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Article

Chemo-Enzymatic Strategy for the Efficient Synthesis of Steroidal Drugs with 10α-Methyl Group and a Side Chain at C17-Position from Biorenewable Phytosterols

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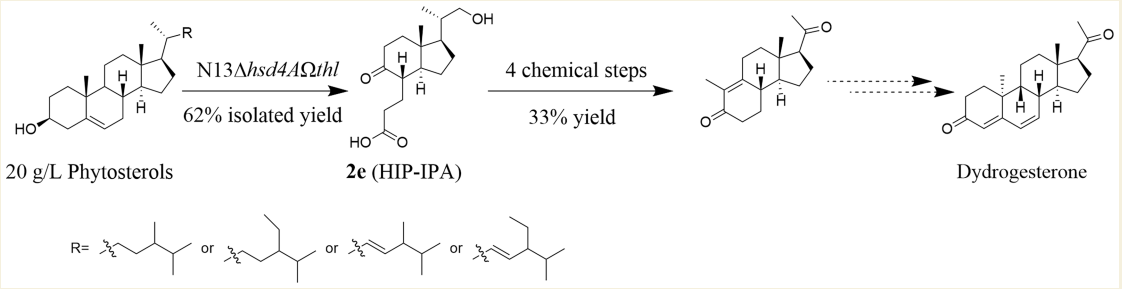

ABSTRACT: Steroidal pharmaceuticals with a 10α-methyl group or without the methyl group at C10-position are important medicines, but their synthesis is quite challenging, due to that the natural steroidal starting materials usually have a 10β-methyl group which is difficult to be inverted to 10α-methyl group. In this study, 3-((1*R*,3a*S*,4*S*,7a*R*)-1-((*S*)-1-hydroxypropan-2-yl)-7a-methyl-5-oxooctahydro-1*H*-inden-4-yl) propanoic acid (HIP-IPA, **2e**) was demonstrated as a valuable intermediate for the synthesis of this kind of active pharmaceutical ingredients (APIs) with a side chain at C17-position. Knockout of a β-hydroxyacyl-CoA dehydrogenase gene and introduction of a sterol aldolase gene into the genetically modified strains of *Mycobacterium fortuitum* (ATCC 6841) resulted in strains N13Δ*hsd4AΩthl* and N33Δ*hsd4AΩthl*, respectively. Both strains transformed phytosterols into **2e**. Compound **2e** was produced in 62% isolated yield (25 g) using strain N13Δ*hsd4AΩthl*, and further converted to (3S,3aS,9aS,9bS)-3-acetyl-3a,6-dimethyl-1,2,3,3a,4,5,8,9,9a,9b-decahydro-7H-cyclopenta[a]naphthalen-7-one, which is the key intermediate for the synthesis of dydrogesterone. This study not only overcomes a challenging synthetic problem by enabling an efficient synthesis of dydrogesterone-like steroidal APIs from phytosterols, the well-recognized cheap and readily available biobased raw materials, but also provides insights for redesigning the metabolic pathway of phytosterols to produce other new compounds of relevance to the steroidal pharmaceutical industry.

KEYWORDS: *steroidal pharmaceuticals, sustainable synthesis, chemo-biocatalytic synthesis, dydrogesterone, HIP-IPA*

INTRODUCTION

Steroidal pharmaceuticals are a class of important medicines second only to antibiotics. There are six steroid medicines with a 10α-methyl group or without the methyl group at the C10-position in the list of top 200 small molecule pharmaceuticals, and they had over a 5.5 billion global market value in 2022.[1] Because the natural steroids usually have a 10β-methyl group and the steroidal active pharmaceutical ingredients (APIs) are semisynthesized from the natural steroidal compounds, the synthesis of steroidal APIs with a 10α-methyl group or without the methyl group at the C10-position is quite challenging and tedious. Recently, 3aα-H-4α-(3′-propionic acid)-7aβ-methyl-hexahydro-1,5-indanedione (HIP) and 3aα-H-4α-(3′-propionic acid)-5α-hydroxy-7aβ-methylhexahydro-1-indanone-δ-lactone (sitolactone, or HIL), which are produced from biorenewable phytosterols by the genetically modified strains, offer an attractive route for the synthesis of a diversity of 19-norsteroids and steroidal APIs with a 10α-methyl group (Scheme 1a).[2−5] Some important steroidal APIs including progestational and adrenocortical hormones have a side-chain at C17, which is normally constructed by introducing a

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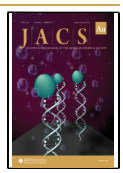
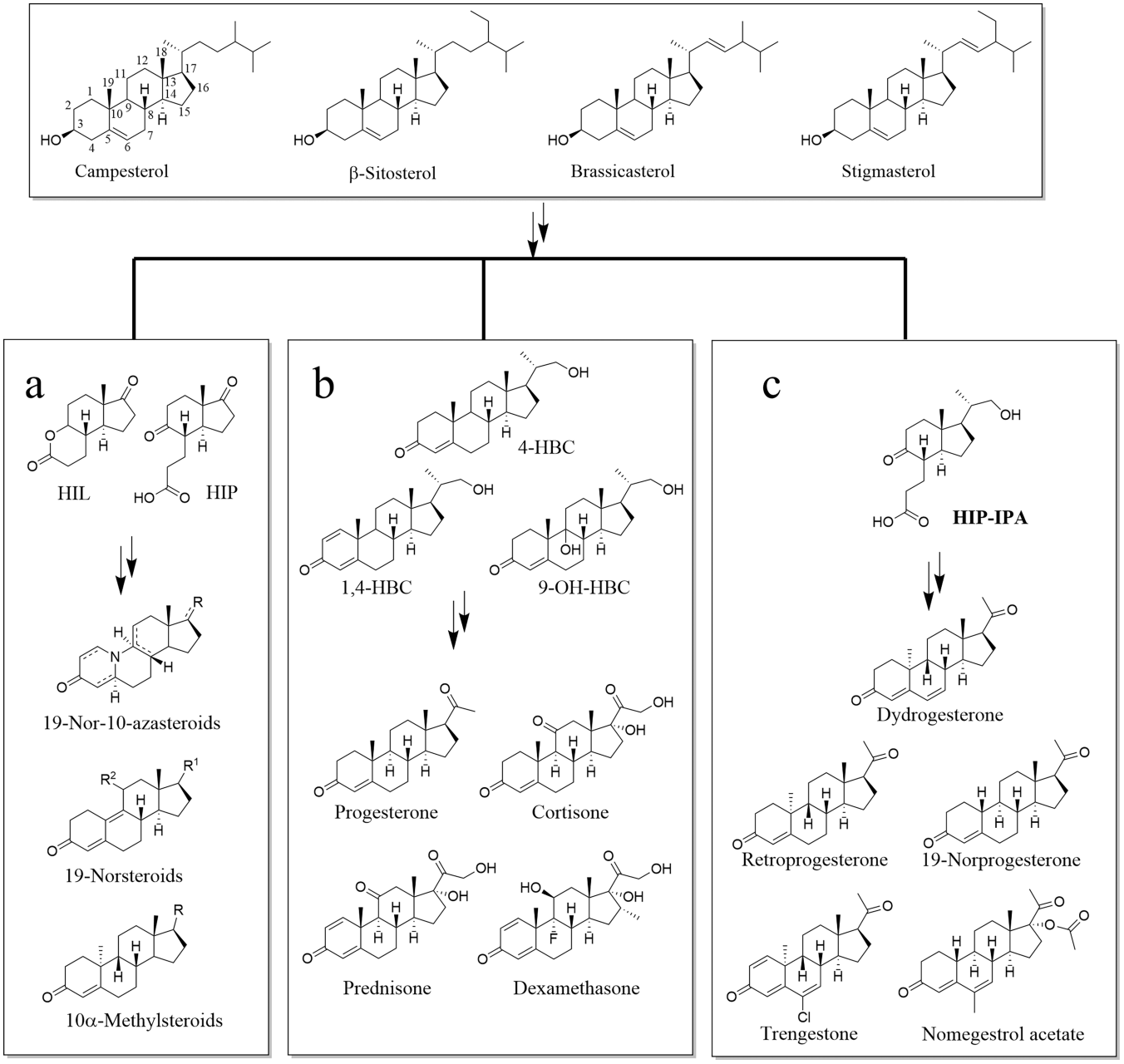


Scheme 1. Transformations of Phytosterols to Key Intermediates for the Synthesis of Various Types of Steroidal Active Pharmaceutical Ingredients (APIs). (a) With 10α-Methyl or Without 10-Methyl Group, (b) With 10β-Methyl Group and Side Chain at C-17, and (c) With 10α-Methyl or Without 10-Methyl Group, and Having C17-Side Chain



nucleophile at the C17 carbonyl group and subsequent transformations. The recently developed 22-hydroxy-23,24-bisnorchol-4-ene-3-one (4-HBC), 22-hydroxy-23,24-bisnorchol-1,4-dien-3-one (1,4-HBC), and 9,22-dihydroxy-23,24-bisnorchol-4-ene-3-one (9-OH-HBC),[6−8] which have a 3-carbon isopropanol side chain at C17-position, provide new intermediates for the concise synthesis of steroidal APIs with C-17 side chain and 10β-methyl group (Scheme 1b).

Many steroidal APIs have C-17 side chains and 10α-methyl group. For example, dydrogesterone (9β,10α-pregna-4,6-diene-3,20-dione) is a progestational hormone with no androgenic or estrogenic properties. The synthesis of this valuable API is quite challenging and one of the synthetic methods involves the A-ring cleavage of progesterone to the key intermediate (3S,3aS,9aS,9bS)-3-acetyl-3a,6-dimethyl-1,2,3,3a,4,5,8,9,9a,9b-decahydro-7H-cyclopenta[a]naphthalen-7-one (**1c**) followed by reconstruction of the A-ring. This chemical process requires expensive starting material and the yield is less than 14% (Scheme 2).[9] It is highly desirable to develop effective synthetic methods for the synthesis of these steroidal APIs with a C-17 side chain and 10α-methyl group from cheap and readily available starting materials. In this context, we envisioned that HIP or HIL with an isopropanol side chain would be valuable intermediates for the facile synthesis of dydrogesterone, 19-norprogesterone, and other steroidal APIs which have a C17 side chain with a 10α- methyl group or without a methyl group at C10-position (Scheme 1c).

Cholesterol/phytosterols catabolic gene clusters had been identified in *Rhodococcus jostii* RNA1, *Mycobacterium tuberculosis* H37Rv, and *Pseudomonas sp. strain Chol1*.[10−12]

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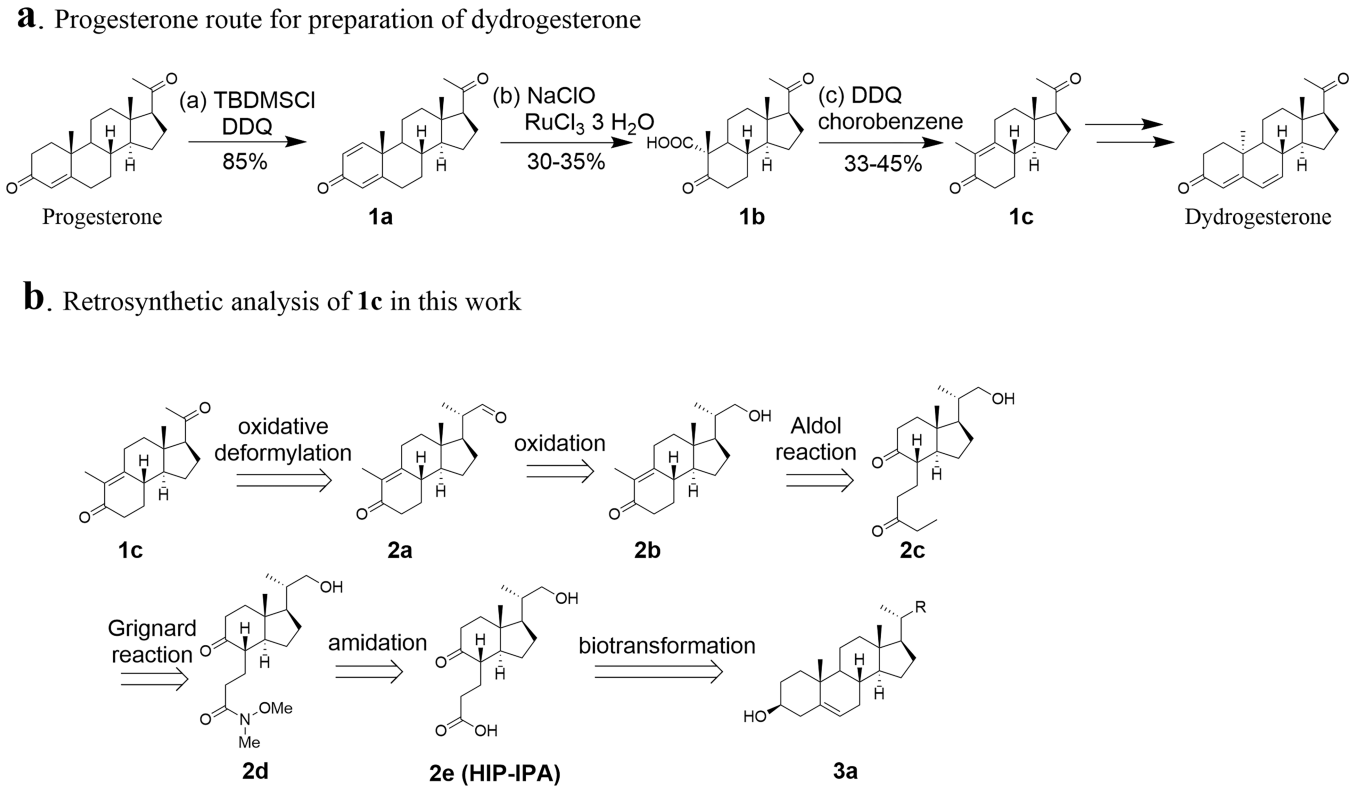
JACS Au 2024, 4, 1356−1364

Scheme 2. Progesterone Route for Preparation of Dydrogesterone and Retrosynthetic Analysis of 1c in This Work[9]



Phytosterols are degraded through two biochemical processes: elimination of the alkyl side-chain and catabolism of the core ring. The metabolism was initiated by the transformation of 3β-ol-5-en- to 3-keto-4-en- moiety and the terminal oxidations of alkyl side-chain.[13,14] The terminal oxidations of alkyl side-chain are proposed to employ a β-oxidation-like mechanism to produce the C-24, C-22, and C-19 steroid intermediates, that mainly involves the sterol-CoA ligases (FadD19, FadD17),[15] dehydrogenases (ChsE1−5, FadE26−29,34 or Scd1−2),[16−19] hydratases (ChsH1−2 or Shy),[11,20] acyl-CoA thiolases (FadA5),[21] β-hydroxyacyl-CoA dehydrogenase (Hsd4A)[6], and aldolase (Ltp2−4 or Sal1−2),[11,22−24] to produce the C-19 steroid intermediate. The ring A/B was degraded via the 9,10 s pathway to produce HIP (Scheme S1). In our previous study, the strains Δ*fadD3*Δ*car1,2* (N13) and Δ*fadE30*Δ*car1,2* (N33) were constructed from *Mycobacterium neoaurum* ATCC 6841 to transform phytosterols to HIP and HIL, respectively.[5] In these strains, two carboxylic acid reductase genes (*car1* and *car2*) and an acyl-CoA synthase gene (*fadD3*) or an acyl-CoA dehydrogenase gene (*fadE30*) were knocked out. Herein, we report the microbial synthesis of 3-((1*R*,3a*S*,4*S*,7a*R*)-1-((*S*)-1-hydroxypropan-2-yl)-7a-methyl-5-oxooctahydro-1*H*-inden-4-yl) propanoic acid (HIP-IPA, **2e**) from the renewable phytosterols by further genetically engineering strains N13 and N33. According to retrosynthetic analysis, **2e** could be converted to **1c**, which is the key intermediate for the synthesis of dydrogesterone in a previous study (Scheme 2).[9]

RESULTS AND DISCUSSION

Construction of N13Δ*hsd4A* and N33Δ*hsd4A* Strains and Identification of Their Metabolite 3e

The β-hydroxyacyl-CoA dehydrogenase (Hsd4A) was characterized as a dual-function enzyme with both 17β-hydroxysteroid dehydrogenase and β-hydroxyacyl-CoA dehydrogenase activities, which played a significant role in sterol side-chain degradation.[6] It is proposed that 22-hydroxy-3-oxo-25,26-bisnorchol-4-en-24-oyl CoA (**3b**) is the most accurate substrate of Hsd4A in *M. neoaurum* strains.[6] The *hsd4A*-null strains of *M. neoaurum* transformed sterols to the C-22 steroids, including 4-HBC, 1,4-HBC, and 9α−OH-HBC, which can be served as the precursors for the production of progesterone and other adrenocortical hormones.[6,25,26] The Hsd4A was found in the strain of ATCC 6841 with the accession number WP_011561997.1 by bioinformatics analysis. As deletion of *hsd4A* gene in *M. neoaurum* strains blocked the dehydrogenation of alcohol **3b**, and Hsd4A enzyme may also be active toward alcohol intermediate **3c**, we proposed that the *hsd4A* gene deletion of N13 and N33 strains would result in the formation of **2e** and **3d**, respectively (Scheme 3). The *hsd4A* gene was then deleted in both N13 and N33 strains by two-step homologous recombination system, respectively.[27] These genetically modified strains were confirmed by PCR using the primers ($hsd4A_{6841del}$−U−F and $hsd4A_{6841del}$-D-R) and sequencing (Figure S1) and were named as N13Δ*hsd4A* and N33Δ*hsd4A*, respectively.

The transformation of phytosterols by strains of N13Δ*hsd4A* or N33Δ*hsd4A* was carried out. The cultures were extracted with ethyl acetate, and the contents of the resulting extracts were determined by GC analysis as previously reported.[5] As shown in Figure S2A, only a trace amount of HIP was detected from the metabolites of the N13Δ*hsd4A* strain, and almost no other product was found. The similar phenomenon was observed from the extract of the N33Δ*hsd4A* culture (Figure S2B). The product of HIL was significantly decreased and nearly none of other metabolites were detected. However, in addition to HIP or HIL, a new spot with the same retention

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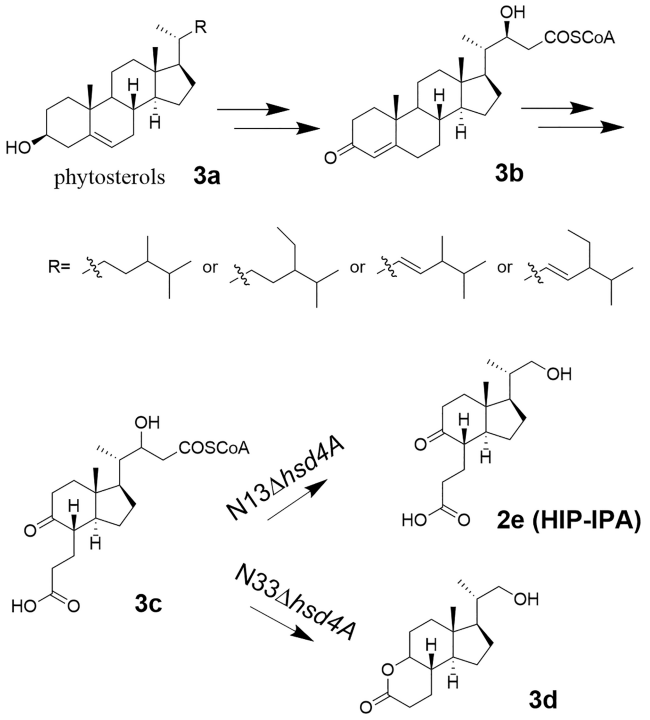
Scheme 3. Proposed Products for the Biotransformation of Phytosterols by Strains N13Δ*hsd4A* and N33Δ*hsd4A*



factor was detected in both cases by TLC (Figure 1). Therefore, the new product was extracted with ethyl acetate

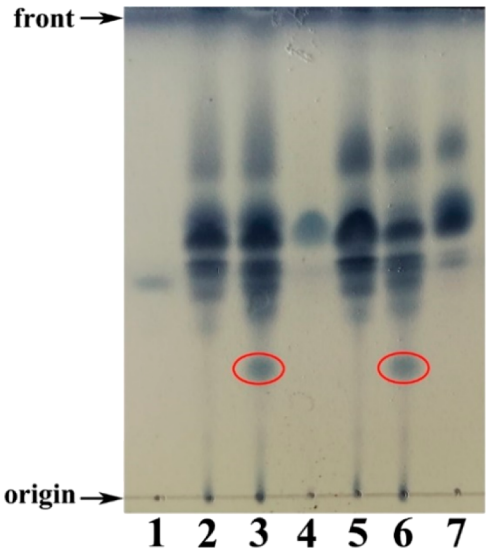
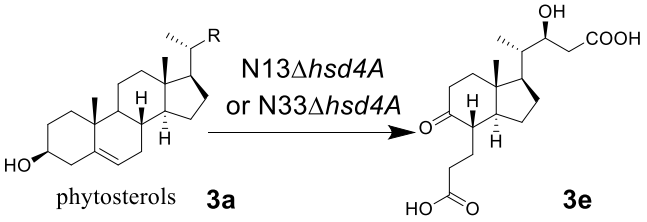


Figure 1. Thin-layer chromatography (TLC) of extracts from cultures of biotransformation of phytosterols by genetically modified strains. 1, the standard of HIP; 2, the extract from the culture of strain N13; 3, the extract from the culture of strain N13Δ*hsd4A*; 4, the standard of HIL; 5, the extract from the culture of strain N33; 6, the extract from the culture of strain N33Δ*hsd4A*; 7, the emulsified phytosterols.

from an acidic culture of N13Δ*hsd4A*. After removing the solvent, dichloromethane was used to wash away impurities. The product was purified by a silica gel column, and HR-MS analysis indicated that the product had an *m*/*z* of 341.1971 $[M + H]^+$. The product was further characterized by ^1H, ^{13}C HMBC, HSQC, and NOESY and COSY NMR spectra in CD_3OD (Table S3, Figures S3−S8) as **3e** (Scheme 4), which has a similar alkyl side-chain with the C-24 steroid

Scheme 4. Biotransformation of Phytosterols to 3e by N13Δ*hsd4A* and N33Δ*hsd4A* Strains



intermediate (**3b**). The appearance of **3e** suggested that **3c** was also the substrate of Hsd4A enzyme and the *hsd4A* gene was knocked out successfully in these two strains.

Construction of N13Δ*hsd4AΩthl* and N33Δ*hsd4AΩthl* Strains and Identification of 2e

It should be noted that aldolases participate in at least two steps in the biodegradation of phytosterols, including the steps from **3b** to **3f** (3-oxo-23,24-bisnorchol-4-ene-20-carbaldehyde) and from **3g** (17-hydroxy-3-oxopregna-4-en-20-carboxylate) to AD (Scheme S2).[11,22−24] In *Pseudomonas sp. strain Chol1*, aldehyde intermediate was identified in the degradation of the steroid compound cholate, and steroid aldolase (Sal) catalyzed the aldolytic cleavage (retroaldol) reaction.[11,28] The deletion of *sal* gene resulted in the accumulation of (22*E*)7α,12α-dihydroxy-3-oxochola-1,4,22*E*-triene-24-oyl-CoA (DHOCTO-CoA) as main product, the dehydration product of 7α,12α,22-trihydroxy-3-oxochola-1,4-diene-24-oyl-CoA (THOCDO-CoA).[11] This might be caused by the interactions between aldolase and hydratase that may affect the activities of both enzymes. In the *M. neoaurum* strains, a reaction catalyzed by an aldolase was carried out to produce an aldehyde (**3f**), followed by a reductive reaction to give 4-HBC in vivo.[6]

The compound **3c** or **3e** is shown to be the substrate for both Hsd4A and aldolase (Schemes S1 and S2). As **3e** was accumulated in the phyotosterol biotransformations using the strain N13Δ*hsd4A* or N33Δ*hsd4A* (Scheme 4), it is reasonable to propose that the aldolase activity is very low or even absent in *Mycobacterium fortuitum* strain. The AMO08280.1 in *M. neoaurum* is described as a transporter and shares 87 and 58% amino acid sequence identities with Ltp2 from *Thermomonospora curvata* and Sal from *Pseudomonas sp. strain Chol1*, respectively. The AMO05741.1 is an OB-fold domain-containing protein and shares 83% identity with the $ChsH2_{DUF35}$ from *T. curvata*. The complex of Ltp2-$ChsH2_{DUF35}$ has the steroid side chain cleaving aldolase activity.[23,24] Therefore, the gene clusters of AMO08280.1 and AMO05741.1were predicted to encode the aldolase, which has the activity to remove the acyl side chain from **3b**. The cluster was cloned into the expressing plasmid pMV261. The resulting plasmid (pMV261-*thl*) was transformed into N13Δ*hsd4A* and N33Δ*hsd4A* strains to afford strains N13Δ*hsd4AΩthl* and N33Δ*hsd4AΩthl*, respectively.

The transformation of phytosterols was performed with strain N13Δ*hsd4AΩthl* or N33Δ*hsd4AΩthl*. The cultures of both strains were extracted with dichloromethane, derivatized with bis(trimethylsilyl)-trifluoroacetamide and trimethylchlorosilane, and analyzed as described in the ref 5. The N13Δ*hsd4AΩthl* strain accumulated a product with an *m*/*z*

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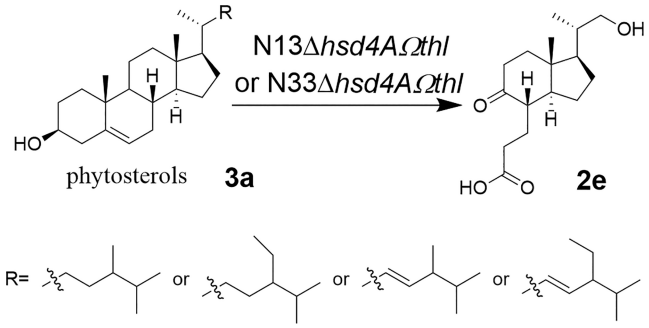
value of 426.2620 as the major product, and a byproduct with an m/z value of 368.2391 based on the GC−MS analyses when the derivative reagent was used (Figure S9A). The byproduct was proposed as methyl 3-((7a*R*)-1-(1-hydroxypropan-2-yl)-7a-methyl-5-oxooctahydro-1*H*-inden-4-yl)propanoate.

To identify the metabolites that accumulated in the N13Δ*hsd4AΩthl* strain, the product was isolated with a silica column to give a white powder. The HR-MS analysis indicated that the compound had an m/z of 305.1735 $[M + Na]^+$. The product was further characterized as 3-((1*R*,3a*S*,4*S*,7a*R*)-1-((*S*)-1-hydroxypropan-2-yl)-7a-methyl-5-oxooctahydro-1*H*-inden-4-yl) propanoic acid (**2e**, Scheme 5) by ^1H, ^{13}C HMBC, and HSQC NMR spectra in CD_3OD (Table S4, Figures S10−S13).

Scheme 5. Biotransformation of Phytosterols to 2e by N13Δ*hsd4AΩthl* and N33Δ*hsd4AΩthl* Strains



In our previous study, strains N13 and N33 were able to transform phytosterols to HIP and HIL, respectively. Since N13Δ*hsd4AΩthl* strain metabolized phytosterols to produce **2e**, we thus hypothesized that N33Δ*hsd4AΩthl* strain would produce **3d** with an m/z value of 266. The culture of strain N33Δ*hsd4AΩthl* was extracted and analyzed as mentioned before. The product had the same molecular weight with an m/z value of 426.2555 (after derivatization) and a retention time of 25.3 min with **2e** (Figure S9B). These results indicated that instead of the expected **3d**, **2e** was also produced from phytosterols with the N33Δ*hsd4AΩthl* strain. Therefore, although the modified genes of strains N13Δ*hsd4AΩthl* and N33Δ*hsd4AΩthl* are different, they produced the same product from phytosterols. The acyl-CoA synthetase (FadD3) uses ATP and Coenzyme A to thioesterify HIP and initiate the degradation of steroid ring C and D.[29,30] Then the 3-oxo group is reduced by 3-oxo HIC-CoA reductase (IpdF) (Scheme S3).[30] The substrates of FadD3 were investigated and no activity was detected when 1β(2′-propanoate)-3aα-*H*-4α(3′′-propanoate)-7aβ-methylhexahydro-5-indanone (HIDP) served as the substrate, which has a partially degraded 3-carbon side chain.[29] Considering a product with longer side chain (C-5) was detected from the N33Δ*hsd4A* strain, it is possible that the FadD3 has no activity on **3c**. The *fadD3* was expressed in the N13Δ*hsd4A* strain, and the **3e** was also detected (data not shown). The Δ*ipdF* strain of *Mycobacterium smegmatis* MC^2155 could not grow on HIP, suggesting the uniqueness of the reductase.[30] While the substrates of IpdF are still unknown, the protein (protein ID: AMD55885.1) from ATCC 6841 shares 89% identity with IpdF from *M. smegmatis* MC^2155. As the main products for N33Δ*hsd4A* and N33Δ*hsd4AΩthl* are compound **3e** and **2e**, respectively, we hypothesized that the IpdF has no activity on these two compounds.

Preparation of 2e and the Key Intermediate 1c for the Synthesis of Dydrogesterone at Preparative Scale. The **2e** may be used as a potential pharmaceutical intermediate for the facile production of steroidal APIs having a C17 side chain and with a 10α-methyl group or without a methyl group at C10-position, such as dydrogesterone. Therefore, the transformation of 20 g/L phytosterols (95% purity) was carried out by N13Δ*hsd4AΩthl* for 7 days in a fermenter with 3 L volume (total of 57 g phytosterols). The fermentation, product extraction, and purification were carried out as described in the experimental section. About 63% yield of **2e** was achieved, along with a little of byproducts. By further recrystallization, 25 g of **2e** (62% isolated yield) was obtained from phytosterols. Therefore, **2e** could be easily produced in a high yield from phytosterols, a readily available renewable starting material.

To demonstrate the practical utility of **2e**, a novel and short synthetic route was designed and performed for the transformation of **2e** into the key intermediate **1c** for the synthesis of dydrogesterone (Scheme 6). The synthesis commenced with the amidation of **2e** to afford Weinreb amide **2d** in 79% yield, which was further protected with glycol and transformed into (3*R*,3a*S*,9a*S*,9b*S*)-3-((*S*)-1-hydroxypropan-2-yl)-3a,6-dimethyl-1,2,3,3a,4,5,8,9,9a,9b-decahydro-7H-cyclopenta[a]-naphthalen-7-one (**2b**) through the Grignard reaction and intramolecular Aldol condensation in 68% yield from **2d**. With **2b** in hand, **1c** could be prepared following a similar procedure as documented, which involved the Dess-Martin oxidation and the copper-mediated catalytic radical oxygenation with 62% yield.[7] The total yield of **1c** was 33% from **2e** (Schemes S4−S7, Figures S14−S17). This synthetic route of **1c** from phytosterols was compared with that in the literature as outlined in Scheme S8. The yields of the intermediate **1c** from phytosterols were approximately 20.8% in our study, which is higher than that of the literature synthetic route through progesterone (8.4%). Therefore, **2e** possibly serves as a valuable intermediate for the synthesis of steroidal APIs that have a C17 side chain with a 10α-methyl group.

CONCLUSIONS

The sterol aldolase gene cluster was expressed in the Δ*hsd4A* strains of N13 and N33, resulting in strains N13Δ*hsd4AΩthl* and N33Δ*hsd4AΩthl*, respectively. Both strains transformed phytosterols into **2e**. At the preparative scale, **2e** was produced from phytosterols with a 62% isolated yield using strain N13Δ*hsd4AΩthl*. Compound **2e** was further transformed into (3S,3a*S*,9a*S*,9b*S*)-3-acetyl-3a,6-dimethyl-1,2,3,3a,4,5,8,9,9a,9b-decahydro-7H-cyclopenta[a]naphthalen-7-one (**1c**), which is the key intermediate in the synthesis of dydrogesterone and other APIs. Therefore, this study not only developed an efficient bioprocess for the production of **2e** from renewable biobased phytosterols but also established a new chemo-biocatalytic route for the facile production of steroidal APIs having a C17 side chain with a 10α-methyl group or without a methyl group at the C10-position. As such, this study has developed a new strategy to address this challenging synthetic problem. In addition, the further understanding of the microbial metabolism of phytosterols would guide the molecular engineering of these industrial strains to produce other valuable steroidal intermediates and APIs from renewable biobased starting materials.

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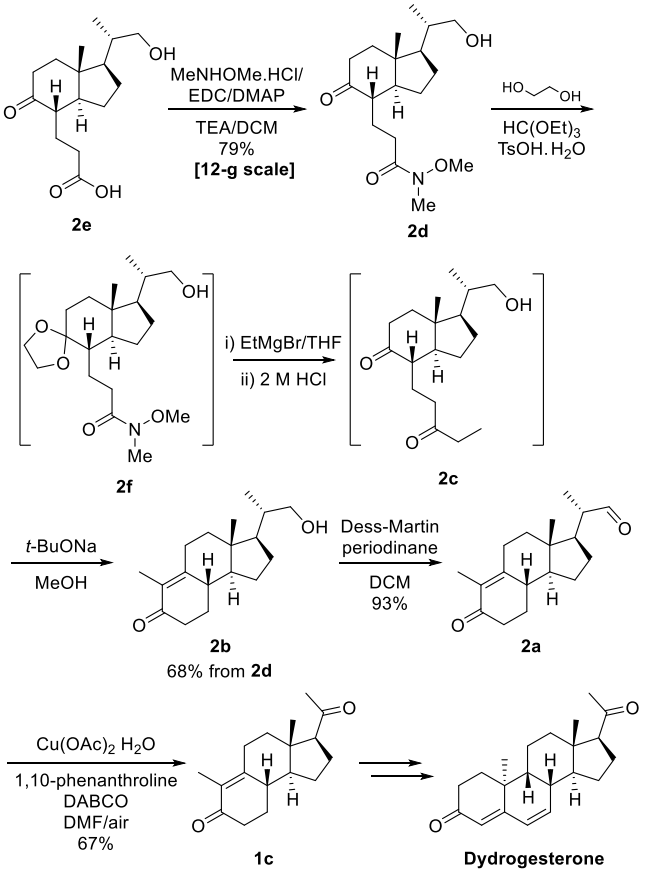
Scheme 6. Chemical Synthesis of the Key Intermediate of Dydrogesterone. Reagents and Conditions: (1) Triethylamine (TEA, 1.5 equiv), Methoxyamine Hydrochloride (1.5 equiv), 4-Dimethylaminopyridine (DMAP, 0.1 equiv), Dichloromethane (DCM), 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide Hydrochloride (EDC, 1.5 equiv), Room Temperature (rt), 20 h; (2) Glycol (5 equiv), Triethyl Orthoformate (7.5 equiv), *p*-Toluenesulfonic Acid Monohydrate (0.1 equiv), 110 °C, 8 h; Then Saturated Aqueous $NaHCO_3$, Ethyl Acetate/Petroleum Ether (4:1) Extracted; Then Ethylmagnesium Bromide (EtMgBr, 3 equiv), THF, 0 °C, 3 h; Then 2 M HCl, Reflux, 3 h; (3) *t*-BuONa (1.3 equiv), Methanol (MeOH), rt, 1 h; (4) DCM, Dess-Martin Periodinane (DMP, 1.5 equiv), rt, 3 h; (5) $Cu(OAc)_2 \bullet H_2O$ (0.2 equiv), 1,10-Phenanthroline (0.2 equiv), DMF, 1,4-diazabicyclo[2.2.2]octane (DABCO, 1.1 equiv), rt, 24 h



METHODS

Strains, Plasmids, and Primers

The strains and mutants used in this study are listed in Table S1. Strains ATCC 6841Δ*fadD3*Δ*car1,2* (N13) and ATCC 6841Δ*fadE30*Δ*car1,2* (N33) were genetically modified strains of ATCC 6841 with the unmarked deletions of two carboxylic acid reductase genes (*car1* and *car 2*) and the acyl-CoA synthase gene (*fadD3*) or the acyl-CoA dehydrogenase gene (*fadE30*), as described previously.[5] All of the plasmids and primers for gene deletion or expression are listed in Tables S1,S2, respectively.

Materials

The phytosterols (95%, a mixture of sitosterol, campesterol, stigmasterol, and brassicasterol with the ratio of about 50:20:20:10), AD, and other chemical reagents and solvents were purchased from chemical companies with reagent grade or the highest purity available. Phusion high-fidelity DNA polymerase and FastDigest restriction enzymes and the plasmid extraction kit and gel extraction kit were supplied by Fermentas (ThermoFisher, USA) and SIGMA (Beijing, China), respectively. The TIANamp bacteria DNA kit and the CloneExpress II (or MultiS) One Step Cloning Kit were purchased from Tiangen Biotech (Beijing, China) and Vazyme (Nanjing, China), respectively.

Gene Deletion and Expression

The unmarked gene deletion strains of ATCC 6841 were constructed by homologous recombination using the plasmid pGOAL19 and p2NIL, as described previously.[27] In brief, two 1 kb fragments were amplified from ATCC 6841 genomic DNA with pairs of primers, $hsd4A_{6841}$−U−F/$hsd4A_{6841}$-U-R, and $hsd4A_{6841}$-D-F/$hsd4A_{6841}$-D-R, respectively. Both of the fragments were ligated with the selection marker cassettes, which were the digested plasmids of p2NIL (with *Kpn*I and *Pst*I) and pGOAL19 (with *Pac*I), to produce the recombinant plasmid pKH_{del}-$hsd4A_{6841}$. The plasmid was electroporated into the strains. The unmarked gene deletion strains were selected after a two-step selection process and were confirmed by PCR and gene sequencing.

The thiolase genes (RefSeq: AMO08280.1 and AMO05741.1) were amplified from *M. neoaurum* B-3805 genomic DNA by PCR using primers thl_{3805}-F and thl_{3805}-R (Table S2). The plasmid pMV261[31] were digested by *Eco*RI and *Hind*III before being ligated by using a ClonExperss II One Step Cloning Kit to produce the recombinant plasmid pMV261-*thl* (Table S1). The constructed expression plasmid was transfected into N13Δ*hsd4A* and N33Δ*hsd4A* by using electroporation, respectively. The strains hosting the plasmid were selected under 50 μg/mL kanamycin.

Phytosterols Biotransformation

All of the Mycobacteria strains were cultured similarly as previously described.[5,32] In a nutshell, the strains were cultured with Luria−Bertani (LB) broth containing 0.5% Tween 80 at 30 °C for 2 days, and then inoculated into the transformation culture, which contained defatted soy flour (10 g/L), corn steep power (5 g/L), $(NH_4)_2HPO_4$ (2 g/L), and emulsified phytosterols (10 g/L),[5] and was adjusted to pH 7.5 with NaOH. All of the strains were cultured at 30 °C for 20 h first, and then at 42 °C for 30 min to induce the expression of thiolase genes. Thereafter, the strains were cultured at 30 °C for an additional 6 days. At the preparation scale, the transformation of 20 g/L of phytosterols (95% purity) was carried out by N13Δ*hsd4A*Ω*thl* for 7 days in a fermenter with 3 L volume. Kanamycin was added to a final concentration of 50 μg/mL when the strains hosting the pMV261 plasmid were cultured.

Isolation and Identification of 2e and 3e

The cultures of *hsd4A* deletion strains were extracted by three volumes of ethyl acetate after acidification using a HCl solution. The organic phase was concentrated under reduced pressure, and the residue was washed twice by dichloromethane. The product was isolated and purified by a silica gel column with methanol-dichloromethane (3:97 [vol/vol]) as eluents. The product was detected using a TLC plate. The plate was developed with dichloromethane-methanol (95:5 [vol/vol]) and visualized by phosphomolybdic acid stain.

The bioconversion mixture of the strains of N13Δ*hsd4A*Ω*thl* and N33Δ*hsd4A*Ω*thl* were extracted using two volumes of dichloromethane under acidic condition and determined with GC and GC−MS, which were carried out as described previously.[5] The extracted samples were derivatized with bis(trimethylsilyl)-trifluoroacetamide (40 μL) and trimethylchlorosilane (5 μL) and then detected on an Agilent GC System equipped with a DB-5 capillary column (30 m × 0.25 mm × 0.25 μm). The column was programmed at 150 °C (maintained for 5 min), 150 to 250 °C, and then to 295 °C at a rate of 5 and 40 °C/min, respectively. At the last, the column was kept at 295 °C for 10 min. The injector temperature was set at 320 °C, and helium (2 mL/min) was served as carrier gas. The retention times of HIP, HIL, and **2e** were 17.8, 18.1, and 25.3 min, respectively. As for

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JACS Au 2024, 4, 1356−1364

the MS detection, an Agilent 7200 Accurate-Mass Q-TOF detector was equipped with the GC system. The ionization energy was set as 70 eV to perform electron ionization.

The ^{1}H, ^{13}C, HSQC, HMBC, and COSY and NOESY NMR spectra of compound **3e**, and the ^{1}H, ^{13}C, HSQC and HMBC NMR spectra of **2e** were recorded at 600 MHz, respectively, with Bruker Avance III devices using CD_3OD or CD_3Cl as the solvent.

Preparation of 2e

The N13Δ*hsd4A*Ω*thl* strain was cultivated with kanamycin at a final concentration of 50 μg/mL. Other culture conditions were the same as described before.[5] The biotransformation mixture was adjusted to pH 3.0 by using an HCl solution. Product **2e** was extracted from the aqueous phase with three volumes of dichloromethane. After concentration under reduced pressure, water and NaOH were added to the residual mixture, and the pH was adjusted to 8.0. The aqueous phase was adjusted to acidic conditions and extracted by dichloromethane again. The product was crystallized after concentration, and the isolated yield of **2e** was 62% (25.0 g).

Chemical Synthesis of 1c

A short chemical route was designed and performed to synthesize key intermediate **1c** in the reported synthesis of dydrogesterone. Triethylamine (TEA, 9.0 mL, 64.0 mmol), methoxyamine hydrochloride (6.24 g, 64.0 mmol), and 4-dimethylaminopyridine (DMAP, 0.78 g, 6.4 mmol) in dry dichloromethane (DCM, 150.0 mL) were added to a stirred solution of 3-((1*R*,3a*S*,4*S*,7a*R*)-1-((*S*)-1-hydroxypropan-2-yl)-7a-methyl-5-oxooctahydro-1H-inden-4-yl)propanoic acid (**2e**, 12.00 g, 42.6 mmol), then 1-ethyl-3-(3-(dimethylamino)-propyl)carbodiimide hydrochloride (EDC, 12.27 g, 64.0 mmol) was added in batches at 0 °C. After that the solution was stirred at room temperature for 20 h. The reaction was quenched by 0.6 mol/L HCl (200.0 mL), extracted with DCM (150.0 mL × 2), and washed with brine (150.0 mL). The combined organic phases were dried over anhydrous Na_2SO_4, concentrated in vacuo, and purified by flash chromatography (petroleum ether/ethyl acetate = 4/1 to 3/2) to afford 3-((1*R*,3a*S*,4*S*,7a*R*)-1-((*S*)-1-hydroxypropan-2-yl)-7a-methyl-5-oxooctahydro-1H-inden-4-yl)-*N*-methoxy-*N*-methylpropanamide (**2d**, Scheme S4).

To a stirred solution of **2d** (10.40 g, 32.0 mmol), glycol (9.92 g, 160.0 mmol), and triethyl orthoformate (35.57 g, 240.0 mmol) was added *p*-toluenesulfonic acid monohydrate (0.61 g, 3.2 mmol). Then the solution was heated to 110 °C and the produced ethanol was removed by distillation in the meanwhile. After stirring at 110 °C for 8 h, the reaction was quenched with saturated aqueous $NaHCO_3$ (150.0 mL) and extracted with ethyl acetate/petroleum ether (4/1, 150.0 mL × 2). The combined organic phases were dried over Na_2SO_4 and concentrated in vacuo to afford the crude product 3-((1*R*,3a*S*,4*S*,7a*R*)-1-((*S*)-1-hydroxypropan-2-yl)-7a-methyl octahydro spiro[indene-5,2′-[1,3]dioxolan]-4 yl)-*N*-methoxy-*N*-methylpropanamide (**2f**) as light brown oil, which was directly used in the next step without further purification.

To a stirred solution of the above crude product **2f** in dry THF (150.0 mL) was dropwise added 1 M EtMgBr in THF (96.0 mL, 96.0 mmol) at 0 °C for 30 min, and then the reaction mixture was stirred at 0 °C for 3 h. The reaction was quenched with 2 M HCl (150.0 mL) and then heated to reflux. After stirring at reflux for 3 h, the organic solvent was removed in vacuo. The residue mixture was extracted with *tert*-butyl methyl ether (150.0 mL × 2) and washed with saturated aqueous $NaHCO_3$ (150.0 mL). The combined organic phases were concentrated in vacuo to afford the crude product (1*R*,3a*S*,4*S*,7a*R*)-1-((*S*)-1-hydroxypropan-2-yl)-7a-methyl-4-(3-oxopentyl)octahydro-5H-inden-5-one (**2c**) as a light brown oil, which was directly used in the next step without further purification.

To a stirred solution of the above crude product **2c** in dry MeOH (150 mL) was added *t*-BuONa (3.99 g, 41.6 mmol) at 0 °C, and then the reaction mixture was stirred at room temperature for 1 h. The reaction was quenched by 10% aqueous NH_4Cl (150.0 mL), extracted with *tert*-butyl methyl ether (150.0 mL × 2), and washed with brine (150.0 mL). The combined organic phases were dried over anhydrous Na_2SO_4, concentrated in vacuo, and purified by flash chromatography (petroleum ether/ethyl acetate = 8/1 to 4/1) to afford (3*R*,3a*S*,9a*S*,9b*S*)-3-((*S*)-1-hydroxypropan-2-yl)-3a,6-dimethyl-1,2,3,3a,4,5,8,9,9a,9b-decahydro-7*H*-cyclopenta[a]naphthalen-7-one (**2b**, Scheme S5).

To a stirred solution of **2b** (5.52 g, 20.0 mmol) in dry dichloromethane (DCM, 100.0 mL) was added Dess-Martin periodinane (DMP, 12.72 g, 30.0 mmol) in batches at room temperature. Then the solution was stirred at room temperature for 3 h. The reaction mixture was filtered through a thin layer of silica-gel and washed with petroleum ether/ethyl acetate (3/2). The combined filtrate was concentrated in vacuo and purified by flash chromatography (petroleum ether/ethyl acetate = 0 to 4/1) to afford (*S*)-2-((3*R*,3a*S*,9a*S*,9b*S*)-3a,6-dimethyl-7-oxo-2,3,3a,4,5,7,8,9,9a,9b-decahydro-1*H*-cyclopenta[a]naphthalen-3-yl)propanal (**2a**, Scheme S6).

To a stirred solution of **2a** (4.38 g, 16.0 mmol), $Cu(OAc)_2 \bullet H_2O$ (0.64 g, 3.2 mmol), and 1,10-phenanthroline (0.58 g, 3.2 mmol) in DMF (64 mL) was added 1,4-diazabicyclo[2.2.2]octane (DABCO, 1.97 g, 17.6 mmol) at room temperature. Then the solution was stirred at room temperature for 24 h in air conditions. The reaction was quenched with 0.5 M HCl (200 mL) and extracted with *tert*-butyl methyl ether (100.0 mL × 2) and washed with brine (100 mL). The combined filtrate was concentrated in vacuo and purified by flash chromatography (petroleum ether/ethyl acetate = 0 to 4/1) to afford (3*S*,3a*S*,9a*S*,9b*S*)-3-acetyl-3a,6-dimethyl-1,2,3,3a,4,5,8,9,9a,9b-decahydro-7*H*-cyclopenta[a]naphthalen-7-one (**1c**, Scheme S7).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacsau.3c00688.

The experimental details, ^{1}H NMR and ^{13}C NMR spectra of the products, and HPLC traces of the products (PDF)

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Notes

The authors declare no competing financial interest.

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