Extensive expansion of the chemical diversity of fusidane-type antibiotics using a stochastic combinational strategy

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Abstract Fusidane-type antibiotics, represented by helvolic acid, fusidic acid and cephalosporin P1, are fungi-derived antimicrobials with little cross-resistance to commonly used antibiotics. Generation of new fusidane-type derivatives is therefore of great value, but this is hindered by available approaches. Here, we developed a stochastic combinational strategy by random assembly of all the post-tailoring genes derived from helvolic acid, fusidic acid, and cephalosporin P1 biosynthetic pathways in a strain that produces their common intermediate. Among a total of 27 gene combinations, 24 combinations produce expected products and afford 58 fusidane-type analogues, of which 54 are new compounds. Moreover, random gene combination can induce unexpected activity of some post-tailoring enzymes, leading to a further increase in chemical diversity. These newly generated derivatives provide new insights into the structure–activity relationship of fusidane-type antibiotics. The stochastic combinational strategy established in this study proves to be a powerful approach for expanding structural diversity of natural products.

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1. Introduction

Fusidane-type antibiotics are a small group of fungi-derived triterpenoid antimicrobials, which are featured with a 4α-methyl group, 16β-acetoxy group and 20-carboxylic acid in the protostadienol framework. Helvolic acid (1), fusidic acid (2) and cephalosporin P1 (3) are the three most representative compounds of this class (Fig. 1A). They exhibit potent antibacterial activity against Gram-positive bacteria, among which fusidic acid has been approved to clinically treat skin infections caused by Staphylococcus aureus. Moreover, fusidic acid is the only clinically used drug that targets elongation factor G (EF-G) to inhibit bacterial protein synthesis. This has made fusidane-type antibiotics have little cross-resistance to other commonly used antibiotics.

Because of the pharmaceutical prospects of fusidane-type antibiotics, the search for new analogues from nature has never been stopped. Recently, several new helvolic acid derivatives are discovered from Aspergillus species. However, this process is highly haphazard and inefficient. On the other hand, chemical derivatization has been extensively employed for diversification of fusidane-type antibiotics. These studies are mainly focused on modification of the reactive groups including 16β-acetoxy group, C17/C20 or C24/C25 double bond, 3-hydroxyl group and 20-carboxylic acid, however, analogues with greater antibacterial activity than that of fusidic acid have not yet been found. Combinational biosynthesis, designed to fuse the capabilities of combinational chemistry with the genetic power and enzymatic prowess of biosynthesis, serves as a promising complementary approach to chemical synthesis. There are two major strategies in combinational biosynthesis: one is to alter the substrate specificity by enzyme engineering, which is usually applied in multi-modular polypeptide synthases (PKSs) and non-ribosomal peptide synthetases (NRPSs) through swapping of the entire domains, modules and subunits of different enzymes; the other is to reprogram the natural biosynthetic pathway by mixing genes from different species. Despite of the great success of combinational biosynthesis in generating natural product analogues, the compatibility between the combined enzymes derived from different origins is a key constraint, and we are still far from programming any desired small molecule at will. To bypass the restriction of the compatibility between enzymes, a strategy using enzymes derived from similar or related biosynthetic pathways have been developed and successfully applied for antitumor indolocarbazole compounds and decalin-containing diterpenoid pyrones. However, the systematic study on the compatibility of the enzymes from related biosynthetic pathways is still lacked.

Recently, we have exhaustively characterized the complete biosynthetic pathway for fusidic acid, helvolic acid and cephalosporin P1. We have shown that these three compounds share an early-stage biosynthetic pathway involving six conserved genes (helA, helB1, helC, helB2, helD2 and helB4) for construction of a common intermediate 16β-acetoxy-29-norprotosta-17(20)Z,24-dien-3-one-21-oic acid, which then diverges to corresponding final products under the action of the rest genes in individual gene clusters (Fig. 1A). Notably, these post-tailoring enzymes exhibit broad substrate promiscuity, allowing for recruiting combinational biosynthetic approach to generate new fusidane-type antibiotics.

Here, we adopt a stochastic combinational strategy by introducing all the possible post-tailoring gene combinations into a strain harboring the six conserved genes. We observe that among a total of 27 gene combinations, 24 combinations produce the expected products. Moreover, random gene combination can trigger novel enzymatic activity of some post-tailoring enzymes and generate unexpected products, leading to a further increase in chemical diversity. Our study has demonstrated that the stochastic combinational biosynthesis based on random combination of post-tailoring genes from pathways sharing a common intermediate is an efficient approach to expand chemical diversity of natural products.

2. Results

2.1. Stochastic combination of post-tailoring genes from hel, fus and cep gene clusters

Despite of the wide application of combinational biosynthesis in diversification of natural products, systematic studies by a stochastic strategy involving all the possible gene combinations have not yet been carried out. To test the potential of compatibility between the post-tailoring enzymes from different pathways sharing a common intermediate and to maximize the chemical diversity of fusidane-type antibiotics, we adopted a stochastic combinational strategy by introducing all the possible combinations of post-tailoring genes from helvolic acid, fusidic acid and cephalosporin P1 biosynthetic pathways into the six-gene expression strain (Fig. 1A). Among the eight post-tailoring genes from hel, fus and cep clusters (Supporting Information Table S1), fusC1 and cepC2 encode the short chain dehydrogenases/reductases (SDRs) with the same function, thus either fusC1 or cepC2 is used for gene combination. Since both helB3 and cepB4 encode P450 enzymes that catalyze dual oxidation at C6 and C7, they are avoided being simultaneously used. In addition, the acyltransferases HelD1 and CepD2 have to be used after HelB3 and CepB4, respectively. Due to the above restrictions, a total of 27 gene combinations have been designed, including 6 two-gene combinations, 11 three-gene combinations, 8 four-gene combinations and 2 five-gene combinations (Fig. IB–E).

2.2. Diversification of fusidane-type antibiotics via random combinational biosynthesis

We have previously established the Aspergillus oryzae NSAR1 transformant harboring the six conserved genes helA, helB1, helC, helB2, helD2 and helB4, which produces the common intermediate of fusidane-type antibiotics. This six-gene transformant, termed as AOS0 (Fig. 2A), was used as the parent strain for construction of all the gene combinations. We first carried out the two-gene combinations. helE is derived from the helvolic acid pathway and responsible for C1/C2 double bond formation, and fusB1 from the fusidic acid pathway accounts for C11α-hydroxylation. When these two genes were combined and introduced into AOS0, a new major peak was observed in the resulting transformant AOS1 (Fig. 2B). Isolation and full NMR analysis proved this compound to be the expected C11α-hydroxylated and C1/C2 dehydrogenated product of 4. Moreover, when we stepwise introduced helB3, helD1 and fusC1 into AOS1 to carry out three-, four- and five-gene combinations, all the resulting transformants AOS7, AOS18 and AOS26 generated the expected products.
Additionally, compounds 1, 23, 27, 60, and 68 arising from the action of partial combinational genes were also observed. Using the above strategy, we have completed the construction of 27 gene combinations in AOS0 (Supporting Information Fig. S1 and Table S2). As shown in Figs. S1, 24 of the total 27 gene combinations gave their target compounds, leading to isolation of 58 fusidane-type analogues, among which 54 are new compounds (Supporting Information Fig. S2). All these new compounds were isolated and structurally determined by full NMR analysis and calculation of $^{13}$C chemical shifts (Supporting methods, Supporting Information Figs. S3–S65 and Tables S3–S69). Though there are still three combinations that did not produce the target products (Fig. 1D and E), the stochastic combination of post-tailoring genes from pathways with a
common intermediate seems to be an effective approach to generate diversified natural products.

2.3. Induction of novel enzymatic activity by random gene combination

Production of 54 new fusidane analogues from 24 gene combinations suggests that many combinations produce more than one new compound. Careful characterization of these combinations reveals that some enzymes in these combinations show novel enzymatic activity. helB3 encodes a P450 enzyme that catalyzes the dual oxidation to afford the 6β-OH and 7-keto in the helvolic acid biosynthesis. However, when this gene is combined with fusB1 and fusC1 derived from the fusidic acid biosynthesis (AOS15), two monooxygenated products 56 and 57 were produced in addition to the predicted dually oxidized product 55 (Fig. 3A and H). To confirm this, we constructed the helB3 expression strain AOS28 and performed a feeding experiment using compound 2. We observed an efficient transformation of 2 to 55, 56 and 57 by AOS28, but not by the untransformed A. oryzae (Fig. 3B and C). Isolation of 56 and 57 allowed us to obtain further insights into the oxidation sequence of the multifunctional HelB3 by feeding AOS28 with 56 and 57, respectively (Fig. 3D–G). These results showed that the multi-step oxidation catalyzed by HelB3 begins with formation of 7α-OH followed by oxidation to 7-keto, and finally terminates with attachment of the 6β-OH group (Fig. 3H).

Aside from HelB3, novel activity of the P450 enzyme CepB4 and the acetyltransferase CepD2 were also observed during random combinatorial biosynthesis. We have previously shown that CepB4 is a multifunctional P450 enzyme that catalyzes the dual hydroxylation at C6 and C7 of 4 to afford 6α-OH and 7β-OH. This process proceeds with C7β-hydroxylation followed by C6α-hydroxylation. In addition, a shunt pathway starting with the C6α-hydroxylation followed by formation of 6-keto is also observed. However, when CepB4 is combined with HelE and FusB1 (AOS10), in addition to exerting the original function to produce the predicted compounds 35, 36 and 40, CepB4 also produced three additional compounds 37, 38 and 39 featured with 7α-OH or 7-keto (Fig. 4A and H). This result suggests that CepB4 is capable of catalyzing C7α-hydroxylation. As 37 and 39 contain 11α-OH and the double bond at C1 and C2, we presumed that the presence of these groups in the substrate might affect its positioning in the active site of CepB4, which, therefore, expands the catalytic function of CepB4. In order to test the hypothesis, we performed feeding experiments using the cepB4-harboring strain CO6. When fed with 5, CO6 indeed yielded 37, 38 and 39 (Fig. 4B and C). Additionally, 35, 36 and 40 were also observed in the feeding experiment. To rule out the possibility that 37 is formed by reduction of 39, feeding CO6 and A. oryzae with 39 was carried out and formation of 37 was not detected in both cases (Fig. 4D and E). On the other hand, feeding CO6 with 37 did not give 39, suggesting 39 is likely to be derived from the C7β-hydroxylated product 40a (Fig. 4F–H). In addition, we observed an efficient conversion of 37 to 38 in both CO6 and control strain (Fig. 4F and G), suggesting this might be a non-enzymatic process. Compounds 35, 38 and 40 bear 1α-OH instead of C1/C2 double bond (Fig. 4H). We reasoned that 7-OH and 11-OH might weaken the stability of products, and the α,β-unsaturated carbonyl in the products were more readily to undergo 1,4-addition reaction to form β-hydroxy ketone. This was confirmed with appearance of 38 when 37 was incubated in Tris-HCl buffer of pH 8.0, but not pH 5.0 or 7.0 (Supporting Information Fig. S66). And we also found that the pH value of culture broth can reach 8.0 when cultured for 3 days, despite that the initial pH of fermentation medium is 5.5.

Another unexpected reaction is the acetylation of 7β-OH catalyzed by CepD2. In cephalosporin P1 biosynthesis, we have revealed that CepD2 specifically transfers acetyl group to 6α-OH, but not to 7β-OH, and the acetyl group at 6α-OH can spontaneously shift to 7β-OH under alkaline condition. However, when CepD2 was combined with CepB4 and HelE, the resulting strain AOS12 generated a product 46 only bearing 7β-acetoxy group (Fig. 5A and H). To determine whether acetylation of 7β-OH is
catalyzed by CepD2 or the endogenous enzyme from A. oryzae NSAR1, the cepD2 harboring strain CO722 and A. oryzae NSAR1 were employed to perform feeding experiments. HPLC‒MS analysis demonstrated that CepD2 can convert the mono-hydroxylated product 11 at 7β-position to 46, while A. oryzae NSAR1 cannot (Fig. 5B and C). Combined with the results that CepD2 acetylates the 6α-hydroxylated product 77 (Fig. 5D and E), but not the 7β-hydroxylated form 80 (Fig. 5F and G), we proposed that the function of CepD2 is expanded with the presence of 3-keto or/and the C1/C2 double bond in the substrate.

2.4. Structure–activity relationship analysis of fusidane-type antibiotics

By systematic combinational biosynthesis, we obtained 58 fusidane-type antibiotics (Fig. S2). Combining with previously isolated 22 analogues during biosynthesis of helvolic acid, fusidic acid and cephalosporin P1 (Supporting Information Fig. S67), we therefore constructed a library consisting of 80 fusidane-type antibiotics, providing an opportunity to systematically analyze the structure–activity relationship of fusidane-type antibiotics. We first tested the anti-S. aureus 209P activity of all these compounds by determining the minimum inhibitory concentration (MIC). As shown in Table 1, most compounds exhibited potent inhibitory activity. Although more active compounds than fusidic acid were not found, we obtained several new insights into the structure–activity relationship. Previous study has shown that 3α-OH has better antibacterial activity than 3-keto, and 3-keto is better than 3β-OH. We confirmed that most compounds follow this rule (Supporting Information Figs. S68 and S69A), however, for compounds derived from helvolic acid pathway that possess 6β-OH/7-keto or C1/C2 double bond or both, reduction of 3-keto to 3α-OH has a negative effect on their antibacterial activity (Fig. S69B). We also observed that C1/C2 double bond generally has a negative effect on the antibacterial activity (Supporting Information Fig. S70A), but it can enhance the activity of compounds from helvolic acid pathway that possess 3-keto or 6β-OH/7-keto or both (Fig. S70B). Similarly, C6 or/and C7 oxidation generally decreases the activity (Supporting Information Figs. S71 and S72A), but 6β-OH/7-keto increase the activity of the compounds possessing 3-keto or C1/C2 double bond or both (Fig. S72B). These results clearly indicate that there exists a synergistic effect of the C1/C2 double bond, 6β-OH/7-keto and 3-keto on anti-S. aureus 209P activity for compounds derived from helvolic acid pathway. This synergistic effect will be destroyed when modified with 3-keto reduction or C11α-hydroxylation from cephalosporin P1 or fusidic acid pathway.
C1α-hydroxylation is the key modification that confers the potent antibacterial activity to fusidic acid (Supporting Information Fig. S73A), however, in most cases, we observed that it has a negative effect when C6 or/and C7 oxidation occurs (S73B). In addition, we have previously shown that acetylation of 6-OH has an opposite effect on the antibacterial activity of helvolic acid and cephalosporin P122,24. In helvolic acid, 6β-OH acetylation decreases the activity whereas acetylation of 6α-OH in cephalosporin P1 increases the activity. Here we further confirmed the opposite effects of 6β-OH and 6α-OH acetylation in a series of compounds including C6/C7 dually oxidized or monooxygenated products (Supporting Information Fig. S74). These results suggest that the configuration of 6-OH is a crucial factor that affects the antibacterial activity of acetylated products. Moreover, we also found that 1α-OH has a negative effect on antibacterial activity (Supporting Information Fig. S75).

In addition, we also evaluated the antibacterial activity of these compounds against Gram-positive bacterium Micrococcus luteus (Table 1). We found that the structure–activity relationship towards M. luteus is slightly different from that against S. aureus. Among them, compound 24 exhibits potent anti-M. luteus activity with a MIC value of 2.0 μg/mL, which is equivalent to that of fusidic acid. This suggests that we can use different activity screening systems to find more active compounds.

3. Discussion

With uncovering of numerous biosynthetic gene clusters and advances in synthetic biology, combinational biosynthesis has received much more attention and becomes a widely used approach for diversification of natural products. Since this approach is mainly based on combination of enzymes, modules and domains from different sources, the compatibility among the combined components is the key constraint, and we are still unable to create any desired molecule at will thus far. In this study, we developed a stochastic combinational strategy based on random combination of the post-tailing genes, which are derived from related biosynthetic pathways sharing a common intermediate. These genes are highly compatible with each other as they directly or indirectly act on their common intermediate. Among a total of 27 gene combinations, 24 combinations afford the expected products though some have low yields. Our study thus demonstrated that the stochastic combinational strategy established in this work would be a powerful approach for chemical diversification of natural products.

The most important finding of this study is that random gene combination does not only maximize the chemical diversity of target compounds, but also induces novel enzymatic activity of the combined enzymes. The multifunctional P450 CepB4 has been previously shown to catalyze 6α- and 7β-dual hydroxylation in cephalosporin P1 biosynthesis22. However, in the present study, when the substrate is equipped with the C1/C2 double bond and 1α-hydroxyl group, we found that CepB4 can simultaneously give 7α- and 7β-monoxyhydroxylated products (Fig. 4H). We inferred that the ferryl-oxo intermediate [FeIV=O, porphyrin πcation radical] locates at an equal distance from 7α-H and 7β-H, and can activate both C–H bonds at C7 to yield a pair of epimers. In addition, the catalytic capability of CepD2 is expanded with functionalization of the substrate as well. Our previous work has confirmed that CepD2 can only region-specifically attach an acetyl group to 6α-OH22. However, when the substrate is processed by HelE, CepD2 is able to achieve acetylation of 7β-OH (Fig. 5H).
Similar phenomena that catalytic capacities of enzymes vary with manipulation of substrates are also observed. The multifunctional P450 enzyme TcP450-1 shows poor reaction specificity, and can catalyze C-11/C-19 hydroxylation of cortexolone. However, when 17α-OH in cortexolone is acetylated, the specificity of TcP450-1 mediated C-19 hydroxylation is dramatically increased. All these results indicate that manipulation of substrates by random gene combination is a feasible option to induce the new function of enzymes for further enriching chemical diversity.

There have been a few reports on the backbone modifications of fusidane-type antibiotics and their resulting effects on the antibacterial activity. In this work, we isolated a number of fusidane-type antibiotics through backbone modifications, and obtained comprehensive insights into the structure–activity relationship of anti-"S. aureus" 209P activity. These results have provided clues to the evolution-guided optimization of helvolic acid, fusidic acid and cephalosporin P1 biosynthesis. Generally, 3α-OH is a key factor for the activity of fusidane-type antibiotics, however, the enzyme to convert 3-keto to 3α-OH is omitted in helvolic acid pathway. Instead, it evolves by introducing the C1/C2 double bond and 6β-OH/7-keto, which exclusively have the synergistic effect on the anti-"S. aureus" 209P activity with 3-keto, to enhance the antibacterial property of helvolic acid. On the other hand, formation of 3α-OH followed by C11α-hydroxylation gives the most potent fusidane-type antibiotic fusidic acid. However, it is puzzling that C6α-C7β-hydroxylation is evolved after 3α-OH formation in cephalosporin P1 biosynthesis, which is detrimental to the anti-"S. aureus" 209P activity. It is worth noting that the structure–activity relationship of fusidane-type antibiotics against M. luteus is slightly different, which might be attributed to the difference of cell permeability or EF–G crystal structure between M. luteus and S. aureus 209P. Although no more potent derivatives than fusidic acid are obtained, the stochastic combinational strategy has enabled us to easily introduce reactive functional groups into the common intermediate in the same time, which provides a library of fusidane-type antibiotics for chemical modification so as to improve antimicrobial properties.

In conclusion, based on the biosynthetic mechanisms of helvolic acid, fusidic acid, and cephalosporin P1, we constructed 27 A. oryzae NSAR1 transformants through the stochastic combinational strategy, and a total of 58 compounds were isolated, including 54 new compounds. Unexpectedly, we found that catalytic functions of CepB4 and CepD2 can be expanded with modification of substrates. With large number of fusidane-type antibiotics in hand, systematic anti-bacterial activity evaluation was performed, and provided new insights into the structure–activity relationship. Our work demonstrates the powerful ability of the stochastic combinational strategy for structural diversification, and provides a series of fusidane-type antibiotics for activity screening and chemical modifications.

4. Experimental

4.1. General materials and experimental procedures

Acetonitrile (CH3CN) was purchased from Oceanpak Alexative Chemical Co., Ltd. (Gothenburg, Sweden). Methanol (MeOH) was purchased from Yuwang Industrial Co., Ltd. (Yucheng, China). Ethyl acetate (EtOAc) was purchased from Fine Chemical Co., Ltd. (Tianjin, China). Formic acid was purchased from Kemiou Chemical Reagent Co., Ltd. (Tianjin, China).

Primer synthesis and DNA sequencing were performed by TSINGKE Biotech Co., Ltd. (Guangzhou, China). Plasmid extraction kits and DNA purification kits were purchased from...
Sangon Biotech Co., Ltd. (Shanghai, China). Both KOD-FX DNA polymerase and KOD-Plus DNA polymerase (TOYOBO, Osaka, Japan) were used for PCR, and ClonExpress® MultiStep Cloning Kit (Vazyme, Nanjing, China) was used to construct recombinant plasmids. Yatalase™ Fungal Cell Lytic Enzyme was purchased from TaKaRa Co., Ltd. (Dalian, China).

UV spectra, IR spectra and optical rotations were recorded on the JANOS V-550 UV/Vis spectrometer, JANSCO FT/IR-480 plus spectrometer and JANSCO P1020 digital polarimeter from JANSCO International Co., Ltd. (Tokyo, Japan), respectively. HRESIMS was performed on the quadrupole orthogonal time-of-flight (Q-TOF) tandem mass spectrometer (Waters, Manchester, U.K.). NMR data were obtained with Bruker AV 400/600 spectrometers (Bruker BioSpin Group, Fällanden, Switzerland) using the following solvent signals (CDCl₃: δH 7.26/δC 77.0; CD3OD: δH 3.30/δC 49.0; pyridine-d₅: δH 7.21/δC 123.5; acetone-d₆: δH 2.05/δC 29.8) as internal standards.

HPLC–MS analysis of metabolites was performed on an Ultimate 3000 HPLC system (Dionex, Germering, Germany) and an amaZon SL ion trap mass spectrometer (Bruker Daltonics Inc., Billerica, Bostone, USA) equipped with an electrospray ionization source using a Cosmosil 5C18-MS-II column (250 mm × 4.6 mm, 5 µm, Nacalai Tesque, Inc., Kyoto, Japan). The mobile phases for preparative HPLC was performed with a UV detector, a dual pump gradient system, and a Dr. Flash II fraction collector system (Lisui E-Tech Co., Ltd., Shanghai, China). Semi-preparative HPLC was carried out on an Ultimate System 1300 HPLC system (Dionex) equipped with a UV detector, using an YMC-Pack ODS-A column (250 mm × 10 mm, 5 µm, YMC Co., Ltd., Tokyo, Japan).

### Table 1. Inhibitory effects of fusidane-type antibiotics on *S. aureus* 209P and *M. luteus*.  

| Compd. MIC (µg/mL) | Compd. MIC (µg/mL) | Compd. MIC (µg/mL) | Compd. MIC (µg/mL) |
|------------------|------------------|------------------|------------------|
| *S. aureus* 209P | *M. luteus* | *S. aureus* 209P | *M. luteus* | *S. aureus* 209P | *M. luteus* |
| 1                | 4                | 64               | 29               | 1                | 4                | 57               |
| 2                | 0.125            | 2                | 30               | 128              | 32               | 58               |
| 3                | 0.5              | 8                | 31               | 8                | 8                | 59               |
| 4                | 16               | 32               | 32               | 32               | 4                | 61               |
| 5                | 16               | 4                | 33               | 34               | 16               | 62               |
| 6                | 4                | 32               | 32               | >128             | 128              | 63               |
| 7                | 4                | 32               | 35               | >128             | 32               | 64               |
| 8                | 2                | 36               | 36               | >128             | 32               | 64               |
| 9                | 32               | 64               | 37               | >128             | 128              | 65               |
| 10               | 128              | >128             | 38               | >128             | 64               | 66               |
| 11               | 16               | 8                | 39               | >128             | 64               | 67               |
| 12               | >128             | >128             | 40               | >128             | >128             | 68               |
| 13               | >128             | >128             | 41               | 32               | 128              | 69               |
| 14               | 64               | 128              | 42               | 128              | 64               | 70               |
| 15               | 32               | 43               | 16               | 8                | 71               | >128             |
| 16               | 32               | 64               | 44               | 16               | 32               | 72               |
| 17               | >128             | >128             | 45               | 64               | 64               | 73               |
| 18               | >128             | >128             | 46               | >128             | >128             | 74               |
| 19               | >128             | >128             | 47               | 64               | >128             | 75               |
| 20               | 32               | 128              | 48               | 8                | 64               | 76               |
| 21               | 8                | 128              | 49               | 32               | 64               | 77               |
| 22               | 128              | 64               | 50               | 16               | >128             | 78               |
| 23               | 16               | 4                | 51               | 8                | >128             | 79               |
| 24               | 2                | 2                | 52               | 8                | 32               | 80               |
| 25               | 16               | 4                | 53               | 16               | 128              | Tobramycin 0.03 |
| 26               | 128              | >128             | 54               | 8                | 32               | Ampicillin —    |
| 27               | 1                | 16               | 55               | 32               | >128             |                  |
| 28               | 8                | 8                | 56               | 2                | >128             |                  |

Not applicable.

4.2. Strains and media

The quadruple auxotrophic *A. oryzae* NSAR1 (*niaD*⁺, *sC*⁺, *ΔargB*, *adeA*⁻) was used as the host for heterologous gene expression. The mycelia of *A. oryzae* transformants were inoculated into 10 mL DPY medium (2% dextrin, 1% polypeptone, 0.5% yeast extract, 0.5% KH₂PO₄, 0.05% MgSO₄·7H₂O) and cultured at 28 °C and 220 rpm for 1–2 days as seed broth. Then the seed broth was transferred to the 100 mL modified Czapek-Dox (CD) medium (0.3% NaNO₃, 0.2% KCl, 0.05% MgSO₄·7H₂O, 0.1% KH₂PO₄, 0.002% FeSO₄·7H₂O, 1% polypeptone, 2% starch, pH 5.5) and grown at 28 °C and 220 rpm for 5 days to induce the expression of exogenous genes under the * amyB* promoter.

*Escherichia coli* DH5α (TaKaRa) was used for molecular cloning. The *E. coli* cells carrying target plasmids were grown in Luria–Bertani (LB) medium supplemented with 100 mg/L ampicillin at 37 °C and 200 rpm.
4.3. Construction of fungal expression plasmids

The gene expression cassette containing the amyB promoter, the target gene, and the amyB terminator was amplified from the pTaex3-based plasmids, which have been established in our previous work, and then inserted into the HindIII-linearized pPTRI or pBarI. All the primers are listed in Supporting Information Table S70, and all the plasmids are listed in Supporting Information Table S71.

4.4. Transformation of A. oryzae NSAR1

The transformation of A. oryzae NSAR1 was conducted via the PEG-mediated strategy. 100 μL spore suspension of the parent strain was inoculated in 10 mL DPY medium and cultured at 28 °C and 220 rpm for 2 days. Subsequently, the seed broth was transferred to 100 mL DPY medium and continues to grow for 24 h. The mycelia were harvested by filtration, and then digested by 1% Yatalase in 0.6 mol/L (NH4)2SO4, 50 mmol/L maleic acid, pH 5.5 at 30 °C for 3 h. The resulting protoplasts were collected by centrifugation, and washed once with Solution 2 (1.2 mol/L sorbitol, 50 mmol/L CaCl2·2H2O, 35 mmol/L NaCl, 10 mmol/L Tris-HCl, pH 7.5), and then the concentration was adjusted to 1 × 10^7 cells/mL. 200 μL protoplast suspension and about 10 μL plasmids (10 μg) were incubated on ice for 30 min followed by addition of 1.3 mL Solution 3 (60% PEG4000, 50 mmol/L CaCl2·2H2O, 10 mmol/L Tris-HCl, pH 7.5) in three times, and then the mixture is placed at room temperature for 20 min. After that, 5 mL Solution 2 was added, and the mixture was subjected to centrifugation at 1500 rpm for 10 min. The precipitates were suspended in 200 μL Solution 2 and spread on the lower selective medium (0.2% NH4Cl, 0.1% (NH4)2SO4, 0.05% KCl, 0.05% NaCl, 0.1% KH2PO4, 0.05% MgSO4·7H2O, 0.002% FeSO4·7H2O, 2% glucose and 1.2 mol/L sorbitol as well as 0.2 μg/mL pyrithiamine hydrobromide and/or 50 μL/mL glufosinate-ammonium, pH 5.5) with 1.5% agar, and then covered with the selective upper medium containing 0.8% agar. The transformants could be obtained after incubation at 30°C for 4–6 days. All the transformants used in the work are listed in Supporting Information Table S72.

4.5. HPLC analysis and isolation of metabolites

To analyze the metabolites from different transformant strains, 10 mL seed broth was inoculated into 100 mL modified CD medium in a 500 mL flask and cultured at 28 °C and 220 rpm for 5 days (CRYSTAL IS-RDS4 incubator shaker, Addison Texas, USA). Then the fermentation broth was collected by filtration and extracted with EtOAc. The resulting crude extract was subjected to HPLC–MS analysis. For isolation and purification of metabolites, extract was subjected to MPLC and semi-preparative HPLC (Supporting Methods).

4.6. Feeding experiments

The A. oryzae NSAR1 transformant strain (CO6, CO7 or AOS28) was inoculated into 10 mL DPY medium and cultured for 1–2 days. The seed broth was then transferred into 100 mL modified CD medium, followed by addition of 1.0 mg substrate in 50 μL DMSO after growing for 12 h. Subsequently, the transformant strain was cultured for additional 4 days, and the fermentation broth was extracted twice with EtOAc and concentrated under reduced pressure. The crude extract was dissolved in methanol for HPLC–MS analysis.

4.7. Stability testing of compound 37 under different pH conditions

To 1 mL of 1 mol/L Tris-HCl buffer (pH 5.0, 7.0 and 8.0) was added 1.0 mg of 37 in 20 μL DMSO, respectively. The mixture was incubated at 28 °C and 220 rpm for 4 days, and then extracted three times with EtOAc. The concentrated crude extract was dissolved in methanol for HPLC–MS analysis.

4.8. Antibacterial assay

In vitro inhibitory effect against Gram-positive strains S. aureus 209P and M. luteus were evaluated by 2-fold dilution assay. After growing on beef extract agar medium at 37 °C for 1 day, S. aureus 209P and M. luteus were collected with normal saline, and then adjusted to a concentration of 5 × 10^8 cells/mL with beef extract medium (0.3% beef extract, 0.2% yeast extract, 1% tryptone and 0.5% NaCl, pH 7.5) and MH liquid medium (purchased from Huankez Microbial Sci. & Tech., Co., Ltd., Guangzhou, China), respectively. 200 μL of seed broth was transferred into the first test well of each line in the 96-well, and 100 μL was added in the other wells in the same line. All compounds were dissolved in DMSO and adjusted to 50 μg/L. 0.5 μL sample solution was added into the first well with the initial concentration of 128 μg/mL, then 100 μL was transferred to the second well, and so on. The concentration of the compound ranged from 128 to 0.0625 μg/mL. Tobramycin and ampicillin were used as the positive control, respectively, and DMSO was used as the negative control. The 96-well microtiter plates were placed at 37 °C for 24 h. The MIC was defined as the minimal concentration at which no turbidity could be observed.

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Author contributions

Dan Hu, Hao Gao, Ikuro Abe and Xinshen Yao designed the research. Xiaojun Song and Jianming Lv performed the experiments. Huiyun Huang and Guodong Chen performed quantum chemical calculation of NMR shifts. Xiaojun Song, Jianming Lv, Zhiqin Cao, Huiyun Huang, Guodong Chen, Takayoshi Awakawa, Dan Hu, Hao Gao, Ikuro Abe and Xinshen Yao analyzed the data, and Xiaojun Song, Jianming Lv, Dan Hu, Hao Gao, Ikuro Abe and Xinsheng Yao wrote the paper.

Conflicts of interest

The authors declare that they have no competing interests.

Appendix A. Supporting information

Supporting information to this article can be found online at https://doi.org/10.1016/j.apsb.2020.12.007.

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