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O-Methylation Steps during Strobilurin and Bolineol Biosynthesis

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Electronic Supplementary Information

1.0 Sequence alignment for Str2 and Str3 2

2.0 Cloning procedure for pTYGS-*niaD-str2/str3* 2

3.0 Transformation and selection of *A. oryzae* 4

3.1 Genetic analysis of transformants 4

3.2 *A. oryzae* Fermentation and Extraction Procedures 5

4.0 Phenotype and LCMS traces of Expt 3 5

4.1 Toxicity test with compound 10 against *A. oryzae* NSAR1 6

5.0 5.0 uv Absorbtion data of compounds 10 and 13 6

6.0 Toxicity test with bolineol against *A. oryzae* NSAR1 7

7.0 References 7

1.0 Sequence alignment for Str2 and Str3

```
Str2      MAAESAKQTPYVLVADEVEWARLDAMHNGIAKFLGNELTPVDLGQPKKILEIGAGSGAWA     60
Str3      -MSSPAAQTPYALVPTDAEWERLDAQHNGIAKFLDHKLAPVDLGQPKKILEIGSGSGAWA     59
            :. * ****.**  :.** **** ********.::*:**************:******

Str2      IQAAKLYPDADVLAIDMNPIPARPLPPNVRYQNINVLEPFPFEAASFDVIHIRLVLCHLP     120
Str3      IQAAKQFPDADVLAVDQNPLPARPLPSNIRFQQLNVLEPFPFPPGSFDIVHIRFVLCHLP     119
          ***** :*******:* **:****** *:*:*::********  .***::***:******

Str2      DGHSVLKRIIDLVAPGGWLLIDDIDWAEAFEGLDKAPGIKRGLTALVRSMEAEAGDPHYG     180
Str3      NGYTVLPRIIELVAPGGWLLVDDIDFLHAFEGLDKAPGVKSGFTGLIKSMESHDADPHFG     179
          :*::** ***:*********:****: .**********:* *:*.*::***:. .***:*

Str2      KTLKPYLEASKELSEVHVREVELPVNPIPEDPALAGLSQMMRKALVGALGAAKQSS---A     237
Str3      KTLKGLLESSSALSEVNVQKVELPINPTPEXPALGPLSRTMRQAFSNAVGAEKLNPDTVT     239
          ****  **:*. ****:*::****:** ** ***. **: **:*: .*:** * .    :

Str2      TVGLTKEVQEGFLSEMAREDMDWSYSCYLYFAAVKKSA   275
Str3      KGGLTREVQQAFLNEMGGDAQDWSYSVHLYFSWSQKRV   277
          . ***:***:.**.**. :  ***** :***:  :* .
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Figure S1: CLUSTAL O(1.2.4) multiple sequence alignment.[1] SAM binding site marked in yellow, identical residue (*), conserved substitutions (:), semi-conserved substitutions (.).

2.0 Cloning procedure for pTYGS-*niaD-str2/str3*

The expression plasmids pTYGS-*niaD-str2/str3* for transformation were constructed using restriction enzyme *Asc*I to cut in between the three promotor/terminator pairs (P/T_{adH}, P/T_{gpdA}, P/T_{eno}) in order to simultaneously add three DNA fragments (genes and plugs) by yeast homologous recombination (Figure S2). The genes *str2* and *str3* were amplified from a previous reported plasmid pTYGS-*niaD-str2-str3.*[2] Plugs to repair the cut vector were amplified from the empty pTYGSniaD expression plasmid. Oligonucelotides used for polymerase chain reaction are summarised in table S2.

Preparation of *S. cerevisiae* competent cells was done using the LiOAc/SS carrier DNA/PEG protocol developed by Gietz and Woods.[3] For each transformation competent yeast cells were freshly prepared. A single colony was inoculated into 10 ml of YPAD medium and grown overnight at 30 °C, 200 rpm. The seed culture was added to 40 ml of YPAD in a 250 ml flask and incubated at 30 °C, 200 rpm, for 4–5 h. Cells were harvested by centrifugation for 5 min at 3000 x g. After washing with 25 ml water the cell pellet was resuspended in 1 ml water and transferred to a 1.5 ml tube. The cells were pelleted at 20000 x g for 15 s, the supernatant discarded and cells resuspended in 400 µl water. Afterwards the suspension was aliquoted (100 µl into 1.5 mL tubes each).

For yeast recombination-based cloning using *S. cerevisiae*, competent cell aliquots were pelleted 20000 x g for 15 s and the supernatant discarded, following addition of 250 µl PEG solution, 36 µl 1 M LiOAc, 50 µl ssDNA (2 mg/mL) and up to 34 µl of DNA was added to the pelleted cells. Cells were resuspended in the transformation mixture by careful pipetting. The mixture was incubated at 30 °C for 50 min and afterwards cells were pelleted at 3000 x g for 15 s. The cell pellet was gently resuspended in 1 ml water before 200 µl of the mixture was spread over SM–URA plates and incubated at 30 °C for 3 to 4 days.

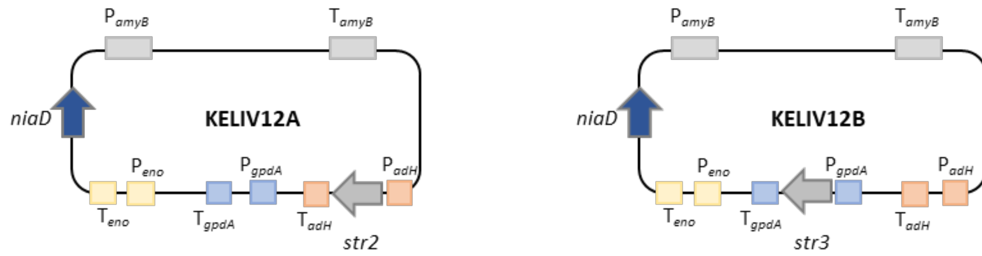

Figure S2: Plasmid maps of constructed plasmids: KELIV12A, pTYGS-*niaD-str2* and KELIV12B, pTYGS-*niaD-str3.*

Table S1: Oligonucleotide sequences for construction of expression plasmids KELIV12A and KELIV12B

Primer name	5'-3' sequence
Str2_F	TTTCTTTCAACACAAGATCCCAAAGTCAAAATGTCTTCTCCTGCTGCTCA
Str2_R	TTCATTCTATGCGTTATGAACATGTTCCCTTTAGACACGCTTCTGCGACC
Str3_F	TAACAGCTACCCCGCTTGAGCAGACATCACATGGCCGCCGAATCTGCTAA
Str3_R	ACGACAATGTCCATATCATCAATCATGACCTTAGGCACTCTTCTTCACCG
Plug gpdA_F	CTTTTCTTTTCTCTTTCTTTTCCCATCTTC
Plug gpdA_R	TGACCTCCTAAAACCCCAGTG
Plug enO_F	CTTCTTAAATATCGTTGTAACTGTTCCTGA
Plug enO_R	CGAAGTATATTGGGAGACTATAGCTACTAG
Plug adH_F	ATTCACCACTATTATTCCCACCCTATAATA
Plug adH_R	GAGACGAAACAGACTTTTTCATCGCTAAAA

3.0 Transformation and Selection of *A. oryzae*.

3.1 Genetic analysis of *A. oryzae* transformants

The genomic DNA of *A. oryzae* transformants was isolated and the correct integration of genes of interest into the genome was tested by PCR using the specific oligonucleotides for each gene (Figure S3, Table S3).

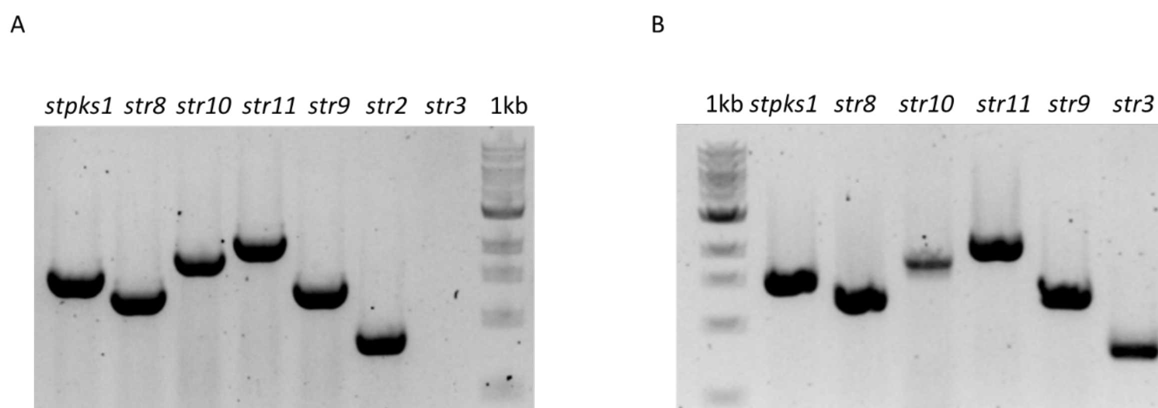

Figure S3: Genetic analysis of transformant *A. oryzae* NSAR1 *+ stPKS1 + str11+ str8 + str10 + str9 + str2* (A), and transformant NSAR1 *+ stPKS1 + str11+ str8 + str10 + str9 + str3* (B); Oligonucleotides used are summarised in table S3.

Table S3: Oligonucleotide sequences for genetic analysis

Primer name	5'-3' sequence
Str11_F	ATGCCATACCCGTCTGACTTG
Str11_R	CAAGCAAATAGTCCAACGACA
Str8_F	ATGATCGTGATGTCTCGCCTG
Str8_R	CTATCGCAGCATCCTCCCTCT
Str10_F	ATGACCATCCTCCGTTCTCGC
Str10_R	ATTGTCTACGATCGGTCGCGC
str9_F	ATGGCCGTTGATCGCAAGA
str9_R	CTAGACAAGGCGTTCCTCA
stPKS1_F	ATGTCACCTACTGCTGAAAT
stPKS1_R	GAATACGAATGGACCATCAG
1105_str2_F	ATGTCTTCTCCTGCTGCTCA
1106_str2_R	TTAGACACGCTTCTGCGACC
1107_str3_F	ATGGCCGCCGAATCTGCTAA
1108_str3_R	TTAGGCACTCTTCTTCACCG

3.2 *A. oryzae* Fermentation and Extraction Procedures

Aspergillus oryzae NSARI transformants were grown on DPY agar plates for 7 days at 28 °C. The mycelium from the plate was used to inoculate the liquid culture (100 ml DPY medium in 500 ml flasks). For LCMS analysis the cultures were grown for 7 days at 28 °C and 120 rpm prior to extraction.

DPY (agar): 2.00%(*w/v*) dextrin from potato starch, 1.00%(*w/v*) polypeptone, 0.50 monopotassium phosphate, 0.50%(*w/v*) yeast extract, 0.05%(*w/v*) $MgSO_4$ x H_2O, (2.50%(*w/v*) agar)

A. oryzae cultures were clarified by Büchner filtration, the supernatant acidified to pH 2 with 2 M HCl and extracted twice with an equal amount of ethyl acetate. Combined organic layers were dried over $MgSO_4$ and solvent removed under vacuum. Extracts were dissolved in methanol to a concentration of 10 mg/ml, filtered over glass wool and directly analysed or purified by LCMS.

4.0 Phenotype and LCMS traces of Expt 3

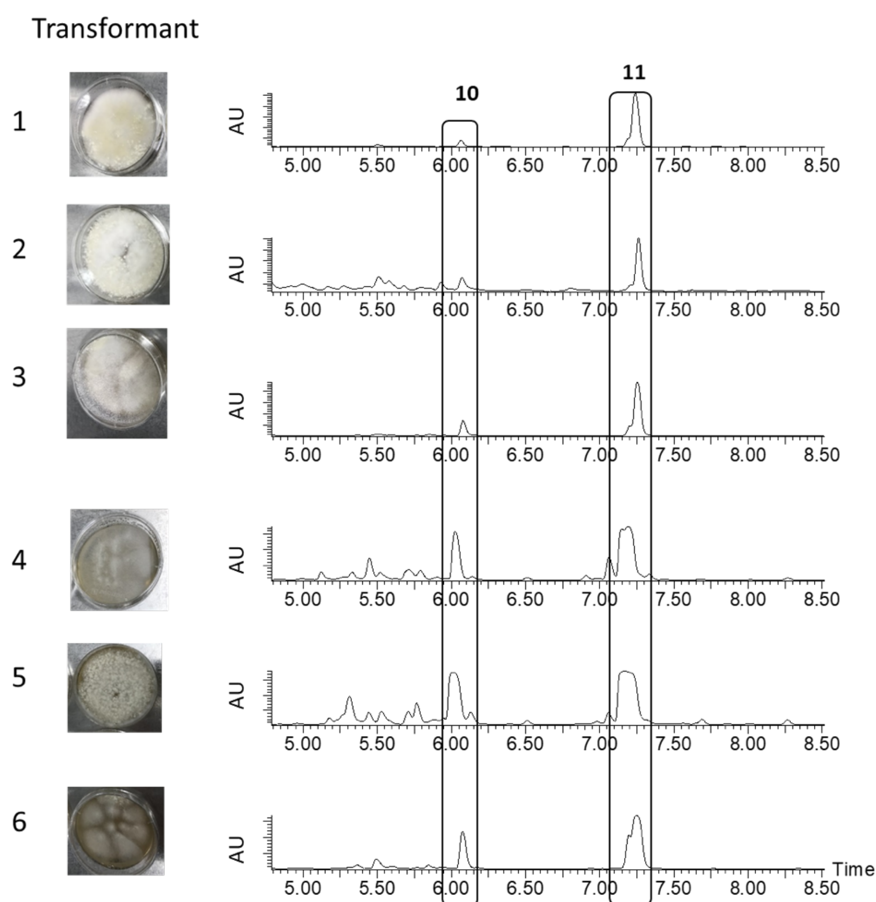

Figure S4: Phenotype and LCMS (DAD) traces of NSAR1 *stPKS1 + str11+ str8 + str10 + str9 + str3* transformants 1-6.

4.1 Toxicity test with compound 10 against *A. oryzae* NSAR1

Toxicity test were carried out on small agar plates filled with 5 ml DPY agar supplemented with different concentrations of compound **10** (0.5, 1, 2, 5 mg). Compound 10 was diluted in 200 µl DMSO prior to addition to the liquid DPY agar. Control plates were supplemented with 200 µl DMSO only. *A. oryzae* NSAR1 spores were streaked on the plates on day 1.

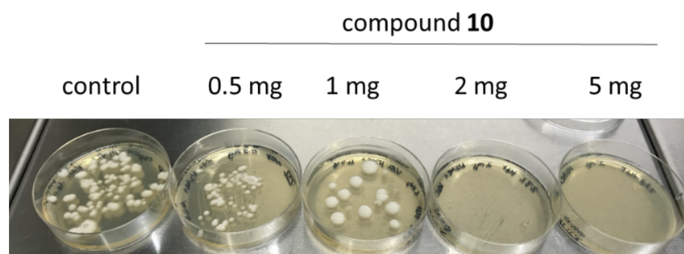

Figure S5: Toxicity test with compound **10** against *A. oryzae* NSAR1. DPY agar (5ml) was supplemented with various concentrations (0.5, 1, 2, 5 mg) of compound **10** diluted in 200 µl DMSO, control only 200 µl DMSO.

5.0 uv Absorbtion data of compounds 10 and 13

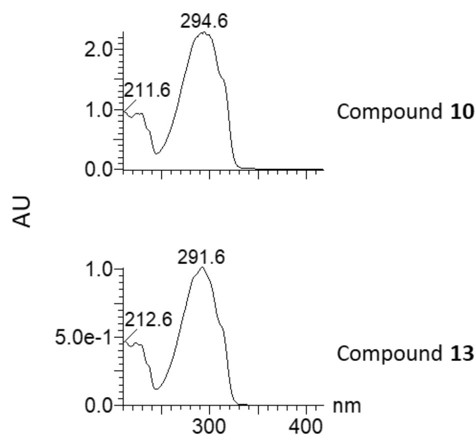

Figure S6: UV spectra of compound **13** in comparison with compound **10**. See main text Figure 2 for MS data.

6.0 Toxicity test with bolineol 4 against *A. oryzae* NSAR1

Toxicity test were carried out on small agar plates filled with 5 ml DPY agar supplemented with different concentrations of bolineol **4** (0.1, 0.2, 0.3, 0.5, 1.0, 2.0 mg). Bolineol was diluted in 200 µl DMSO prior to addition to the liquid DPY agar. Control plates were supplemented with 200 µl DMSO only. *A. oryzae* NSAR1 spores were streaked on the plates on day 1.

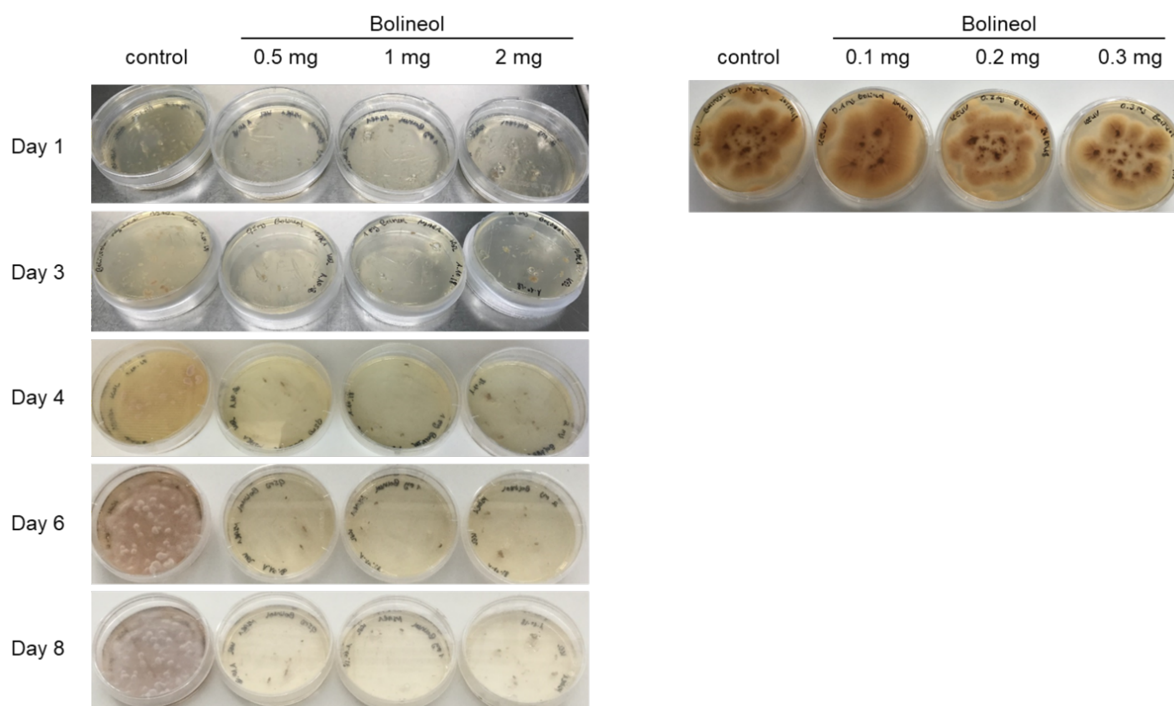

Figure S7: Toxicity test with bolineol 4 against *A. oryzae* NSAR1. DPY agar (5ml) was supplemented with various concentrations (0.1, 0.2, 0.3, 0.5, 1.0, 2.0 mg) of compound **4** diluted in 200 µl DMSO, control only 200 µl DMSO.

7.0 References

1. F. Sievers, A. Wilm, D. Dineen, T. J. Gibson, K. Karplus, W. Li, R. Lopez, H. McWilliam, M. Remmert, J. Söding, J. D. Thompson, D. G. Higgins, *Mol Syst Biol.*, 2011, **7**, 539.

2. R. Nofiani, K. de Mattos-Shipley, K. E. Lebe, L.-C. Han, Z. Iqbal, A. M. Bailey, C. L. Willis, T. J. Simpson & R. J. Cox, *Nat. Commun.*, 2018, **9**, 3940.

3. Gietz, R. D.; Woods, R. A. *Methods Enzymol.* 2002, **350**, 87–96.