Supplemental Material

Genetic and biochemical characterization of halogenation and drug-transportation genes encoded in the albofungin biosynthetic gene cluster

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† These two authors contributed equally to this work. Zhe-Chong Wang and I-Wen Lo implemented respectively the genetic and biochemical portions of the work.
Table S1. Strains and plasmids used in this study.

| Bacterial strains and plasmids | Properties | Source or reference |
|-------------------------------|------------|--------------------|
| E. coli DH5a                  | Competent cells for routine cloning | Thermo Fisher |
| E. coli BL21(DE3)             | Competent cells for routine cloning | Thermo Fisher |
| 10GBC                         | Construction of high molecular weight BAC | Lucigen |
| E. coli ET12567               | DNA methylation deficient strain | (1) |
| E. coli ET12567[pUZ8002]      | Conjugal transfer of DNA | (1) |
| S. tumenacerans JCM5050       | Albofungin producing strain | JCM Resources |
| S. albus J1074::erm*-crpA    | Heterologous expression host | (2) |
| pET28a (+)                    | T7 vector for E. coli BL21(DE3) | Novagen |
| pBelOBAC11                    | Single-copy E. coli plasmid vector used for constructing E. coli-Streptomyces shuttle BAC | New England Biolabs (NEB) |
| pGUSRolRPA3                   | Promoter probe vector containing the gusA gene under the control of PA3-rolO promoter | (4) |
| pLUS970                       | E. coli–Streptomyces shuttle vector for the expression of genes | (1) |
| pNX24                         | Plasmid containing the promoter of xysA gene for expression in Streptomycetes | (5) |
| pMKBAC02                      | BAC for carrying truncated abf BGC segment | This study |
| pMKBAC02-tnsR                 | Modified BAC for capturing abf BGC | This study |
| pMKBAC02-tnsR-H               | BAC for capturing abf BGC | This study |
| pMKBAC07                      | BAC for carrying truncated abf BGC segment | This study |
| pmk01                         | BAC carrying abf BGC segment orfX–abf61 | This study |
| pSL01                         | BAC carrying abf BGC segment abf5–abf61 | This study |
| pSL02                         | BAC carrying abf BGC segment abf5–abf58 | This study |
|   |   | BAC carrying \( \text{abf} \) BGC segment \( \text{abf5-} \text{abf56} \) | This study |
|---|---|---|---|
| pN1 |   | BAC carrying \( \text{abf} \) BGC segment \( \text{abf2-} \text{abf4} \) | This study |
| pN2 |   | BAC carrying \( \text{abf} \) BGC segment \( \text{orfB-} \text{abf4} \) | This study |
| pN3 |   | BAC carrying \( \text{abf} \) BGC segment \( \text{orfI-} \text{abf4} \) | This study |
| pN4 |   | BAC carrying \( \text{abf} \) BGC segment \( \text{orfO-} \text{abf4} \) | This study |
| pN5 |   | BAC carrying \( \text{abf} \) BGC segment \( \text{orfL-} \text{abf4} \) | This study |
| pN6 |   | BAC carrying \( \text{abf} \) BGC segment \( \text{orfK-} \text{abf4} \) | This study |
| pC9-DA |   | Construction of \( \text{orfA} \)-deficient mutant | This study |
| pGM1202 |   | Vector for gene expression in \textit{Streptomyces} | Addgene\# 69615 |
| pGM1202-\( \text{orfA} \) |   | For His\(6\)-tagged OrfA expression | This study |
| pGM1202-\( \text{orfA}_{\text{K144A}} \) |   | For His\(6\)-tagged OrfA\(_{\text{K144A}} \) expression | This study |
| wzc-\( \text{orfL} \) |   | Plasmid for expressing \( \text{orfL} \) in \textit{Streptomyces} | This study |
| wzc-\( \text{orfA} \) |   | Plasmid for expressing \( \text{orfA} \) in \textit{Streptomyces} | This study |
| wzc -\( \text{orfA}_{\text{K144A}} \) |   | K144A mutant of wzc-\( \text{orfA} \) | This study |
| pET28a-\( \text{Fre} \) |   | For His\(6\)-tagged Fre expression | This study |
| pET28a-\( \text{TGase} \) |   | For His\(6\)-tagged TGase expression | This study |
| Primers | Sequence 5'→3' |
|---------|----------------|
| P80     | TCCCTTCGTTGGACGGTGCTGAACA |
| P81     | ATTATTATGCTAGCCGGACGGCCGCCC |
| P82     | ATTATTATAATAGGCATTCGCCCTGGCAGACTGA |
| P83     | ATTATTATTTTTTATCCCACTACCCGCTTCGACCTGA |
| P84     | ATTATTATTTTTTATCCCACTACCCGCTTCGACCTGA |
| P85     | ATTATTATTTTTTATCCCACTACCCGCTTCGACCTGA |
| P86     | ATTATTATTTTTTATCCCACTACCCGCTTCGACCTGA |
| P87     | ATTATTATTTTTTATCCCACTACCCGCTTCGACCTGA |
| P88     | ATTATTATTTTTTATCCCACTACCCGCTTCGACCTGA |
| P89     | ATTATTATTTTTTATCCCACTACCCGCTTCGACCTGA |
| P129    | ATTATTATTTTTTATCCCACTACCCGCTTCGACCTGA |
| P130    | ATTATTATTTTTTATCCCACTACCCGCTTCGACCTGA |
| P175    | ATTATTATTTTTTATCCCACTACCCGCTTCGACCTGA |
| P176    | ATTATTATTTTTTATCCCACTACCCGCTTCGACCTGA |
| P325    | ATTATTATTTTTTATCCCACTACCCGCTTCGACCTGA |
| P326    | ATTATTATTTTTTATCCCACTACCCGCTTCGACCTGA |
| P327    | ATTATTATTTTTTATCCCACTACCCGCTTCGACCTGA |
| P328    | ATTATTATTTTTTATCCCACTACCCGCTTCGACCTGA |
| P517    | ATTATTATTTTTTATCCCACTACCCGCTTCGACCTGA |
| P518    | ATTATTATTTTTTATCCCACTACCCGCTTCGACCTGA |
| P498    | ATTATTATTTTTTATCCCACTACCCGCTTCGACCTGA |
| P499    | ATTATTATTTTTTATCCCACTACCCGCTTCGACCTGA |
| P574    | ATTATTATTTTTTATCCCACTACCCGCTTCGACCTGA |
| P577    | ATTATTATTTTTTATCCCACTACCCGCTTCGACCTGA |
| P329    | ATTATTATTTTTTATCCCACTACCCGCTTCGACCTGA |
| P330    | ATTATTATTTTTTATCCCACTACCCGCTTCGACCTGA |
| P380    | ATTATTATTTTTTATCCCACTACCCGCTTCGACCTGA |
| P386    | ATTATTATTTTTTATCCCACTACCCGCTTCGACCTGA |
| P389    | ATTATTATTTTTTATCCCACTACCCGCTTCGACCTGA |
| P413    | ATTATTATTTTTTATCCCACTACCCGCTTCGACCTGA |
| P414    | ATTATTATTTTTTATCCCACTACCCGCTTCGACCTGA |
| P571    | ATTATTATTTTTTATCCCACTACCCGCTTCGACCTGA |
| P572    | ATTATTATTTTTTATCCCACTACCCGCTTCGACCTGA |
P863  ATTATTATTTCTAGAGAAGATCCTTTGATCTTTTCTACGGGGTCTGA
P864  ATTATTATTTCTAGACTAGCGATTCCAGACGTCCCGAAGG
P877  ACGCCCGAAGCCCGAAGAAGGA
P878  AAACCTCTTCTCGGGCTGGTG
P901  ATTATTATTATTAATATGGACCACAAGGTACTGAACACGGGC
P902  ATTATTATTCTCGAGCCACATCCCCATCCGCGCGCC
P905  ATTATTATTCTAGATACTGGTAGGTGGTGCCGATATTTCCTCCTT
P906  ATGACGCTCCTCCTCACGACTACCTGCTGCTGGCCAGCA
P907  AGCAGGTAGTCGATGAAAGAGCGTCATGTACGTCACGGT
P908  ATATTAATATCTAGCTCTCCTCGTGTAATGGTTCGTGAGTAGTTCT
P909  TTCAGCATGTCCGTACGAAAGGGTT
P910  TGGAAGCTGGGCATCGGTTGACT
| proteins (aa) | Putative product | Homologs | Identities |
|--------------|------------------|----------|------------|
| OrfL (501)   | proton-dependent oligopeptide transporter | WP_206505980.1 | 484/501(97%) |
| OrfK (107)   | antibiotic biosynthesis monoxygenase | WP_031001543.1 | 107/107(100%) |
| OrfJ (236)   | putative transcriptional regulator | WP_206505981.1 | 151/168(90%) |
| OrfI (227)   | short-chain dehydrogenase | WP_206505983.1 | 214/227(94%) |
| OrfH (126)   | hypothetical protein | WP_050510192.1 | 118/126(94%) |
| OrfG (460)   | FAD-linked oxidase | WP_206505984.1 | 435/460(95%) |
| OrfF (369)   | L-lysine 6-monooxygenase | WP_206505985.1 | 340/369(92%) |
| OrfE (457)   | methionyl-tRNA synthetase | WP_206505986.1 | 426/457(93%) |
| OrfD (116)   | cupin | WP_206505987.1 | 110/116(95%) |
| OrfC (171)   | isochorismatase | WP_206505988.1 | 149/159(94%) |
| OrfB (518)   | hypothetical protein | WP_206505989.1 | 454/518(88%) |
| OrfA (583)   | tryptophan halogenase | WP_206505990.1 | 543/582(93%) |
| Abf1 (452)   | glutamate-ammonia ligase | WP_206505991.1 | 437/452(97%) |
| Abf2 (614)   | asparagine synthase | WP_206505992.1 | 608/621(98%) |
| Abf3 (232)   | putative transcriptional regulator | WP_206505993.1 | 214/232(92%) |
| Abf4 (527)   | 2,4-dichlorophenol 6-monooxygenase | WP_206505994.1 | 511/527(97%) |
| Abf5 (337)   | methyltransferase | WP_206505995.1 | 328/337(97%) |
| Abf6 (508)   | lysine N6-hydroxylase | WP_206505996.1 | 422/442(95%) |
| Abf7 (217)   | acetoacetyl-CoA reductase | WP_206505997.1 | 209/216(97%) |
| Abf8 (404)   | monoxygenase | WP_206506268.1 | 376/404(93%) |
| Abf9 (284)   | putative F420-dependent reductase | WP_206505998.1 | 276/284(97%) |
| Abf10 (31)   | transposase-like protein | WP_096624216.1 | 31/31(100%) |
| Abf11 (80)   | transposase IS4 family protein | WP_238545538.1 | 62/102(61%) |
| Abf12 (260)  | methyltransferase | MBO082939.1 | 134/257(52%) |
| Abf13 (313)  | sugar kinase | KOT91395.1 | 196/296(66%) |
| Abf14 (33)   | hypothetical protein | WP_211275163.1 | 22/33(67%) |
| Abf15 (175)  | glycosyltransferase | WP_206505999.1 | 163/175(93%) |
| Abf16 (226)  | UDP-glucuronosyltransferase | WP_206505999.1 | 214/226(95%) |
| Abf17 (287)  | 3-hydroxyisobutyrate dehydrogenase | WP_206506000.1 | 272/287(95%) |
| Abf18 (108)  | heme-degrading monoxygenase | WP_031001509.1 | 98/103(95%) |
| Abf19 (107)  | antibiotic biosynthesis monoxygenase | WP_031001507.1 | 101/107(94%) |
| Abf20 (250)  | short-chain dehydrogenase | WP_206506001.1 | 237/250(95%) |
| Abf21 (151)  | putative monoxygenase | WP_206506002.1 | 134/139(96%) |
| Abf22 (153)  | polyketide cyclase | WP_031001503.1 | 144/153(94%) |
| Abf23 (410)  | Minimal PKS chain-length factor | WP_206506003.1 | 387/410(94%) |
| Abf24 (194) | type II PKS ketosynthase alpha subunit | WP_206506004.1 | 184/194(95%) |
|------------|-------------------------------------|----------------|--------------|
| Abf25 (274) | 3-oxoacyl-(Acyl-carrier-protein) synthase | WP_206506004.1 | 225/227(99%) |
| Abf26 (144) | putative (Acyl-carrier-protein) synthase | WP_206506005.1 | 139/144(97%) |
| Abf27 (111) | polyketide synthase | WP_031001497.1 | 104/111(94%) |
| Abf28 (132) | SchA/CurD | WP_031001496.1 | 124/131(95%) |
| Abf29 (141) | putative membrane protein | WP_031001495.1 | 110/115(96%) |
| Abf30 (129) | SchA/CurD | WP_031001493.1 | 126/129(98%) |
| Abf31 (237) | short-chain dehydrogenase | WP_206506006.1 | 232/237(98%) |
| Abf32 (89) | phosphopantetheine-binding protein | WP_031001489.1 | 88/89(99%) |
| Abf33 (274) | AfsR family transcriptional regulator | WP_206506007.1 | 269/274(98%) |
| Abf34 (253) | thioesterase | WP_206506269.1 | 203/225(90%) |
| Abf35 (285) | 4'-phosphopantetheinyl transferase | WP_206506270.1 | 255/270(94%) |
| Abf36 (87) | type I polyketide synthase | WP_206506008.1 | 81/87(93%) |
| Abf37 (514) | type I polyketide synthase component | WP_206506008.1 | 371/418(89%) |
| Abf38 (72) | hypothetical protein | WP_206506009.1 | 66/72(92%) |
| Abf39 (196) | hypothetical protein | WP_206506010.1 | 187/224(83%) |
| Abf40 (71) | Acyl-CoA carboxylase subunit epsilon | WP_206506111.1 | 58/71(82%) |
| Abf41 (579) | polyketide synthase | WP_206506012.1 | 565/579(98%) |
| Abf42 (413) | hypothetical protein | WP_206506013.1 | 375/413(91%) |
| Abf43 (204) | DNA-binding response regulator | WP_206506014.1 | 201/204(99%) |
| Abf44 (323) | histidine kinase | WP_206506015.1 | 308/322(96%) |
| Abf45 (264) | regulatory protein | WP_206506016.1 | 256/264(97%) |
| Abf46 (529) | methylmalonyl-CoA carboxyltransferase | WP_206506017.1 | 504/529(95%) |
| Abf47 (260) | hydroxyneurosporene-O-methyltransferase | WP_206506018.1 | 249/260(96%) |
| Abf48 (48) | hypothetical protein | WP_206506018.1 | 23/28(82%) |
| Abf49 (336) | carminomycin 4-O-methyltransferase | WP_206506019.1 | 315/336(94%) |
| Abf50 (399) | cytochrome P450 | WP_206506020.1 | 389/402(97%) |
| Abf51 (80) | ferredoxin | WP_050510183.1 | 77/80(96%) |
| Abf52 (102) | transcriptional regulator | WP_238783843.1 | 101/102(99%) |
| Abf53 (65) | transcriptional regulator, MarR family | WP_063759224.1 | 36/37(97%) |
| Abf54 (54) | hypothetical protein | WP_206506021.1 | 41/44(93%) |
| Abf55 (126) | MarR family transcriptional regulator | WP_206506021.1 | 123/126(98%) |
| Abf56 (254) | NAD(P)-dependent dehydrogenase | WP_031001458.1 | 245/254(96%) |
| Abf57 (109) | FAD-binding monooxygenase | WP_206506022.1 | 75/77(97%) |
| Abf58 (350) | FAD-binding monooxygenase | WP_206506022.1 | 337/350(96%) |
### Table S4. $^1$H-NMR (600 MHz) spectroscopic data of albofungins 1–3. 

| No. | 1 $\delta_H$ (mult, $J$ in Hz) | 2 $\delta_H$ (mult, $J$ in Hz) | 3 $\delta_H$ (mult, $J$ in Hz) |
|-----|-------------------------------|-------------------------------|-------------------------------|
| 10  | 4.82 (br t, 6.0)              | 4.83 (br dd, 7.7, 3.8)        | 4.83 (br dd, 7.9, 3.7)        |
| 11  | 1.81 (ddd, 13.8, 7.8, 3.5)    | 1.81 (m)                      | 1.82 (m)                      |
|     | 1.71 (m, overlapping)         | 1.73 (m)                      | 1.74 (m)                      |
| 12  | 2.05 (dd, overlapping)        | 2.05 (m)                      | 2.07 (overlapping)            |
|     | 2.05 (dd, overlapping)        | 2.05 (m)                      | 2.07 (overlapping)            |
| 13  | 4.43 (dd, 8.7, 6.8)           | 4.43 (br d, 6.8)              | 4.45 (dd, 8.6, 7.0)           |
| 14  |                               |                               |                               |
| 15  |                               |                               |                               |
| 16  |                               |                               |                               |
| 17  |                               |                               |                               |
| 18  |                               |                               |                               |
| 19  | 4.96 (dd, 13.0, 4.6)          | 4.99 (dd, 13.1, 4.7)          | 5.00 (dd, 13.1, 4.7)          |
| 20  | 3.22 (dd, 13.0, 4.6)          | 3.37 (dd, 13.1, 4.7)          | 3.38 (overlapping)            |
|     | 2.75 (t, 13.0)                | 2.82 (t, 13.1)                | 2.82 (t, 13.1)                |
| 21  |                               |                               |                               |
| 22  | 7.01 (s)                      | 7.33 (s)                      | 7.32 (s)                      |
| 23  |                               |                               |                               |
| 24  | 6.61 (s)                      |                               |                               |
| 25  |                               |                               |                               |
| 26  | 2.44 (s)                      | 2.66 (s)                      | 2.74 (s)                      |
| 27  | 3.55 (s)                      | 3.56 (s)                      | 3.57 (s)                      |
| 28  | 5.60 (d, 5.9)                 | 5.62 (d, 5.9)                 | 5.63 (d, 5.9)                 |
|     | 5.42 (d, 5.9)                 | 5.43 (d, 5.9)                 | 5.44 (d, 5.9)                 |
| 3-OH| 13.57 (s)                     | 13.76 (s)                     | 13.82 (s)                     |
| 6-OH| 12.94 (s)                     | 13.00 (s)                     | 13.00 (s)                     |
| 10-OH| 5.14 (br s)                  | 5.15 (d, 4.7)                 | 5.15 (d, 4.6)                 |
| N-NH$_2$| 5.90 (br s)               | 6.01 (s)                      | 6.04 (s)                      |

*Coupling constants ($J$) in Hz were given in parentheses. The assignments were determined by $^1$H, $^{13}$C, COSY, HMQC, and HMBC NMR spectra.*
Table S5. $^{13}$C-NMR (150 MHz) spectroscopic data of albofungins 1–3.\textsuperscript{a}

| No. | 1 $\delta_{c}$ (type) | 2 | 3 |
|-----|----------------------|---|---|
| 1   | 163.3 (C)            | 162.7 (C) | 162.2 (C) |
| 2   | 109.2 (C)            | 108.7 (C) | 108.8 (C) |
| 3   | 156.8 (C)            | 157.1 (C) | 158.0 (C) |
| 4   | 112.8 (C)            | 114.6 (C) | 114.4 (C) |
| 5   | 109.8 (C)            | 109.1 (C) | 109.8 (C) |
| 6   | 149.5 (C)            | 149.8 (C) | 149.7 (C) |
| 7   | 111.6 (C)            | 111.0 (C) | 111.2 (C) |
| 8   | 182.1 (C)            | 182.1 (C) | 182.1 (C) |
| 9   | 120.3 (C)            | 120.4 (C) | 120.5 (C) |
| 10  | 58.7 (CH)            | 58.8 (CH) | 58.8 (CH) |
| 11  | 27.9 (CH$_2$)        | 27.9 (CH$_2$) | 27.9 (CH$_2$) |
| 12  | 22.8 (CH$_2$)        | 22.8 (CH$_2$) | 22.8 (CH$_2$) |
| 13  | 74.7 (CH)            | 74.3 (CH) | 74.5 (CH) |
| 14  | 165.2 (C)            | 165.3 (C) | 165.3 (C) |
| 16  | 142.7 (C)            | 143.0 (C) | 143.0 (C) |
| 17  | 130.3 (C)            | 130.4 (C) | 130.5 (C) |
| 18  | 130.1 (C)            | 130.1 (C) | 130.2 (C) |
| 19  | 72.1 (CH)            | 71.9 (CH) | 72.4 (CH) |
| 20  | 35.9 (CH$_2$)        | 36.0 (CH$_2$) | 36.4 (CH$_2$) |
| 21  | 140.4 (C)            | 141.7 (C) | 141.6 (C) |
| 22  | 113.8 (CH)           | 111.5 (CH) | 114.2 (CH) |
| 23  | 136.3 (C)            | 133.6 (C) | 134.6 (C) |
| 24  | 105.2 (CH)           | 109.9 (C) | 100.2 (C) |
| 25  | 141.7 (C)            | 139.8 (C) | 141.2 (C) |
| 26  | 19.0 (CH$_3$)        | 16.3 (CH$_3$) | 20.3 (CH$_3$) |
| 27  | 57.8 (CH$_3$)        | 57.9 (CH$_3$) | 57.9 (CH$_3$) |
| 28  | 90.6 (CH$_2$)        | 90.7 (CH$_2$) | 90.7 (CH$_2$) |

\textsuperscript{a} Carbon type were given in parentheses. The assignments were determined by $^1$H, $^{13}$C, COSY, HMQC, and HMBC NMR spectra.
Table S6. Recovery screening of immobilized TGase from *S. aureus* ATCC 29213<sup>a</sup>

| Compound | Concentration (µM) | Recovery Concentration (nmol) |
|----------|--------------------|-------------------------------|
| 1        | 2.0                | 0.1176                        |
|          | 0.8                | 0.1176                        |
|          | 0.4                | 0.0588                        |
|          | 0.2                | 0.0294                        |
|          | 0.1                | 0.0147                        |
|          | 0.05               | <0.0147                       |
| 2        | 2.0                | 0.1176                        |
|          | 0.8                | 0.1176                        |
|          | 0.4                | 0.0588                        |
|          | 0.2                | 0.0588                        |
|          | 0.1                | 0.0147                        |
|          | 0.05               | <0.0147                       |
| 3        | 2.0                | 0.1176                        |
|          | 0.8                | 0.1176                        |
|          | 0.4                | 0.0588                        |
|          | 0.2                | 0.0588                        |
|          | 0.1                | 0.0147                        |
|          | 0.05               | <0.0147                       |

<sup>a</sup>TGase: 0.4 (nmol)
FIG S1. Construction of His<sub>6</sub>-tagged OrfA. (A) Agarose gel of orfA amplicons and restriction enzyme digested pET28a vectors. (B) SDS-PAGE of His<sub>6</sub>-tagged OrfA by E. coli BL21(DE3)-chaperone (groES/groEL) system. BI, protein expression before IPTG induction; AI-S, the supernatant of protein expression after IPTG induction. (C) FPLC (left) of His<sub>6</sub>-tagged OrfA from the E. coli BL21(DE3) chaperone co-expression (groES/groEL) system and SDS-PAGE (right) of fractions 42–50. (D) SDS-PAGE (left) and FPLC (right) of the His<sub>6</sub>-tagged OrfA from S. lividans TK64 [pGM1202-orfA]. (E) SDS-PAGE (left) and FPLC (right) of the His<sub>6</sub>-tagged OrfA<sub>K144A</sub> (an inactive OrfA) from S. lividans TK64 [pGM1202-orfA<sub>K144A</sub>]. (F) SDS-PAGE of the His<sub>6</sub>-tagged TGase of S. aureus ATCC 29213 from E. coli BL21(DE3). (G) SDS-PAGE of the His<sub>6</sub>-tagged Fre protein from E. coli BL21(DE3). DNA
ladder: GeneRuler 1kb (Thermo Scientific); Protein ladder: Precision Plus Protein™ Dual Color standards (Bio-Rad).
FIG S2. Construction of pMK01ΔorfA mutant strains used in this study. (A) Scheme for the construction of orfA-deficient mutants using pC9-DA plasmids via in vivo CRISPR-Cas9 genome editing and homology directed repairing. P905/P908/P909/P910 are primers used for verifying the mutants. (B) Amplifying DNA fragments from the genome of S. albus J1074::erm*-crpsc integrated with pMK01 or pMK01ΔorfA BAC and analyzing the amplicons with 1% agarose gel. Amplicons of 3.8 and 2.7 kb were produced from the heterologous host carrying pMK01 and pMK01ΔorfA BAC using primer sets P905/P908, respectively. 1.2-kb PCR fragments and no specific amplicons were produced from pMK01 and mutant strain individually using primer sets P909/P910.
FIG S3. Chemical data of albofungins 1–3. (A) Chemical information of compounds 1–3, including the assignment numbering, structure formulas, and molecular weights. (B) COSY and HMBC correlations of compounds 1–3. COSY correlations, the bold blue lines; HMBC correlations, the red arrows. (C) NOESY correlations of compounds 1–2. NOESY correlations, pink double-head dashed arrows.
FIG S4. Bioinformatic analysis of OrfA and nine selected FAD-dependent halogenases (FDHs), MibH, PyrH, PrnA, RebH, ThaI, PltA, CndH, XanH, and LlpH. (A) The evolutionary relationship of OrfA with nine selected FDHs shown in phylogenetic tree. (B) Overall multi-sequence alignment of OrfA and nine selected FDHs. The red stars indicate catalytic residues K144 and E397 in OrfA.
FIG S5. LC-MS spectra of crude extracts from NaCl-feeding fermentations of *S. tumemacerans* JCM5050. (A) LC traces of broth extracts at a given NaCl concentration, 0.02%, 0.20%, or 0.25%. (B) MS of albofungin (1). (C) MS of chloroalbofungin (2).
FIG S6. LC-MS spectra of crude extracts from NaBr-feeding fermentations of *S. tumenacerans* JCM5050. (A) LC traces of broth extracts at a given NaBr concentration, 0.02%, 0.20%, or 0.25%. (B) MS of albofungin (1). (C) MS of bromoalbofungin (3).
FIG S7. LC-MS spectra of crude extracts from KI-feeding fermentations of *S. tumenceras* JCM5050. (A) LC traces of broth extracts at a given KI concentration, 0.02%, 0.20%, or 0.25%. (B) MS of albofungin (1). (C) MS of iodoalbofungin (4). The mass intensity ratio of halide albofungins CI/I = 10:1 in the 0.25% KI condition, suggesting the iodination is low.
**FIG S8.** Kinetics of OrfA with varying concentrations of albofungin (1).
## TC-BLAST RESULTS

| Query | >Ort. |
|-------|-------|
| Query Length | 542 |
| # of TMSs in Query (HMMTOP) | 14 |

### Version: blastp

### Reference:
Atschul, Stephen F., Thomas L. Madden, Alejandro A. Schaffer, Jinghui Zhang, Zheng Zhang, Yeebo Miller, and David J. Lipman (1997). "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs." *Nucleic Acids Res.* 25:3389-3402.

### Perform PSI-BLAST with iterations

![Graph showing TC-BLAST results with various iterations and hits]
**FIG S9.** The TCDB-BLAST result of OrfL. The BLAST result indicates that OrfL is a homologous enzyme of the nitrate/chloride transporter family.
FIG S10. The multi-sequence alignment of the TGase-domains from different TGases, including S. aureus MtgA, S. aureus PBP2, Aquifex aeolicus PBP1a, and E. coli PBP1b.
FIG S11. AutoDock simulation of PBP1b and albofungins 1–3. (A) The H-bonds between PBP1b and albofungin (1) are observed from the NH$_2$ and C=NH$_2^+$ on the guanidine of Arg$_{94}$ to 10-OH within 2.2 (3.9) and 3.0 (4.7) Å; the NH on indole of Trp$_{100}$ to 10-OH within 4.4 Å; the δ-C=O of Glu$_{211}$...
the 6-OH within 4.1 Å; the δ-OH of Glu211 to the 6-OH and O atom of C-8 ketone within 3.1 and 3.1 Å; the C=NH₂⁺ on the guanidine of Arg213 to the O atoms of C-8 ketone within 3.8 (4.2) Å; the C=O of Asn275 to the 3-OH, 6-OH, and 10-OH within 3.6, 3.3, and 2.8 Å; the γ-NH₂ of Asn275 to the O atom of C-1 ketone, 3-OH, 6-OH, and N-NH₂ within 2.3 (2.5), 2.5 (3.7), 4.4, and 3.9 (4.7) Å; the C=O of Leu276 to 10-OH within 2.5 Å; the α-NH of Ser280 to the O atom of (C-19)-O-(C-28) ether within 4.6 Å; the δ-C=O of Glu313 to 6-OH within 3.7 Å; the δ-OH of Glu313 to 10-OH within 4.5 Å; the C=O of Tyr315 to the N-NH₂ within 4.5 Å; the α-NH₂ of Tyr315 to the N-NH₂ and C-1 ketone within 4.3 and 3.5 Å; the NH₂ on the guanidine of Arg325 to the O atom of C-1 ketone, 3-OH, 6-OH, and C-8 ketone within 2.7 (3.3), 2.0 (3.6), 2.7 (4.2), and 4.5 Å; the C=NH₂⁺ on the guanidine of Arg325 to the O atoms of C-1 ketone, 3-OH, 6-OH, and C-8 ketone within 4.2, 3.2, 2.1 (3.5), and 3.9 (4.5) Å, respectively; the α-NH₂ of Ala357 to the N-NH₂ within 4.8 Å. (B) The H-bonds between PBPⅠb and chloroalbofungin (2) are observed from the NH₂ and C=NH₂⁺ on the guanidine of Arg94 to 10-OH within 3.1 (4.3) and 4.7 Å; the NH on indole of Trp100 to 10-OH within 3.4 Å; the δ-C=O of Glu211 to the 10-OH within 4.7 Å; the δ-OH of Glu211 to the 6-OH and O atom of C-8 ketone within 4.4 and 3.1 Å; the C=NH₂⁺ on the guanidine of Arg213 to the O atoms of C-8 ketone and 10-OH within 4.0 (4.1) and 3.3 (3.35) Å; the C=O of Asn275 to the 3-OH, 6-OH, and 10-OH within 4.3, 4.7, and 2.2 Å; the γ-NH₂ of Asn275 to the O atom of C-1 ketone, 3-OH, 6-OH, C-8 ketone, and N-NH₂ within 2.3 (3.1), 2.8 (3.2), 3.2 (4.6), 4.3, and 4.5 Å; the C=O of Leu276 to 10-OH within 4.1 Å; the α-NH of Ser280 to the O atom of (C-19)-O-(C-28) ether within 4.2 Å; the δ-C=O of Glu313 to 6-OH and 10-OH within 4.8 and 3.1 Å; the δ-OH of Glu313 to 10-OH within 3.1 Å; the α-NH₂ of Tyr315 to the O atom of C-1 ketone within 4.6 Å; the NH₂ on the guanidine of Arg325 to the O atom of C-1 ketone, 3-OH, 6-OH, and C-8 ketone within 4.2 (4.8), 3.5 (4.7), 2.1 (3.8), and 2.8 (3.7) Å; the C=NH₂⁺ on the guanidine of Arg325 to the O atoms of 3-OH, 6-OH, and C-8 ketone within 4.9, 2.5 (4.3), and 2.7 (4.5) Å, the δ-NH of Arg325 to the O atom of C-8 ketone within 4.6 Å; the C=O of Val354 to N-NH₂ within 4.6 (4.9) Å; the C=O of Lys355 to N-NH₂ within 4.0 Å; the α-NH₂ of Ala357 to the N-NH₂ within 4.6 Å. (C) The H-bonds between PBPⅠb and chloroalbofungin (2) are observed from the NH₂ and C=NH₂⁺ on the guanidine of Arg94 to 10-OH within 2.1 (3.7) and 2.9 (4.6) Å; the NH on indole of Trp100 to 10-OH within 4.4 Å; the δ-C=O of Glu211 to the 10-OH within 4.4 Å; the δ-OH of Glu211 to the 6-OH and O atom of C-8 ketone within 3.2 and 3.3 Å; the C=NH₂⁺ on the guanidine of Arg213 to the O atoms of C-8 ketone within 3.8 (4.2) Å; the C=O of Asn275 to the 6-OH and 10-OH within 4.6 and 3.4 Å; the γ-NH₂ of Asn275 to the O atom of C-1 ketone, 3-OH, 6-OH, and N-NH₂ within 2.3 (2.6), 2.6 (3.8), 4.4, and 2.9 (4.3) Å; the C=O of Leu276 to 10-OH within 3.7 Å; the α-NH of Ser280 to the O atom of (C-19)-O-(C-28) ether within 4.7 Å; the δ-C=O of Glu313 to 6-OH within 4.9 Å; the δ-OH of Glu313 to 10-OH within 4.2 Å; the C=O of Tyr315 to the N-NH₂ within 4.4 Å; the α-NH₂ of Tyr315 to the N-NH₂ and C-1 ketone within 4.4 and 3.7 Å; the NH₂ on the guanidine of Arg325 to the O atom of C-1 ketone, 3-OH, 6-OH, and C-8 ketone within 2.6 (3.4), 2.1 (3.7), 2.8 (4.3), and 4.6 Å; the C=NH₂⁺ on the guanidine of Arg325 to the O atoms of C-1 ketone, 3-OH, 6-OH, and C-8 ketone within 4.1, 3.2, 2.2 (3.6), and 4.0 (4.6) Å, respectively.
FIG S12. $^1$H NMR spectrum (600 MHz) of albofungin (1) in DMSO-$d_6$. 

$^1$H NMR of JCM5050F2-12 (albofungin) in D6
FIG S13. $^{13}$C NMR spectrum (150 MHz) of albofungin (1) in DMSO-$d_6$. 
FIG S14. $^1$H-$^1$H COSY spectrum of albofungin (1) in DMSO-$_6$. 
FIG S15. HSQC spectrum of albofungin (1) in DMSO-\textit{d}_6.
FIG S16. HMBC spectrum of albofungin (1) in DMSO-$d_6$. 
FIG S17. NOESY spectrum of albofungin (1) in DMSO-$d_6$. 
FIG S18. $^1$H NMR spectrum (600 MHz) of chloroalbofungin (2) in DMSO-$d_6$. 
FIG S19. $^{13}$C NMR spectrum (150 MHz) of chloroalbofungin (2) in DMSO-$d_6$. 
FIG S20. $^1$H-$^1$H COSY spectrum of chloroalbofungin (2) in DMSO-$d_6$. 
FIG S21. HSQC spectrum of chloroalbofungin (2) in DMSO-$d_6$. 
FIG S22. HMBC spectrum of chloroalbofungin (2) in DMSO-$d_6$. 
FIG S23. NOESY spectrum of chloroalbofungin (2) in DMSO-d$_6$. 
FIG S24. $^1$H NMR spectrum (600 MHz) of bromoalbofungin (3) in DMSO-$d_6$. 

![NMR Spectrum](image_url)
FIG S25. $^{13}$C NMR spectrum (150 MHz) of bromoalbofungin (3) in DMSO-$d_6$. 
FIG S26. $^1$H-$^1$H COSY spectrum of bromoalbofungin (3) in DMSO-$d_6$. 
FIG S27. HSQC spectrum of bromoalbofungin (3) in DMSO-$d_6$. 
FIG S28. HMBC spectrum of bromoalbofungin (3) in DMSO-\textit{d}_6.
FIG S29. NOESY spectrum of bromoalbofungin (3) in DMSO-d$_6$.

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