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BRIEF COMMUNICATION

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A carbonate-forming Baeyer-Villiger monooxygenase

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Despite the remarkable versatility displayed by flavin-dependent monooxygenases (FMOs) in natural product biosynthesis, one notably missing activity is the oxidative generation of carbonate functional groups. We describe a multifunctional Baeyer-Villiger monooxygenase, CcsB, which catalyzes the formation of an in-line carbonate in the macrocyclic portion of cytochalasin E. This study expands the repertoire of activities of FMOs and provides a possible synthetic strategy for transformation of ketones into carbonates.

FMOs catalyze an enormous variety of substrate oxidations and reductions in both primary and secondary metabolism[1]. During natural product biosynthesis, following the assembly of the structural framework by upstream enzymes, FMOs have essential roles in introducing structural complexity and biological activity[2]. FMOs are versatile enzymes that can catalyze the formation of different types of C-O bonds[3–5]; absent from the FMO product portfolio is the carbonate moiety. To date, no enzymatic oxidation of a ketone or an ester to the corresponding carbonate has been described, although there are abundant examples of oxidation of ketones to esters catalyzed by BV monooxygenases (BVMOs)[6,7]. Nonenzymatic transformation of an ester to a carbonate is a similarly challenging synthetic transformation. The difficulties in generation of the carbonate by synthetic and enzymatic BV mechanisms are similar and include increased electron density of the ester carbonyl that deters a second peroxide attack and the unlikelihood that the resulting Criegee complex will collapse via C-C bond migration to form an additional C-O bond.

The carbonate functionality is therefore very rarely found in natural products[8]. Remarkably, several members of the cytochalasin family of fungal natural products contain an in-line carbonate moiety in the macrocycle portion of the molecules (**Fig. 1** and **Supplementary Results**, **Supplementary Fig. 1**). Both cytochalasin E (**1**) and cytochalasin K (**2**) are polyketides produced by *Aspergillus clavatus*. Related compounds such as phenochalasin B (**3**)[9] and scoparasin A (**4**)[10] also contain a vinyl carbonate. Other members of the large cytochalasin family are less oxidized at the corresponding carbonate carbon than **1**–**4**, including esters such as rosellichalasin (**5**) and ketones, as seen in cytochalasin G (**6**; **Fig. 1** and **Supplementary Figs. 2** and **3**)[11].

To study the enzymatic basis of the carbonate group in **1**, we first investigated the biosynthetic origin of the oxygen atoms in **1** by growing *A. clavatus* either in medium supplemented with sodium [1-^{13}C, 1-$^{18}O_2$]acetate or in a closed system in which consumed oxygen was replaced by $^{18}O_2$. A slight upfield shift ($\Delta\delta_c$ ~0.05 p.p.m.) for ^{13}C connected to ^{18}O was used as an indicator of the source of oxygen atoms in **1** (ref. 12). Results showed that the carbonyl oxygen at C21 of **1** is derived from acetate during polyketide assembly. In contrast, both carbonate oxygen atoms attached to C21 are derived from molecular oxygen, thereby pointing to an insertion pathway catalyzed by an oxygenase (**Supplementary Figs. 4** and **5**).

The *ccs* biosynthetic gene cluster for **1** and **2** from *A. clavatus* is centered on a PKS-nonribosomal peptide synthetase CcsA[13]. CcsB (ACLA_078650) is the only predicted FMO in the gene cluster, with moderate sequence identity to well-characterized type I BVMOs[7] (**Supplementary Figs. 6** and **7**). CcsB contains the conserved fingerprint motif FXGXXXHXXXW[14] and the strictly conserved active site arginine (Arg421) that stabilizes the Fl-4a-OO$^-$ anion through electrostatic interactions[15]. The two remaining oxygenases encoded in the gene cluster, CcsG and CcsD, are both P450 monooxygenases and are possibly involved in the oxidation of other sites in **1**, as reported for the related chaetoglobosin[16]. We therefore propose CcsB is involved in generation of the carbonate group in **1** starting from a ketone precursor, via a mechanism previously not observed among BVMOs.

To investigate the role of CcsB, we sought to inactivate the gene *ccsB* in *A. clavatus*. First, the positive regulatory gene *ccsR* was overexpressed in a Δ*lig4* modified *A. clavatus* strain to improve the titer of **1** and **2** (ref. 13). We also detected and structurally verified the presence of **5** in the culture extract (**Fig. 2a** and **Supplementary Fig. 8**), which is consistent with other co-isolation reports of **1** and **5** in fungal strains that can produce **1** (ref. 17). Using the overproducing strain (OE::*ccsR*, Δ*lig4*), the *ccsB* gene was deleted, and one of the desired mutants (Δ*ccsB*-37) was confirmed by PCR (**Supplementary Fig. 9**). Deletion of the *ccsB* gene abolished the production of **1**, **2** and **5** and led to the production of 1,5 diketone–containing compound **7** (*m*/*z* 454[M + Na]$^+$; **Fig. 2a**). The structure of **7** (named ketocytochalasin; **Fig. 2b**) was characterized by UV, MS and NMR techniques (**Supplementary Note 1**, **Supplementary Table 1** and **Supplementary Figs. 10**–**13**) and was confirmed by X-ray diffraction (**Supplementary Fig. 12** and **Supplementary Data Sets 2** and **4**; Cambridge Crystallographic Data Centre (CCDC) 970431). The abolishment of **1**, **2** and **5** upon *ccsB* inactivation and the recovery of **7** strongly indicate that CcsB is the enzyme involved in the oxidation reactions at C21. The structure of **7** also points to a relatively early action of CcsB in the tailoring of the cytochalasin scaffold en route to **1** and **2**. Additional modifications, such as C18 hydroxylation and C6-C7 epoxidation, should take place subsequent to the oxidative ring expansion steps (**Supplementary Fig. 14**).

To examine whether CcsB can generate the ester or carbonate product starting from **7**, recombinant CcsB was cloned, expressed and purified to homogeneity from *Escherichia coli* (**Supplementary Fig. 15**). CcsB was purified with a light yellow hue, and its UV spectrum showed the characteristic absorption of FAD (**Supplementary Fig. 16**). LC/MS analysis of the supernatant of denatured protein confirmed the presence of FAD at ~10% CcsB concentration, which could be reconstituted with addition of FAD (**Supplementary Figs. 16** and **17**). We then performed an *in vitro* reaction with CcsB and **7** in the presence of NADPH, FAD and SsuE (which functions as an FMN reductase; Online Methods). Whereas the control reaction without CcsB did not lead to new products, CcsB catalyzed the

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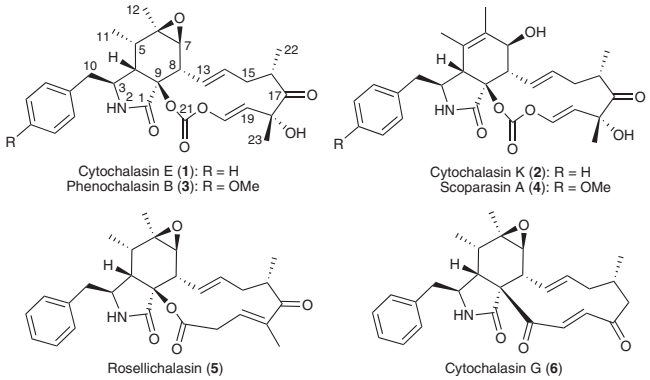

Figure 1 | Cytochalasins with different oxidation outcomes in the macrocyclic portion. Compounds **1**–**4** contain a vinyl carbonate group of interest at C21 within the 13-membered macrocycle that is fused to an isoindolone bicyclic scaffold. Other members of the large cytochalasin family are less oxidized at the corresponding carbonate carbon than **1**–**4**, including esters such as rosellichalasin (**5**) and ketones as in cytochalasin G (**6**).

conversion of **7** into two new products, **8** and **9**, as evident in the time-course analysis (**Fig. 3a**). The masses of **8** (*m*/*z* 448 $[M+H]^+$) and **9** (*m*/*z* 464 $[M+H]^+$) are consistent with that of **7** containing one and two additional oxygen atoms, respectively. Removal of NADPH from the reaction abolished the conversion of **7**. The ratio of **9** to **8** formed in the reaction strongly correlated to the molar ratio of NADPH to **7** in the reaction (**Supplementary Fig. 18**).

Complete NMR and X-ray characterization (**Supplementary Note 1**, **Supplementary Table 2**, **Supplementary Figs. 19** and **20** and **Supplementary Data Sets 1** and **3**; CCDC 970432) of **9** (**Fig. 3b**) verified the compound to indeed be the carbonate-containing cytochalasin Z_{16} (ref. 18). The identity of **9** confirmed that CcsB is the only enzyme required to convert the ketone **7** into carbonate **9**, an unprecedented example in FMO chemistry. Mutation of the Fl-4a-OO^-–stabilizing Arg421 to alanine (**Supplementary Fig. 21**) completely abolished the conversion of **7** to **8** and **9** (**Fig. 3a** and **Supplementary Fig. 15b**), thereby confirming the catalytic role of CcsB in the reaction. Feeding of pure **9** to the *ΔccsB-37* strain led to the restored production of **1** and **2** (but not **5**; **Fig. 3c** and **Supplementary Fig. 22**), which verified that **9** is indeed an on-pathway intermediate in the *ccs* biosynthetic pathway.

Initially, we expected that **8** would be an ester corresponding to **11**. However, structural elucidation revealed a double bond shift to isoprecytochalasin (**Fig. 3b**, **Supplementary Table 3**, **Supplementary Figs. 23** and **24** and **Supplementary Note 1**). No conversion to **9** was observed when pure **8** was incubated with CcsB (**Supplementary Fig. 25**), suggesting that the β-γ unsaturated ester **8** may be a shunt product formed from the spontaneous isomerization of the true intermediate **11** (**Fig. 3b**). Feeding of **8** to *A. clavatus ΔccsB-37* strain led to the restored production of **5**, along with a new product cytochalasin Z_{17} (**10**)[18] (**Fig. 3c**). Structurally, **5** and **10** represent the variations observed at C6-C7 in **1** and **2**, respectively. The simultaneous production of both **8** and **9** from the CcsB assay therefore rationalizes the co-isolation of **5** and **1** in all fungal producers (**Supplementary Fig. 14**).

The proposed isomerization of **11** to yield **8** unveils a reactive vinylogous β-diketo–type system facilitated through deprotonation of the distal α-carbon C18, followed by C20 proton abstraction from solution. As expected, when the CcsB reaction was performed in D_2O buffers, we observed the increase in the molecular weight of **8** by 1 Da (**Supplementary Fig. 26**). Under aqueous conditions, conjugation of the β-diketo system is expected to be favored for the ketone (**8**) over the ester (**11**). Indeed, density functional theory calculations (DFT; **Supplementary Note 2**) indicate that **8** is roughly 6 kcal mol^{-1} (Δ*G*) more stable than **11** (**Supplementary Fig. 27**). With shorter reaction times and lower NADPH concentrations, we did detect an unstable compound with the same molecular weight and UV absorbance as **8** (**Supplementary Fig. 28**). However, attempts to purify this compound resulted in rapid conversion to **8**, thereby indicating the possible identity as **11** (**Supplementary Fig. 29**).

Examination of known cytochalasin compounds revealed that the vinylogous 1,5-diketo system is absolutely required for carbonate formation (**Supplementary Fig. 1**). Only esters or ketone compounds are found when such configuration is altered (**Supplementary Figs. 2** and **3**). Our experiments here also support that both an α,β-unsaturated ester and an acidic proton in the γ position (α to a distal carbonyl) are required for this unique reactivity. Testing CcsB with numerous cytochalasin analogs lacking one or more of these features, including cytochalasin B and dehydrozygosporin[19] (**Supplementary Note 3**, **Supplementary Table 4** and **Supplementary Fig. 30**), did not lead to formation of carbonate products (**Supplementary Fig. 25**). These observations, combined with the lack of precedent for a second BV oxidation of an ester moiety in both synthetic and biosynthetic literature, suggest alternative mechanisms to generate the carbonate **9** (**Supplementary Figs. 31**–**33**). The first oxygen insertion step that oxidizes **7** to **11** follows that of the classic BVMO mechanism and is in line with the rules of migratory aptitude. We propose that insertion of the second oxygen into **11** to form the carbonate **9** could be initiated through a Michael addition from the peroxy-flavin anion (Fl-4a-OO^-) on the β-carbon of the vinyl ester to yield an α-β epoxide. (**Supplementary Fig. 31**). Epoxide opening facilitated by abstraction of the C18 α-proton leads to an α-alcohol intermediate, which can form the epoxy alkoxide[20] and readily rearrange across the 1,5-diketone system to afford **9**. This last step is analogous to the reported Lewis acid–promoted conversion of an α-hydroxy β-dicarbonyl compound to a carbonate[21,22]. Alternatively, direct addition of Fl-4a-OO^- at C21 of **11** may also take place (**Supplementary Figs. 32** and **33**). Direct testing of these proposals, including trapping of the conjugated flavin intermediate or testing of molecules containing similarly tuned functional groups, will be needed to conclusively define the mechanism.

Sequence analysis of CcsB revealed that it is unremarkably grouped with well-characterized BVMOs (**Supplementary Fig. 7**). An intriguing question is therefore how intrinsically different

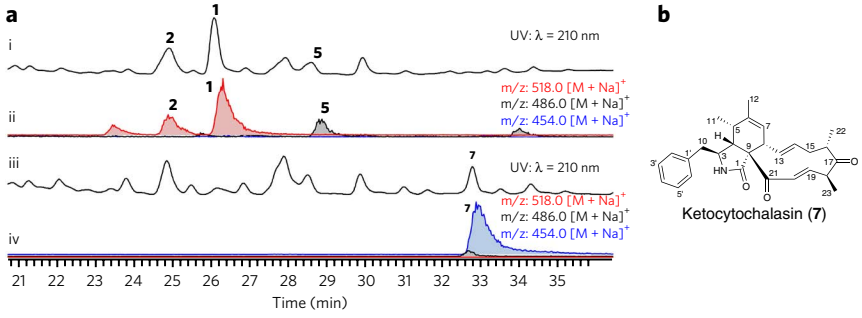

Figure 2 | Genetic confirmation of CcsB activity. (**a**) Traces i and iii show HPLC analysis (λ = 210 nm) of metabolites extracted from the OE::*ccsR Δlig4* strain and from *ΔccsB*-37 mutant, respectively. Traces ii and iv show selected ion monitoring of *m*/*z* 518 (**1** and **2**), 486 (**5**) and 454 (**7**) of traces i and iii, respectively. All of the *m*/*z* values are $[M + Na]^+$. The metabolites are extracted from the day 4 cultures. The experiments were performed three times, and representative results are shown here. (**b**) Chemical structure of **7**.

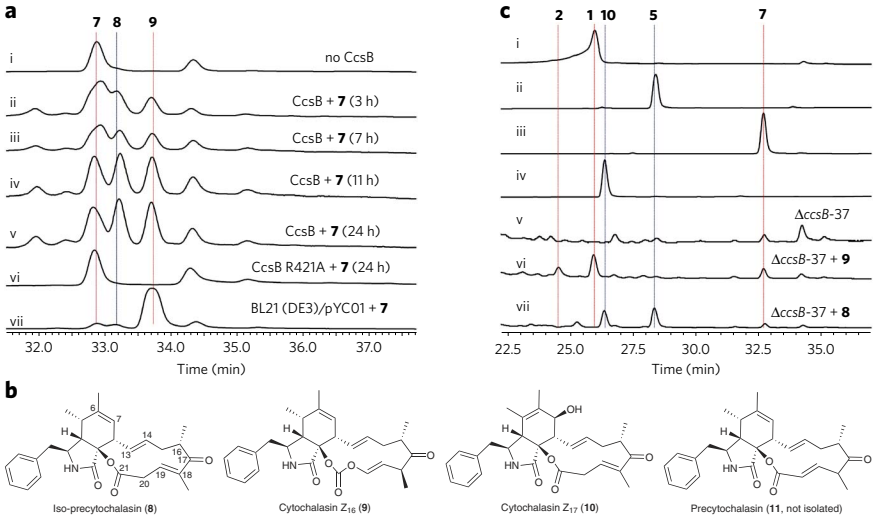

Figure 3 | Reactions catalyzed by CcsB and chemical complementation of *ΔccsB-37*. (**a**) HPLC analysis of products of the CcsB reaction (λ = 210 nm). *In vitro* reaction conditions are 50 mM potassium phosphate buffer (pH 7.0), 4 mM NADPH, 20 μM FAD, 6 μM SsuE, 10 μM CcsB and 0.4 mM **7**. Trace i shows control reaction without CcsB, traces ii–v show time course analysis of the conversion of ketone **7** to ester **8** and carbonate **9**, trace vi shows the reaction with the CcsB mutant R421A, and trace vii shows the hexane extract of *E. coli* used for biotransformation of **7** to **8** and **9**. (**b**) Chemical structures of compounds **8**–**11**. Compound **11** was not isolated in this study because of its isomerization to **8**. (**c**) HPLC analysis (λ = 210 nm) of extract from the chemical complementation of **8** and **9** to *A. clavatus ΔccsB*-37. Traces i–iv show standards of **1**, **5**, **7** and **10**; trace v shows *A. clavatus ΔccsB*-37; and trace vi and vii show *A. clavatus ΔccsB*-37 complemented with **9** or **8** in growth medium, respectively. **9** is a precursor to **1** and **2**, whereas **8** is a precursor to **5** and **10**. All of the experiments were performed three times, and representative results are shown here.

CcsB is from the canonical BVMO in catalytic function. Close homologs of CcsB are widely found in sequenced fungal genome databases, with several highly homologous hits embedded in gene clusters that resemble the *ccs* pathway. It is also expected that for all of the ester-containing cytochalasins, such as cytochalasin B, a CcsB-like enzyme must be present to perform the first BV oxidation. Such activities were demonstrated in the classical feeding studies of deoxaphomin to *Phoma* sp. and the recovery of cytochalasin B[23]. We therefore propose that formation of the carbonate product by CcsB is the fortuitous result of the perfectly arranged functional groups near the ketone site in **7** and **11**. The only comparable BVMO examples are those that can convert an acyl-carrier protein–bound thioester into a thiocarbonate for subsequent product release, such as that catalyzed by FR9H-Ox in the FR901464 pathway[24,25].

In conclusion, we identified the enzymatic basis for forming the in-line carbonate moiety in cytochalasins isolated from fungi. The conversion of a ketone to a carbonate is unprecedented in natural product biosynthesis and is a completely new type of transformation discovered for the large, well-characterized FMO family.

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Methods

Methods and any associated references are available in the online version of the paper.

Accession codes. CCDC: Crystallographic data of **7** and **9** were allocated under deposition numbers CCDC 970431 and CCDC 970432, respectively.

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Author contributions

Y.H., D.D., J.C.V. and Y.T. developed the hypothesis and designed the study. D.D. performed the isotopic labeling studies in *A. clavatus*, and J.A.J.T. and D.D. prepared selected substrate analogs. Y.H. performed the compound isolation and characterization. Y.H. performed the *in vitro* analysis of CcsB functions. A.P. and K.N.H. performed and interpreted the density functional theory calculations. All of the authors analyzed and discussed the results. Y.H., J.C.V. and Y.T. prepared the manuscript.

Competing financial interests

The authors declare no competing financial interests.

Additional information

Supplementary information and chemical compound information is available in the online version of the paper. Reprints and permissions information is available online at http://www.nature.com/reprints/index.html. Correspondence and requests for materials should be addressed to J.C.V. or Y.T.

ONLINE METHODS

Strains and culture condition. The *A. clavatus* strain was obtained from Agriculture Research Service (NRRL 1) Culture Collection and was used as the parental strain in our study. Both the wild-type and the mutant strains were grown on malt extract peptone agar (MEPA) (30 g/l malt extract, 3 g/l papaic digest of soybean meal and 15 g/l agar) at 25 °C. *E. coli* strain XL1-Blue (Stratagene) and *E. coli* TOPO10 (Invitrogen) were used for cloning. *E. coli* strain BL21(DE3) (Novagen) was used for protein expression.

DNA manipulation and construction of plasmids. Genomic DNA of *A. clavatus* NRRL1 was isolated from mycelium grown in stationary liquid culture supplied with malt extract peptone for 48 h at 25 °C. The mycelia collected were lyophilized overnight and were ground to a fine powder using toothpicks. Then, 700 µl LETS buffer (10 mM Tris-HCl, pH 8.0, 20 mM EDTA, 0.5% SDS, 0.1 M HCl) was added, and the samples were mixed by using the toothpick as well as by inverting the tubes several times before leaving the sample on the bench for 5 min. After that, 700 µl of phenol/chloroform/isoamyl alcohol (25:24:1) was added and mixed by inverting 10–15 times before centrifuging at 13,200 r.p.m. for 10 min. The supernatant was then transferred into a new 1.5-µl centrifuge tube and an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added and mixed by inverting 10–15 times. The mixture was centrifuged at 13,200 r.p.m. for 10 min, and the supernatant was then transferred into a new 1.5-µl centrifuge tube and 1 ml 95% ice-cold EtOH was added. DNA was pelleted by centrifugation at 4 °C (13,200 r.p.m., 10 min), the supernatant was removed and the pellet was washed twice with ice-cold 70% ethanol, centrifuging briefly and discarding supernatant after each wash. The resulting pellet was allowed to air dry for 10 min before resuspending in 40 µl of 10 mM Tris buffer (pH 8.0). 0.5 µl RNase (10 mg/ml stock) was added, and the mixture was incubated at 50 °C for 30 min before using for molecular cloning. Platinum Pfx DNA polymerase (Invitrogen) was used to perform PCR reactions from genomic DNA. The sequences of PCR products were confirmed by DNA sequencing (Retrogen, CA). The primers for cloning *ccsB* and for the construction of knockout cassettes are shown in **Supplementary Table 4**. The gene encoding *ccsB* was amplified and inserted into pET23 to yield pYC01. The *ccsB* gene was fused to an N-terminal Flag peptide for protein purification. The first 36 residues at the N terminus of CcsB were removed in this construct as these were predicted to be a membrane signal peptide. To perform site-directed mutagenesis of *ccsB*, PCR-directed mutagenesis was used. To construct the knockout cassette for *ccsB*, the selection marker *bar* gene with the *trpC* promoter was amplified from the plasmid pBARKS1. This marker was then flanked by two fragments of *ccsB* using fusion PCR and inserted into the pCR-Blunt (Invitrogen) vector. PCR was then used to amplify up to 10 µg of the entire knockout cassette for fungal transformation.

Fungal transformation and genetic manipulation in *A. clavatus*. Preparation of *A. clavatus* protoplasts and polyethylene glycol–mediated transformation of *A. clavatus* was performed as described previously[26] with some modification. In detail, *A. clavatus* spores were collected from culture grown for 2–3 d and inoculated in 50 ml sterile liquid minimal medium and incubated at 28 °C (280 r.p.m.) for 14 h. The culture was harvested by centrifuging at 4 °C (3,750 r.p.m.) for 10 min. The supernatant was decanted, and the aggregated germlings were transferred to a 250-ml flask containing 3 mg/ml lysing enzymes (Sigma-Aldrich) and 2 mg/ml yatalase (Sigma-Aldrich) in 12 ml of osmotic medium (1.2 M $MgSO_4$, 10 mM sodium phosphate buffer) and incubated at 25 °C (80 r.p.m.) for 12 h. Cells were poured directly in a sterile 30-ml glass Corex tube and overlaid very gently with 10 ml of trapping buffer (0.6 M sorbitol, 0.1 M Tris-HCI, pH 7.0) before centrifuging at 4 °C (5,000 r.p.m.) for 15 min. Protoplasts at the buffer interface were removed with a pipette and diluted with 1 volume of STC buffer (1.2 M sorbitol/10 mM Tris HCl, pH 7.5/10 mM $CaCl_2$) before centrifuging at 4 °C (6,000 r.p.m.) for 5 min. The supernatant was then decanted, and the protoplasts were diluted with 1 volume of STC buffer before using for transformation. Then 100 µl of PEG solution at pH 8.0 (400 mg/ml polyethylene glycol 4000, 50 mM calcium chloride and 10 mM Tris-HCl) was added to the protoplast suspension. The mixture was subsequently combined with 10 µg of the DNA fragment with which the cells were to be transformed. The mixture was incubated at 4 °C for 20 min to allow the transformation to proceed. After incubation on ice, 1 ml of the PEG solution was added to the reaction mixture, and the mixture was incubated at room temperature for an additional 5 min. The resulting cells were plated on a SMMT agar medium ($(NH_4)_2C_4H_4O_6$ 1.84 g/l, KCl 0.52 g/l, KH_2PO_4 1.52 g/l, $MgSO_4{\cdot}7H_2O$ 0.52 g/l, glucose 10 g/l, sorbitol 218.6 g/l and agar 18 g/l, supplemented with a mixture comprising $ZnSO_4{\cdot}7H_2O$ 22.0 mg/l, H_3BO_3 11.0 mg/l, $MnCl_2{\cdot}4H_2O$ 5 mg/l, $FeSO_4{\cdot}7H_2O$ 5 mg/l, $CoCl_2{\cdot}5H_2O$ 1.6 mg/l, $CuSO_4{\cdot}7H_2O$ 1.6 mg/l, $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ 1.1 mg/l and Na_2EDTA 50.0 mg/l and adjusted to pH 6.5 with 1 N KOH) with a suitable selection agent and overlaid with an SMMT soft agar medium. The transformants were transferred to fresh SMMT agar plates and grown at 25 °C for another 7 d. For selection of transformants, zeocin (200 µg/ml), hygromycin (100 µg/ml) or glufosinate (8 mg/ml) was used as the selection marker for *ccsR* overexpression integration, *lig4* knockout or *ccsB* knockout, respectively. Correct insertion of the *bar* marker in the *ccsB* gene among transformants was screened using PCR, as shown in **Supplementary Figure 9**. *ΔccsB-37* was found to be the correct mutant (**Supplementary Table 6**).

Protein expression and purification. The expression plasmid for CcsB pYC01 (**Supplementary Table 7**) was transformed into *E. coli* strain BL21 (DE3) for expression of CcsB. Cells in LB medium (1 l) supplemented with ampicillin (100 mg/l) inoculated with BL21(DE3)/pYC-1 were grown to an OD_{600} of 0.6. Protein expression was then induced with 0.12 mM of isopropylthio-β-D-galactoside (IPTG, Sigma-Aldrich), followed by further incubation with shaking at 250 r.p.m. at 16 °C for 16 h. All of the enzyme purification steps were conducted at 4 °C. *E. coli* cells were harvested by centrifugation (3,750 r.p.m., 15 min, 4 °C), resuspended in 20 ml TBS buffer and lysed with sonication on ice. Cellular debris was removed by centrifugation (14,000*g*, 0.5 h, 4 °C). Flag-tagged proteins were purified by using Anti-Flag M1 Agarose Affinity Gel (Sigma-Aldrich), following the supplied protocols. The cleared cell lysate was applied onto a gravity flow column with packed anti-Flag Agarose Affinity Gel. After extensive washing steps, CcsB was eluted with the Flag peptide elution buffer (100 µg/ml Flag peptide, 50 mM Tris-HCl, pH 7.4, 100 mM NaCl). Purified proteins were concentrated, buffer exchanged into 50 mM potassium phosphate buffer (pH 7.0) plus 20% glycerol, concentrated, aliquoted and flash frozen. Protein concentrations were determined using the Bradford dye-binding assay (Bio-Rad).

***In vitro* activity test for CcsB.** CcsB activity was assayed by monitoring the conversion of substrates into products as analyzed by LC/MS. A typical 100-µl assay solution contained 50 mM potassium phosphate buffer (pH 7.0), 4 mM NADPH (Sigma-Aldrich), 20 µM FAD (Sigma-Aldrich), 6 µM SsuE and 10 µM CcsB. The *in vitro* reaction was initiated by adding 0.4 mM of substrate, such as rosellichalasin (**5**), cytochalasin B (**6**; Sigma-Aldrich), ketocytochalasin (**7**), iso-precytochalasin (**8**) or cytochalasin D derivative (**12**). The reactions were performed at 25 °C for 3 h, 7 h, 11 h and 24 h. At each time point, the reaction was terminated by the addition of an equal volume of MeOH. Protein precipitate was removed by centrifugation. The substrates and products were extracted with 200 µl hexane twice, dried and redissolved in 60 µl methanol. A 20-µl sample was then analyzed by LC/MS. LC/MS was conducted with a Shimadzu 2010 EV liquid chromatography mass spectrometer by using both positive and negative electrospray ionization and a Phenomenex Luna 5 µm, 2.0 mm × 100 mm C18 reverse-phase column. Samples were separated on a linear gradient of 5% to 95% acetonitrile (CH_3CN) (v/v) in H_2O at a flow rate of 0.1 ml/min. To examine the effect of the relative concentrations of NADPH and **7** on the distribution of products **8** and **9**, separate assays containing **7** (0.4 mM) and NADPH of varying concentrations (3.2 mM, 2.0 mM, 1.2 mM, 0.8 mM, 0.4 mM and 0.2 mM) were performed and analyzed. The products were extracted and monitored by LC/MS after 1 h, 3 h, 7 h and 12 h. To perform the reaction in D_2O, all of the buffers and stock solutions were prepared with D_2O. The remaining conditions were kept the same as above.

Chemical analysis and characterization of compounds from *A. clavatus* and *A. clavatus ΔccsB*-37 mutant. For small-scale analysis, *A. clavatus* strains were grown in stationary liquid surface culture as a surface mat on 100 × 15 mm^2 Petri dishes with 15-ml malt extract peptone (MEP) medium for 4 d at 25 °C. The cultures were extracted with the same volume of ethyl acetate (EtOAc) and evaporated to dryness. The dried extract was dissolved in methanol and analyzed by LC/MS (for conditions, see above). To purify intermediate **7**, *A. clavatus ΔccsB-37* mutant strain was grown in stationary liquid surface culture condition in 4 l MEP liquid medium divided into 120 large 150 × 15 mm^2 Petri dishes for 5 d at 25 °C. The resulting mycelia were extracted two times with equal volumes of acetone, whereas the culture medium was treated with XAD16 resin. Absorbed compounds on XAD16 were eluted by washing

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with acetone. The organic extracts were combined and evaporated by Rotovap. The dried extract was partitioned between methanol and hexane. The hexane fraction was evaporated to dryness and purified by ISCO-CombiFlash Rf 200 (Teledyne Isco, Inc.) with a gradient of hexane and acetone (linear gradient of 0% to 30% acetone over 30 min at 10 ml/min) on normal phase silica gel. The fractions containing **7** were combined and further purified by reverse-phase HPLC (Phenyl-Hexyl 5µm, 250 × 10 mm) on a linear gradient of 60% to 90% CH_3CN (v/v, free of acid) over 30 min at a flow rate of 2.5 ml/min to give a pure white solid (**7**, t_R = 16.2 min, 10 mg/12 l). ^1H, ^{13}C and 2D NMR spectra were obtained using $CDCl_3$ as solvent on Bruker AV500 spectrometer with a 5-mm dual cryoprobe at the UCLA Molecular Instrumentation Center. Pure **7** was crystallized from MeOH/$CHCl_3$ (5:1) with an initial concentration of 2 mg/ml. Ketocytochalasin (**7**) is a colorless crystal with $[\alpha]_D$ –148° (*c* 1.2, $CHCl_3$); ^1H NMR (500 MHz, $CDCl_3$) and ^{13}C NMR(125 MHz, $CDCl_3$) (**Supplementary Table 1** and attached spectra); ESI-MS *m/z* 432 $[M + H]^+$ and 454 $[M + Na]^+$. HRESIMS *m/z* 432.25183 $[M + H]^+$ (for 432.25387, $C_{28}H_{34}NO_3$); 430.23815$[M - H]^-$(calcd. for 430.23877, $C_{28}H_{32}NO_3$).

Biotransformation of 7 to yield 8 and 9. To obtain sufficient amount of **8** and **9** for structural characterization, *in vivo* biotransformation using *E. coli* that overproduces CcsB was performed. The *E. coli* BL21(DE3)/pYC01 strain was grown in 0.5 l of LB medium supplemented with ampicillin (100 mg/l) to an OD_{600} = 0.6 at 37 °C and 250 r.p.m. Expression was induced with 0.12 mM IPTG. After induction, the culture was shaken for an additional 16 h at 16 °C and then concentrated 20-fold to 25 ml. To initiate the biotransformation, 2 mg/l of **7** was added to the concentrated culture, and the culture was shaken at 250 r.p.m. for an additional 24 h at 16 °C. To monitor the conversion of **7**, 1-ml aliquots were removed at 3 h, 7 h, 13 h and 24 h and extracted with EtOAc for LC/MS analysis. After 24 h, the culture was extracted with 25 ml of hexane twice, dried and redissolved in 1 ml of MeOH. The crude extract was then purified by reverse-phase HPLC (phenyl-hexyl 5 µm, 250 × 10 mm) on a linear gradient of 60% to 90% CH_3CN (v/v, free of acid) over 30 min at a flow rate of 2.5 ml/min, to give **8** (t_R = 16.7 min, 0.3 mg) and **9** (t_R = 17.7 min, 1.5 mg). Small needles of **9** were obtained from MeOH/$CHCl_3$ (10:1) by slow evaporation with an initial concentration of 1 mg/ml. Iso-precytochalasin (**8**) was a white solid with $[\alpha]_D$ +83° (*c* 0.2, $CHCl_3$); ^1H NMR (500 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$); (**Supplementary Table 3** and attached spectra). ESI-MS *m/z* 448 $[M + H]^+$ and 470 $[M + Na]^+$. HRESIMS *m/z* 448.24674 $[M + H]^+$ (calcd for 448.24878, $C_{28}H_{34}NO_4$,); 446.23307$[M - H]^-$ (calcd for 446.23368, $C_{28}H_{32}NO_4$,). Cytochalasin Z_{16} (**9**) was a colorless needle with $[\alpha]_D$ +37° (*c* 0.8, $CHCl_3$); ^1H NMR (500 MHz, $CDCl_3$) and ^{13}C NMR(125 MHz, $CDCl_3$) (**Supplementary Table 2** and attached spectra). ESI-MS *m/z* 464 $[M + H]^+$ and 486 $[M + Na]^+$.

Isotopic labeling study: feeding with 1-^{13}C, 1-$^{18}O_2$ acetate or $^{18}O_2$ gas. To test the origin of oxygen at C17 in cytochalasin E (**1**), doubly labeled acetate (1-^{13}C and $^{18}O_2$) was fed to *A. clavatus* grown in potato-dextrose (PD) liquid stationary cultures. Labeled **1** was isolated by extraction with EtOAc, purified by SiO_2 gel column chromatography (97-3, CH_2Cl_2-MeOH) and characterized by ^1H and ^{13}C-NMR. To confirm the molecular origin of oxygen at C17, **1** was isolated from *A. clavatus* grown in a closed system in which the oxygen consumed was replaced by $^{18}O_2$ (apparatus is shown in **Supplementary Fig. 4a**). *A. clavatus* was inoculated in fresh PD medium (4 × 50 ml) and incubated in a closed system at 30 °C. The closed system is connected via Tygon tubing to a condensation trap, a stirring 3 M KOH trap (to remove excess CO_2) and an aquarium pump (controlled by a Variac, 3–5 V). A pressure-equalizing burette (containing 0.5 g/l $CuSO_4$) was filled with either $^{16}O_2$ or $^{18}O_2$ to create a slightly over-pressurized system. As the oxygen was consumed, more was added. In the first 24 h of growth, 50 ml residual $^{16}O_2$ was consumed. In the following 49 h, 1,130 ml $^{18}O_2$ was consumed, and, finally, the system is purged with 170 ml $^{16}O_2$ in the final 14 h. Labeled **1** was isolated and characterized by ^1H and ^{13}C-NMR.

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