Electronic Supplementary Material (ESI) for ChemComm.

Supplementary information

Thioether Bond Formation By SPASM Domain Radical SAM Enzymes: C_α H-Atom Abstraction In Subtilosin A Biosynthesis

A. Benjdia[a], A. Guillot[a], B. Lefranc[b,c], H. Vaudry[b,c], J. Leprince[b,c] and O. Berteau[a*]

[a] Micalis Institute, ChemSyBio, INRA, AgroParisTech, Université Paris-Saclay, 78350 Jouy-en-Josas, France
[b] INSERM U982, F-76821 Mont-Saint-Aignan, France
[c] Institute for Research and Innovation in Biomedicine (IRIB), Regional Platform for Cell Imaging, PRIMACEN
University of Rouen, F-76821 Mont-Saint-Aignan, France

*E-mail: Olivier.Berteau@jouy.inra.fr

Experimental procedure

Peptides synthesis. All peptides, Ac-SIGAACLVDGPIPDFEIAGA-NH_2 (**$SboA_{8-27}$**), Ac-ATGLFGLWGNKGCAT-NH_2 (**$SboA_{27-6}$**), Ac-ATGL(phenyl-D_5, α,β,β-D_3)FGLWGNKGCAT-NH_2 (**$SboA_{27-6}$-F**) and Ac-ATGLFGLWGNKG(β,β-D_2)CAT-NH2 (**$SboA_{27-6}$-C**) were synthesized (0.1-mmol scale) by the solid phase methodology on a Rink amide 4-methylbenzhydrylamine resin (VWR, Fontenay-Sous-Bois, France) using a 433A Applied Biosystems peptide synthesizer (Applera-France, Courtaboeuf, France) and the standard Fmoc manufacturer's procedure. All Fmoc-amino acids (1 mmol, 10 eq., Christof Senn Laboratories, Dielsdorf, Switzerland), including the per-deuterated phenylalanyl residue Fmoc-(phenyl-D_5, α,β,β-D_3)Phe-OH and Fmoc-(β,β-D_2)Cys(Trt)-OH (Sigma-Aldrich, Saint-Quentin-Fallavier, France) were coupled by *in situ* activation with HBTU/HOBt (1.25 mmol:1.25 mmol, 12.5 eq.) and DIEA (2.5 mmol, 25 eq.) in NMP. Peptides were deprotected and cleaved from the resin by adding 10 mL of the mixture TFA/phenol/H_2O/thioanisole/ethanedithiol (82.5:5:5:5:2.5, v/v/v/v/v; reagent K) for 120 min at room temperature. After filtration, crude peptides were precipitated by addition of tertbutylmethylether (TBME), centrifuged (4,500 rpm), washed twice with TBME, and freeze-dried. The synthetic peptides were purified by reversed-phase HPLC on a 2.2 x 25-cm Vydac 218TP1022 C_{18} column (Grace, Epernon, France) by using a linear gradient (20-50% over 45 min) of CH_3CN/TFA (99.9:0.1 ; v/v) at a flow rate of 10 $mL.min^{-1}$. Analytical HPLC, performed on a 0.46 x 25-cm Vydac 218TP54 C_{18} column (Grace), showed that the purity of the peptides was >99.5%. The purified peptides were characterized by MALDI-TOF mass spectrometry on a Voyager DE-PRO (Applera-France) in the reflector mode with α-cyano-4-hydroxycinnamic acid as a matrix.

AlbA expression and purification. Plasmid containing the albA gene was constructed by amplifying albA from *B. subtilis* 168 strain. The oligonucleotides alba-F (GGTCTCAGCGCCTTGTTTATAGAGCAGATGTTTCCATTTATT) and alba-R (GGTCTCATATCCTAAATAAGCTGGACCACGTCTTCTAATTG) were used to amplify albA. The 1.8 kb BsaI/BsaI fragment containing the gene was ligated into pASK17+ plasmid to produce the recombinant plasmid pASK-albA expressing AlbA fused with an N-terminal strep-Tag. The entire cloned gene was sequenced to ensure that no error was introduced. *E. coli* BL21 (DE3) star transformed with pASK-albA were used to inoculate fresh LB medium (9 L) supplemented with ampicillin (100 $\mu g.mL^{-1}$) and bacterial growth proceeded at 37°C until the OD600 reached between 0.6-0.8. Protein expression was induced by adding 9 mL of anhydrotetracycline (0.2 $mg.mL^{-1}$). After 24 hours at 18°C, the cells were collected by centrifugation at 5,000 x g for 20 minutes. The cells were suspended in 10 mL of buffer A (Tris 50 mM, KCl 300 mM, NaCl 0.5 mM, glycerol 10%, pH 8). The cells were disrupted by sonication on ice after addition of Triton X100 and 0.5% of 2-mercaptoethanol. The protein extract was centrifuged at 45,000 x g at 4°C for 1.5 hour. The solution obtained was loaded onto a streptactin high capacity column (IBA) previously equilibrated with buffer A. The column was washed with 5 column volumes of buffer A. Proteins were eluted with 9 mL of buffer A containing 1 mM of desthiobiotin and dithiothreitol (DTT, 3 mM). The purified protein, after concentration, was stored at -80°C and the purity was assayed on a 12% SDS PAGE.

Spectrophotometric measurement. UV-visible absorption spectra were recorded on a Beckman DU 640 spectrophotometer.

AlbA assay. Enzymatic assays were carried out in an anaerobic chamber (Bactron IV) with an atmosphere constituted of 5% H_2, 10% CO_2 and 85% N_2. AlbA was reconstituted by adding 3 mM of

DTT at 12°C during 15 minutes. Then, Na_2S and $(NH_4)2Fe(SO_4)_2$ (12 equivalents) were added and the solution was incubated at 12°C overnight. The protein was then desalted to remove unbounded iron and sulfide and concentrated. The reaction mixture was composed of AlbA (20 µM), peptide substrate (1 mM), S-adenosyl-L-methionine (1 mM) and DTT (6 mM). Incubations were performed at 25°C under strict anaerobic conditions with a one-electron donor (*i.e.* sodium dithionite or flavodoxin/flavodoxin reductase/NADPH+).

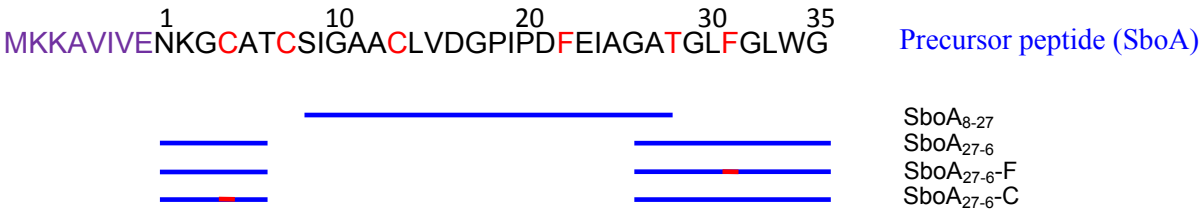
HPLC analysis. Samples (3 µL) were diluted with 27 µL of trifluoroacetic acid solution (TFA 0.1%). HPLC analysis was carried out on an Agilent 1200 series Infinity chromatographic system with a reversed phase column (LiChroCART RP-18e 5 µm). The column was equilibrated with 100% solvent A (H_2O, 0.1% TFA) and solvent B (80% CH_3CN, 0.1% TFA) was applied with the following gradient: 0-1 min 0% B; 1-15 min, a linear gradient to 20% B; 15-50 min, a linear gradient to 60% B; 50-55 min, a linear gradient to 0% B at a flow rate of 1 $mL.min^{-1}$.

LC-MS/MS analysis. LC-MS analysis was performed on an NanoLC-Ultra system (Eksigent). Each reaction was diluted 100 times and 4 µL were loaded at 7.5 $\mu L.min^{-1}$ on a cartridge (stationary phase: PepMap 100 C_{18}, 5 µm; column: 300 µm i.d., 5 mm; Dionex) and desalted with 0.1% formic acid (FA). After 3 min, the cartridge was connected to the separating C_{18} column (stationary phase: C_{18} Bio-sphere, 3 µm; column: 75 µm i.d., 150 mm; Nanoseparations). Buffers were H_2O, 0.1% FA (A) and CH_3CN, 0.1% FA (B). The peptide separation was achieved with a linear gradient from 5 to 65% for 30 min of buffer B at 300 $nL.min^{-1}$. Eluted peptides were analyzed on-line with a QExactive mass spec-trometer (Thermo Electron) using a nanoelectrospray interface piloted by Xcalibur 2.1 version. Peptide ionization was performed with picotip ermitters (20 µm i.d.; New objective) in a liquid junction with an ionization potential of 1.4 KV. Peptides were detected by the Orbitrap mass analyser in full MS scan mode with a range of mass-to-charge ratio (m/z) between 400 to 1400, at a resolution potency of 70000. The doubly charged ions corresponding to cyclic and linear peptides were specifically selected for fragmentation (MS/MS) during the complete separation by the HCD fragmentation mode with a normalized collision energy of 20%, at a resolution of 17,500. The spectra were merged and analyzed manually in Qualbrowser software (Thermo Xcalibur version 2.2.44).

Model of AlbA iron-sulfur centers. Using the structure of anSME (PDB: 4k38) and i-Tasser program a model for AlbA from residues 117 to 446 was generated (estimated r.m.s.d is of 4.1±2.8 Å, i-Tasser source). Based on i-Tasser, the best identified structural analog is anSME (PDB: 4k36). The structure superposition was performed with Coot using the SSM program and all residues.

Figure S1

Figure S1 - Peptides used in this study: The leader peptide which is cleaved after subtilosin A cyclization is indicated by purple residues while the residues in red are those involved in thioether bonds.



Based on SboA sequence, peptides covering the entire sequence of mature SboA have been synthesized.

$SboA_{8-27}$: Ac-SIGAACLVDGPIPDFEIAGA-NH_2
$SboA_{27-6}$: Ac-ATGLFGLWGNKGCAT-NH_2
$SboA_{27-6}$-F: Ac-ATGLFGLWGNKGCAT-NH_2
$SboA_{27-6}$-C: Ac-ATGLFGLWGNKGCAT-NH_2

All synthetic peptides had a N-terminal acetylation and a C-terminal amidation. In $SboA_{27-6}$-F, the letter F in red indicates a per-deuterated residue while in $SboA_{27-6}$-C, the letter C in red indicates a (β,β-D_2) cysteine residue.

The theoretical molecular weights of singly- and doubly-charged parent ions (including post-translational modifications) of the peptides used in this study are:

Peptide	**Molecular formula**	**$[M+H]^+$**	**$[M+2H]^{2+}$**
$SboA_{8-27}$	$C_{87}H_{137}N_{21}O_{28}S$	1956.974	978.991
$SboA_{8-27}$* (with a thioether bond)	$C_{87}H_{135}N_{21}O_{28}S$	1954.958	977.983
$SboA_{27-6}$	$C_{69}H_{105}N_{19}O_{19}S$	1536.763	768.886
$SboA_{27-6}$* (with a thioether bond)	$C_{69}H_{103}N_{19}O_{19}S$	1534.747	767.878
$SboA_{27-6}$-F (containing a per-deuterated phenylalanine)	$C_{69}H_{97}D_8N_{19}O_{19}S$	1544.812	772.910
$SboA_{27-6}$-F* (with a thioether bond)	$C_{69}H_{96}D_7N_{19}O_{19}S$	1541.886	771.447
$SboA_{27-6}$-C (containing a (β,β-D_2) cysteine)	$C_{69}H_{103}D_2N_{19}O_{19}S$	1538.763	769.886
$SboA_{27-6}$-C* (with a thioether bond)	$C_{69}H_{101}D_2N_{19}O_{19}S$	1536.747	768.878

Figure S2

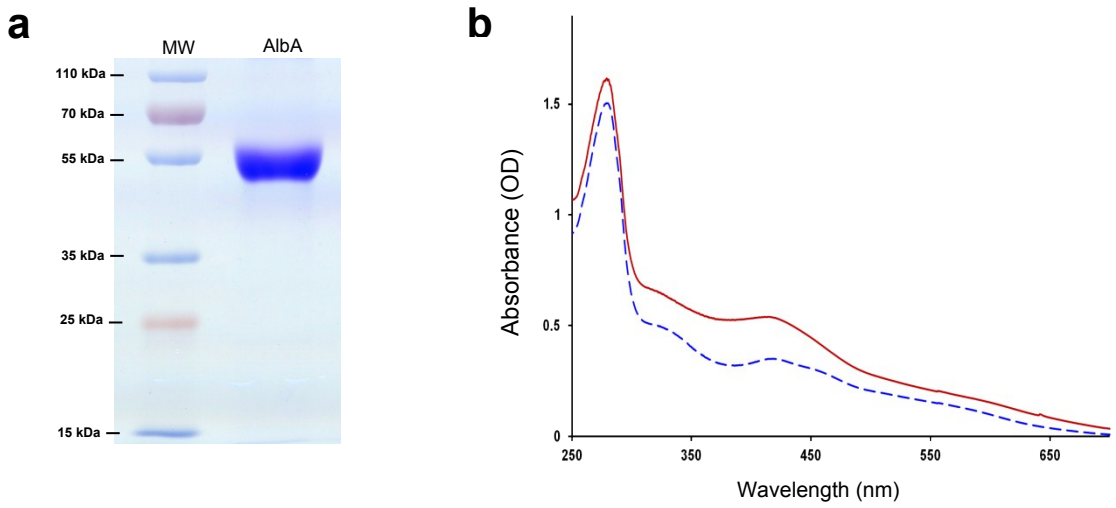


Figure S2 - (a) Gel electrophoresis analysis of AlbA. Molecular weight markers (MW), and purified AlbA. **(b) UV-visible analysis of AlbA before (dashed blue line) and after (solid red line) anaerobic reconstitution.**

Figure S3

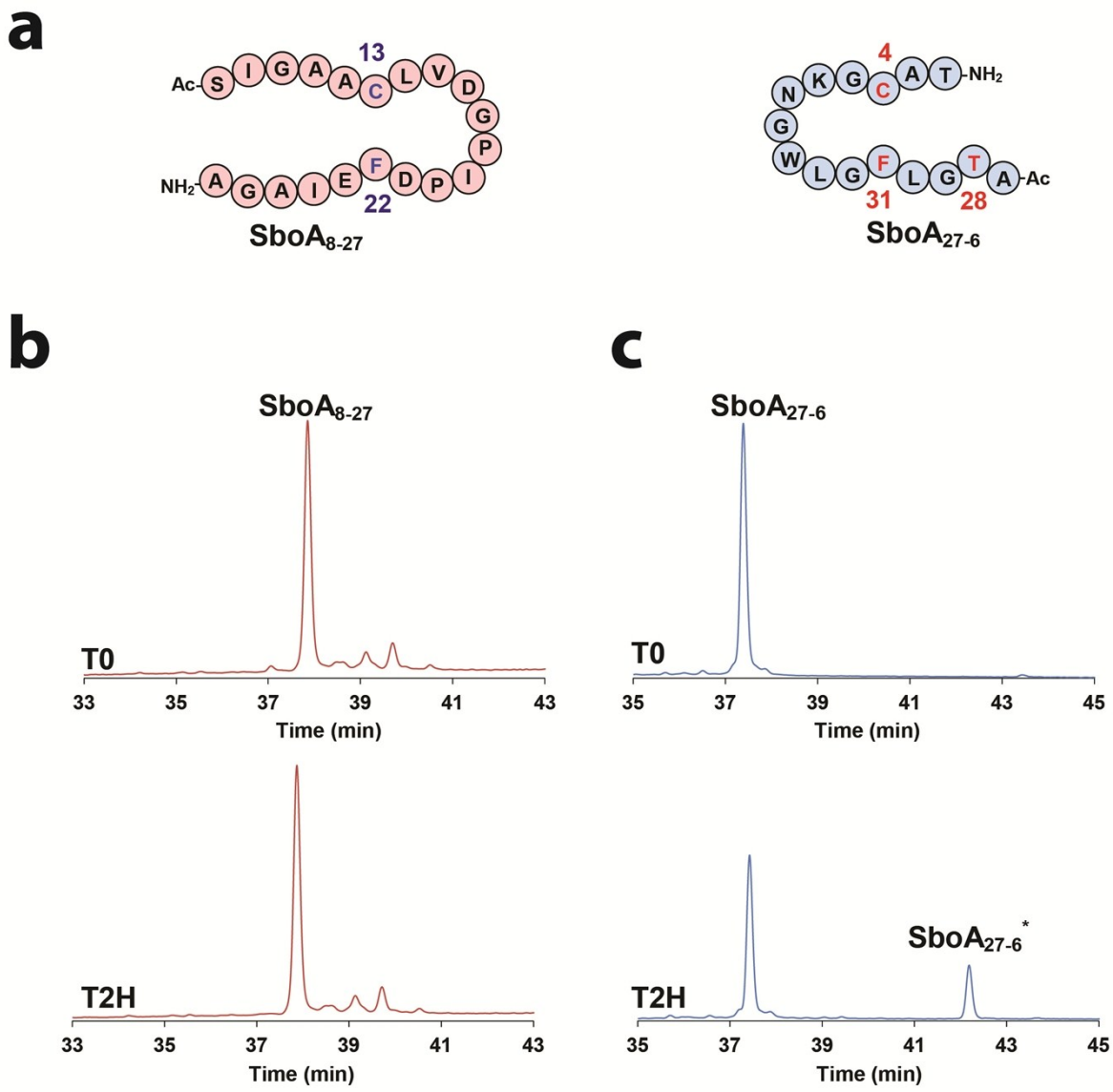


Figure S3 – (a) Sequence of the synthetic peptides assayed as substrates: $SboA_{8-27}$ and $SboA_{27-6}$. Amino acids involved in thioether bonds are labeled in blue or red. **HPLC analysis of incubation of AlbA with (b) $SboA_{8-27}$ and (c) $SboA_{27-6}$ at T0 and after 120 min** (UV detection at 215 nm). AlbA (20 µM) was incubated under anaerobic and reducing conditions in the presence of SAM (1 mM), peptide substrate (1 mM) and sodium dithionite (2 mM). Only in the presence of $SboA_{27-6}$, a new product $SboA_{27-6}$* was formed.

Figure S4

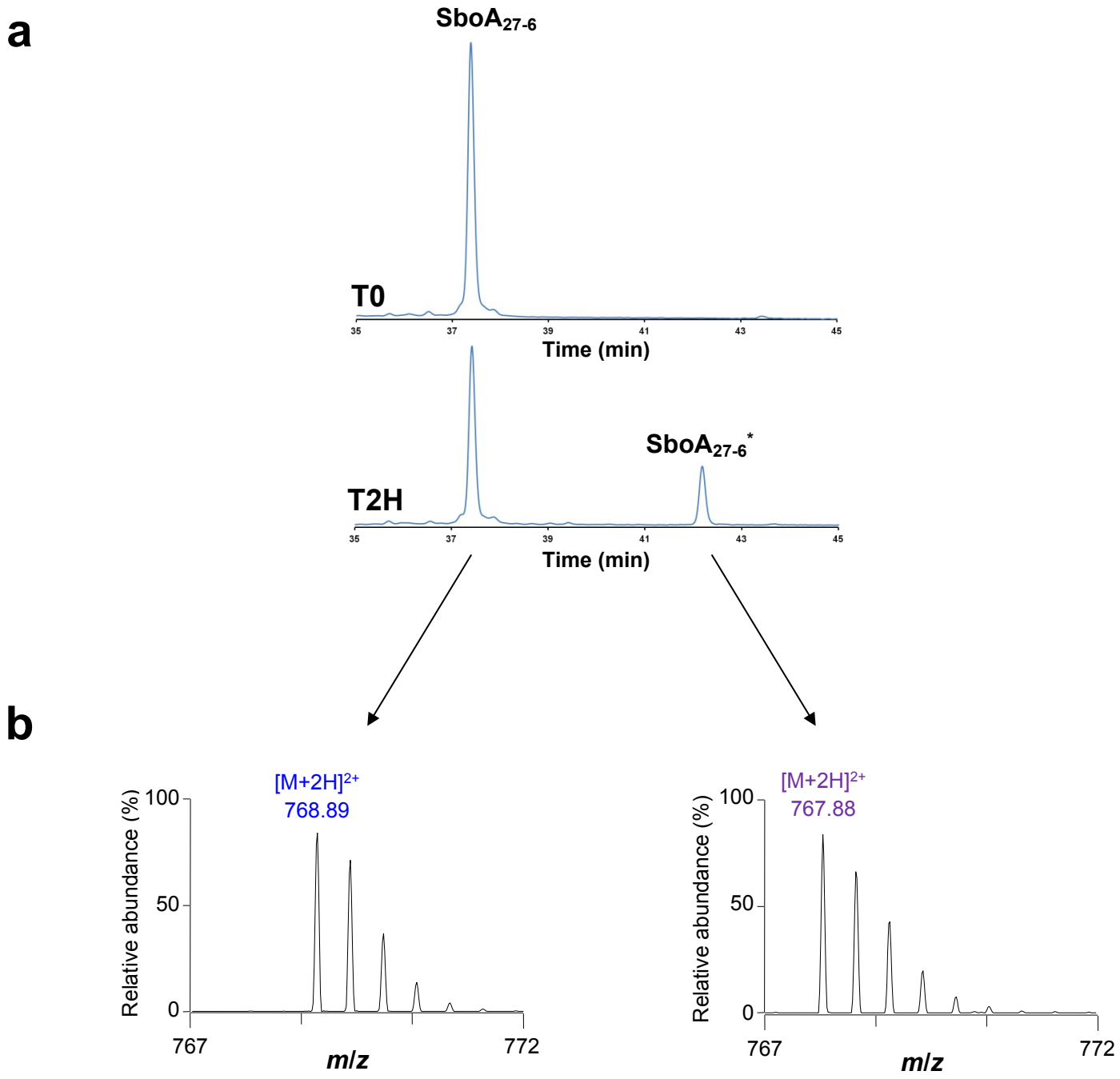


Figure S4 - Activity of AlbA incubated with $SboA_{27\text{-}6}$. (a) HPLC analysis of $SboA_{27\text{-}6}$ with AlbA (UV detection at 215 nm). **(b)** LC-MS analysis of $SboA_{27\text{-}6}$ and the corresponding product $SboA_{27\text{-}6}^{*}$. AlbA was incubated with $SboA_{27\text{-}6}$ (1 mM) under anaerobic and reducing conditions in the presence of SAM (1mM) and sodium dithionite (2 mM).

Figure S5

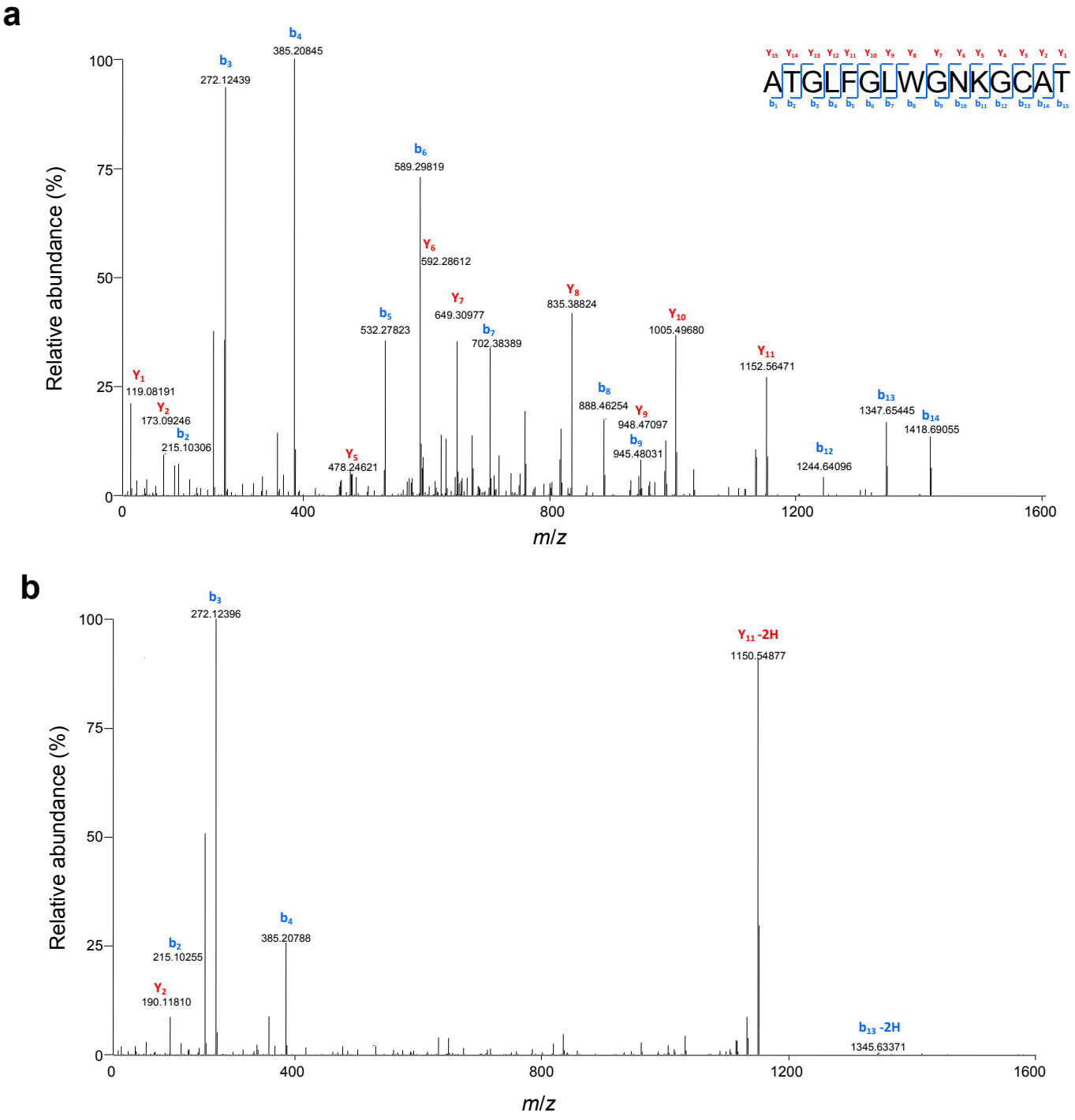


Figure S5 - LC-MS^2 analysis of SboA$_{27\text{-}6}$ (a) and the cyclic peptide produced (b) by AlbA. AlbA (20 µM) was incubated in the presence of SAM (1 mM) and SboA$_{27\text{-}6}$ (1 mM) under anaerobic conditions with sodium dithionite (2 mM) as one-electron donor. Relevant fragments of the *b* and *y* series are indicated (see Table S2 for complete assignment).

Figure S6

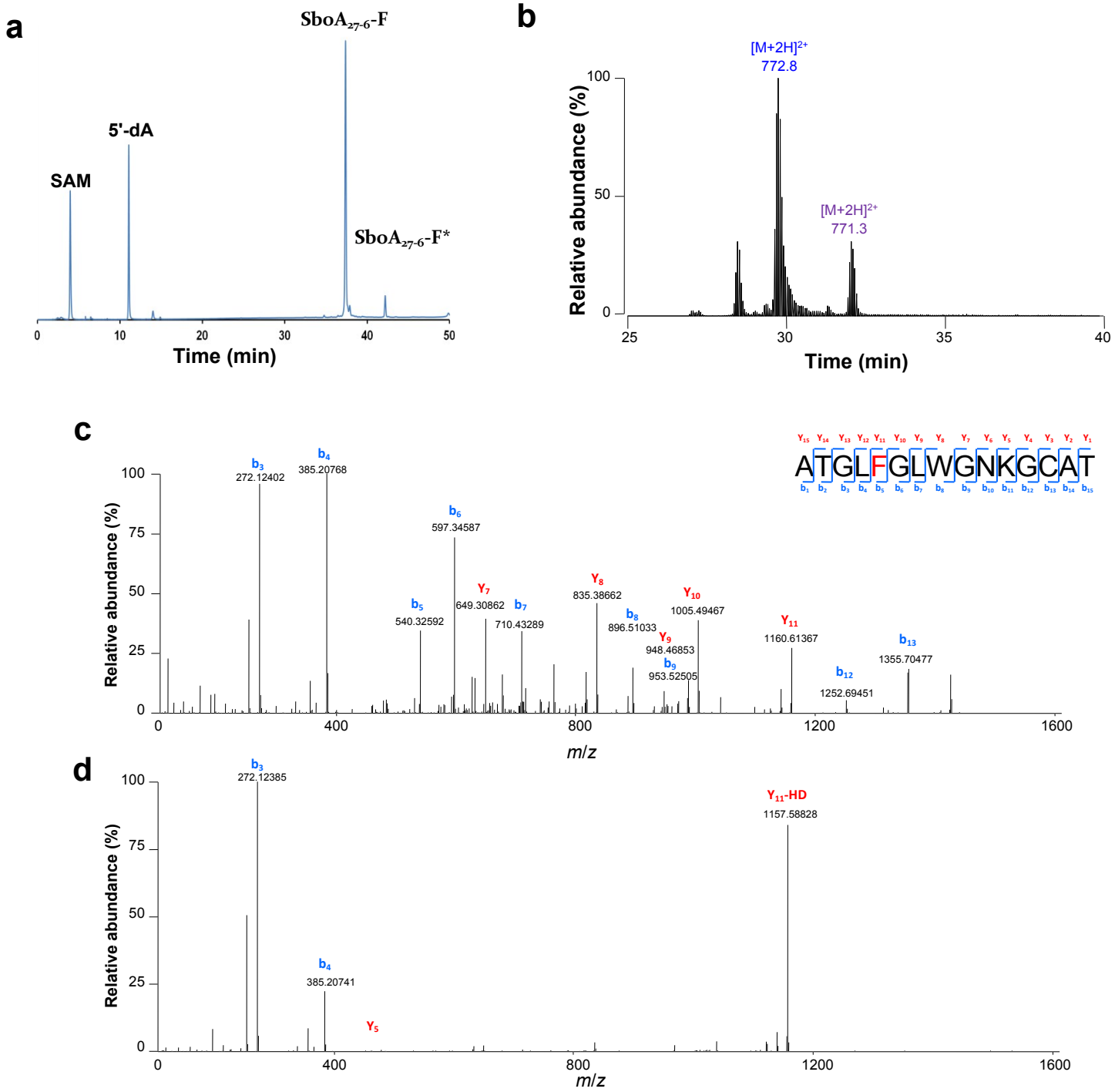


Figure S6 - Activity of AlbA incubated during 2 hours with SboA$_{27\text{-}6}$-F: (a) HPLC analysis of AlbA incubated in the presence of SboA$_{27\text{-}6}$- F (UV detection at 280 nm). (b) LC-MS analysis of SboA$_{27\text{-}6}$-F incubated with AlbA. (c) LC-MS^2 analysis of SboA$_{27\text{-}6}$-F and (d) the cyclic peptide SboA$_{27\text{-}6}$-F* formed. SboA$_{27\text{-}6}$-F (1 mM) was incubated with AlbA (20 µM) with SAM (1 mM) and sodium dithionite (2 mM) under anaerobic conditions. Relevant fragments of the *b* and *y* series are indicated (**see Table S3** for complete assignment).

Figure S7

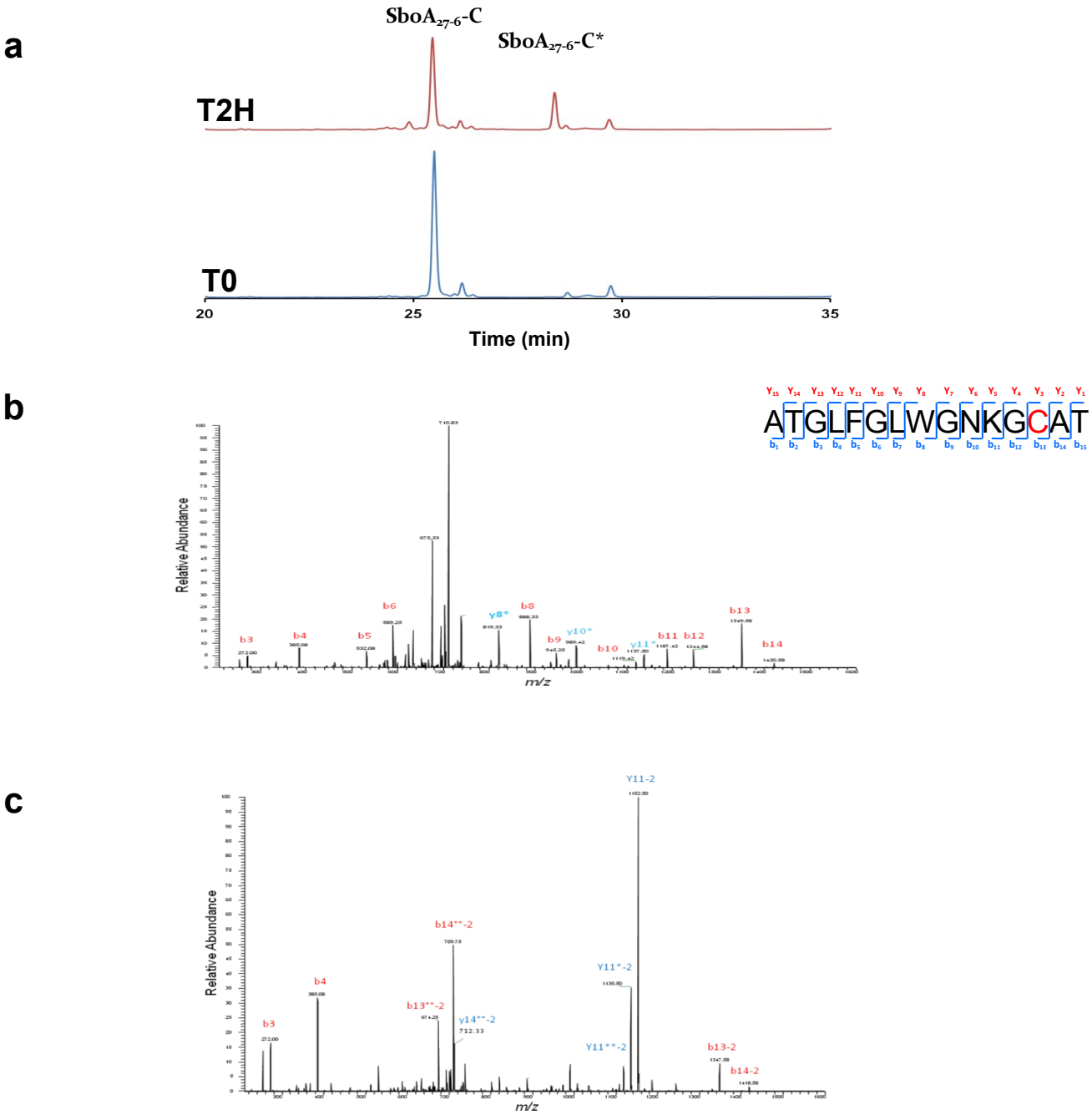


Figure S7 - Activity of AlbA incubated during 2 hours with $SboA_{27\text{-}6}$-C: (a) HPLC analysis of AlbA incubated in the presence of $SboA_{27\text{-}6}$-C (215 nm). (b) LC-MS^2 analysis of $SboA_{27\text{-}6}$-C and (c) of the cyclic peptide $SboA_{27\text{-}6}$-C* formed. $SboA_{27\text{-}6}$-C (1 mM) was incubated with AlbA (20 µM) under anaerobic conditions with flavodoxin/flavodoxin reductase/$NADPH^+$ as one electron donor. Relevant fragments of the *b* and *y* series are indicated (**see Table S4** for complete assignment).

Figure S8

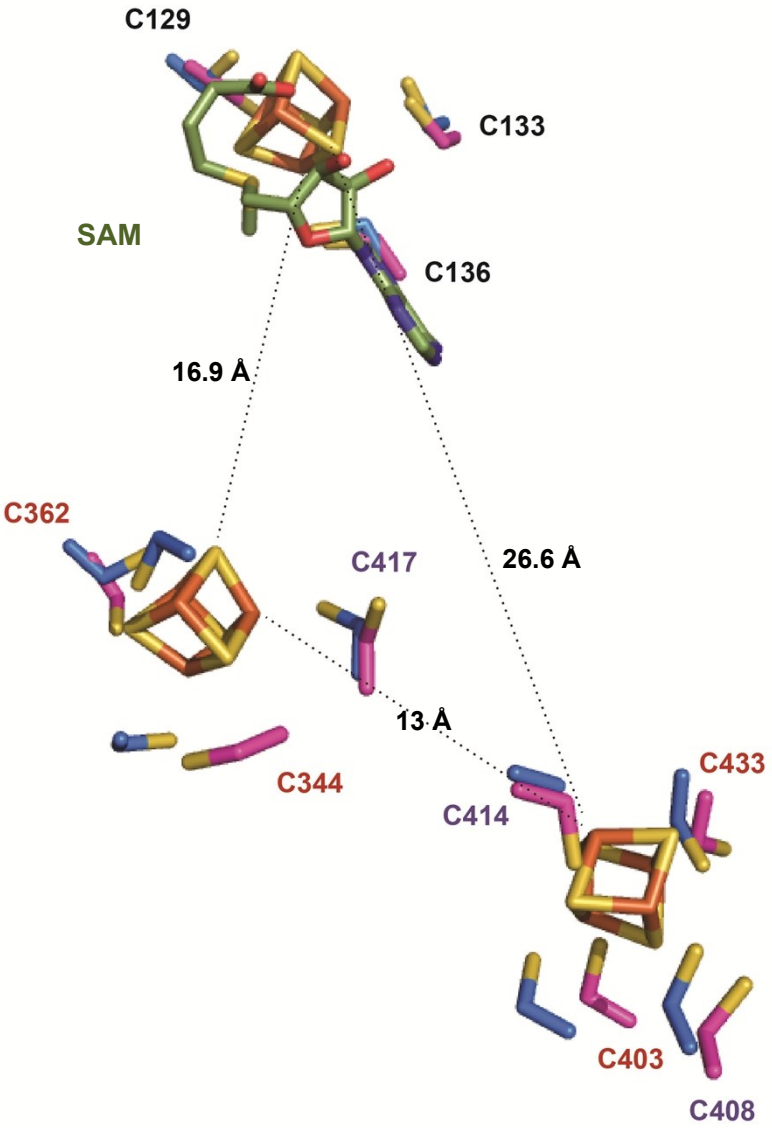


Figure S8 –Structural model of AlbA iron-sulfur centers. The cysteine residues involved in the coordination of [4Fe-4S] centers are depicted in blue for anSME and in pink for AlbA. Numbers in purple indicate cysteine residues of AlbA from the SPASM-domain previously mutated. Estimated distances between the [4Fe-4S] centers are indicated.

Figure S9

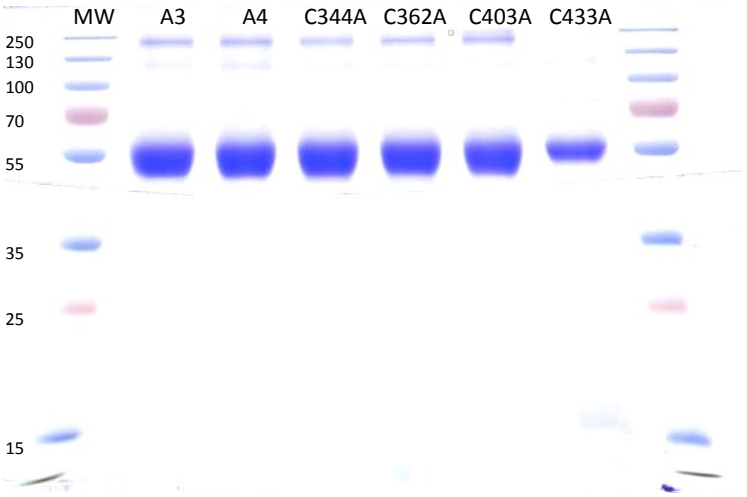


Figure S9 - Gel electrophoresis analysis of purified AlbA mutants.

A3: AlbA lacking radical SAM binding motif: Cx3Cx2C
A4: AlbA mutated on residues Cys^{344}, Cys^{362}, Cys^{403} and Cys^{433}
C344A: AlbA mutated on residue Cys^{344}
C362A: AlbA mutated on residue Cys^{362}
C403A: AlbA mutated on residue Cys^{403}
C433A: AlbA mutated on residue Cys^{433}
MW: molecular weight markers

Figure S10

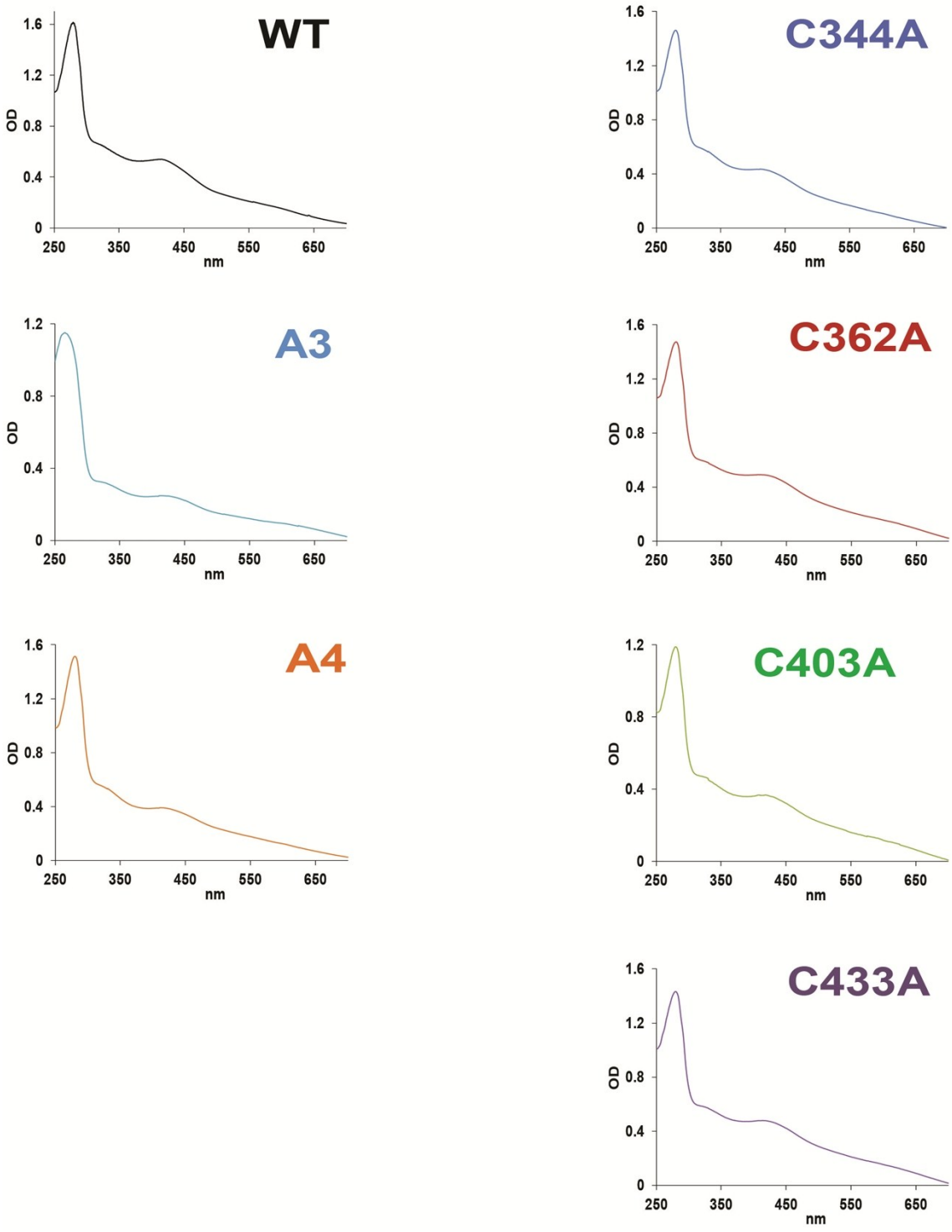


Figure S10 - UV-visible spectrum of purified wild-type (WT) AlbA and cysteine mutants after anaerobic reconstitution. C344A, C362A, C403 and C433A indicate the spectrum corresponding to AlbA single mutants: **C344A**, **C362A**, **C403A** and **C433A.** The **A3 mutant** lacks the radical SAM motif **(**Cx3Cx2C) while the **A4 mutant** is mutated on the four cysteine residues C344A, C362A, C403A and C433A.

Figure S11

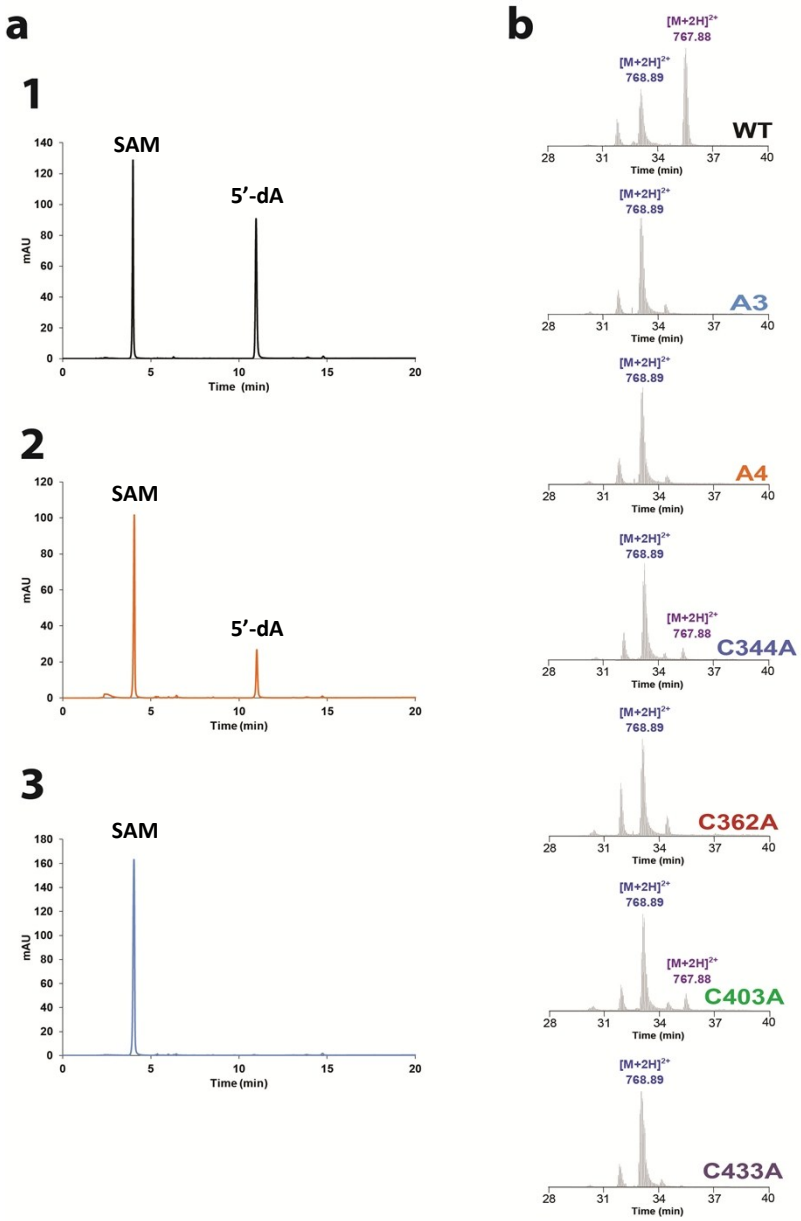


Figure S11 - (a) SAM cleavage activity of (trace 1) AlbA, (trace 2) AlbA mutated on residues Cys^{344}, Cys^{362}, Cys^{403} and Cys^{433} (A4 mutant) and (trace 3) AlbA lacking radical SAM binding motif: Cx3Cx2C (A3 mutant). HPLC analysis performed on reverse phase column with UV detection at 257 nm. Proteins were incubated with SboA$_{27\text{-}6}$ (1 mM) under anaerobic and reducing conditions in the presence of SAM (1mM) and sodium dithionite (2 mM). **(b) LC-MS analysis of the peptide substrate SboA$_{27\text{-}6}$ incubated with WT AlbA and the corresponding mutants.** The **SboA$_{27\text{-}6}$ peptide** $[M+2H]^{2+}$: 768.89 is converted into the cyclic peptide SboA$_{27\text{-}6}$* $[M+2H]^{2+}$: 767.88 only in the presence of the WT enzyme or the C344A and C403A mutants. Each protein (20 µM) was incubated under anaerobic and reducing conditions in the presence of SAM (1 mM), peptide substrate (1 mM) and sodium dithionite (2 mM).

Table S1

Fragmentation pattern of **$SboA_{8-27}$**

$SboA_{8-27}$	Sequence	#	B	Y	# (+1)
	S	1	130.05046	1956.97412	20
	I	2	243.13452	1827.93153	19
	G	3	300.15599	1714.84747	18
	A	4	371.1931	1657.82601	17
	A	5	442.23021	1586.78889	16
	C	6	545.2394	1515.75178	15
	L	7	658.32346	1412.74259	14
	V	8	757.39188	1299.65853	13
	D	9	872.41882	1200.59012	12
	G	10	929.44028	1085.56317	11
	P	11	1026.49305	1028.54171	10
	I	12	1139.57711	931.48895	9
	P	13	1236.62987	818.40488	8
	D	14	1351.65682	721.35212	7
	F	15	1498.72523	606.32518	6
	E	16	1627.76782	459.25676	5
	I	17	1740.85189	330.21417	4
	A	18	1811.889	217.13011	3
	G	19	1868.91046	146.09299	2
	A	20	1938.96356	89.07153	1

Table S2

Fragmentation pattern of **$SboA_{27-6}$**

$SboA_{27-6}$	Sequence	#	B	Y	# (+1)
	A	1	114.05554	1536.76334	15
	T	2	215.10322	1423.71567	14
	G	3	272.12469	1322.66799	13
	L	4	385.20875	1265.64653	12
	F	5	532.27716	1152.56246	11
	G	6	589.29863	1005.49405	10
	L	7	702.38269	948.47258	9
	W	8	888.462	835.38852	8
	G	9	945.48347	649.30921	7
	N	10	1059.52639	592.28775	6
	K	11	1187.62136	478.24482	5

	G	12	1244.64282	350.14986	4
	C	13	1347.652	293.12839	3
	A	14	1418.68912	190.11921	2
	T	15	1518.75278	119.08209	1

Table S3

Fragmentation pattern of **$SboA_{27\text{-}6}$-F**

$SboA_{27\text{-}6}$-F*	Seq	#	B	Y	# (+1)
	A	1	114.05554	1544.81246	15
	T	2	215.10322	1431.76479	14
	G	3	272.12469	1330.71711	13
	L	4	385.20875	1273.69565	12
	F	5	540.32628	1160.61158	11
	G	6	597.34775	1005.49405	10
	L	7	710.43181	948.47258	9
	W	8	896.51112	835.38852	8
	G	9	953.53259	649.30921	7
	N	10	1067.57551	592.28775	6
	K	11	1195.67048	478.24482	5
	G	12	1252.69194	350.14986	4
	C	13	1355.70112	293.12839	3
	A	14	1426.73824	190.11921	2
	T	15	1526.8019	119.08209	1

Table S4

Fragmentation pattern of **$SboA_{27\text{-}6}$-C**

$SboA_{27\text{-}6}$-C*	Sequence	#	B	Y	# (+1)
	A	1	114.05554	1538.77734	15
	T	2	215.10322	1425.72967	14
	G	3	272.12469	1324.38199	13
	L	4	385.20875	1267.66053	12
	F	5	532.27716	1154.57646	11
	G	6	589.29863	1007.50805	10
	L	7	702.38269	950.48658	9
	W	8	888.462	837.40252	8

	G	9	945.48347	651.32321	7
	N	10	1059.52639	594.30175	6
	K	11	1187.62136	480.25882	5
	G	12	1244.64282	352.16386	4
	C	13	1349.666	295.14239	3
	A	14	1420.70312	190.11921	2
	T	15	1520.76678	119.08209	1