Controlling a Structural Branch Point in Ergot Alkaloid Biosynthesis

Johnathan Z. Cheng, Christine M. Coyle, Daniel G. Panaccione*, Sarah E. O'Connor*

Massachusetts Institute of Technology, Department of Chemistry, 77 Massachusetts Ave., Cambridge, MA 02139; West Virginia University, Division of Plant & Soil Sciences, Morgantown, WV 26506
Dan.Panaccione@mail.wvu.edu, soc@mit.edu

Supporting Information

Table of Contents

S1. General materials and methods

General recombinant DNA cloning procedures were performed using pGEM-T vector (Promega) propagated in *E. coli* Top10 (Invitrogen). Protein expression for *N. lolii* EasA was conducted in *E. coli* Rosetta (DE3) pLysS (Novagen). Protein expression for EasG was conducted in *E. coli* BL-21(DE3) (Invitrogen). PCR amplification utilized Platinum Taq DNA Polymerase (Invitrogen). Recombinant DNA plasmids were prepared using Qiaprep Spin Miniprep and Qiaquick Gel Extraction kits (Qiagen). Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs. Primers for cloning were synthesized by Integrated DNA Technologies and DNA sequencing was conducted by the MIT Biopolymers Laboratory (Cambridge, MA).

LCMS analysis was conducted using an Acquity Ultra Performance BEH C18 column with a 1.7 mm particle size, 2.1 x 100 mm dimension, with a gradient of acetonitrile/0.1% formic acid in water mobile phase(10:90 to 20:80 from 0-5 min, 20:80 to 90:10 from 5-6 min, and 90:10 to 10:90 from 6-7min at a constant flow rate of 0.5 mL/min). The column elution was coupled to MS analysis carried out using a Micromass LCT Premier TOF Mass Spectrometer with an ESI source (Waters). Accurate mass data were acquired using reference compound leucine enkephalin for lock mass correction. A Varian Cary 50 Bio Scanning Spectrometer was used to acquire UV-Vis spectra.

The chanoclavine-I aldehyde **1** substrate was isolated from the Δ*easA* deletion strain of *A. fumigatus* as previously described.[1]

S2. Cloning, overexpression, and purification of *Neotyphodium lolii* EasA

The *N. lolii easA* gene was PCR amplified using *N. lolii* genomic DNA. Primers were designed based on the nucleotide sequence of *N. lolii easA* from the NCBI database

(EF125025.1). The following pair of oligonucleotide primers were used to amplify the *easA* gene: forward primer 5'- TTGGC**CATATG**TCAACTTCAAATCTTTTCACGCCGC-3' (with NdeI restriction site in bold) and reverse primer 5'-GA**CTCGAG**TGCTAGAACTGCCTGCTTCTTGTTC-3' (XhoI restriction site in bold). The PCR amplified *easA* gene was inserted into pGEM-T vector (Promega) for propagation and sequencing. Subsequently, the *easA* sequence was excised from pGEM-T by restriction digest and ligated into the NdeI/XhoI site of pET-24a(+) (Novagen) expression vector as a C-His_6 tagged construct.

The F176Y mutant of *N. lolii easA* was constructed using the Quickchange II Site Directed Mutagenesis Kit (Qiagen). The following pair of oligonucleotide primers were used: forward primer 5'- TCCACGGTGCCAATGGATATCTCATCGATCAGTTT-3' and reverse primer 5'-AAACTGATCGATGAGATATCCATTGGCACCGTGGA-3'. Expression of EasA_AfY178F was conducted according to previously published methods described for expression of EasA_Af.[1] The Y178F mutant of *A. fumigatus easA* was constructed using the Quickchange II Site Directed Mutagenesis Kit (Qiagen). The following pair of oligonucleotide primers were used: forward primer 5'-CATGGTGCCAATGGGTTCCTCATCGACCAGT-3' and reverse primer 5'-ACTGGTCGATGAGGAACCCATTGGCACCATG-3'.

Expression for both wild type and mutant *N. lolii* EasA was carried out in LB medium supplemented with kanamycin (50 μg/mL) and chloramphenicol (34 μg/mL). Rosetta (DE3) pLysS cells were grown to an OD_{600} of 0.7 prior to induction with IPTG (1 mM) and grown for 30 hours at 15°C prior to harvesting. Cells were resuspended in buffer (20mM Tris-HCl, 300 mM NaCl, 10% (v/v) glycerol, pH = 8.0) and incubated on ice for 30 minutes with added lysozyme (1 mg/mL) and DNAseI (10 μg/mL) and lysed by sonication. Cellular debris were pelleted by centrifugation (15,000 x g for 1 hour). EasA enzyme was purified through Ni-NTA agarose (Qiagen). Previous work established that all Old Yellow Enzyme homologs, as well as EasA from *A. fumigatus* co-purifies with an FMN flavin cofactor.[1-8] The yield of active (holo) EasA from *N. lolii* wild type and mutant was estimated to be 0.3 mg per liter culture by measuring the UV absorbance of flavin at 446 nm (FMN extinction coefficient of 12,200 $M^{-1}cm^{-1}$).[9] Assays were conducted with enzyme that eluted in the 50 mM imidazole fractions from the Ni-NTA column (Figure S1). The enzyme in these fractions co-purified with flavin cofactor. Final EasA_Nl enzyme stock concentration was at 6 μM. Enzyme eluted in the 150-300 mM imidazole fractions lacked the flavin cofactor as evidenced by UV-Vis spectra (Figure S3). These fractions were also shown to convert chanoclavine-I aldehyde **1** to agroclavine **3** when assayed with added FMN (1 μM) and apo-enzyme EasA_Nl (0.1 μM).

S3. Cloning, overexpression, and purification of *Aspergillus fumigatus* EasG

The *eas*G gene from *A. fumigatus* was PCR amplified using *A. fumigatus* cDNA. Total RNA was extracted from *A. fumigatus* mycelia tissue using the Trizol RNA extraction procedure (Invitrogen). Using Creator SMART MMLV reverse transcriptase (Clontech), cDNA was constructed from the extracted total RNA. Primers were designed based on the nucleotide sequence of *easG* from the NCBI database (XM_751041). A pair of oligonucleotide primers were used to amplify the *easG* gene: forward primer 5'-TTGGC**CATATG**ACTATCCTCGTGCTGGGTGGCCGCG-3' (with NdeI restriction site in bold) and reverse primer 5'- GA**AAGCTT**ATGCCGCATCCAGCGCGCTTTTTCC -3' (HindIII restriction site in bold). The PCR amplified *easG* gene was inserted into pGEM-T vector (Promega) for propagation and sequencing. Subsequently, the *easG* sequence was excised from

pGEM-T by restriction digest and ligated into the NdeI/HindIII site of pET-24a(+) (Novagen) expression vector as a C-His_6 tagged construct.

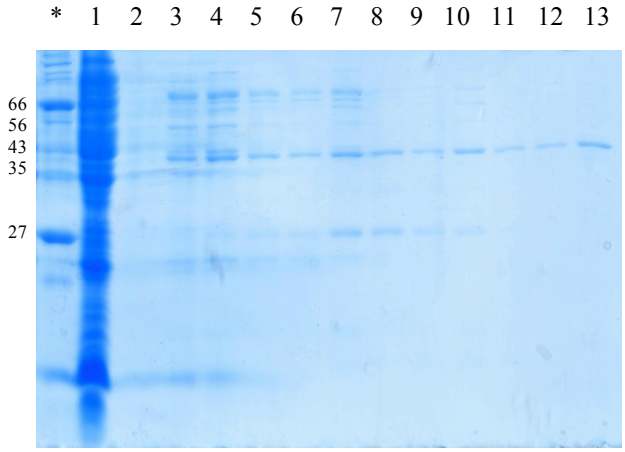
Expression was carried out in *E. coli* BL-21(DE3) cells grown in LB medium supplemented with kanamycin (50 μg/mL). Cells were grown to an OD_{600} of 0.7 prior to induction with IPTG (5 μM) and grown for 60 hours at 15°C prior to harvesting. Cells were resuspended in buffer (20mM Tris-HCl, 300 mM NaCl, 10% (v/v) glycerol, pH = 8.0) and incubated on ice for 30 minutes with added lysozyme (1 mg/mL) and DNAseI (10 μg/mL) and lysed by sonication. Cellular debris was pelleted by centrifugation (15,000 x g for 1 hour). Crude EasG enzyme was purified by incubating with Ni-NTA agarose in batch (Qiagen). Fractions containing pure EasG, as demonstrated by SDS-PAGE (Figure S2), were collected and buffer exchanged (50 mM K_2HPO_4, 100 mM NaCl, 10% (v/v) glycerol, pH = 7.0). The yield of EasG was estimated by Bradford assay as 6 mg per liter culture. Final purified EasG enzyme stock concentration was 20 μM.

S4. Endpoint assays for EasA and EasG

Endpoint assays incubated EasA (0.1 μM, final concentration), EasG (0.1 μM, final concentration), chanoclavine-I aldehyde (10 μM, final concentration), and NADPH (500 μM, final concentration) in 100 mM K_2HPO_4 buffer (pH = 7.0) at 25°C for 30 minutes. Aliquots (3 μL) were quenched by dilution in 0.1% formic acid (in water) and analyzed by LCMS.

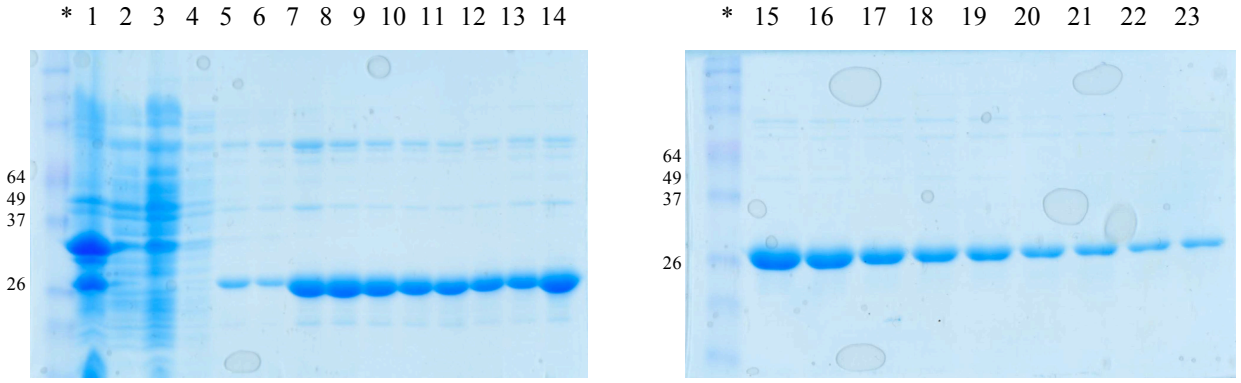
S5. Supporting Table and Figures

Figure S1. SDS-PAGE of EasA (*N. lolii*) (42 kDa)



*. NEB Broad Range Protein Ladder (2-212kDa)
1. column flow-through
2. buffer wash 10 mM imidazole
3. 25 mM imidazole
4-6. 50 mM imidazole
7. 100 mM imidazole
10-12. 150 mM imidazole
13. 300 mM imidazole

Figure S2. SDS-PAGE of EasG (*A. fumigatus)* (32kDa)



*. Invitrogen BenchMark Pre-Stained Protein Ladder
1. column flow-through
2-3. buffer wash 10 mM imidazole
4. 25 mM imidazole
5-11. 50 mM imidazole
12-19. 100 mM imidazole
20-23. 150 mM imidazole

Figure S3. Flavin absorbance spectrum (300 nm to 550 nm).

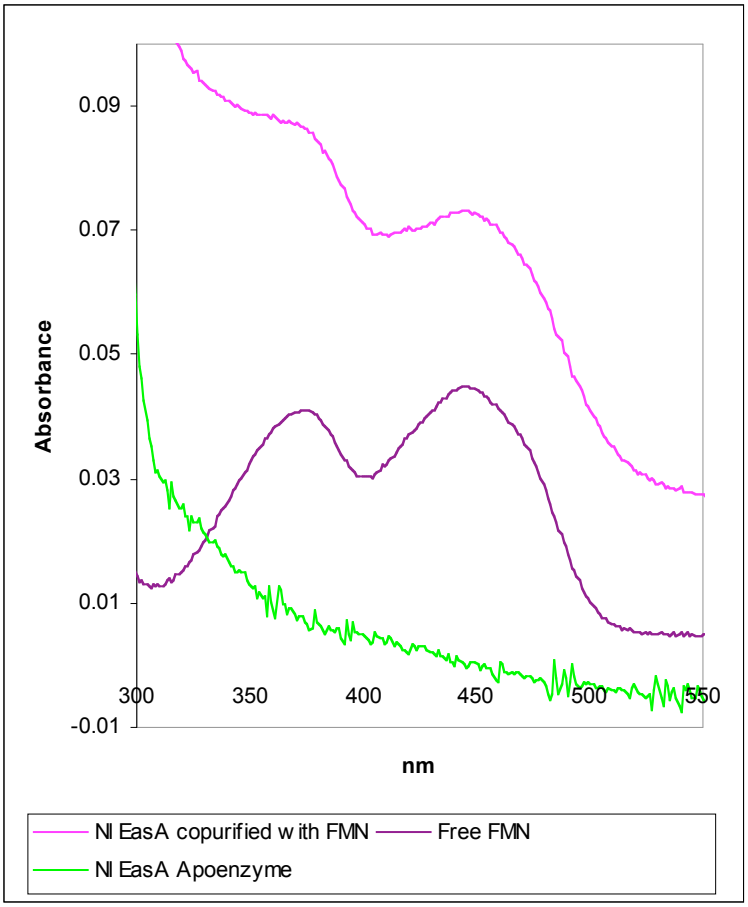


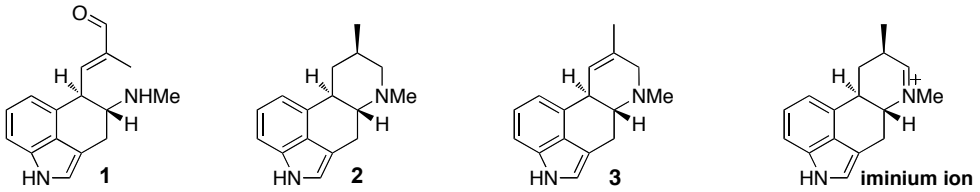
Figure S4. Alignment of partial EasA sequences from several ergot alkaloid-producing fungi. Names of fungi that produce festuclavine derivatives are shown in blue; names of those that produce agroclavine derivatives are in red. The Tyr-Phe mutation that correlates with production of festuclavine **2** (blue) versus agroclavine **3** (red) is indicated. Numbers correspond to the position, in *A. fumigatus* EasA, of the last residue in each row. The symbol * marks residues implicated in the mechanism of 1OYA of *Saccharomyces carlsbergensis* (Brown et al., 1998; Brown et al., 2002; Kholi et al., 1998; Meah et al., 2001; Xu et al., 1999). Accessions: *A. fumigatus*, XP_756133; *C. africana*, HM535795; *C. gigantea*, HM535794; *N. lolii*, ABM91449; *Epichloë festucae*, scaffold 00549 http://lims.ca.uky.edu/2368blast/blast.html; *C. purpurea* strain P1, CAG28312; *C. purpurea* A2 (strain ATCC20102), HM535793; and, *C. fusiformis*, ABV57819.

```
                                                                      82
A.fumigatus     HRMIMAPTTRFRADGQGVPLPFVQEYYGQRASVPGTLLITEATDITPKAMGYKHVPGIWS
C.africana      HKIVLSPMTRIRADDDGVPLPCVQTYYAQRASVRGTLLITEAVAICPRAKGIPNIPGIWN
C.gigantea      HKIVLSPMTRIRADDHGVPLPYVKTYYAQRACVRGTLLITEAVAVCPRAKGLPNIPGIWS
N.lolii         HKLVLSPMTRFRADNEGVPLPYVKTYYCQRASLPGTLLLTEATAISRRARGFPNVPGIWS
E.festucae      HKLVLSPMTRFRADNEGVPLPYVKTYYCQRASLPGTLLLTEATAISRRARGFPNVPGIWS
C.purpurea P1   HKIVLSPMTRFRADDNGVPLSYMKTFYAQRASVRGTLLVTDAVAICPRTKGFPNVPGIWH
C.purpurea A2   HKIVLSPMTRFRADDNGVPLSYMKTFYAQRASVRGTLLVTDAVAICPRTKGFPNVPGIWH
C.fusiformis    HKVVLSPMTRFRADNDGVPLSYVKSYYGQRASIRGTLLITEAVAICPRAKGFSNCPGIWH
                         *
                                                                     141
A.fumigatus     EPQREAWREIVSRVHSKKCFIFCQLWATGRAADPDVLADMK-DLISSSAVPVEEKGPLPR
C.africana      HDQMVAWKEVVDQVHARGCFIWVQLWATGRASEMETLRRNGFELESSSDVPIAAGDAVPR
C.gigantea      DSQIAAWKDVVDEVHSMGCSIWIQLWATGRASEMETLSSLGFELESSSDVPIASGDPVPR
N.lolii         QEQIAGWKEVVDAVHAKGSYIWLQLWATGRAAEVGVLKANGFDLVSSSAVPVSPGEPTPR
E.festucae      QEQIAGWKEVVDAVHAKGSYIWLQLWATGRAAEVGVLKANGFDLVSSSAVPVSPGEPTPR
C.purpurea P1   KDQIAAWKEVVDEVHSKGSFIWLQLWATGRAADLEALTSQGLKLESSSEVPVAPGEPTPR
C.purpurea A2   KDRIAAWKEVVDEVHSKGSFIWLQLWATGRAADLEALTSRGLKLGSSSEVPVAPGEPTPR
C.fusiformis    QDQIAAWKEVVDEVHSKGSVIWLQLWATGRASDADTLKESGFHLESSSDVPVAPGEPVPR
                                  *
                                                                     200
A.fumigatus     ALTEDEIQQCIADFAQAARNAIN-AGFDGVEIHGANGYLIDQFTQKSCNHRQDRWGGSIE
C.africana      PLSDQDIQAYIREYAQAARNAVHGAGFDGVEIHGAHGYQVDQFLQSSCNRRTDGWGGSIP
C.gigantea      PLSSEDIQLYIREYAQAARNAVHGAGFDGVEIHGAHGYQIDQFLQSSCNRRTDKWGGSIP
N.lolii         ALSDDEINSYIGDFVQAAKNAVLEAGFDGVELHGANGFLIDQFLQSPCNQRTDQWGGCIE
E.festucae      ALSDDEINSYIGDFVQAAKNAVLEAGFDGVELHGANGFLIDQFLQSPCNQRTDQWGGCIE
C.purpurea P1   ALDEDEIQQYILDYVQGAKNAVHGAGFDGVEIHGANGFLIDQFLQSSCNRRTDQWGGSIE
C.purpurea A2   ALDEDEIQQYILDYVQGAKNAVHGAGFDGVEIHGANGFLIDQFLQSSCNRRTDQWGGSIE
C.fusiformis    PLSEDEIESYIRDYVTGAINAVQGAGFDGIEIHGANGFLVDQFLQASCNTRADQWGGSIE
                                          *   *  *
                                                                     260
A.fumigatus     NRARFAVEVTRAVIEAVGADRVGVKLSPYSQYLGMGTMDELVPQFEYLIAQMRRLDVAYL
C.africana      ARSRFGLDVVRSVIDAVSKDRVGVKLSPWSTFQGMGTMDDLVPQFQHFISRLREMDVAYL
C.gigantea      GRSKFGLDITQSIIDAVGKDRVGMKLSPWSTFQGMGTMVDLLPQFQHFITCLREMDIAYL
N.lolii         NRSRFGLEITRRVIDAVGKDHVGMKLSTWSTFQGMGTMDDLIPQFEHFIMRLREIGIAYL
E.festucae      NRSRFGLEITRRVIDAVGKDHVGMKLSTWSTFQGMGTMDDLIPQFEHFIMRLREIGIAYL
C.purpurea P1   NRSRFGLEITRGVVDAVGHDRVGMKLSPWSTFQGMGTMDDLVPQFEHFITCLREMDIAYL
C.purpurea A2   NRSRFGLEITRGVVDAVGHDRVGMKLSPWSTFQGMGTMDDLVPQFEHFITCLREMDIAYL
C.fusiformis    NRSRFGLEITRRVVDAVGKDRVGVKLSPWSTFQGMGTMDDLVAQFEHFISRLREMDIAYI

                                                                     316
A.fumigatus     HLANSRWLDEEKP----HPDPNHEVFVRVWGQSSPILLAGGYDAASAEKVTEQMAAATYT
C.africana      HLTNSRWLQEEQPGIKIHPDVHNATFVRMWGREKPIVLAGGYDADSAKRLVDETYSD-QN
C.gigantea      HLTNSRWLQEEQPGVKIHPDVNNATFVRMWGLEKPIVLAGGYDADSAKRLVDETYSE-HN
N.lolii         HLANSRWVEEEDPTIRTHPDIHNETFVRMWGKEKPVLLAGGYGPESAKLVVDETYSD-HK
E.festucae      HLANSRWVEEEDPTIRTHPDIHNETFVRMWGKEKPVLLAGGYGPESAKLVVDETYSD-HK
C.purpurea P1   HLANSRWVEEEDPSIRTHPDFHNQTFVQMWGKKRPILLAGGYDPDSARRLVDQTYSD-RN
C.purpurea A2   HLANSRWVEEEDPSIRTHPDFHNQTFVQMWGKKRPILLAGGYDPDSARRLVDQTYSD-RN
C.fusiformis    HLVNTRWLEEEEPGIKTHPDVDNQTFVRMWGNKTPILLAGGYDADSARRLVDETYSD-QN


                                    340
A.fumigatus     NVAIAFGRYFISTPDLPFRVMAGI
C.africana      NVLVAFGRHYISNPDLPFRLKMGI
C.gigantea      NVLVAFGRHYISNPDLPFRLKMGI
N.lolii         NIGVVFGRHYISNPDLPFRLKMGL
E.festucae      NIGVVFGRHYISNPDLPFRLKMGL
C.purpurea P1   NVLVVFGRHYISNPDLPFRLRMGI
C.purpurea A2   NVLVVFGRHYISNPDLPFRLRMGI
C.fusiformis    NIMVVFGRHYISNPDLPFRLRLGI
```

Figure S5. Alignment of EasG sequences. Accessions: *A. fumigatus*, XM_751041; *N. lolii*, EF125025.1.

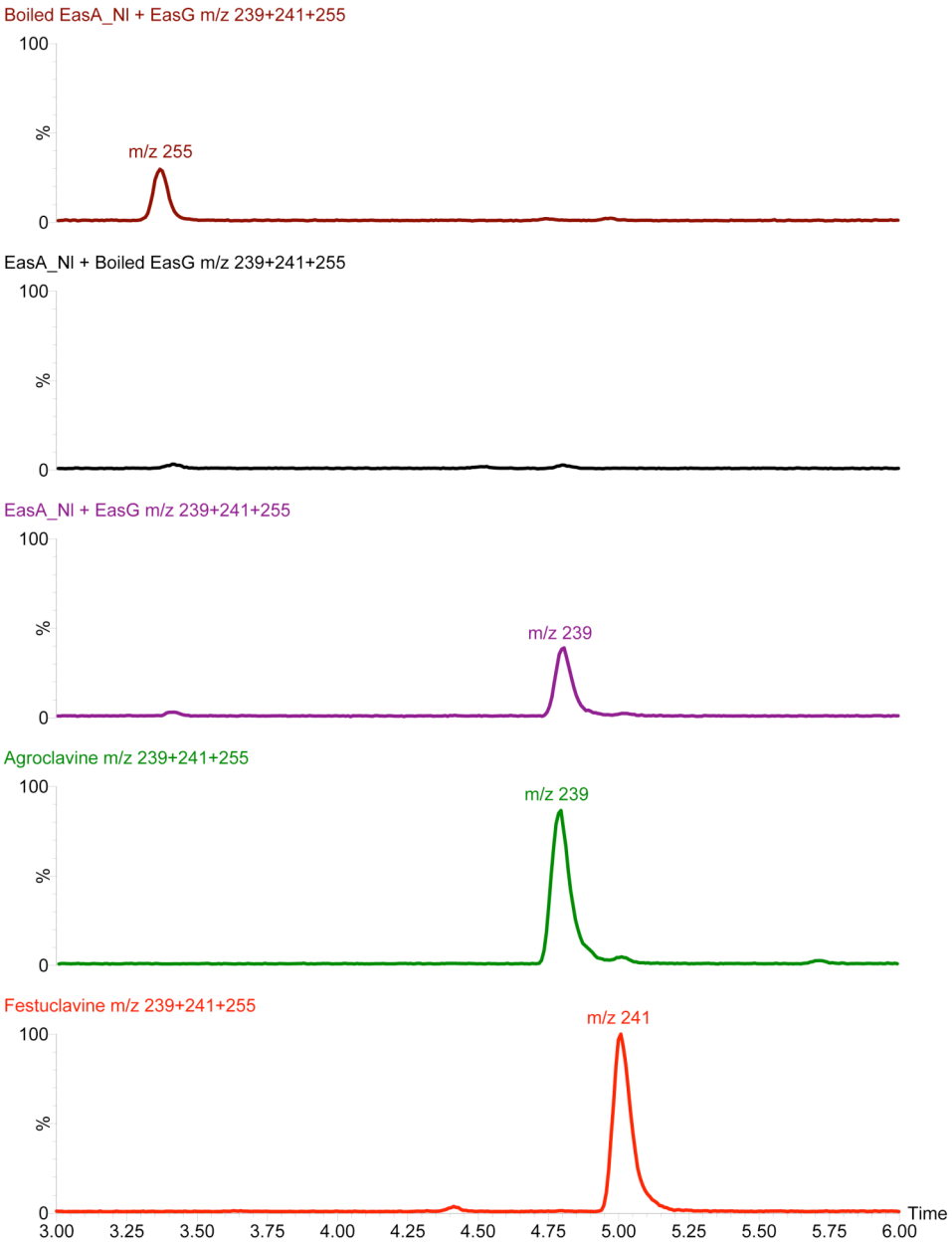
```
EasG_Af_XM_751041       ----------------------MTILVLGGRGKTASRLSLLLDNAGVPFLVGSSST--SYV 37
EasG_Nl_EF125025.1      MVQIYLPRLSMRLGYHDKNNKMTILLTGGRGKTASHIASLLQAAKVPFIVASRSSDPSSS 60
                                         ****: ********::: **: * ***:*.* *:  *

EasG_Af_XM_751041       GPYKMTHFDWLNEDTWTNVFLRASLDGIDPISAVYLVGGHAPELVDPGIRFINVARAQGV 97
EasG_Nl_EF125025.1      SPYYQNCFDWLDEKTYGDVLT--SKDSMQPISTIWLVPPPIFDLAPLMIKFVDFASRKGV 118
                        .**  . ****:*.*: :*:   * *.::***:::**     :*.   *:*::.*  :**

EasG_Af_XM_751041       NRFVLLSASNIAKGTHSMGILHAHLDSLEDVQYVVLRPTWFMENLLEDPHVS--WIKKED 155
EasG_Nl_EF125025.1      KRFVLLSASTIKKGGPAMGQVHEYLASLGGIEYAVLRPTWFMENFSYPQELQRLAIKNEN 178
                        :********.* **  :** :* :* ** .::*.**********:    .:.   **:*:

EasG_Af_XM_751041       KIYSATGDGKIPFISADDIARVAFSVLTEWKSQRAQEYFVLGPELLSYDQVADILTTVLG 215
EasG_Nl_EF125025.1      KIYSAAGDGKLPFVSVADIARVAFRTLTDEKS-HNTDYVLLGPELITYDQVAETLSTVLG 237
                        *****:****:**:*. ******* .**: ** :  :*.:*****::*****: *:****

EasG_Af_XM_751041       RKITHVSLAEADLARLLRDDVGLPPDFAAMLASMETDVKHGTEVRNSHDVKKVTGSLPCS 275
EasG_Nl_EF125025.1      RTITHIKLTEEELVKRL-ENSGMPAEDAKMLAGMDTSISDGAEDRLNNVVKHVTGADPRT 296
                        *.***:.*:* :*.: * :: *:*.: * ***.*:*.:..*:* * .: **:***: * :

EasG_Af_XM_751041       FLDFAEQEKARWMRH 290
EasG_Nl_EF125025.1      FLDFATHQKATWG-- 309
                        ***** ::** *
```

Figure S6. Endpoint Assays of EasA_Nl, EasA_Af, and EasA_Nl F176Y in combination with EasG (30 minute reaction).
LCMS chromatograms showing selected ion monitoring of chanoclavine-I aldehyde **1** ($[M+H]^+$ 255), cyclized iminium intermediate ($[M]^+$ 239), agroclavine **3** ($[M+H]^+$ 239), and festuclavine **2** ($[M+H]^+$ 241).

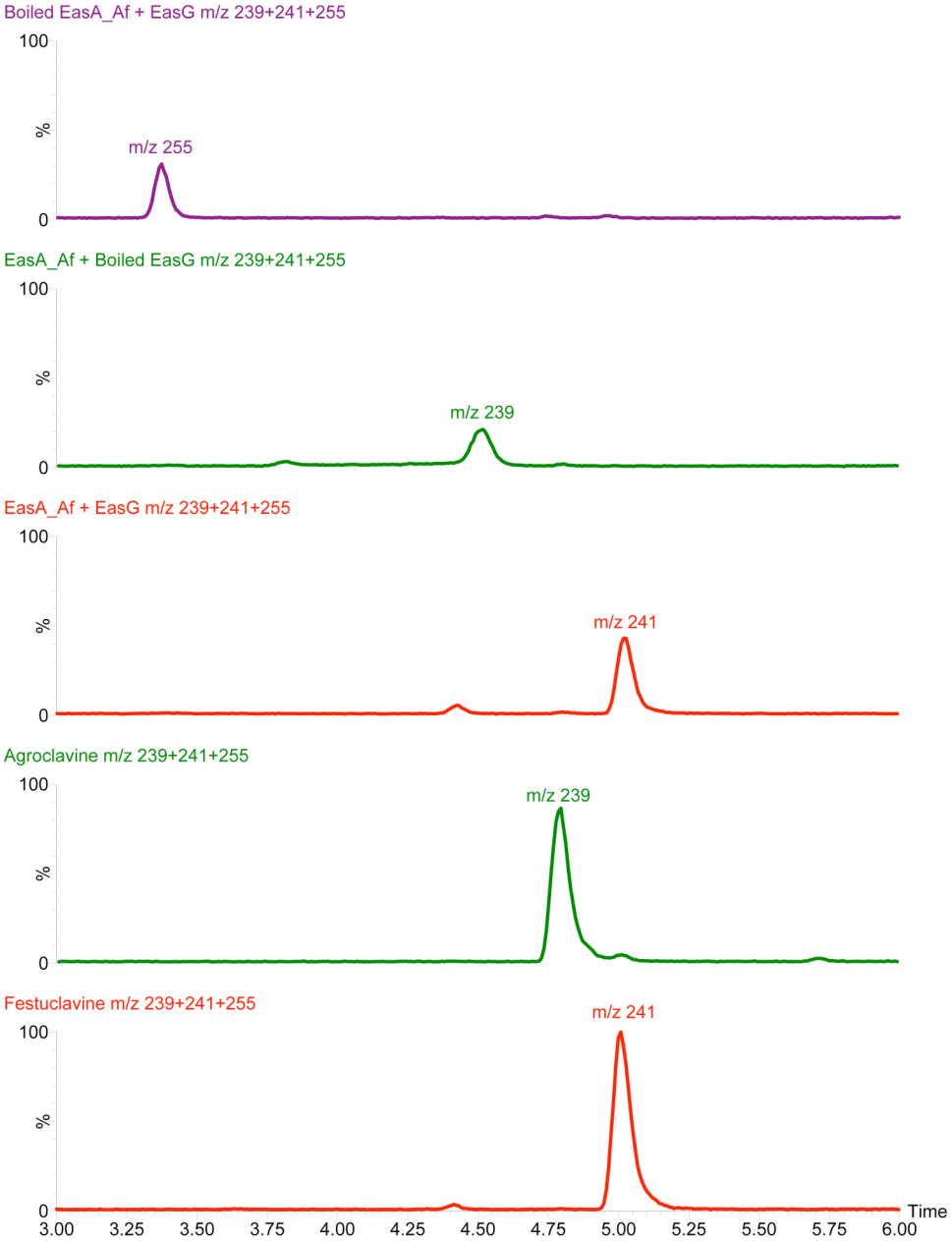


$C_{16}H_{18}N_2O$ $[M+H]^+$ 255 | $C_{16}H_{20}N_2$ $[M+H]^+$ 241 | $C_{16}H_{18}N_2$ $[M+H]^+$ 239 | $C_{16}H_{19}N_2^+$ $[M]^+$ 239

(a) Peak intensities for this set of chromatograms have been normalized to allow relative comparison of compound masses present.
EasA_Nl boiled + Eas G (negative control): only *m/z* 255 observed
EasA_Nl + EasG boiled (negative control): negligible products observed (see also Figure S9)
EasA_Nl + EasG: only *m/z* 239 observed
Agroclavine **3**: only *m/z* 239 observed
Festuclavine **2**: only *m/z* 241 observed



(b) Peak intensities for this set of chromatograms have been normalized to allow relative comparison of compound masses present.
EasA_Af boiled + Eas G (negative control): only *m/z* 255 observed
EasA_Af + EasG boiled (negative control): only *m/z* 239 observed
EasA_Af + EasG: only *m/z* 241 observed
Agroclavine **3**: only *m/z* 239 observed
Festuclavine **2**: only *m/z* 241 observed



(c) Peak intensities for this set of chromatograms have been normalized to allow relative comparison of compound masses present.
EasA_Nl F176Y boiled + Eas G (negative control): only *m/z* 255 observed
EasA_Nl F176Y + EasG boiled (control): *m/z* 255 and *m/z* 239 (iminium ion) observed
EasA_Nl F176Y + EasG: *m/z* 239 (agroclavine **3**) and *m/z* 241 (festuclavine **3**) observed
Agroclavine **3**: only *m/z* 239 observed
Festuclavine **2**: only *m/z* 241 observed

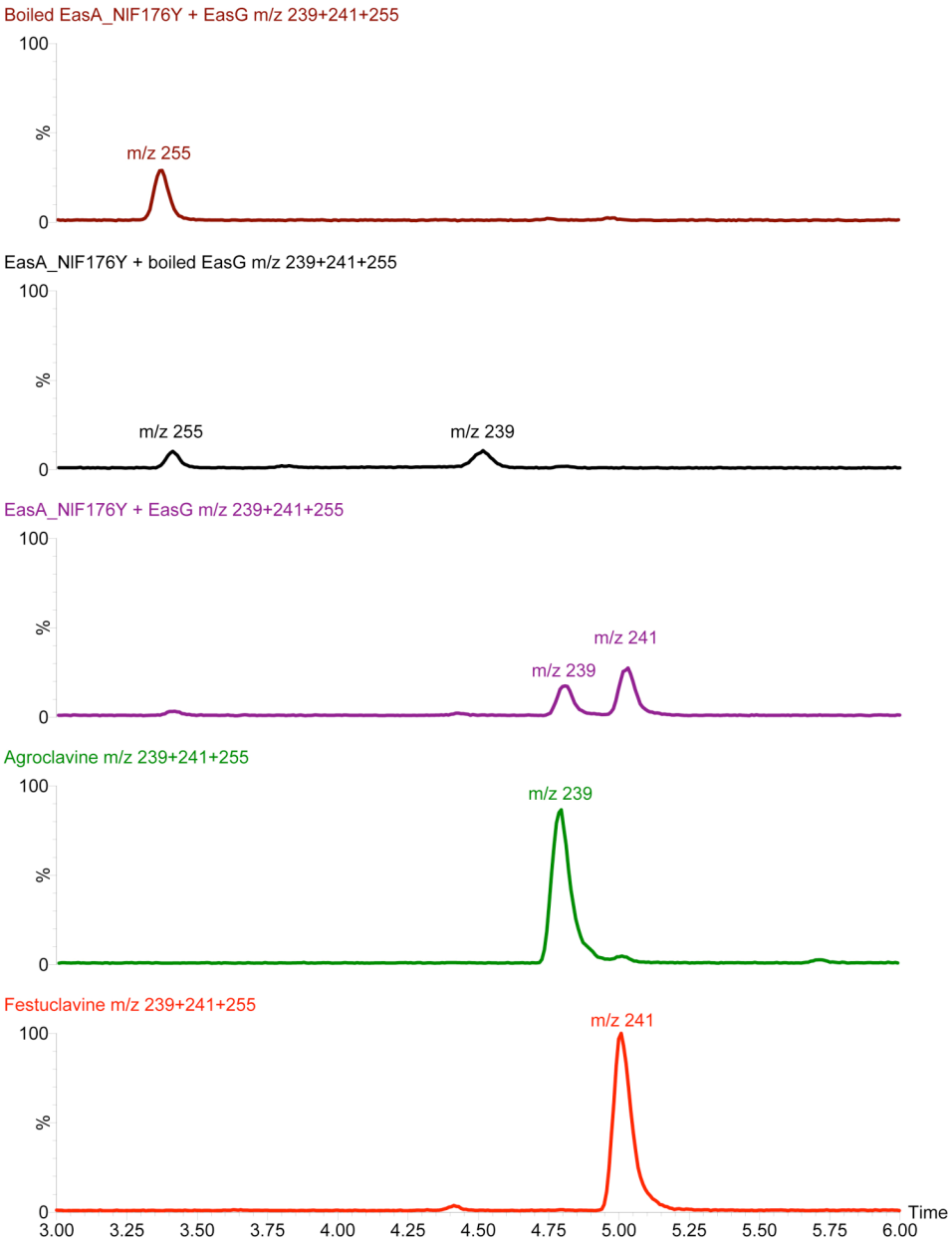


Figure S7. Endpoint Assay (30 minutes) of EasA_Af Y178F and EasG showing only formation of festuclavine 2. Assay conditions are described above in the methods. Peak intensities for this set of chromatograms have been normalized to allow relative comparison of compound masses present.

EasA_Af Y178F + Eas G: only *m/z* 241 (festuclavine **2**) observed; product at *m/z* 239 (expected for agroclavine **3**) is not observed

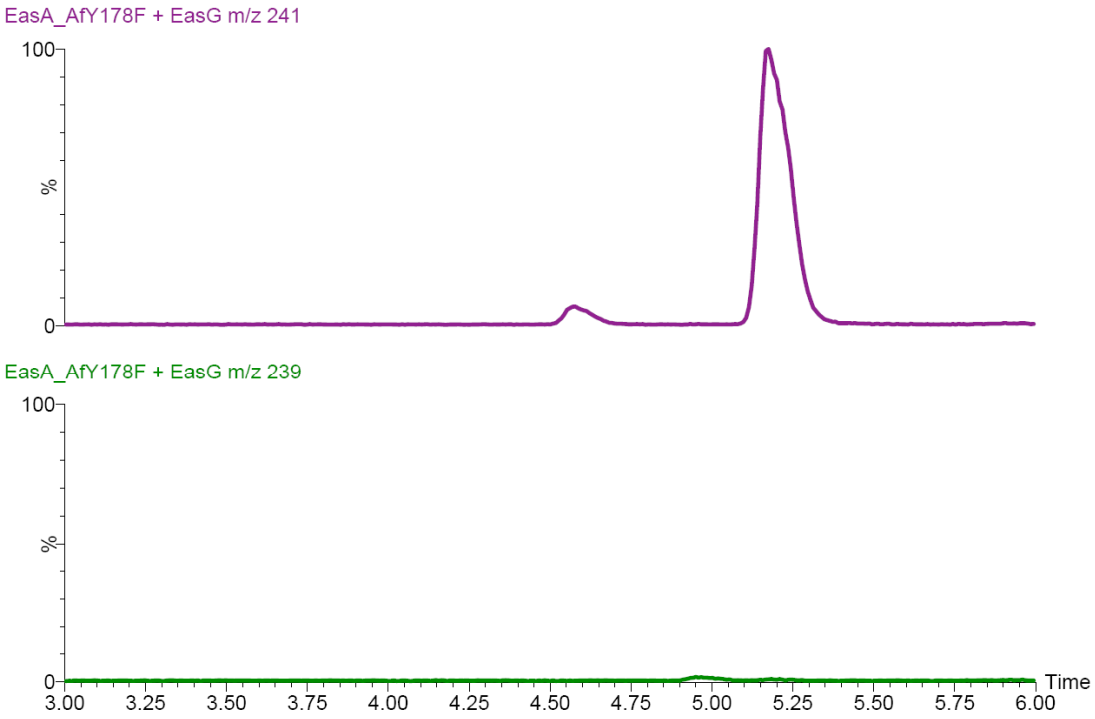


Figure S8. LCMS chromatograms showing accumulation of agroclavine 3 for EasA_Nl in the presence of EasG, chanoclavine-I aldehyde 1 and NADPH. Assay conditions are described above in the methods. Peak intensities for this of chromatograms have been normalized to allow relative comparison of compound masses present. Selected ion monitoring of agroclavine **3** ($[M+H]^+$ 239)

EasA_Nl + EasG, time = 0.5 hour
EasA_Nl + EasG, time = 12 hours
Agroclavine **3** standard

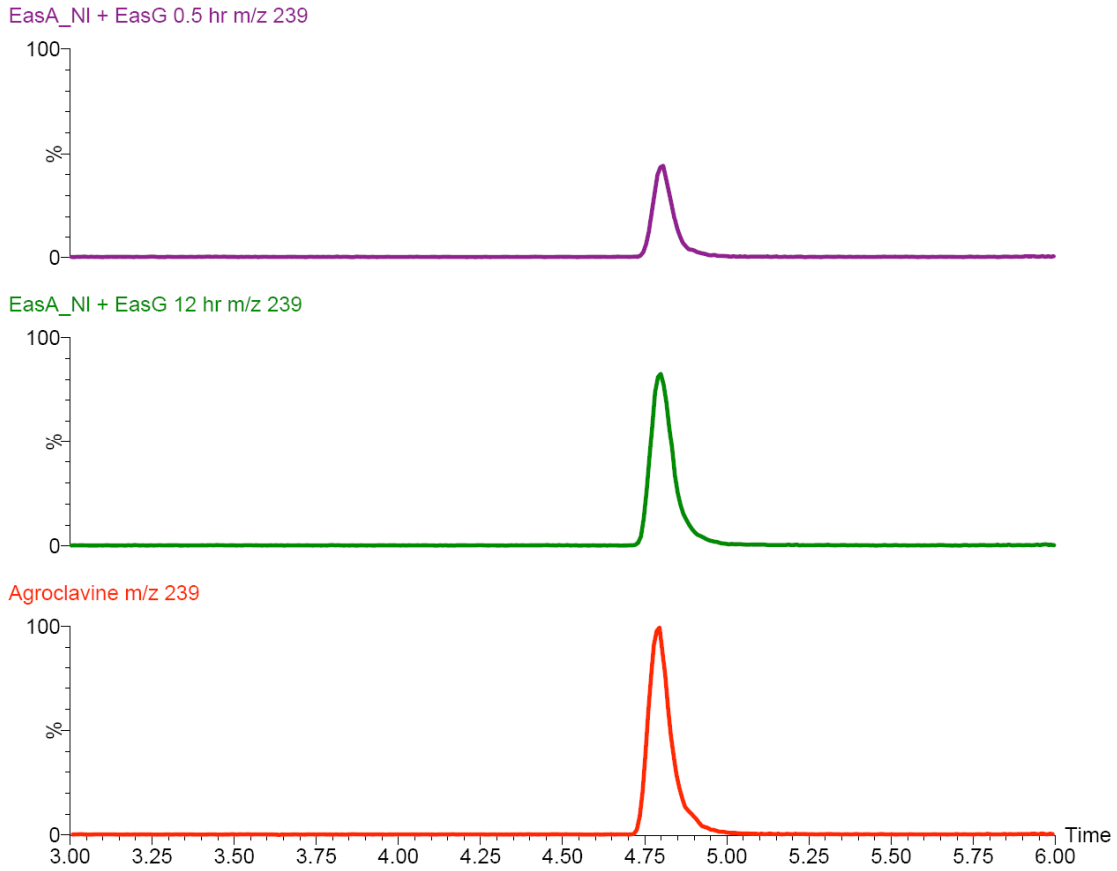
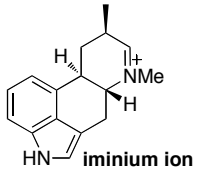
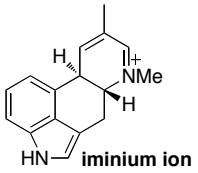


Figure S9. LCMS Selected ion monitoring chromatograms comparing the intermediates produced by EasA_Af and EasA_Nl. Selected ion monitoring of hypothetical cyclized iminium intermediates ($[M]^+$ 239 and $[M]^+$ 237). Peak intensities for this set of chromatograms have been normalized to allow relative comparison of compound masses present.

Selected ion monitoring at *m/z* 239 of EasA_Af + boiled EasG
Selected ion monitoring at *m/z* 237 of EasA_Af + boiled EasG
Selected ion monitoring at *m/z* 239 of EasA_Nl + boiled EasG
Selected ion monitoring at *m/z* 237 of EasA_Nl + boiled EasG
Selected ion monitoring at *m/z* 239 of EasA_Nl F176Y mutant + boiled EasG
Selected ion monitoring at *m/z* 237 of EasA_Nl F176Y mutant + boiled EasG



$C_{16}H_{19}N_2^+$
$[M]^+$ 239



$C_{16}H_{17}N_2^+$
$[M]^+$ 237

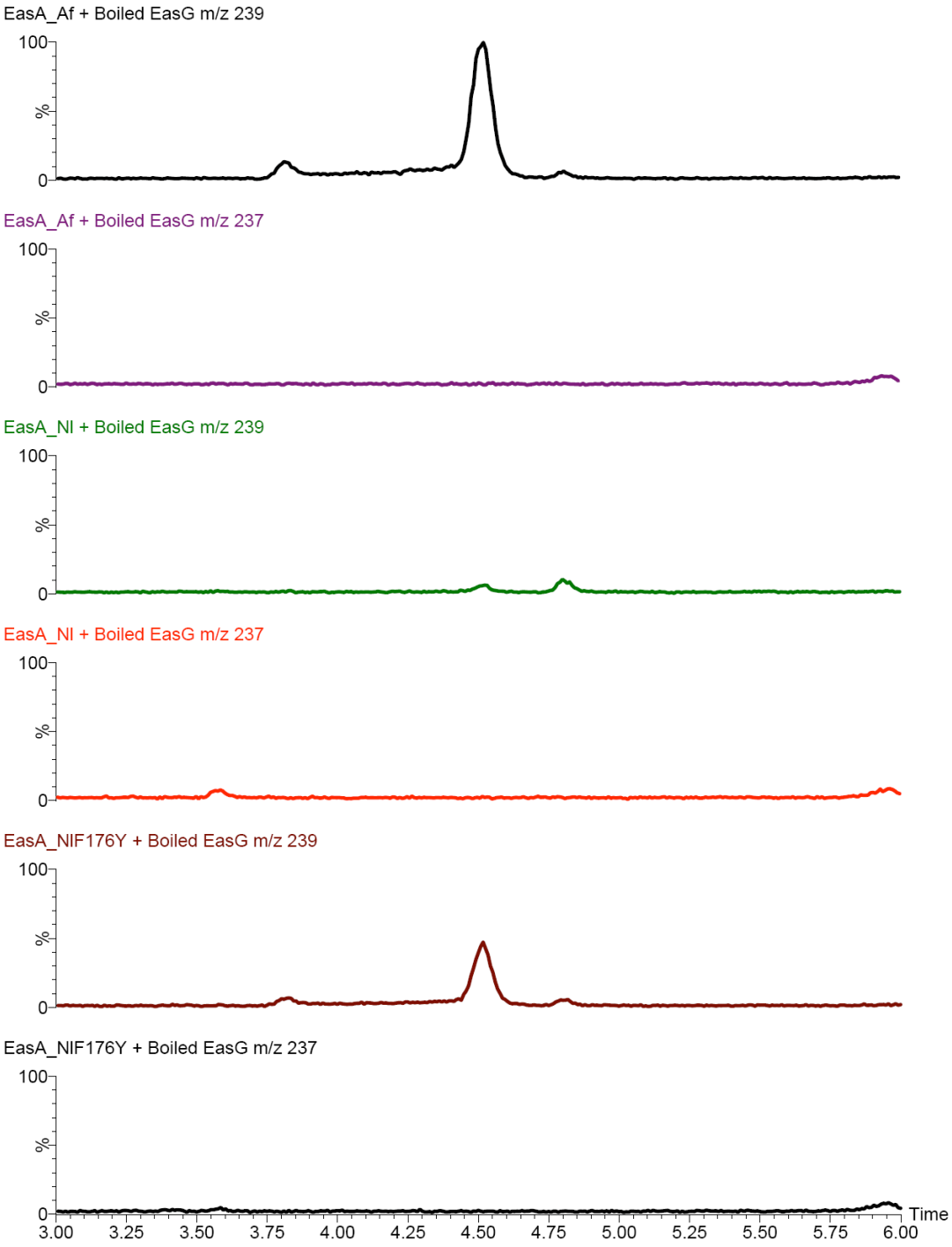

EasA_Af + Boiled EasG m/z 239
100
%
0
EasA_Af + Boiled EasG m/z 237
100
%
0
EasA_NI + Boiled EasG m/z 239
100
%
0
EasA_NI + Boiled EasG m/z 237
100
%
0
EasA_NIF176Y + Boiled EasG m/z 239
100
%
0
EasA_NIF176Y + Boiled EasG m/z 237
100
%
0
Time
3.00
3.25
3.50
3.75
4.00
4.25
4.50
4.75
5.00
5.25
5.50
5.75
6.00

Table S1. Exact Mass of Compounds Determined by High Resolution MS

Compound	Observed Mass	Theoretical Mass	Deviation of Theoretical from Observed Mass (ppm)	Molecular Formula
EasA_Af + EasG Product Festuclavine $[M+H]^+$	*m/z* 241.1695	*m/z* 241.1705	-4.1	$C_{16}H_{21}N_2$
EasA_Nl + EasG Product Agroclavine $[M+H]^+$	*m/z* 239.1541	*m/z* 239.1548	-2.9	$C_{16}H_{19}N_2$
EasA_NlF176Y + EasG Product Agroclavine $[M+H]^+$	*m/z* 239.1556	*m/z* 239.1548	3.3	$C_{16}H_{19}N_2$
EasA_NlF176Y + EasG Product Festuclavine $[M+H]^+$	*m/z* 241.1708	*m/z* 241.1705	1.2	$C_{16}H_{21}N_2$

S6. Supporting References

1. Cheng, J.; Coyle, C.; Panaccione, D. and O'Connor, S. *J. Am. Chem. Soc.* **2010**, *132*, 1776-1777.

2. Brown, B. J.; Hyun, J.; Duwuri, S.; Karplus, P. A.; Massey, V. *J. Biol. Chem.* **2002**, *277*, 2138–2145.

3. Williams, R. and Bruce, N., *Microbiology*. **2002**, *148*, 1607-1614.

4. Meah, Y.; Brown, B. J.; Chakraborty, S.; Massey, V. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 8560–8565.

5. Xu, D.; Kohli, R.; Massey, V. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 3556–3561.

6. Brown, B. J.; Deng, Z.; Karplus, P. A.; Massey, V. *J. Biol. Chem.* **1998**, *273*, 32753–32762.

7. Kohli, R.; Massey, V. *J. Biol. Chem.* **1998**, *273*, 32763–32770.

8. Karplus, P.; Fox, K. and Massey, V. *Fed. Am. Soc. Exp. Biol.* **1995,** *9*, 1518-1526.

9. Macheroux, P. In *Methods in Molecular Biology: Flavoprotein Protocols*, Chapman, S.; Reid, G., Eds. Humana Press Inc.: Totowa, NJ, 1999; Vol. 131.