacek L, Smith LL. Biotransformations of steroids. Crit Rev Biochem 1998;38(3):187–236.
7. Nesbitt NM, et al. A thiolase of Mycobacterium tuberculosis. Nat Prod Res. 2012;26(11):1004–11.
8. Peng H, et al. A dual role reductase from phytosterols catabolism enables the efficient production of valuable steroid precursors. Angew Chem Int Ed. 2021;60(10):5414–20.
9. Sun BB, et al. A CRISPR-Cpf1-assisted non-homologous end joining genome editing system of Mycobacterium smegmatis mc²155. Environ Microbiol. 2011;13(4):943–59.
10. Peng H, et al. Molecular characterization of three 3-ketosteroid-(3)-dehydrogenase isozymes of Rhodococcus ruber strain Chol-4. J Steroid Biochem Mol Biol. 2012;132(3–5):271–81.
11. Mathieu JM, et al. 7-Ketosteroid-steroid dehydrogenase isoenzymes of Mycobacterium smegmatis mc²155. Environ Microbiol. 2021;13(7):385–96.
12. Liu H-H, et al. Engineered 3-Kketosteroid carboxylases in Mycobacterium neoauratum: an efficient platform for production of steroid drugs. Appl Environ Microbiol. 2018;84(14):e02777-e2817.

Publisher's Note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.