Supporting Information

Andexer et al. 10.1073/pnas.1015773108

SI Text

General Methods. NMR spectra were acquired on a Bruker Avance 500 spectrometer fitted with a 5 mm triple resonance inverse automatic tuning and matching (TCI ATM) cryoprobe with Z gradients running at 298 K and operating at 500 MHz and 125 MHz for ^{1}H and ^{13}C, respectively. For enzyme assays a Bruker Avance III 400 spectrometer fitted with a QNP cryoprobe was used. Standard Bruker pulse programs were used to acquire all spectra. Chemical shifts are reported in parts per million and are referenced relative to the solvent resonance. Coupling constants are given in hertz. Liquid chromatography (LC)-MS was performed on an integrated Agilent HP1100 HPLC system in combination with a Bruker Daltonics Esquire 3000+ spectrometer fitted with an electrospray source. High-resolution MS were measured on a Bruker BioApex II 4.7e Fourier Transform Ion Cyclotron Resonance spectrometer fitted with an electrospray source and operating in positive ion mode.

All purchased chemicals and solvents were of reagent or HPLC grade unless otherwise stated and were used directly as obtained from the manufacturer: benzoic acid (Sigma), [$^{2}H_5$] sodium benzoate (QMX Labs), 3-hydroxybenzoic acid (Sigma), 4-hydroxybenzoic acid (Sigma), chorismic acid [Sigma, ≥80%; or generated in house (see below)], cyclohexanecarboxylic acid (Sigma; ≥98%), (4*R*,5*R*)-4,5-dihydroxycyclohexa-1,5-dienecarboxylic acid (DCDC) (Jülich Fine Chemicals GmbH; ≥95%). D-(-)-[1,7-$^{13}C_2$] Shikimic acid was a kind gift of Professors Heinz G. Floss (University of Washington, Seattle, WA) and Taifo Mahmud (Oregon State University, Corvallis, OR) and was prepared using published methods (1). (±)-(1*R**,3*R**,4*R**)-3,4-dihydroxycyclohexanecarboxylic acid was synthesized from racemic (±)-cyclohex-3-enecarboxylic acid following published methods (2). Rapamycin, FK506, and FK520 standards were isolated from their known producer organisms (at Biotica) following standard published methods.

Escherichia coli strains were grown in 2× tryptone-yeast extract (2TY) medium under standard conditions (3). DNA manipulation, PCR, and transformation procedures were carried out using standard procedures (3). *E. coli* transformants were selected with ampicillin (100 μg/mL), kanamycin (50 μg/mL), chloramphenicol (34 μg/mL), or apramycin (50 μg/mL). For PCR reactions Phusion polymerase (Finnzymes) was used in a two-step protocol according to the manufacturer's manual; oligonucleotides were purchased from Invitrogen. Restriction enzymes. ligase (T4 DNA Ligase), and pUC19 were obtained from New England Biolabs (NEB). Oliognucleotides were obtained from Sigma or Invitrogen. DNA sequencing was performed at the University of Cambridge, Department of Biochemistry DNA Sequencing Facility. DNA synthesis was performed at Genscript.

Production of Chorismate. Chorismate was isolated from a mutant *E. coli* strain (KA12, donated by Professors Donald Hilvert and Peter Kast, Swiss Federal Institute of Technology Zurich, Zurich, Switzerland), as previously described (4). Chorismate was further purified by preparative HPLC using an Agilent 1200 Series HPLC. Chromatography was achieved over a Phenomenex Luna C_{18} column (250 × 21.2 mm; 10 μm) eluting with a gradient from water +0.1% formic acid (100%) to acetonitrile +0.1% formic acid (100%) over 30 min at a flow rate of 20 mL/min. The retention time for chorismate was approximately 10.5 min.

Preparation of Genomic DNA from *Streptomyces*. The mycelium from 3 d culture (500 μL) was collected by centrifugation and washed in sterile sucrose (10.3%) before being resuspended in SET buffer (500 μL; 75 mM NaCl, 25 mM EDTA pH 8, 20 mM Tris-HCl pH 7.5) with Lysozyme (2 mg/mL) and incubated at 37 °C for 1 h. Proteinase K solution (14 μL; 20 mg/mL) and SDS solution (60 μL; 10%) were added before incubating at 55 °C for 1 h, or until the solution was completely clear. NaCl solution (200 μL; 5 M) was then added and mixed by inversion. Chloroform (500 μL) was added and the solution was mixed on a rotary mixer at room temperature for 15 min before centrifuging at 15,000 rpm for 10 min. The aqueous layer was removed and the DNA was precipitated by adding an equal volume of isopropanol. The DNA was pelleted and washed twice with 70% ethanol before being dried and resuspended in sterile Milli-Q water (100 μL).

Construction of Plasmids for Heterologous Expression of $FkbO_{520}$ and Hyg5. Genes for $FkbO_{520}$ (5) (AF235504) and Hyg5 (6) (AF007101) were amplified by PCR from genomic DNA isolated from *Streptomyces hygroscopicus* var. *ascomyceticus* and *Streptomyces hygroscopicus*, respectively, using the following primers: FkbOF 5′-GGGCATATGACCGATGCCGGACGCCA-3′ and FkbOR 5′- GGGGGAATTCTCACGCCACCATGCCTTCGA-3′ for *fkbo*; and Hyg5F 5′-TATATATACATATGAACCCGT-CATCGCTTGTCCTGAATGG-3′ and Hyg5R 5′- TATACTC-GAGCTACATGACCACGCCCTCG-3′ for *hyg5*. The products were digested with the appropriate restriction endonucleases (*Nde*I/*EcoR*I for *fkbo*; *Nde*I/*Xho*I for *hyg5*) and ligated into pET28a. The resulting plasmids (pET28a-FkbO and pET28a-Hyg5) were verified by sequencing.

Heterologous Expression and Purification of $FkbO_{520}$ and Hyg5. A single colony of a fresh transformation was used to inoculate LB medium (5 mL) supplemented with kanamycin and chloramphenicol, and the culture was grown at 37 °C, with shaking at 300 rpm. These cells were used to inoculate 2TY medium (1 L) containing the same supplements and the culture grown at 30 °C, 200 rpm to an A_{600} of 0.4–0.6. The cells were then induced with IPTG (0.1 mM) and grown for a further 5 h before harvesting by centrifugation (5,000 × *g*, 4 °C, 15 min). For purification, the cells were resuspended in Binding Buffer (20 mL; 40 mM Tris-HCl pH 7.8, 100 mM NaCl) and broken by sonication on ice using a Mysonix Incorporated Sonicator fi Ultrasonic Processor XL2020 sonicator (5 s sonication intervals, 10 s cooling, 5 min total). After centrifugation at 35,000 × *g* for 60 min, the resulting crude extract was passed through a Ni-NTA column (2 mL bed volume), the column was washed sequentially with 5 column volumes each of binding buffer containing 0 mM imidazole, 20 mM imidazole, and 40 mM imidazole, respectively. The target protein was eluted with 5 column volumes each of 100 and 200 mM imidazole in Binding Buffer 1 (Fig. S3). Finally, the eluate was concentrated (Sartorius Vivaspin 20–30,000 MW cutoff), the buffer replaced by Storage Buffer (50 mM potassium phosphate pH 7, 300 mM NaCl) using a PD-10 desalting column (GE Healthcare), supplemented with 50% glycerol and stored at −20 °C.

$FkbO_{520}$ and Hyg5 in Vitro Assays. *NMR assays.* Assays (1 mL total volume) were carried out in deuterated potassium phosphate buffer (20 mM, pD 7) containing chorismate (10 mg/mL, 44.2 mM) and enzyme (0.5 mg/mL) at 20 °C. The samples were analyzed immediately and after 6 h by ^{1}H NMR. An experiment without enzyme served as control to monitor chorismate degradation. Data are shown in Table S1.

LC-MS assays. Enzyme reactions (1 mL total volume) were carried out in potassium phosphate buffer (100 mM, pH 7) containing chorismate (1 mM) and enzyme (100 mg/mL) at 20 °C for 2 h. After removal of the enzyme by filtration through a Sartorius Vivaspin concentrator (cutoff 10 kDa), the samples were frozen pending LC-MS analysis. LC-MS analysis was performed on an Agilent HP1100 equipped with a Gemini NX C_{18} 110 Å column (150 mm × 4.6 mm, 3 μm, Phenomenex) heated to 40 °C. The gradient elution was from 8% mobile phase B for 10 min followed by a linear increase to 100% mobile phase B over 2 min at a flow rate of 1 mL/min. Mobile phase A was water containing 0.1% trifluoroacetic acid; mobile phase B was acetonitrile containing 0.1% trifluoroacetic acid. The HPLC system described above was coupled to a Bruker Daltonics Esquire 3000 electrospray mass spectrometer, scanning from 50 to 1,500 amu and tuned to 150 amu, operating in negative mode.

Retention time and ionization of standards: chorismate, 5.0 min (450.5 $[2M-H]^-$, 206.4 $[M-H_2O-H]^-$); DCDC, 1.5 min (154.5 $[M-H]^-$, 310.6 $[2M-H]^-$); 3-hydroxybenzoic acid (3HBA), 9.6 min (136.5 $[M-H]^-$); 4HBA, 6.5 min (136.5 $[M-H]^-$).

Assay results (see Fig S4):

Chorismate (1 mM) in buffer, no enzyme: chorismate (double peak, 5.0 and 5.4 min), 4-hydroxybenzoic acid (4HBA) (degradation product, 6.5 min); no DCDC.
Chorismate (1 mM) in buffer, FkbO: DCDC (1.5 min), 4HBA (degradation product, 6.5 min).
Chorismate (1 mM) in buffer, FkbO (denatured): chorismate (double peak, 5.0 & 5.4 min), 4HBA (degradation product, 6.5 min); no DCDC.

Assay results (see Fig S8):

Chorismate (1 mM) in buffer, no enzyme: chorismate (double peak, 5.0 and 5.4 min), 4HBA (degradation product, 6.5 min); no 3HBA.
Chorismate (1 mM) in buffer, Hyg5: 3HBA (9.6 min), 4HBA (degradation product, 6.5 min).
Chorismate (1 mM) in buffer, Hyg5 (denatured): chorismate (double peak, 5.0 and 5.4 min), 4HBA (degradation product, 6.5 min); no 3HBA.

Spectrophotometric assays. For determination of kinetic parameters, the resulting pyruvate was determined spectrophotometrically based on a LDH (lactate dehydrogenase) coupled assay described elsewhere (7). Various amounts of chorismate (0–4 mM) were preincubated in potassium phosphate buffer (100 mM) containing LDH (2.5 U/mL; Sigma) and NADH (0.5 mM). After the absorption measured at 340 nm stabilized (initial decrease due to phenylpyruvate impurities in chorismate preparations), enzyme (100 μg/mL) was added and the decrease in absorption followed for 5 min. Kinetic data (Fig. S5) were fitted according to the Michaelis–Menten model using Origin software (ORIGIN® 7G, OriginLab Corporation).

Genetic Manipulation and Fermentation of *S. hygroscopicus* Mutants. Plasmid DNA was introduced into *S. hygroscopicus* by conjugal transfer as described previously (8). Fermentation was performed in 50 mL Falcon tubes (8 mL working volume) or Braun 22 L Bioreactors (15 L working volume) using the media and methods described previously (8).

In Vivo Feeding Experiments. These were performed routinely in 50 mL Falcon tubes, with the required carboxylic acid feed being added after 24 h growth in production media. Feeds were typically prepared as a 0.32 M stock solution in methanol and 50 μL was added to each tube to give a final concentration of 2 mM (25 μL for 1 mM).

Chemical Analysis of Cultures. Fermentation broth was shaken vigorously with an equal volume of acetonitrile for 20 min and then clarified by centrifugation. The resulting supernatant was examined by LC-MS/MS-UV using methods described previously (8). The content of rapamycin analogs was calculated by comparison to a standard calibration curve.

Construction of Expression Vector pGP9. pGP9 is an *E. coli*-actinomycete shuttle plasmid, containing the *oriT* gene for conjugal transfer, the apramycin gene for resistance selection, the φBT1 integrase for site-specific integration into the actinomycete chromosome, and the strong heterologous *act*I promoter (under the control of its cognate activator, *actII*-orfIV), which has been shown highly effective for gene expression in *S. hygroscopicus* (9). This was achieved by digesting pSGset1 (10) with *Spe*I and *Bst*EII and ligating the resulting 2.4-kbp fragment with the 3.9-kbp fragment of pRT801 (11) that had been digested with *Spe*I and *Bst*EII. Site-directed mutagenesis was then used to remove an undesired additional *Xba*I site thus allowing genes to be placed under the control of the *act*I promoter by cloning them directly using the unique *Nde*I-*Xba*I sites.

Construction of the *mxcF* Expression Plasmid pSGK217 and Strain BIOT-4199. The *mxcF* gene was specifically amplified as an *Nde*I-*Xba*I fragment suitable for inserting into pGP9 using the oligos mcxFfor 5′-AACATATGGCACTTCCCGCCATCGCCCCCTATCGCATGC-3′ and mcxFrev 5′-TTTCTAGAACTACGCGGTGGGGCGGACGCCCCGCTGAAG-3′. The plasmid pTYB1-MxcF was used as template [supplied by Professor Rolf Müller (Saarland University, Saarbrüken Germany) (12)]. The resulting product was treated with T4 kinase and cloned into *Sma*I cut pUC19 (dephosphorylated). Resulting clones were sequenced to confirm the database sequence. The final plasmid was designated pSGK215. The *Nde*I-*Xba*I fragment containing *mxcF* was excised and ligated into pGP9 prepared with the same enzymes. The resulting clone was verified by restriction digestion and by sequencing from the *act*I promoter. The resulting construct was designated pSGK217. pSGK217 was introduced into BIOT-4010 by conjugal transfer to give BIOT-4199.

Construction of the *rapK* Expression Plasmid pSGK261 and Strain BIOT-4549. The *Nde*I-*Xba*I fragment of pSGset1RapK (13) containing *rapK* was excised and ligated into pGP9 that had been digested with the same enzymes. The resulting clone was verified by restriction digestion and by sequencing from the *act*I promoter and was designated pSGK261. pSGK261 was introduced into BIOT-4010 by conjugal transfer to give strain BIOT-4549.

Construction of the $fkbO_{506}$ Expression Plasmid pSGK264 and Strain BIOT-4576. The $fkbO_{506}$ gene was amplified as an *Nde*I-*Xba*I fragment suitable for inserting into pGP9 using the oligos FKBO506f: 5′-CCTCTAGACCTCACGACACCACTCCTTCGATCTCCACG-3′ and FKBO506r: 5′-TGCATATGCCTGTCGCGGCACCGTACTGCCGCTTCG-3′ as reported previously (14): 5′-CCTCTAGACCTCACGACACCACTCCTTCGATCTCCACGAGCAGGTCGCCACGGGCGATGTCGGTGTGCAGAAACGCGACGGTCGCGGTGCTCGCCAAGCGGGCGGCGCAGACCCGGCGGACCGCGGGGAGGTCCGCGCGGCGCCGGACGTAGACCTTGAGGTGATCGACGTCGGTGAGGGCGTAACCCCGCCGGACACCGTGCCGGTGCAGGTTCTCGGCGGCCACGACCCGGGCGATGTTGTCCAGGGCGACCTCGCACTGACCGGTCACATCACCGTGGTGCGTGGTCTCGTGCCCGAGGATTCCGGCCGTCGCGGACACGAACAGCCGGCCGTCGCCCGGCGGGCCCAGCCAGGTGGCCCGTGCGAAGACCGGGGGGCGCGGGCCGTACGCCGTGGGGTAGTGGTGTGCCGTGAGGACGGCAGGGTTCTCGATGTTGATCCGGGTTCCGCCGCGGGCGGCGAGGAACACGCAGGTGATACCACCCCCGTGCGTGCCGATGCCGGTGGCCGCGGG-

CAGGCCGGCCGGGTCGATCCCGCCCGCGTCCAGCGCC-TGCGCACGGCCCACGCAGAAGTCCCGGTACACCTCCA-GCCCGTCCGCGTTCGCCGCGTTGATCCCACTGATGTAG-TTCCACGTCCGGGGCGAGCAGCGGATGTCCCAGGGACC-GGGTCAGCCCGAAGACCCGCGTGTAGAGCGCCGCGAC-GGCATCGGCGTACCCCCGGCTCTCGGGGACGCGGCCG-ACACCGAACAGGTACTCGTCGGTGCGGGCCCATGCGAT-GCCTCCGTCCCGGCCGGACTCGACGGGCGGCTGCGCG-CGCCACACCTCGGCGAACGCCTCCTCCTCGAAGGTCGT-GGTGTGCACCGCGGCACGCGGGACACCGTCCGTGAGC-GACACCTCGGCATGACCGGTGCCGTGCTCGATGACGC-CGAGCACGGTCTCGTCCCCTTCGAGGTCCGACGGCAC-GAGCTTCTCGAAGCGGCAGTACGGTGCCGCGACAGG-CATATGCA-3′. The resulting product was treated with T4 kinase and ligated into *Sma*I cut pUC19 and clones sequenced to verify the amplified DNA sequence. The resulting construct was designated pSGK262. The *Nde*I-*Xba*I fragment containing $fkbO_{506}$ was excised and ligated into pGP9 prepared with the same enzymes. The resulting clone was verified by restriction digestion and by sequencing from the *act*I promoter and was designated pSGK264. pSGK264 was introduced into BIOT-4010 by conjugal transfer to give strain BIOT-4576.

Construction of the $fkbO_{520}$ Expression Plasmid pSGK265 and Strain BIOT-4577. The $fkbO_{520}$ gene (5) (AF235504) was amplified as an *Nde*I-*Xba*I fragment suitable for inserting into the conjugative *Streptomyces* expression vector pGP9 using the oligos FKBO520f: 5′-GGACATATGGAGGCGTTGTCCATATCTGTCACGGCGC-3′ and FKBO520r: 5′-CCTCTAGACCTCACGCCACCATGCC-TTCGATTTCGACG-3′. pET28a-FkbO (see above) was used as template. The resulting product was treated with T4 kinase and ligated into *Sma*I cut pUC19 and clones sequenced to verify the amplified DNA sequence. The resulting construct was designated pSGK263. The *Nde*I-*Xba*I fragment containing $fkbO_{520}$ was excised and ligated into pGP9 prepared with the same enzymes. The resulting clone was verified by restriction digestion and by sequencing from the *act*I promoter and was designated pSGK265. pSGK265 was introduced into BIOT-4010 by conjugal transfer to give strain BIOT-4577.

Construction of the *hyg5* Expression Plasmid pSGK260 and Strain BIOT-4545. The *hyg5* gene was excised from plasmid pET28a-Hyg5 (see above) by digesting with *Nde*I and *Xho*I and ligating into pUC19 that had been digested with *Nde*I and *Ava*I. The resulting construct was designated pSGK258. The *Nde*I-*Xba*I fragment containing *hyg5* was excised and ligated into pGP9 prepared with the same enzymes. The resulting clone was verified by restriction digestion and by sequencing from the *act*I promoter and was designated pSGK260. pSGK260 was introduced into BIOT-4010 by conjugal transfer to give strain BIOT-4545.

Construction of the *bra8* Expression Plasmid pSGK259 and Strain BIOT-4538. The *bra8* gene (15) (AB264550) was obtained by gene synthesis (Genscript) and was obtained on an engineered *Nde*I-*Xba*I fragment. 5′-CATATGACCGGGCGGCGCGCCCTGGCCG-TCGTGGAATTCGGCGCCGAGGAGGGCCCCGCGGAGC-TGCGCGACGGCGTCCCGGTGGTCCGGATCCGCGCCG-CCGCAACCGAATCCGCGGGTTTCCGCGAGGTGTGGAT-CACCGACGGCGACGCGCGGACCGGCCACACCGACGG-CCTGGTCTACGCGCACGACGACGATATCCTGTTCTGCG-CCGGGCACATTCGGCACGGCGACCGGTACGCGGCAC-ACACCCGCGAGCGGTACGCGGCCGCCTTCGGCCTCGC-CGACGCGCTCGGCTACCCCACGCTGGTCCGGATGTGG-AACTACGTCGGCGACATCAACGCGGCCAATGCCGACG-GCCTGGAGATCTACCGCGACTTCTGCCTGGGACGGGC-GGAAGCCTTTGCCGCACACGGGAAGGCGATGCCCGC-CGCGACCGGTGTCGGCACGCACGGCAGCGGCGTGGT-CTTCTACTTCCTGAGCTGCCGGTCCGGCGGCGTCACC-CACATCGAGAACCCGCGCCAGATCCCGGCCTACCGGT-ATCCGCGCCGCTACGGTCCGAAGGCGCCGACCTTCGC-GCGGGCGACCCACCTGCTGCCCGACGGCGGCAGCGA-CACCCTGTTCGTCTCGGGCACCGCCGCGATCCTCGGG-CACGAGACGGTGTGCCCGGGCGATGTGGCGCGGCAG-TGCGAGGTGGCGCTCGCCAATATCCGGGAACTGGTCG-GCGAGCAGAATCTGGGCCGGTACGGGATCGCCAAAC-CCTATGGGCTGTCGGCGATTCGGCGCGCCAAGGTGTA-CGTCCGCCACCGCGAGCACCTGGAGGTGGTGCGCCG-GGCCTGCGCCGCCGCGCTGCCGCCGGAGGCCGAGAT-CGCGTACCTGGTCGTCGACATCTGCCGCAGGGACCTG-CTGGTCGAGATCGAGGCCGTGGCATGAGTCTAGAGG-3′. The resulting construct was designated pSGK257. The *Nde*I-*Xba*I fragment containing *bra8* was excised and ligated into pGP9 prepared with the same enzymes. The resulting clone was verified by restriction digestion and by sequencing from the *act*I promoter and was designated pSGK259. pSGK259 was introduced into BIOT-4010 by conjugal transfer to give strain BIOT-4538.

Fermentation, Isolation, and Structural Characterization of BC325. Fermentation of BIOT-4010 in a Braun 22 L bioreactor (×2) and subsequent processing to give a crude extract containing BC325 was performed as described previously (8). The resulting material (13.0 g) was suspended in a methanol:water mix (80:20; 300 mL) and extracted twice with hexane (300 mL). The methanol:water fraction was then concentrated under reduced pressure to yield a crude oil (8.2 g; 17 mg BC325). This was dissolved in methanol (40 mL), silica gel (10 g; silica gel 60 for column chromatography, 230–400 mesh ASTM) was added to the extract and then dried to a free running powder. This was loaded onto a silica gel column (20×5 cm) and eluted first with chloroform (1 L), and then with an increasing methanol gradient (every one liter of chloroform, 1% of MeOH was added to increase the polarity, then 2% MeOH, and so on, up to 5% MeOH). Approximately 12 fractions (each 250 mL) were collected and monitored by TLC and HPLC. Fractions 4 and 5 contained BC325 and the combined fractions (2.8 g total weight after removal of solvent) were loaded onto a second silica gel column (15×2 cm) and eluted with a mixture of hexane and ethyl acetate (6:4). Approximately 20 fractions (150 mL each) were collected and checked by TLC and HPLC. Fractions 2 and 3 were combined, and the solvent was removed to yield 489 mg of material containing BC325 (15 mg). This was dissolved in acetonitrile (4 mL) and subjected to preparative HPLC (five injections) using a Gilson 306 HPLC system. Chromatography was achieved over a Waters XTerra MSC_{18} column (250×19 mm; 10 μm particle size) eluting with a water-acetonitrile gradient over 30 min at a flow rate of 21 mL/min ($T = 0$, 55% acetonitrile; $T = 30$, 80% acetonitrile). BC325 eluted at 27 min. The solvent from BC325 containing fractions was removed under reduced pressure and to yield BC325 (11.0 mg), which was chromatographically pure as assessed by UV and MS analysis using two different HPLC columns/elution systems, and by inspection of the ^{1}H and ^{13}C NMR spectra acquired in chloroform-d_1 containing 0.1% pyridine-d_5.

UV (Diode Array Detector) λ_{max} 280 nm (typical rapamycin triene chromophore); MS: ESI$^+$, m/z 870.7 $[C_{49}H_{69}NNaO_{11}]^+$, 798.6, 780.6; high resolution MS: $[M + NH_4]^+$ requires $(C_{49}H_{73}N_2O_{11}) = 865.5214$, observed 865.5244, $\Delta = 3.5$ ppm. The expected LC-MS/MS fragmentation [method published (8)] and data obtained are shown in Table S2.

The NMR data for BC325 are presented in Table S3 and are for the major rotameric form observed. The ^{1}H NMR spectra showed four characteristic signals resonating at δ7.14 (t, 7.7 Hz), δ6.73 (br. d 7.7 Hz), δ6.65 (dd 7.7 and 2.3 Hz). and δ6.48 (br. s). These were assigned to H41, H42, H40, and H38, respectively. The ^{13}C NMR spectra showed six signals characteristic for an aromatic ring not usually present in rapamycin. The signals were

at δ140.3, δ115.8, δ156.5, δ113.4, δ130.1, and δ120.1 and were assigned to C37, C38, C39, C40, C41, and C42, respectively. These and all other assignments were confirmed through extensive use of COSY and heteronuclear multiple bond correlations (HMBCs), and compared those for rapamycin. Strong HMBCs were observed for H38 and H42 with C36, which confirmed the identity of the aromatic ring.

Isolation of BC325 after Feeding [1,7-$^{13}C_2$] Shikimic Acid. BIOT-4010 was grown in 70 Falcon tubes using methods described previously (8). After 24 h growth in production media, an aliquot (25 μL) of [1,7-$^{13}C_2$]shikimic acid dissolved in methanol (0.32 M) was added to each tube and fermentation continued for a further 5 d (final concentration 1 mM). The combined fermentation broth (approximately 570 mL) was extracted twice with an equal volume of ethyl acetate, the extracts combined, and the solvent removed under reduced pressure to yield a solid extract (373 mg). This was dissolved into ethyl acetate : hexane (4 : 6; 1 mL) and applied to an Isolute SPE column (20 g, Flash Silica), then eluted with ethyl acetate : hexane (4 : 6) collecting 20 mL fractions. Fractions 5 and 6 were combined and the solvents removed to yield a solid yellow material (1.5 mg BC325 in 14 mg). The presence of BC325 was confirmed by TLC and HPLC. This was further purified by HPLC on an Aglient 1100 system. Chromatography was achieved over a Hypersil C_{18} BDS column (ThermoQuest; 150 × 10 mm; 3 μm particle size), eluting with a gradient of water and acetonitrile (45 : 55) to (20 : 80) over 30 min at a flow rate of 5 mL/min. BC325 eluted at 21 min and was collected and taken to dryness (1 mg). This was analyzed by ^{13}C NMR (10,000 scans) in chloroform-d_1 containing 0.1% pyridine-d_5. The resulting ^{13}C NMR spectra (Fig. S7) showed four signals, two in the aromatic and two in the aliphatic region. The first coupled pair was assigned to C36 (δ39.3, $J = 43.0$ Hz) and C37 (δ140.3, $J = 43.0$ Hz) and correspond to incorporation of an intact molecule of [1,7-$^{13}C_2$] shikimic acid. The second pair of coupled signals corresponds to a rotameric form of the molecule and was observed at δ38.2 and δ141.5 ($J = 43.3$ Hz). Note, ^{13}C signals for 0.1% pyridine-d_5 cosolvent are also observed in the spectra.

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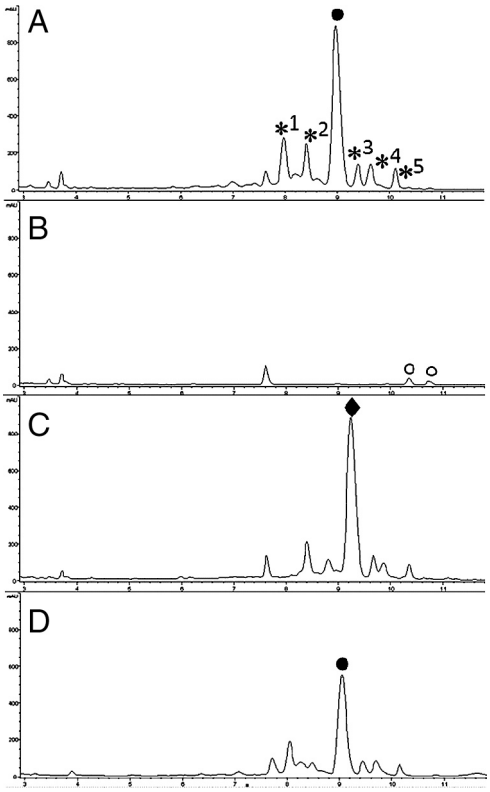

Fig. S1. LC-UV chromatograms of representative samples displaying response at 280 nm from 2.9 to 11.8 min, and 0 to 1000 mAU. •, rapamycin; ○, BC325; ◆, 39-desmethoxyrapamycin: (*A*) BIOT-3410; (*B*) BIOT-4010; (*C*) BIOT-4010 + 2 mM cyclohexanecarboxylic acid; (*D*) BIOT-4010 + 2 mM DCDC. Asterisks refer to rapamycin isomers and congeners; $*^1$,$*^4$, rapamycin isomers (1); $*^2$, rapamycin congener (+2 amu); $*^3$,$*^5$, 27-desmethoxyrapamycin isomers (2).

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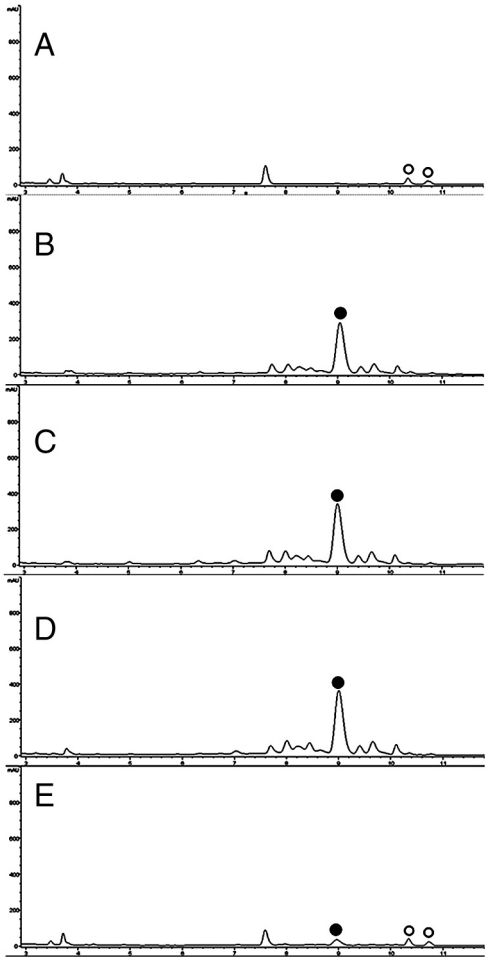


Fig. S2. LC-UV chromatograms of representative samples displaying response at 280 nm from 2.9 to 11.8 min, and 0 to 1000 mAU. •, rapamycin; ○, BC325: (*A*) BIOT-4010; (*B*) BIOT-4010 + *rapK*; (*C*) BIOT-4010 + $fkbO_{506}$; (*D*) BIOT-4010 + $fkbO_{520}$; (*E*) BIOT-4010 + *mcxF*.

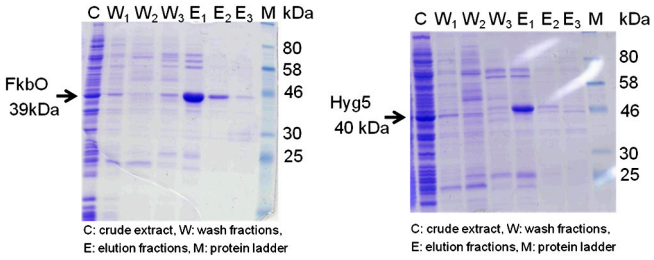


Fig. S3. SDS-PAGE analysis of FkbO and Hyg5 purification.

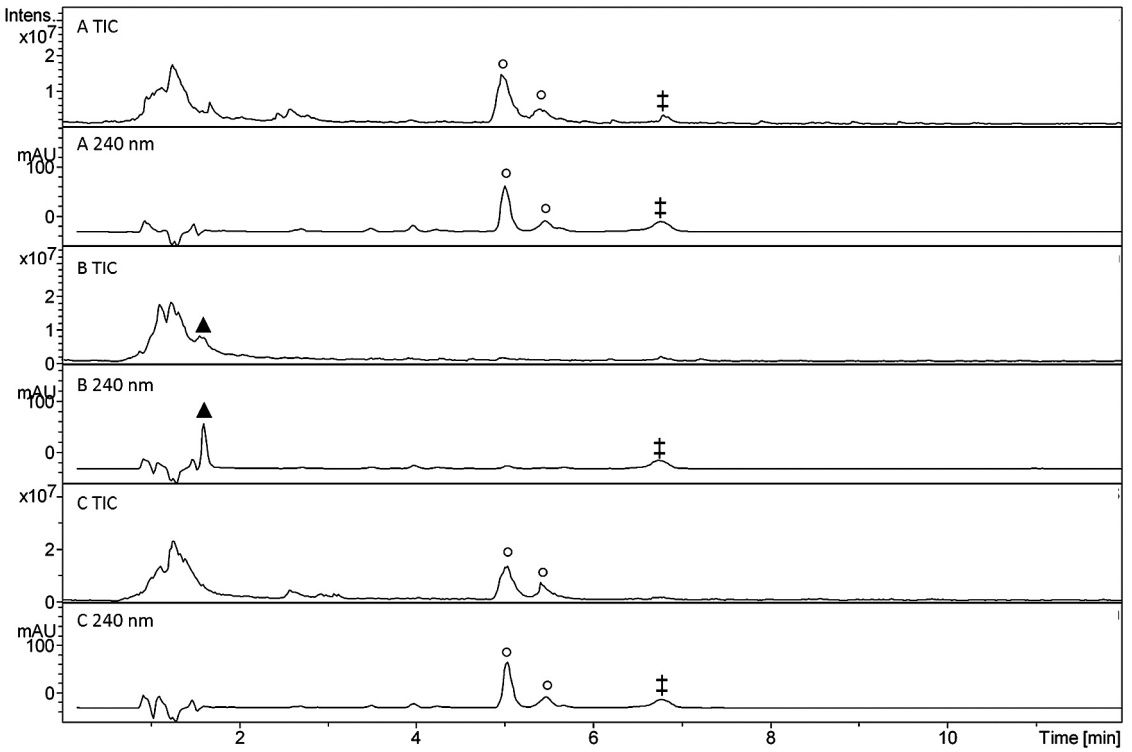


Fig. S4. LC-MS traces of (*A*) chorismate in buffer, (*B*) chorismate in buffer plus FkbO, and (*C*) chorismate in buffer plus denatured FkbO. The negative mode total ion count (TIC) and UV traces (at 240 nm) are shown between 0 and 12 min. Retention times: DCDC (▲), 1.5 min; chorismate (double peak, ○), 5.0 and 5.4 min; 4HBA (degradation product, ‡), 6.5 min.

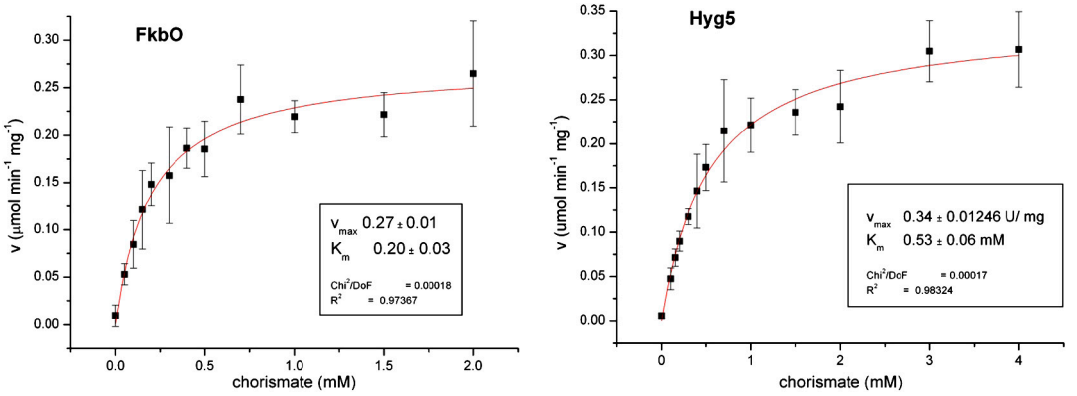


Fig. S5. Kinetic parameters for FkbO and Hyg5.

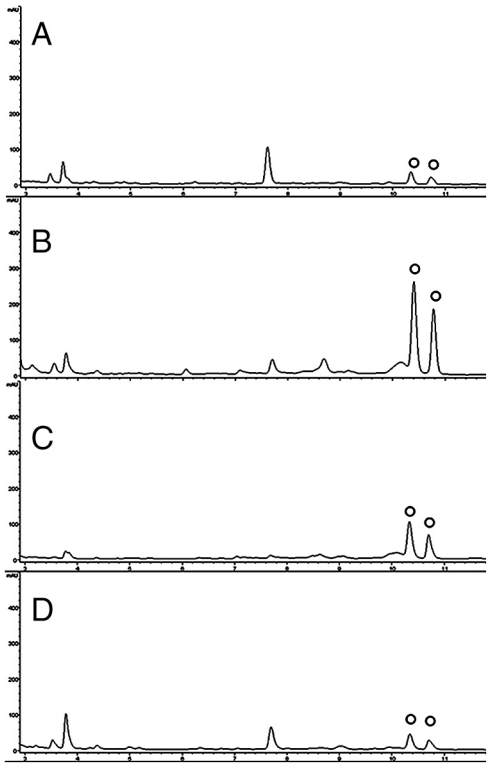


Fig. S6. LC-UV chromatograms of representative samples displaying response at 280 nm from 2.9 to 11.8 min, and 0 to 500 mAU. ○, BC325: (*A*) BIOT-4010; (*B*) BIOT-4010 + 1 mM 3HBA; (*C*) BIOT-4010 + *hyg*5; (*D*) BIOT-4010 + *bra*8.

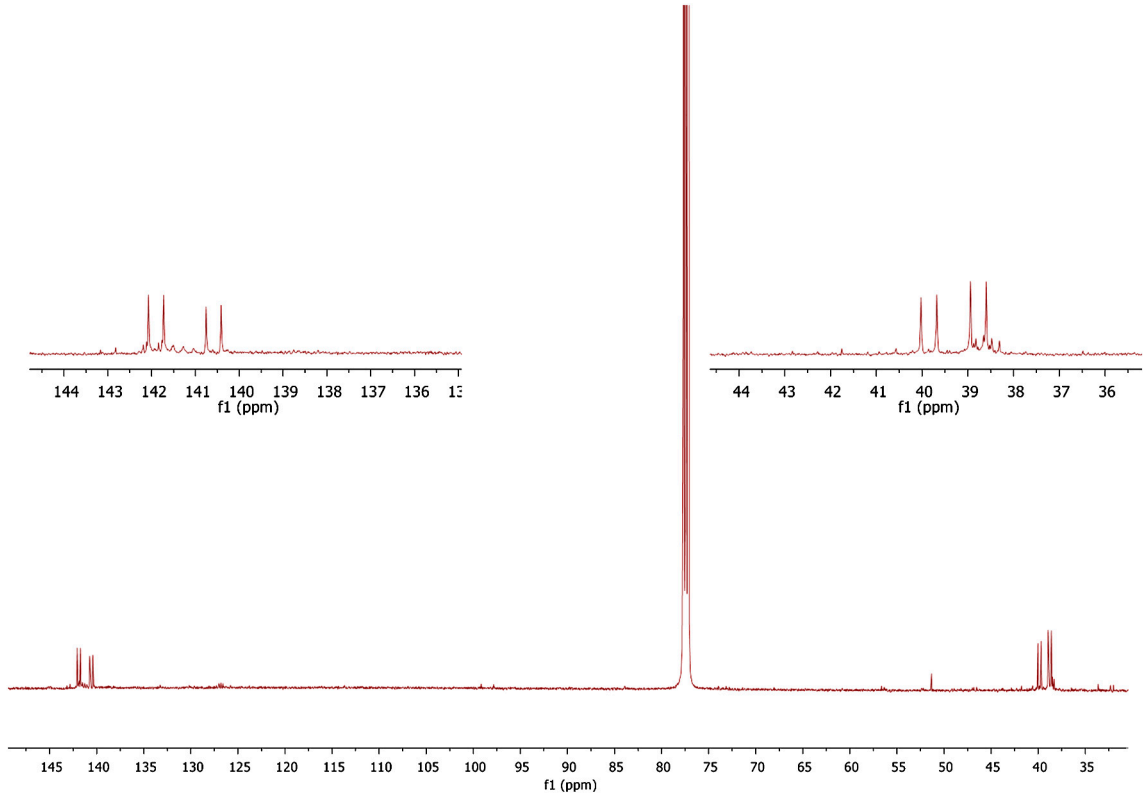


Fig. S7. ^{13}C NMR (10,000 scans) of BC325 in chloroform-d_1 (containing 0.1% pyridine-d_5) isolated after feeding [1,7-$^{13}C_2$]shikimic acid to BIOT-4010.

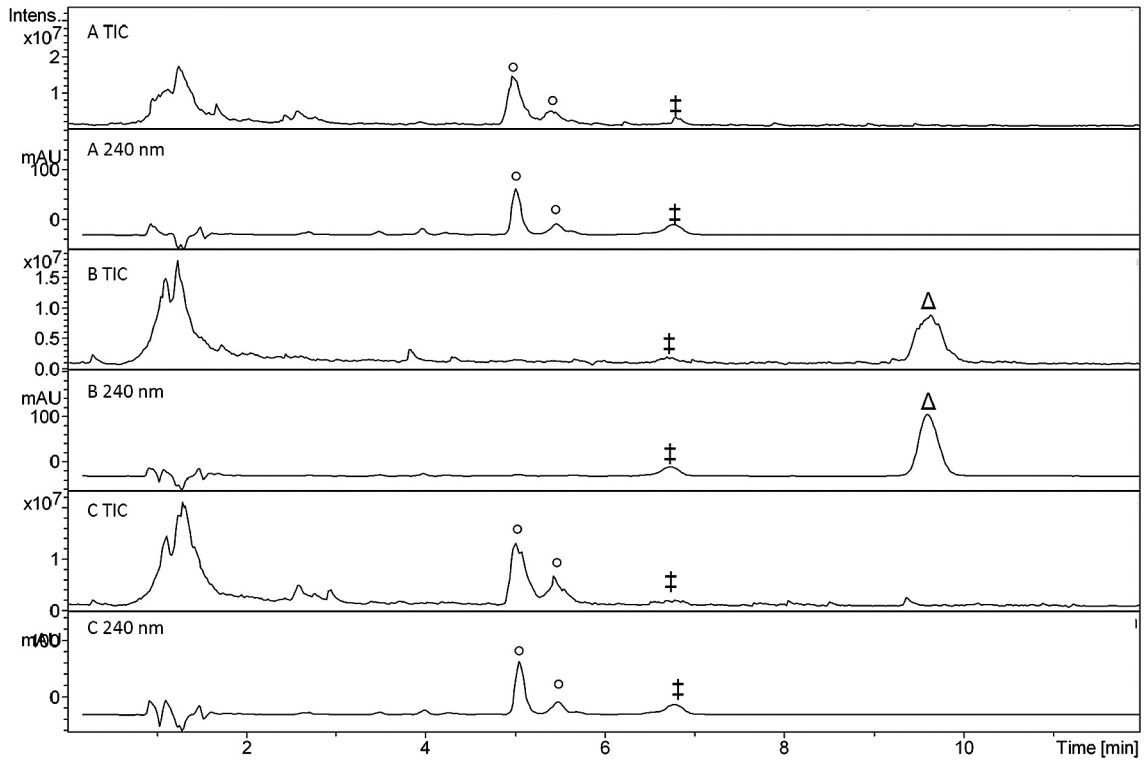


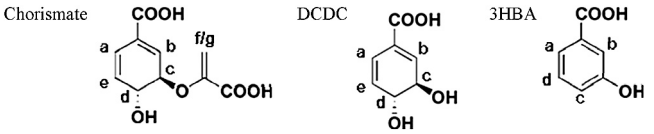
Fig. S8. LC-MS traces of (*A*) chorismate in buffer, (*B*) chorismate in buffer plus Hyg5, and (*C*) chorismate in buffer plus denatured Hyg5. The negative mode TIC and UV traces (at 240 nm) are shown between 0 and 12 min. Retention times: chorismate (double peak, ○), 5.0 and 5.4 min; 4HBA (degradation product, ‡), 6.5 min; 3HBA (Δ), 9.6 min.

Table S1. NMR data for chorismate, and the products of chorismate incubated in buffer with no enzyme (control), FkbO and Hyg5

^{1}H NMR (400 MHz, D_2O)		Standard (D_2O)	No enzyme control (deuterated buffer)
		δ/ppm	δ/ppm
Chorismate	a	6.27 (dt, J = 10.4, 2.0 Hz, 1H)	6.34 (dt, J = 10.0, 2.0 Hz, 1H)
	b	6.86 (m, 1H)	6.57 (m, 1H)
	c	4.98 (dd, J = 11.4, 2.8 Hz, 1H)	4.92 (dd, J = 11.6, 2.5 Hz, 1H)
	d	4.71* (dtd, J = 11.2, 2.4, 0.4 Hz, 1H)	Obscured (water peak)
	e	5.96 (dd, J = 10.1, 2.8 Hz, 1H)	5.98 (dd, J = 10.0, 2.5 Hz, 1H)
	f/g	5.50 (d, J = 3.5 Hz, 1H)	5.22 (d, J = 2.8 Hz, 1H)
		4.85 (d, J = 3.5 Hz, 1H)	4.59 (d, J = 2.8 Hz, 1H)
DCDC	a	6.35 (dt, J = 10.0, 1.8 Hz, 1H)	
	b	6.90 (dd, J = 2.9, 1.2 Hz, 1H)	
	c	4.54 (dd, J = 10.6, 3.3 Hz, 1H)	
	d	4.45 (ddd, J = 10.6, 2.8, 2.3 Hz, 1H)	
	e	6.01 (dd, J = 10.0, 3.0 Hz, 1H)	
3HBA	a	7.53 (dt, J = 7.6, 1.2 Hz, 1H)	
	b	7.42 (dd, J = 2.3, 1.6 Hz, 1H)	
	c	7.37 (t, J = 7.9 Hz, 1H)	
	d	7.12 (ddd, J = 8.2, 2.6, 0.8 Hz, 1H)	

^{1}H NMR (400 MHz, D_2O)		FkbO reaction (deuterated buffer) δ/ppm	Hyg5 reaction (deuterated buffer) δ/ppm
Chorismate	a	6.34 (dt, J = 10.0, 1.6 Hz, 1H)	6.34 (dt, J = 10.4, 2.0 Hz, 1H)
	b	6.57 (m, 1H)	6.57 (m, 1H)
	c	4.92 (dd, J = 11.7, 2.5 Hz, 1H)	4.92 (dd, J = 11.7, 2.4 Hz, 1H)
	d	Obscured (water peak)	Obscured (water peak)
	e	5.98 (dd, J = 10.8, 2.8 Hz, 1H)	5.98 (dd, J = 10.0, 2.6 Hz, 1H)
	f/g	5.22 (d, J = 2.8 Hz, 1H)	5.22 (d, J = 2.8 Hz, 1H)
		4.59 (d, J = 2.8 Hz, 1H)	4.59 (d, J = 2.8 Hz, 1H)
DCDC	a	6.34 (dt, J = 10.0, 1.6 Hz, 1H)	
	b	6.50 (d, J = 2.8 Hz, 1H)†	
	c	4.48 (dd, J = 10.0, 3.3 Hz, 1H)	
	d	4.40 (ddd, J = 10.0, 3.0, 2.0 Hz, 1H)	
	e	5.95 (dd, J = 10.4, 3.2 Hz, 1H)	
3HBA	a		7.42 (dt, J = 7.6, 1.2 Hz, 1H)
	b		Hidden under triplet
	c		7.35 (t, J = 8.0 Hz, 1H)
	d		7.03 (ddd, J = 7.9, 2.5, 0.8 Hz, 1H)

The original spectra are shown in Fig. 3.



*Partly obscured by water peak.
†Further splitting not assignable.

Table S2. The MS/MS fragmentation data for BC325

Fragment	Expected *m/z*	Observed *m/z*
A	870.5	870.7
B	741.4	741.6
B′	697.4	697.6
C	606.3	606.4
D	584.3	584.4
E	320.1	320.1

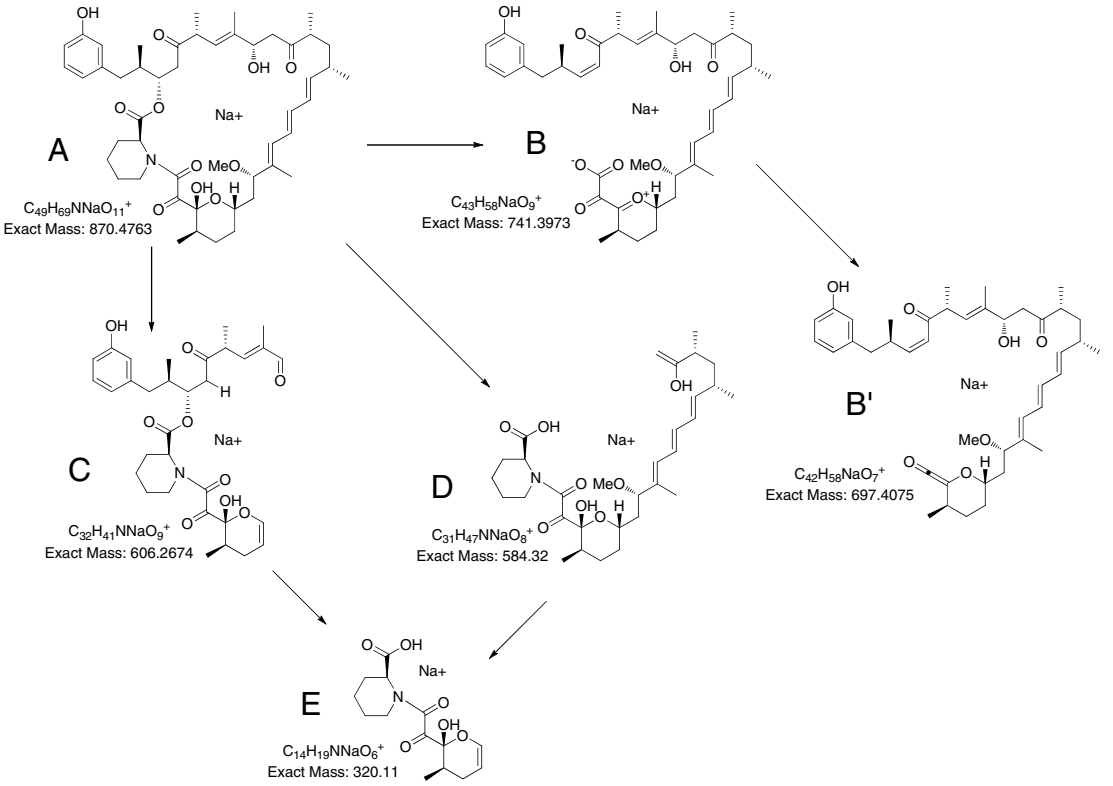


Table S3. ^{1}H and ^{13}C NMR data for BC325 in chloroform-d_1 containing 0.1% pyridine-d_5.

Position	^{1}H		^{13}C δ ppm
	δ ppm	Multiplicity, J Hz	
1	—	—	169.1
2	5.18	br. d, 5.5	51.9
3	2.35/1.65	m, complex/m, complex	26.6
4	1.69/1.47	m, complex/m, complex	20.9
5	1.55/1.41	m, complex/m, complex	25.0
6	3.44/2.90	m, complex/m, complex	44.2
7	—	—	N
8	—	—	166.1
9	—	—	195.6
10	—	—	98.7
11	2.13	m, complex	34.9
11-CH_3	0.93	d, 6.5	16.2
12	1.64	m, complex	27.0
13	1.79/1.22	m, complex/m, complex	30.6
14	4.18	m, complex	67.6
15	2.89/2.43	m, complex/m, complex	39.5
16	3.58	m, complex	83.2
16-OCH_3	3.11	s	55.9
17	—	—	137.6
17-CH_3	1.65	s	10.4
18	6.07	d, 10	128.5
19	6.32	dd, 15, 10	127.1
20	6.21	dd, 15, 10.5	133.1
21	6.04	dd, 15, 10.5	129.7
22	5.32	dd, 15, 8.5	141.1
23	2.14	m, complex	39.2
23-CH_3	1.00	d, 6.5	22.0
24	1.85/1.35	m, complex/m, complex	40.1
25	2.99	m, complex	46.1
25-CH_3	1.02	d, 6.5	18.5
26	—	—	217.1
27	2.73/2.46	m, complex/m, complex	47.8
28	4.41	m, complex	73.3
29	—	—	139.3
29-CH_3	1.47	s	11.9
30	5.56	d, 10	125.2
31	3.00	m, complex	46.3
31-CH_3	1.02	d, 6.5	14.6
32	—	—	206.5
33	2.91/2.44	dd, 17, 6/dd, 17, 6.5	40.7
34	5.18	m, complex	72.7
35	2.17	m, complex	35.2
35-CH_3	0.90	d, 6.5	15.4
36	2.58/2.35	m, complex/m, complex	39.3
37	—	—	140.3
38	6.48	br. s	115.8
39	—	—	156.5
40	6.65	dd, 7.7, 2.3	113.4
41	7.14	t, 7.7	130.1
42	6.73	br. d, 7.7	120.1