Chinese Chemical Letters 35 (2024) 108818

Contents lists available at ScienceDirect

Chinese Chemical Letters

journal homepage: www.elsevier.com/locate/cclet

A fungal CYP from *Beauveria bassiana* with promiscuous steroid hydroxylation capabilities

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ARTICLE INFO

Article history:
Received 5 May 2023
Revised 9 July 2023
Accepted 14 July 2023
Available online 17 July 2023

Keywords:
Fungal CYPs
Steroid hydroxylase
Beauveria bassiana
Promiscuous biocatalyst
Molecular dynamics

ABSTRACT

Hydroxylation of steroid core is critical to the synthesis of steroid drugs. Direct sp^3 C–H hydroxylation is challenging through chemical catalysis, alternatively, fungal biotransformation offers a possible solution to this problem. However, mining and metabolic engineering of cytochrome P450 monooxygenases (CYPs) is usually regarded as a more eco-friendly and efficient strategy. Herein, we report the mining and identification of a new steroid CYP (CYP68BE1) from *Beauveria bassiana* by transcriptomics, heterologous expression, *in vivo* and *in vitro* functional characterization. The catalytic promiscuity of CYP68BE1 was explored, and CYP68BE1 showed promiscuously and catalytically versatile, which is qualified for monohydroxylation on C11α, C1α, C6β and dihydroxylation on C1β,11α and C6β,11α of six steroids, leading to the production of key steroid intermediates required in the industrial synthesis of some indispensable steroid drugs. Molecular dynamics simulations were performed, revealing the molecular basis of different binding orientations of CYP68BE1 with different substrates. The discovery of CYP68BE1 offers a promising biocatalyst for enriching the steroid structural and functional diversity, which also can be applied to biosynthesize valuable steroid drug intermediates.

Steroids are widely distributed in natural organisms and have great significance on clinical application due to their important and broad pharmacological activities, such as anti-microbial [1], anti-tumor [2], anti-inflammatory [3], and anti-human immunodeficiency virus (HIV) effects [4]. Currently, over 400 steroid-based drugs are extensively used for treatment on inflammation, cancer, rheumatic, arthritis, and contraception [5–8]. The bioactivity of steroid drugs largely depends on the type and number of functional groups in specific positions and stereo-configurations on the skeleton [9]. In that way, the hydroxyl groups in specific positions become the vital building blocks in the synthesis of steroid drugs. However, such hydroxylations are challenging through chemical catalysis due to the predominance of inert C−H bonds in steroids.

Microbial steroid transformation has been utilized for years as a powerful tool to generate novel steroidal drugs and routinely incorporated into the synthetic routes as a key to affect specific oxygenation of the steroids for the reason of the high regio- and stereo-selectivity, better process efficiency, and eco-friendliness [7,10-15]. More importantly, fungal biotransformation occupies an important position in steroid biotransformation for its affluent enzyme system, and some fungal cytochrome P450 monooxygenases (CYPs) with varying steroid hydroxylation activities have been identified (Fig. S2 in Supporting information) [16]. However, the number of fungal steroidal CYPs and their range of hydroxylation positions remain limited, so digging more fungal CYPs and applying them to the steroid production will undoubtedly drive the industrial progress of steroidal drugs forward.

Beauveria bassiana is a crucial microbial catalyst used in a variety of chemical biotransformation, which is capable of accepting multiple types of substrates and catalyzing diverse types of reactions [17–21], especially the hydroxylation of steroids [22]. Despite the completion of the genome sequence of *B. bassiana*, which revealed 83 putative CYPs [23], the related steroid CYPs have not yet been identified. Therefore, there is a considerable interest in mining the putative CYPs of *B. bassiana* that have high catalytic hydroxylation ability.

Androst-4-ene-3,17–dione (4-AD, **1**) is a key intermediate in steroid synthesis, whose hydroxylation products usually have pharmacological activities or play important roles in industrial production of steroid drugs [24,25]. Firstly, we carried out the biotransformation of 4-AD by *B. bassiana* in order to give more detailed time-course for steroid transformation and lay a foundation for the

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https://doi.org/10.1016/j.cclet.2023.108818
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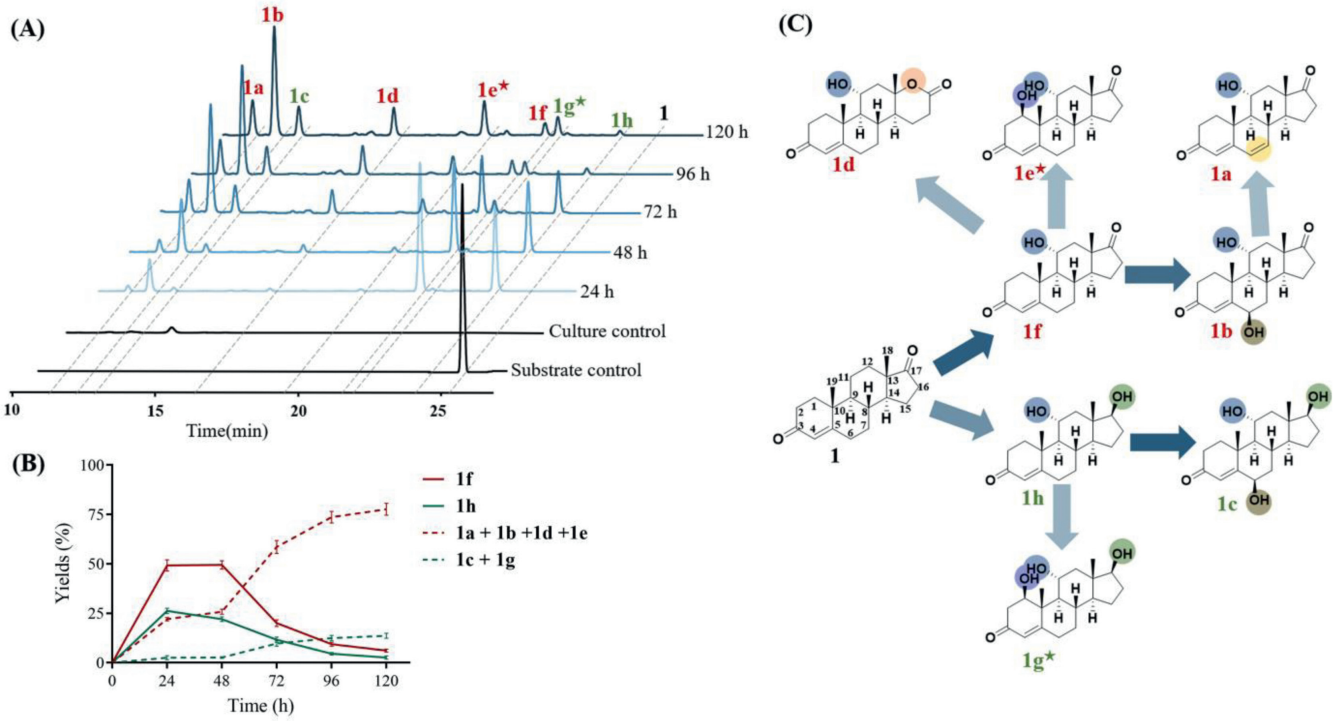

Fig. 1. Biotransformation of 4-AD (**1**) by *B. bassiana*. (A, B) Time course profile for the biotransformation of **1** by *B. bassiana*. Culture control consisted of fermentation blanks in which microorganisms were grown under identical conditions but without the substrate. Substrate control consisted of sterile medium containing the same amount of substrate and were incubated under the same conditions. (C) A proposed biotransformation pathway of **1** by *B. bassiana*. The color depth of arrowhead represents the conversion rate, the darker the color, the higher the conversion rate. * New compounds.

discovery of steroidal CYPs from *B. bassiana*. When 4-AD was fed to *B. bassiana*, eight metabolites (**1a**–**1h**) were observed after 3 days incubation (Fig. 1A), and thus were isolated from the extracts of the culture medium. We identified their structures by mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectra, and divided them into two groups as the hydroxylated derivatives of 4-AD (**1f**, **1a**, **1b**, **1d**, **1e**) and those of testosterone (TES, **2**) which was the C17 keto reduction product of 4-AD (**1h**, **1c**, **1g**) (Fig. 1C). It is worth mentioning that 1β,11α-dihydroxyandrost-4-ene-3,17–dione (**1e**) and 1β,11α-dihydroxytestosterone (**1g**) were two new compounds (the detailed structure elucidation of new compounds and the spectral data of known compounds were described in Figs. S3 and S4 in Supporting information). The position and relative configuration of two hydroxyl groups in **1e** and **1g** were determined by analysis of ^{1}H NMR, ^{13}C NMR, heteronuclear single quantum coherence (HSQC), heteronuclear multiple-bond correlation (HMBC) and ^{1}H–^{1}H correlation spectroscopy (^{1}H–^{1}H COSY) (Figs. S20–S31 in Supporting information).

The transformation route of 4-AD by *B. bassiana* was summarized by analyzing the time-course changes of the products. Initially, the substrate was almost completely transformed to 11α-hydroxylated products **1f** and **1h** after 24 h incubation. The second oxidation of **1f** and **1h** including 6β-hydroxylation, 1β-hydroxylation or Baeyer-Villiger oxidation occurred gradually in the next 96 h, leading to the corresponding production of **1b**, **1d**, **1e** and **1c**, **1g**, respectively. Besides, **1b** underwent an elimination to produce **1a** (Fig. 1C). The above results clearly indicated the presence of at least one efficient steroid oxygenase in *B. bassiana*.

Since steroidal hydroxylases of *B. bassiana* may be induced by substrate at the transcriptional level, the substrate 4-AD was added to the culture medium to up-regulate the expression of related hydroxylases. Strategy of RNA sequencing was chosen to screen for the genes with differential expression levels in different states, especially up-regulated genes after the substrate induction.

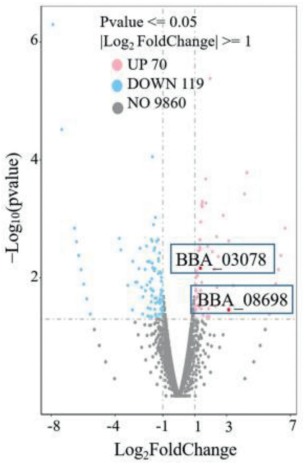

Fig. 2. Volcano map of differential genes. Points filled with light-pink or light-blue represent gene expressions with increased or decreased abundance (|$\log_2$ Fold change| $\geq$ 1) in response to 4-AD induction, while gray points represent genes with no differences in the expression. Two CYP genes are colored as red, respectively.

Moreover, we focused on CYPs, because almost all steroidal hydroxylases reported are CYPs [16]. Among the 70 up-regulated genes (Fig. S5 in Supporting information), two CYP genes were selected for further analysis which exhibited a 2.48-fold increase (BBA_03078) and 8.75-fold (BBA_08698) increase in their transcript abundance after induction with 4-AD (**1**) (Fig. 2, Table S1 in Supporting information).

The two genes were successfully amplificated from the total cDNA of *B. bassiana*, which were named CYP68BE1 (BBA_03078) and CYP58A5 (BBA_08698) by International CYP Nomenclature Committee. To explore the steroid hydroxylation activities of two

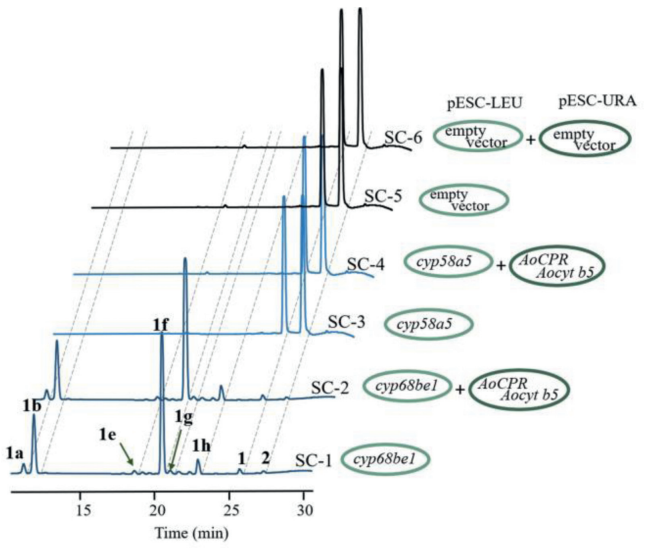

Fig. 3. HPLC results of *in vivo* functional verification of two CYPs in recombinant *S. cerevisiae*.

candidate CYP genes, *cyp68be1* and *cyp58a5* (Fig. S6 and Table S3 in Supporting information) were respectively introduced into *S. cerevisiae* INVSc1 cells, yielding the recombinant strains SC-1 (harboring *cyp68be1*) and SC-3 (harboring *cyp58a5*). Catalytic activities of the vast majority of CYPs require redox partner proteins to sequentially deliver electrons from nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) [26]. To successfully perform the function of the two CYPs, a recombinant vector containing AoCYP reductase (AoCPR) and cyt b5 genes was constructed and transformed into SC-1 and SC-3, respectively, resulted in the generation of the recombinant strains SC-2 and SC-4 (Tables S4 and S5, Figs. S7 and S8 in Supporting information). Additionally, the mock transformants harboring the empty vectors were also generated as the controls.

Biotransformations of 4-AD were performed with the aforementioned recombinants for 48 h (Fig. S8), and the results were analyzed by high-performance liquid chromatography (HPLC)-MS. Compared with the controls SC-5 and SC-6, the SC-3 and SC-4 strains did not produce any products, indicating that CYP58A5 was not the target CYP. To our delight, the SC-1 and SC-2 recombinant yeasts produced two major products (**1f** and **1b**) and four minor ones (**1h**, **1a**, **1e** and **1g**) based on comparison with the standards, demonstrating that CYP68BE1 is a multifunctional CYP performing consecutive catalytic processes. This enzyme could hydroxylate 4-AD at the C11α, C6β or C1β-positions (Fig. 3). Meanwhile, SC-1 and SC-2 had the same capacities to hydroxylate substrate **1**, which indicated that CYP68BE1 could interact with the endogenous CPR of *S. cerevisiae*.

To explore the catalytic promiscuity of CYP68BE1 on different steroids, we selected TES (**2**), 1,4-androstenedione (1,4-AD, **3**), progesterone (P4, **4**), estrone (E1, **5**), and estra-4,9-diene-3,17–dione (E-4,9, **6**) (Fig. S1 in Supporting information) for *in vivo* biotransformation assays by the recombinant strain SC-1. All the above five substrates are frequently used as the sexual hormones or vital industrial intermediates. The HPLC-MS results (Figs. S9–S16 in Supporting information) showed that all of them could be mono- or di-hydroxylated with high capabilities by SC-1. To elucidate the structures of the products, the large-scale fermentations were carried out. A total of 12 mono- or di-hydroxylated products were isolated, in which three products (**5b**, **6b**, and **6d**) were novel compounds. These structures were identified by MS, 1D and 2D NMR spectroscopic data analysis (structure elucidation section was showed in Figs. S13–S16 and S32–S49 in Supporting information). Besides, the NMR data of other three metabolites (**5a**, **6a** and **6c**) were reported for the first time. The above results indicated that C11α was the most common position for steroid oxidation by CYP68BE1, but different binding orientations (C6β, C1α or C1β) of CYP68BE1 could be observed with different substrates. Since some accessory products (**1a**, **4b**, **5b**) yielded *in vivo* affected the functional research of CYP68BE1, we conducted an *in vitro* functional assay on CYP68BE1 by extracting microsomes to avoid the influence of internal factors of *S. cerevisiae* on the experimental results.

For substrates **1–4**, with highly structural similarity, 11α-monohydroxylation process occurred in catalysis of the all four substrates by CYP68BE1 (Figs. 4A–D, Figs. S7–S10). Similarly, the monohydroxylated products of the four substrates continued to be hydroxylated under the catalysis of CYP68BE1, generating the corresponding 6β,11α-dihydroxylated products. But the regioselectivity of the enzyme is slightly different in the process of catalyzing the second-hydroxylation step of **1** and **2**. TES (**2**) was also hydroxylated by CYP68BE1 to yield extra **1g** both *in vivo* and *in vitro* (Fig. 4B, Fig. S10). The dihydroxylation product of **1** also included small amount of 1β,11α-type (**1e**). Possibly due to the decrease in the activity of CYP68BE1, no **1e** was detected in the HPLC analysis of *in vitro* biotransformation. (Fig. 4A, Fig. S9). Based on the yields of different products, it was suggested that the regioselectivity of CYP68BE1 preferred the C6β position of these four substrates during the dihydroxylation process.

Certainly, the structure of substrate **5** is quite different from other substrates, characterized by a benzene ring in the A ring and the absence of 19-CH_3. The catalytic activity of CYP68BE1 on this substrate is noteworthy, as it produced not only the expected 11α-monohydroxylated metabolite (**5a**) and 6β,11α-dihydroxylated metabolite (**5d**), but also a 6β-monohydroxylated metabolite (**5c**) (Fig. 4E, Fig. S13). The catalytic hydroxylation of substrate **6** only produced the monohydroxylated products: 11α-hydroxyestra-4,9-diene-3,17–dione (**6a**), 1α-hydroxyestra-4,9-diene-3,17–dione (**6b**), 11β,17β-dihydroxyestra-4,9-diene-3-one (**6c**) and 1α,17β-dihydroxyestra-4,9-diene-3-one (**6d**), which were significantly different from those of the other substrates (Fig. 4F, Fig. S14).

To systematically explain the mechanisms of the production of different hydroxylated products above, we used Alphafold2 to perform three-dimensional structure prediction, and molecular dynamics (MD) simulation to explore the molecular basis for regioselectivity of CYP68BE1 to different steroid substrates (Figs. S17–S19 in Supporting information). The 11α-hydroxylation product (**1h**) of **2** was docked into the pocket of CYP68BE1 with C11 pointing to the ferrous of heme. Then 200 ns MD simulations were performed and the evolution of distances of Fe-C11 and Fe-C6 was recorded. The distance of Fe-C11 approximately maintained within 5 Å (4.58 ± 0.33 Å) from 0 to 75 ns, followed by the distance of Fe-C11 increased immediately with Fe-C6 distance decreased to about 5 Å (4.58 ± 0.33 Å) (Fig. 5A). The above-mentioned change of distance held the possibility that **1h**, as the intermediate of modification reaction to **2**, could flip to the C6 near-attack pose in favor of the next modification of C6-hydroxylation. However, we could not exclude another possibility that the intermediate **1h** firstly dissociated from the pocket and then bound into the pocket again with C6 near-attack pose.

Due to the identification of **5a** and **5c**, both of which were monohydroxylated products, it is suggested that **5** could take two different near-attack poses in the pocket of CYP68BE1. MD simulations based on two initial near-attack poses of C11 and C6 were conducted respectively (Figs. 5B and C, Fig. S18). Within the 500 ns of trajectories of two systems, C11 and C6 near-attack poses were basically retained with both distances keeping around 5 Å (5.02 ± 0.30 Å and 4.05 ± 0.30 Å). Compared with simulations of **1h**, the flip of conformation was not observed in simulation of **5**. The relative stable conformations of **5** suggested that the substrate

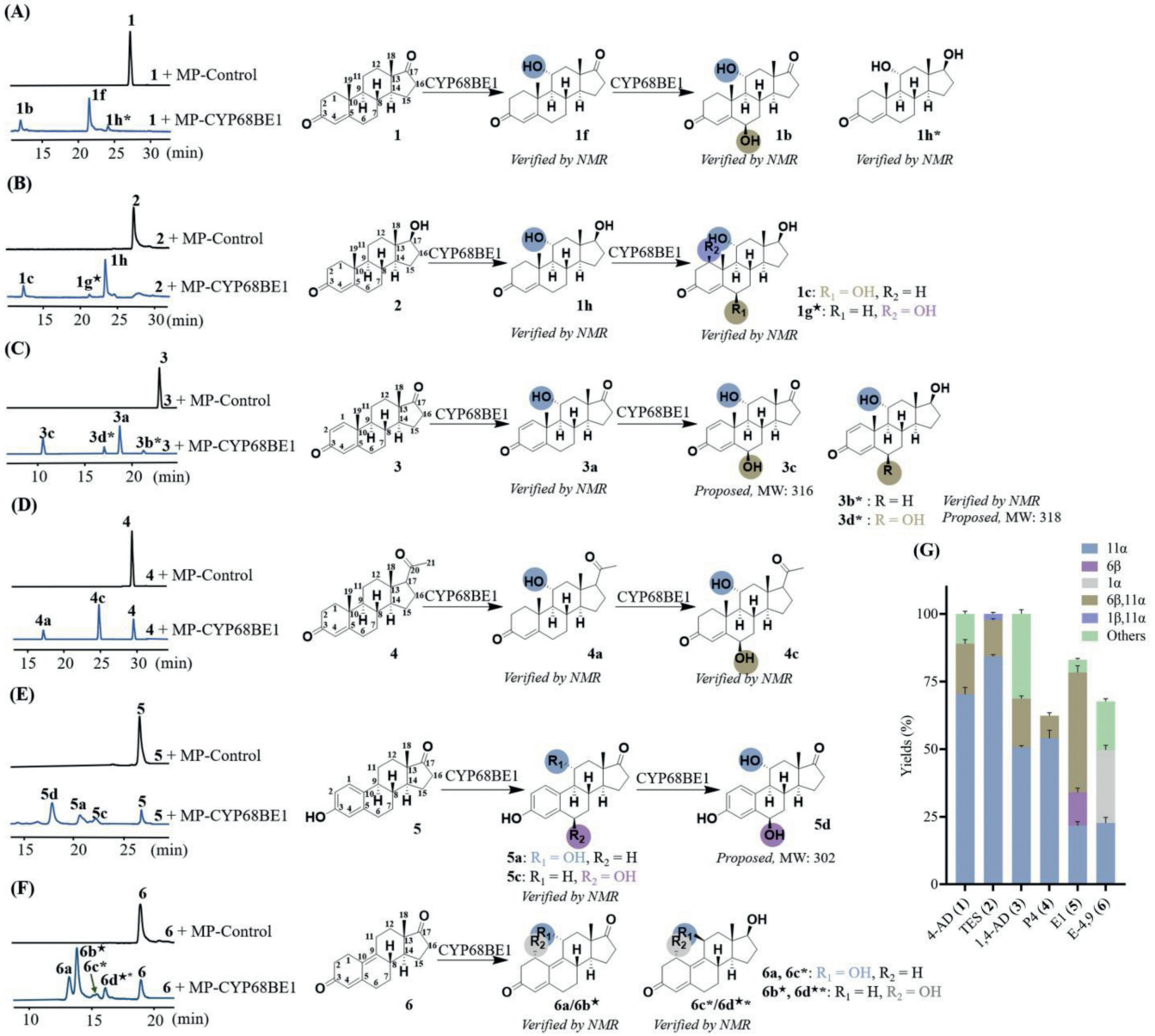

Fig. 4. Results of catalytic promiscuity tests of CYP68BE1. (A–F) *In vitro* functional verification and conversion route of CYP68BE1 to substrates **1**–**6.** (G) Conversion products distribution of **1**–**6** from CYP68BE1 *in vitro* assay. MP-Control refers to the microsome protein of SC-5, and MP-CYP68BE1 refers to the microsome protein of SC-1. * New compounds. * Hydroxylated steroids products in which C17 keto has been reduced. Products **3c**, **3d** and **5d** were not determined due to separation problems, but their structures were deduced to be hydroxylated products, considering their molecular weights.

could adopt a constant near-attack pose regardless of C6 or C11 once binding into the protein pocket. However, no observations of transformation between different near-attack poses were likely caused by insufficient time scale of MD simulations.

E-4,9 (**6**) preserved permanent conformation with Fe-C11 (4.75 ± 0.30 Å) and Fe-C1 (4.74 ± 0.37 Å) distance keeping around 5 Å, implying that CYP68BE1 could modify C11 or C1 sites of **6** (Fig. 5D, Fig. S19). Lacking obvious distinction between Fe-C11 and Fe-C1 distances, CYP68BE1 did not have regioselectivity to the two sites of **6**. The substrate probably was hydroxylated at either C11 or C1 site, followed by being released from the pocket, due to obtaining of only monohydroylated products from experiments. There was the possibility that steric hindrance owing to formation of monohydroxylated products hampered the further attack to either C11 or C1 from heme.

In summary, *B. bassiana* showed a strong capacity for 4-AD (**1**) transformation, forming two new 1β,11α-hydroxylated metabolites (**1e** and **1g**), except for its previously reported function to catalyze C11α and C6β,11α hydroxylation [17]. Employing strategies of transcriptomics, heterologous expression, *in vivo* and *in vitro* functional characterization, we excavated and identified a new steroid hydroxylase CYP68BE1, which was responsible for the hydroxylation on multiple positions (11α; 1α; 6β; 1β,11α; 6β,11α) of six steroid substrates with more than 70% conversion rate (Fig. 4G), indicating that CYP68BE1 was promiscuous and catalytically versatile. We obtained 12 mono- or di-hydroxylated products, and three of them are new compounds. Notably, of the products involved in this work, 11α-hydroxyandrostenedione (**1f**) is an important intermediate used to produce diuretics eplerenone [27]; 1β,11α-dihydroxytestosterone (**1g**) is expected to be a significant intermediate in the synthesis of ouabain; 11β,17β-dihydroxyestra-4,9-diene-3-one (**6c**) is regarded as a vital intermediate for the production of trenbolone [28]; 11α-hydroxyprogesteron (**4c**) is a potent inhibitor of 11β-hydroxysteroid dehydrogenase type 2 (11β

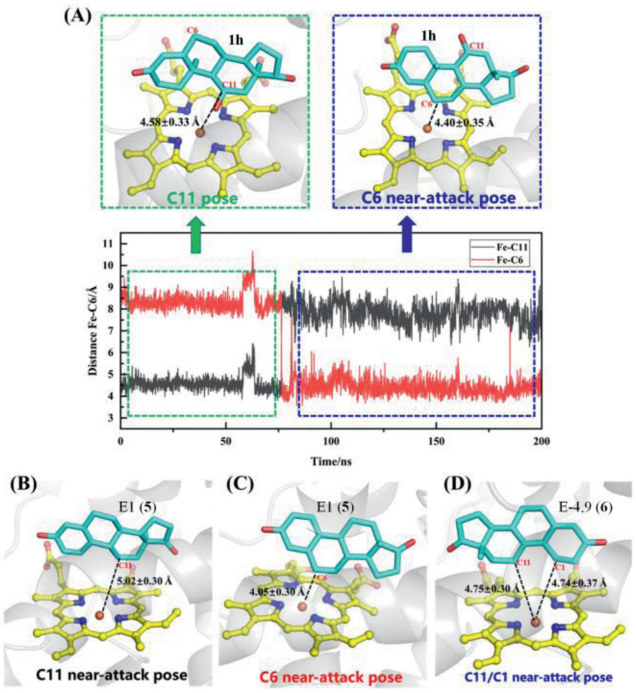

Fig. 5. MD simulations of CYP68BE1 in the reaction with different substrates. (A) The evolution of distance over simulation times of compound **1h**. (B) Representative C11 near-attack pose generated by cluster analysis from MD simulations of **5**. (C) Representative C6 near-attack pose generated by cluster analysis from MD simulations of **5**. (D) Representative C11/C1 near-attack pose from MD simulations of **6**.

HSD-2) [29]. Clearly, steroidal CYP with sufficient substrate promiscuity and high regioselectivity is usually regarded as eco-friendly and efficient biocatalyst for the synthesis of high-value steroid drugs and the enrichment of nature product library by enzymatic way or metabolic engineering technology. Therefore, we performed MD simulation with the aim of understanding the structural basis of the regioselectivity and substrate specificity of CYP68BE1. On the one hand, further designing mutation studies are needed to bias the catalytic reactions toward desired products, facilitating its industrial applications. On the other hand, combining with other modification enzymes, such as methylases and glycosyltransferases, more structurally diverse steroids could be created for the pharmacological screening.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The study was supported by the National Key Research and Development Program of China (Nos. 2020YFA0908003 and 2018YFA0901900), CAMS Innovation Fund for Medical Sciences (No. CIFMS2021-I2M-1–029). We are grateful to the Department of Instrumental Analysis of our institute for measurement of the NMR and HRESIMS data. We acknowledge the Network and Information Center of our institute for providing computing resources. We would like to thank Yufan Chou for support with MD services.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cclet.2023.108818.

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