Supporting information

A Fluorogenic Screening Platform Enables Directed Evolution of an Alkyne Biosynthetic Tool.

Xuejun Zhu[a], Peyton Shieh[b], Michael Su[a], Carolyn R. Bertozzi[b, c], Wenjun Zhang[a,d]*

[a]Department of Chemical and Biomolecular Engineering, University of California, Berkeley, CA 94720. [b]Department of Chemistry, Stanford University, Stanford, CA 94305. [c]Howard Hughes Medical Institute, Stanford University, Stanford, CA 94305. [d]Physical Biosciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA 94720, USA.

Table of Contents

Supplementary methods

Ep-PCR and Construction of Mutant Library. Ep-PCR procedure was modified from established protocols[1]. The reaction consisted of 0.35 mM dATP, 0.4 mM dCTP, 0.2 mM dGTP, 1.35 mM dTTP, 1.5 mM $MgCl_2$, 1.5 mM $MnCl_2$, and 1 U Taq polymerase. The reaction mixture was submitted to 25 cycles of PCR: 95°C for 1 min, 50°C for 1 min, and 68°C for 1 min. The resulting PCR products were digested with DpnI, then Gibson assembled into pXZ44 that was pre-digested with NdeI/XhoI. The Gibson mixture was desalted, co-transformed with pXZ27 to electro-competent BAP1 strain, and plated on LB agar containing 100 μg mL^{-1} carbenicillin and 100 μg mL^{-1} spectinomycin.

Mutagenesis of JamB. Site-directed mutagenesis were performed using the standard QuikChange strategy using relevant templates. To make JamB with CamB N-terminus substitution, the first 4 amino acids of JamB were deleted. To make JamB with TtuB N-terminus substitution, the first 4 amino acids of JamB were replaced with the first 15 amino acids of TtuB. All mutations were verified by DNA sequencing.

Culture Conditions. For quantification platform set up or identified JamB mutant verification, engineered BAP1 strains were grown in LB medium with 100 μg mL^{-1} carbenicillin and 100 μg mL^{-1} spectinomycin at 37 °C to an OD_{600} of ~0.4-0.6. The cells were harvested and concentrated 5-fold into fresh F1 medium supplemented with 100 μg mL^{-1} carbenicillin, 100 μg mL^{-1} spectinomycin, 0.5 mM IPTG, and 1 mM fatty acid (hexanoic acid or 5-hexenoic acid). After 40 hr of growth at 20 °C, cell-free supernatants were used for subsequent plate-reader fluorescence measurements or extracted with ethyl acetate for LC-HRMS analysis. To monitor the alkyne titers in the cultures, we collected cell-free supernatants every a few hours during fermentation for subsequent fluorogenic assays. For directed evolution, BAP1 library were grown in 96-well plates. Each well contained 1 ml of LB medium supplemented with 100 μg mL^{-1} carbenicillin and 100 μg mL^{-1} spectinomycin. When cultures were grown at 37 °C to an OD_{600} of ~0.4, the cell pellets were separated from LB medium and suspended in 200 μL F1 medium supplemented with 100 μg mL^{-1} carbenicillin, 100 μg mL^{-1} spectinomycin, 0.5 mM IPTG, and 1 mM 5-hexenoic acid. After ~2 days of growth at 20 °C, cell-free supernatants were separated from cell pellets, and were transferred to 96-well black plates for subsequent plate-reader fluorescence measurements.

Preparation of membrane fractions of the engineered *E.coli*. Membrane fractions were prepared using a standard protocol[2].

Plate-Reader Fluorescence Measurements. Reactions were run in a 96-well black plate, and measurements were performed using a Tecan Safire Microplate Reader. Each well had 200 μL of sample containing 0.75 μM of probe, 5 mM ascorbic acid, 500 μM $CuSO_4$, 100 μM BTTAA, and 190 μL of cultures. The reactions were performed in dark for 10 min at room temperature. For linear detection range determination, different concentrations of **4** (0.75, 3, 10 μM) and alkynes, and similar conditions were used.

LC-HRMS Analysis. LC-HRMS was performed on an Agilent Technologies 6520 Accurate Mass QTOF LC-MS with an Agilent Eclipse Plus C18 column (4.6 × 100 mm). A linear gradient

of 2-95% CH_3CN (v/v) over 20 min and 95% CH_3CN for a further 5 min in H_2O supplemented with 0.1% (v/v) formic acid at a flow rate of 0.5 mL min^{-1} was used.

Sequence Alignment Analysis. Amino acid sequence alignment of JamB, CamB, and TtuB was performed using on-line program Clustal Omega.

Protein Structure Modeling and docking. Protein structures of JamB, JamC were modeled using on-line program HHpred[3] using SCD1[4, 5] and CurA[6] (Protein Data Bank entry 4ZYO/4YMK and 2LIU, respectively). JamC was then docked to the electro-positive surface of JamB near the substrate entrance using ClusPro[7, 8].

References

1. X. Gao, X. Xie, I. Pashkov, M. R. Sawaya, J. Laidman, W. Zhang, R. Cacho, T. O. Yeates and Y. Tang, *Chem Biol*, 2009, **16**, 1064-1074.
2. X. Zhu, M. Su, K. Manickam and W. Zhang, *ACS Chem. Biol.*, 2015, **10**, 2785-2793.
3. J. Söding, A. Biegert and A. N. Lupas, *Nucleic Acids Res.*, 2005, **33**, W244-W248.
4. H. Wang, M. G. Klein, H. Zou, W. Lane, G. Snell, I. Levin, K. Li and B. C. Sang, *Nat. Struct. Mol. Biol.*, 2015, **22**, 581-585.
5. Y. Bai, J. G. McCoy, E. J. Levin, P. Sobrado, K. R. Rajashankar, B. G. Fox and M. Zhou, *Nature*, 2015, **524**, 252-256.
6. A. Busche, D. Gottstein, C. Hein, N. Ripin, I. Pader, P. Tufar, E. B. Eisman, L. Gu, C. T. Walsh, D. H. Sherman, F. Lohr, P. Guntert and V. Dotsch, *ACS Chem. Biol.*, 2012, **7**, 378-386.
7. S. R. Comeau, D. W. Gatchell, S. Vajda and C. J. Camacho, *Bioinformatics (Oxford, England)*, 2004, **20**, 45-50.
8. S. R. Comeau, D. W. Gatchell, S. Vajda and C. J. Camacho, *Nucleic Acids Res.*, 2004, **32**, W96-99.

Supplementary Tables

Table S1. Primers used in this study.

Primer	Sequence (5'→3')	Note
pXZ43-B-F	agtatattagttaagtataagaaggagatatacat	Used for JamB mutant library generation.
pXZ43-B-R	aatttcgcagcagcggtttctttaccagactcgag	
pXZ167_F	atgtcaatgccaatAgatgtgagcaaaaaa	Used for creation of JamB (M5I)
pXZ167_R	ttttttgctcacatcTattggcattgacat	
pXZ168_F	ttaatcgcaattatgCagtgtggatagttt	Used for creation of JamB (V189A)
pXZ168_R	aaactatccacactGcataattgcgattaa	
pXZ169_F	ccctttcgatagtaaTgaccagagtcggaa	Used for creation of JamB (K250N)
pXZ169_R	ttccgactctggtcAttactatcgaaaggg	
pXZ205_F	atgtcaatgccaGAGgatgtgagcaaaa	Used for creation of JamB (M5E)
pXZ205_R	ttttgctcacatcCTCtggcattgacat	
pXZ206_F	atgtcaatgccaACCgatgtgagcaaaa	Used for creation of JamB (M5T)
pXZ206_R	ttttgctcacatcGGTtggcattgacat	
pXZ207_F	atgtcaatgccaTGCgatgtgagcaaaa	Used for creation of JamB (M5C)
pXZ207_R	ttttgctcacatcGCAtggcattgacat	
pXZ208_F	atgtcaatgccaTTTgatgtgagcaaaa	Used for creation of JamB (M5F)
pXZ208_R	ttttgctcacatcAAAtggcattgacat	
pXZ209_F	atgtcaatgccaGCGgatgtgagcaaaa	Used for creation of JamB (M5A)
pXZ209_R	ttttgctcacatcCGCtggcattgacat	
pXZ210_F	gtcaaaaaccaAAtcaaatgattac	Used for creation of JamB (I23K)
pXZ210_R	gtaatcatttgaTTtggtttttgac	
pXZ211_F	aagtcaaaaaccGAGtcaaatgattac	Used for creation of JamB (I23E)
pXZ211_R	gtaatcatttgaCTCggtttttgactt	
pXZ212_F	aagtcaaaaaccACCtcaaatgattaca	Used for creation of JamB (I23T)
pXZ212_R	tgtaatcatttgaGGTggtttttgactt	
pXZ213_F	aagtcaaaaaccTGCtcaaatgattaca	Used for creation of JamB (I23C)
pXZ213_R	tgtaatcatttgaGCAggtttttgactt	
pXZ214_F	aagtcaaaaaccTACtcaaatgattaca	Used for creation of JamB (I23Y)
pXZ214_R	tgtaatcatttgaGTAggtttttgactt	
pXZ215_F	aagtcaaaaaccGTGtcaaatgattaca	Used for creation of JamB (I23V)
pXZ215_R	tgtaatcatttgaCACggtttttgactt	
pXZ216_F	CGCAGCCGCAAACCGGGatggatgtgagcaaaaaatc	Used for creation of JamB-TtuB
pXZ216_F1	AAAcatATGAGTAATACATTTGACAACGCCCTCGCGCAGCCGCAAACCGGGatg	
pXZ217_F	AAAcatatggatgtgagcaaaaaatc	Used for creation of JamB-CamB

Table S2. Plasmids used in this study.

Plasmid	Derived from	Function
pXZ27	pCDFDuet-1	Coexpression of JamA and HsPKS1
pXZ43	pETDuet-1	Coexpression of JamC and JamB
pXZ44	pETDuet-1	Expression of JamC
pXZ167	pETDuet-1	Coexpression of JamC and JamB-M5I
pXZ168	pETDuet-1	Coexpression of JamC and JamB-V189A
pXZ169	pETDuet-1	Coexpression of JamC and JamB-K250N
pXZ204	pETDuet-1	Coexpression of JamC and JamB-M5K
pXZ205	pETDuet-1	Coexpression of JamC and JamB-M5E
pXZ206	pETDuet-1	Coexpression of JamC and JamB-M5T
pXZ207	pETDuet-1	Coexpression of JamC and JamB-M5C
pXZ208	pETDuet-1	Coexpression of JamC and JamB-M5F
pXZ209	pETDuet-1	Coexpression of JamC and JamB-M5A
pXZ210	pETDuet-1	Coexpression of JamC and JamB-I23K
pXZ211	pETDuet-1	Coexpression of JamC and JamB-I23E
pXZ212	pETDuet-1	Coexpression of JamC and JamB-I23T
pXZ213	pETDuet-1	Coexpression of JamC and JamB-I23C
pXZ214	pETDuet-1	Coexpression of JamC and JamB-I23Y
pXZ215	pETDuet-1	Coexpression of JamC and JamB-I23V
pXZ216	pETDuet-1	Coexpression of JamC and JamB-TtuB
pXZ217	pETDuet-1	Coexpression of JamC and JamB-CamB

Table S3. Strains used in this study.

Strain	Genes	Plasmids
XZ1	*Hspks1*, *jamA*, *jamB*, *jamC*	pXZ27, pXZ43
XZ5	*Hspks1*, *jamA*, *jamC*	pXZ27, pXZ44
XZ1_1	*Hspks1*, *jamA*, *jamB*-M5I/V189A/K250N, *jamC*	pXZ27, pXZ43 mutant (from library)
XZ1_2	*Hspks1*, *jamA*, *jamB*-M5V, *jamC*	pXZ27, pXZ43 mutant (from library)
XZ1_3	*Hspks1*, *jamA*, *jamB*-I23M, *jamC*	pXZ27, pXZ43 mutant (from library)
XZ1_4	*Hspks1*, *jamA*, *jamB*-M5I, *jamC*	pXZ27, pXZ167
XZ1_5	*Hspks1*, *jamA*, *jamB*-V189A, *jamC*	pXZ27, pXZ168
XZ1_6	*Hspks1*, *jamA*, *jamB*-K250N, *jamC*	pXZ27, pXZ169
XZ1_7	*Hspks1*, *jamA*, *jamB*-M5T, *jamC*	pXZ27, pXZ206
XZ1_8	*Hspks1*, *jamA*, *jamB-ttuB*, *jamC*	pXZ27, pXZ216
XZ1_9	*Hspks1*, *jamA*, *jamB-camB*, *jamC*	pXZ27, pXZ217
XZ1_10	*Hspks1*, *jamA*, *jamB*-M5K, *jamC*	pXZ27, pXZ204
XZ1_11	*Hspks1*, *jamA*, *jamB*-M5E, *jamC*	pXZ27, pXZ205
XZ1_12	*Hspks1*, *jamA*, *jamB*-M5C, *jamC*	pXZ27, pXZ207
XZ1_13	*Hspks1*, *jamA*, *jamB*-M5F, *jamC*	pXZ27, pXZ208
XZ1_14	*Hspks1*, *jamA*, *jamB*-M5A, *jamC*	pXZ27, pXZ209
XZ1_15	*Hspks1*, *jamA*, *jamB*-I23K, *jamC*	pXZ27, pXZ210
XZ1_16	*Hspks1*, *jamA*, *jamB*-I23E, *jamC*	pXZ27, pXZ211
XZ1_17	*Hspks1*, *jamA*, *jamB*-I23T, *jamC*	pXZ27, pXZ212
XZ1_18	*Hspks1*, *jamA*, *jamB*-I23C, *jamC*	pXZ27, pXZ213
XZ1_19	*Hspks1*, *jamA*, *jamB*-I23Y, *jamC*	pXZ27, pXZ214
XZ1_20	*Hspks1*, *jamA*, *jamB*-I23V, *jamC*	pXZ27, pXZ215

Table S4. Probes used in this study.

Probe	λ_{ex}	λ_{em}	Folds of fluorescence enhancement of XZ1*	
			Cell-containing cultures	Cell-free supernatants
3	500	515	--	2x
4	500	520	12x	20x
5	592	610	14x	39x
6	660	675	4x	12x

*Folds of fluorescence enhancement were calculated as the ratio of the fluorescence of XZ1 to that of the medium-only control.

Supplementary Figures

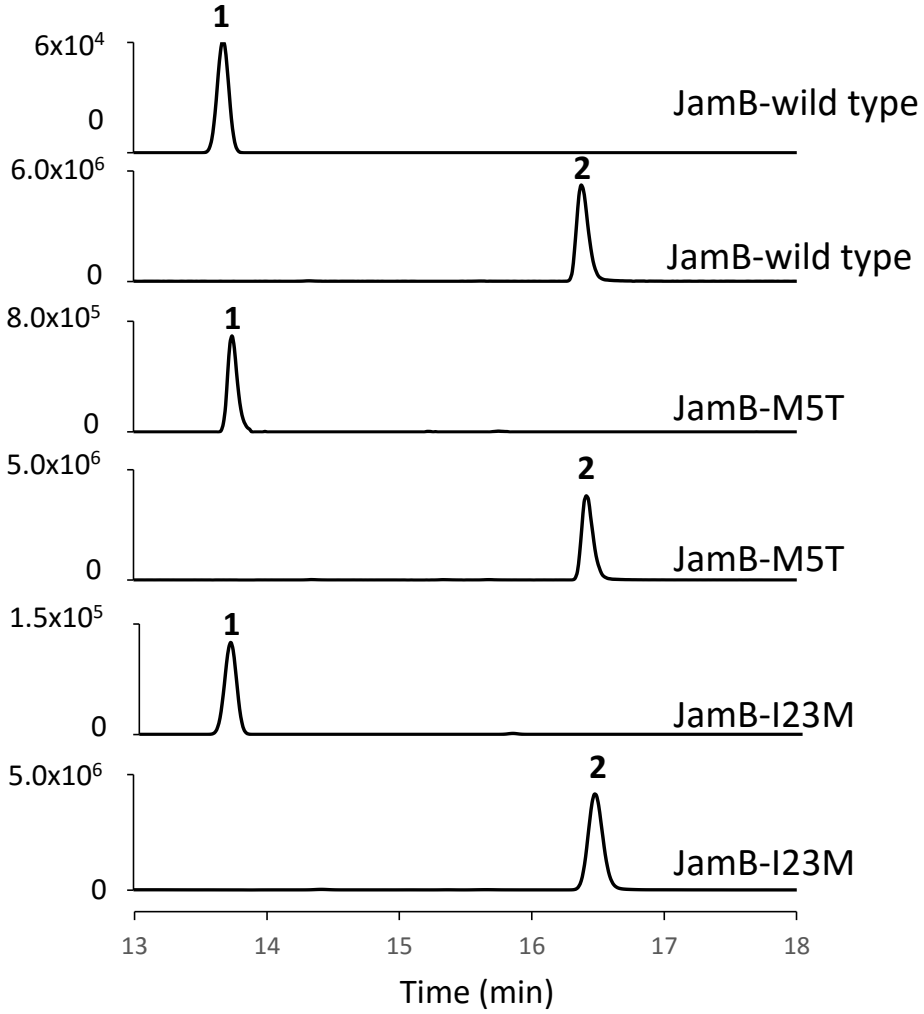

Figure S1. Efficiencies in the production of **1** compared to the wild-type strain (XZ1) when all cultures were fed with hexanoic acid. Extracted ion chromatograms (**1**: m/z 179.0703 $[M+H]^+$, **2**: m/z 183.1016 $[M+H]^+$) showed that compared to the wild type, the ratios between compounds **1** and **2** were increased by ~20-fold and ~2-fold from the strains expressing JamB-M5T and JamB-I23M, respectively. The calculated mass with 10 ppm mass error tolerance was used.

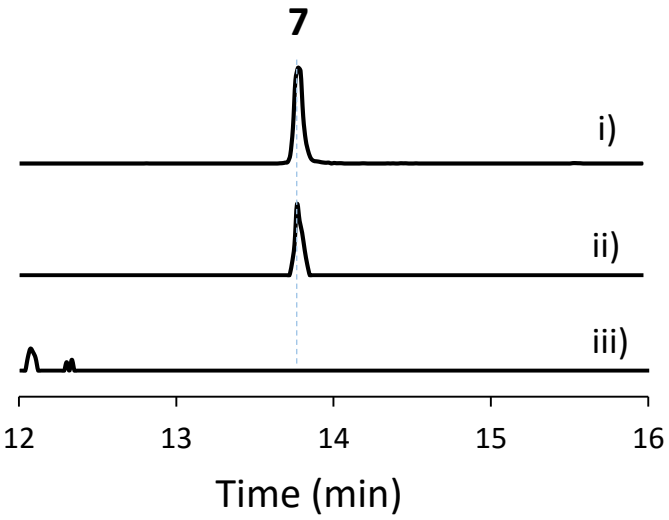

Figure S2. Extracted ion chromatograms (**7**: m/z 832.3287 $[M+H]^+$) showing production of **7** upon the reaction with XZ1 supernatant (trace ii), but not with the XZ5 supernatant (traces iii). The compound synthesized by a chemical reaction using pure **1** and **4** is shown for comparison (trace i). The calculated mass with 10 ppm mass error tolerance was used.

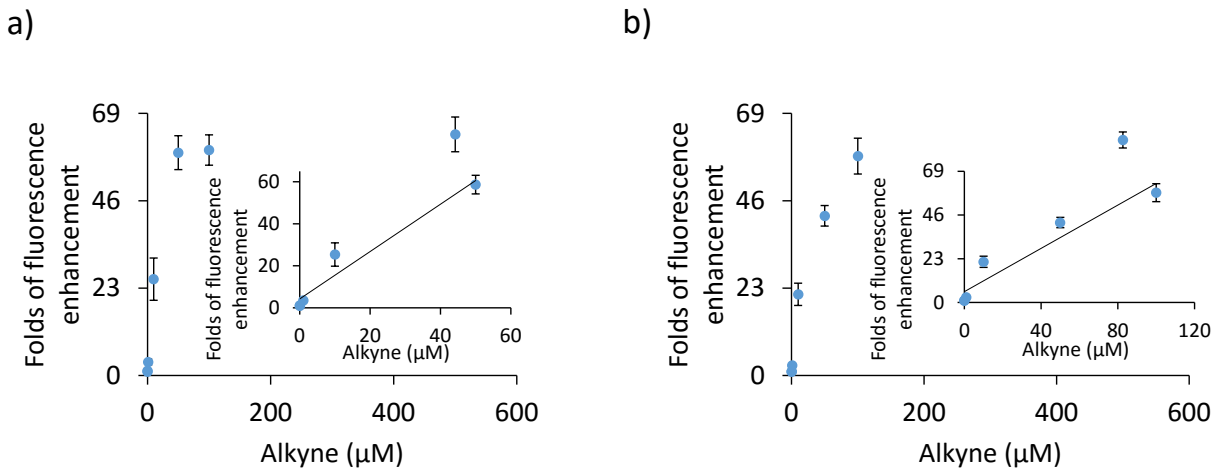

Figure S3. By varying the concentrations of probe **4** used in the assays, the linear detection range can be adjusted. One-fold fluorescence enhancement corresponds to the signal obtained from the reaction with pure medium. (a) When 3 μM of **4** was used, 0.05-50 μM of alkynes can be detected in a linear range, R^2=0.95. (b) When 10 μM of **4** was used, 0.05-100 μM of alkynes can be detected in a linear range, R^2=0.92. Error bars represent standard deviations from three measurements.

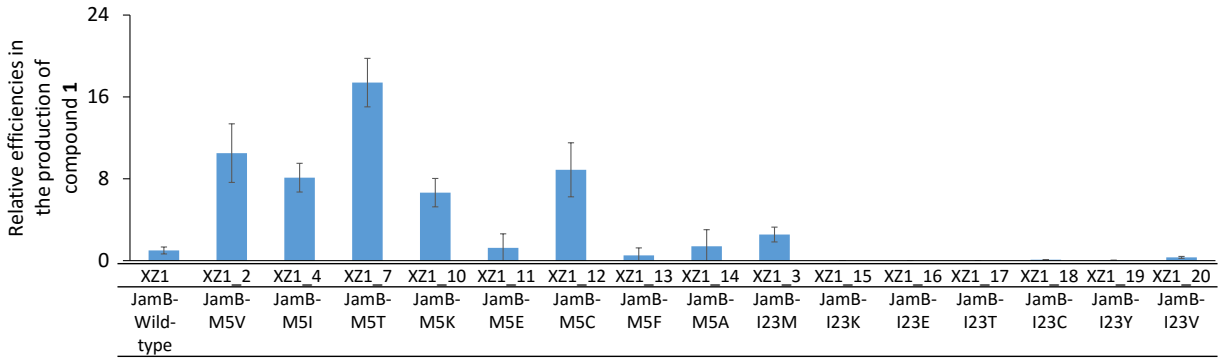

Figure S4. Relative efficiencies in the production of **1** compared to the wild-type strain (XZ1) when all cultures were fed with hexanoic acid. The efficiency of the wild-type JamB in the production of compound **1** in XZ1 was set to be 100%. Error bars represent standard deviations from three measurements.

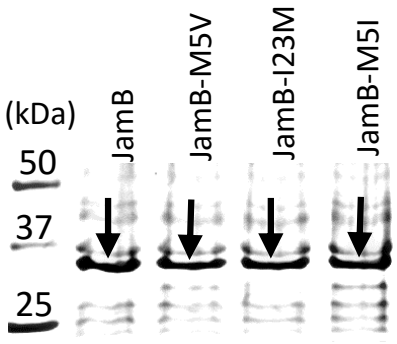

Figure S5. SDS-PAGE analysis of membrane fractions of the engineered *E. coli*. Representative SDS-PAGEs showed that JamB-M5I/V and JamB-I23M were expressed at a level comparable to the wild-type (~36 kDa). Mini-PROTEAN® gel (4-15% precast, Biorad) was used.

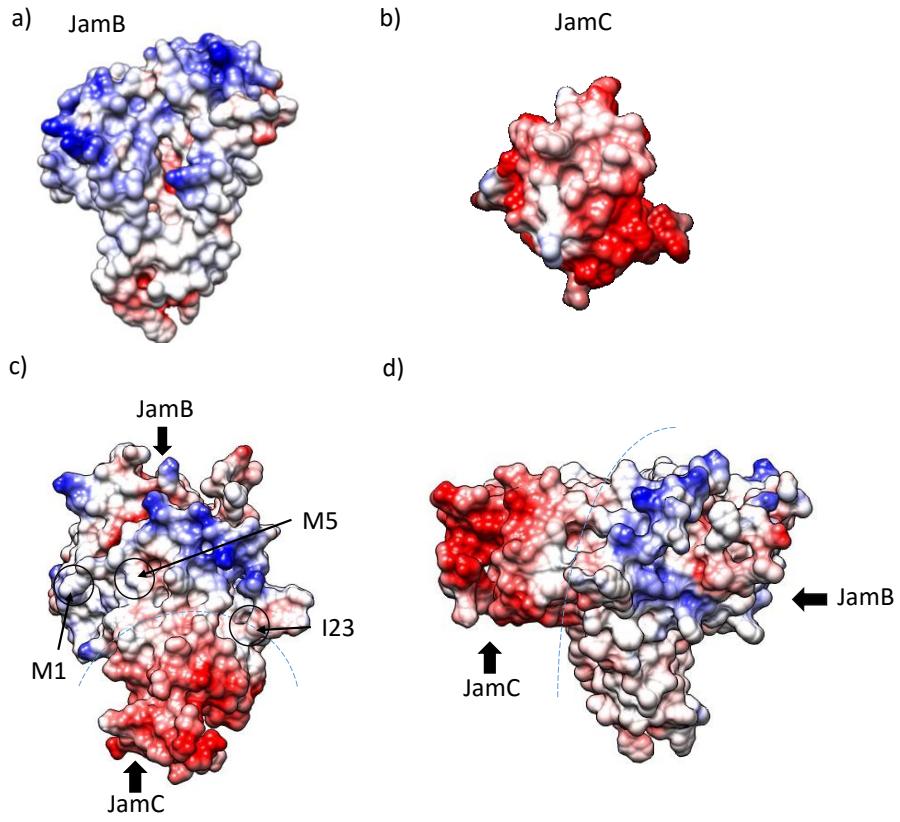

Figure S6. Modeled structures of JamB, JamC, and JamB-JamC complex. Coulombic surfaces of JamB (a), and JamC (b) are oriented with their proposed interaction surface towards the viewer. Coulombic surfaces of the JamB-JamC complex are shown at different angles in (c) and (d). Electro-negative surface is shown in red, and electro-positive surface is shown in blue.

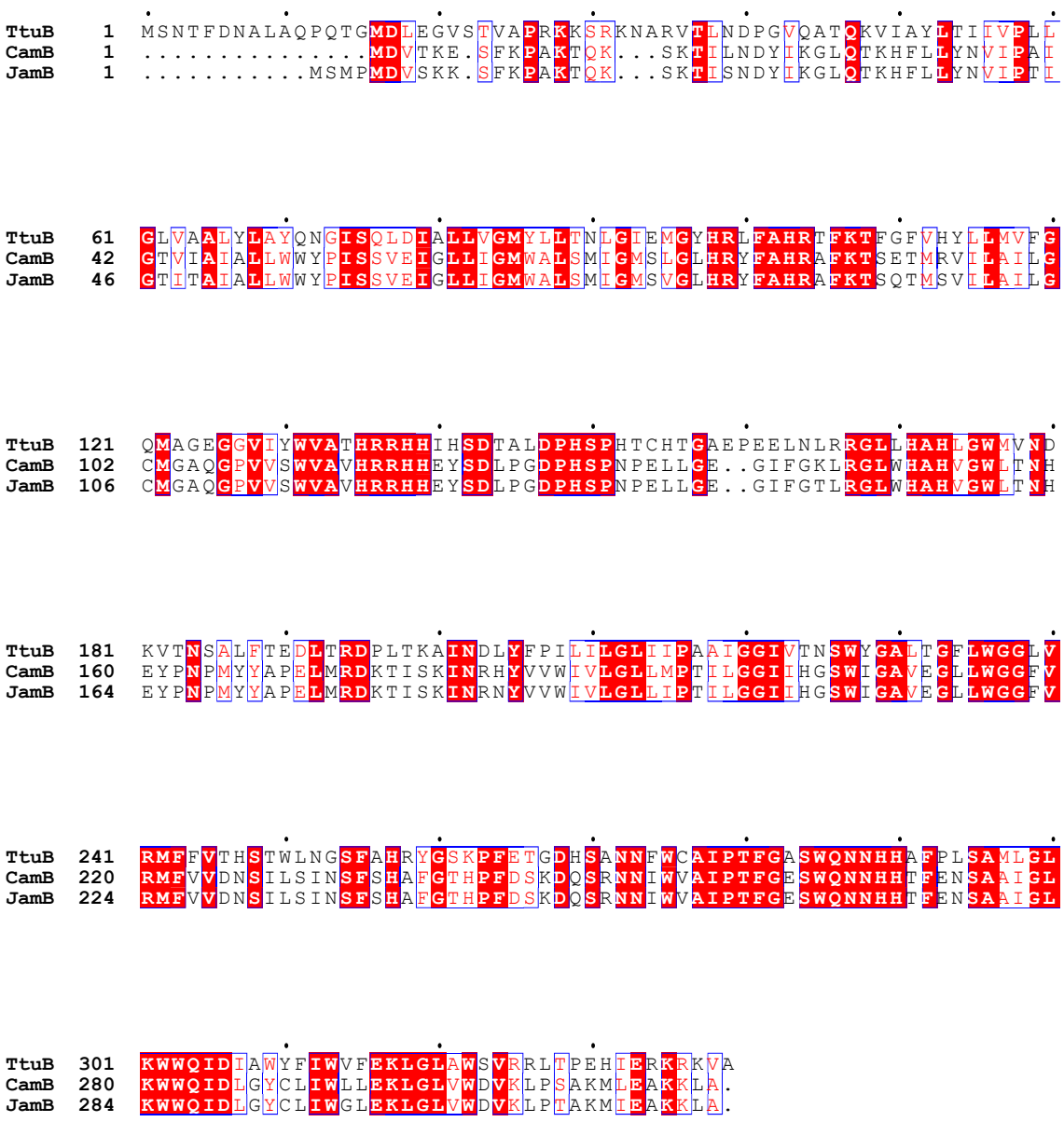

Figure S7. Sequence alignment of TtuB, CamB, and JamB.

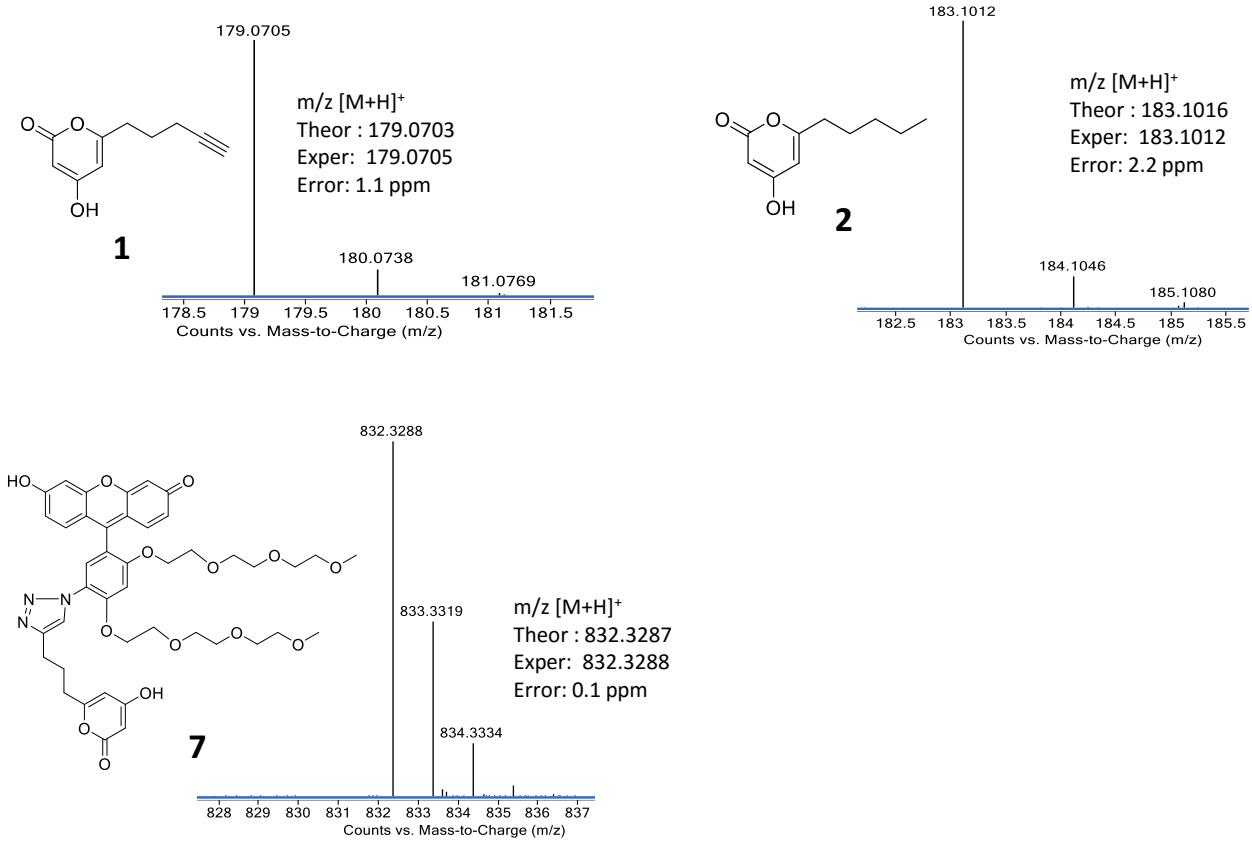

Figure S8. HRMS of compounds **1**, **2**, **7**.