
Supplementary information

β -NAD as a building block in natural product biosynthesis

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β -NAD as a Building Block in Natural Product Biosynthesis

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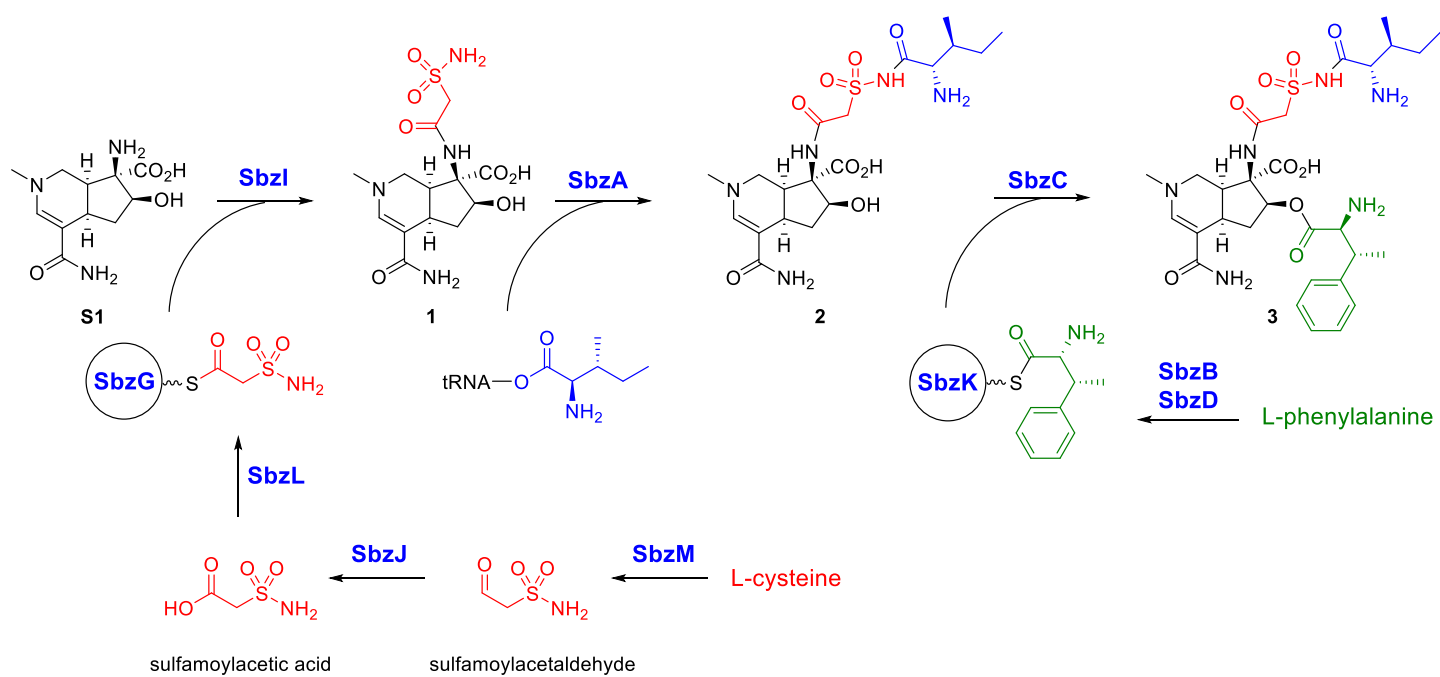
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1. Supplementary Figures and Notes to Main Text.

Supplementary Table 1 The *sbz* BGC. ID = protein sequence identity. SI = protein sequence similarity.

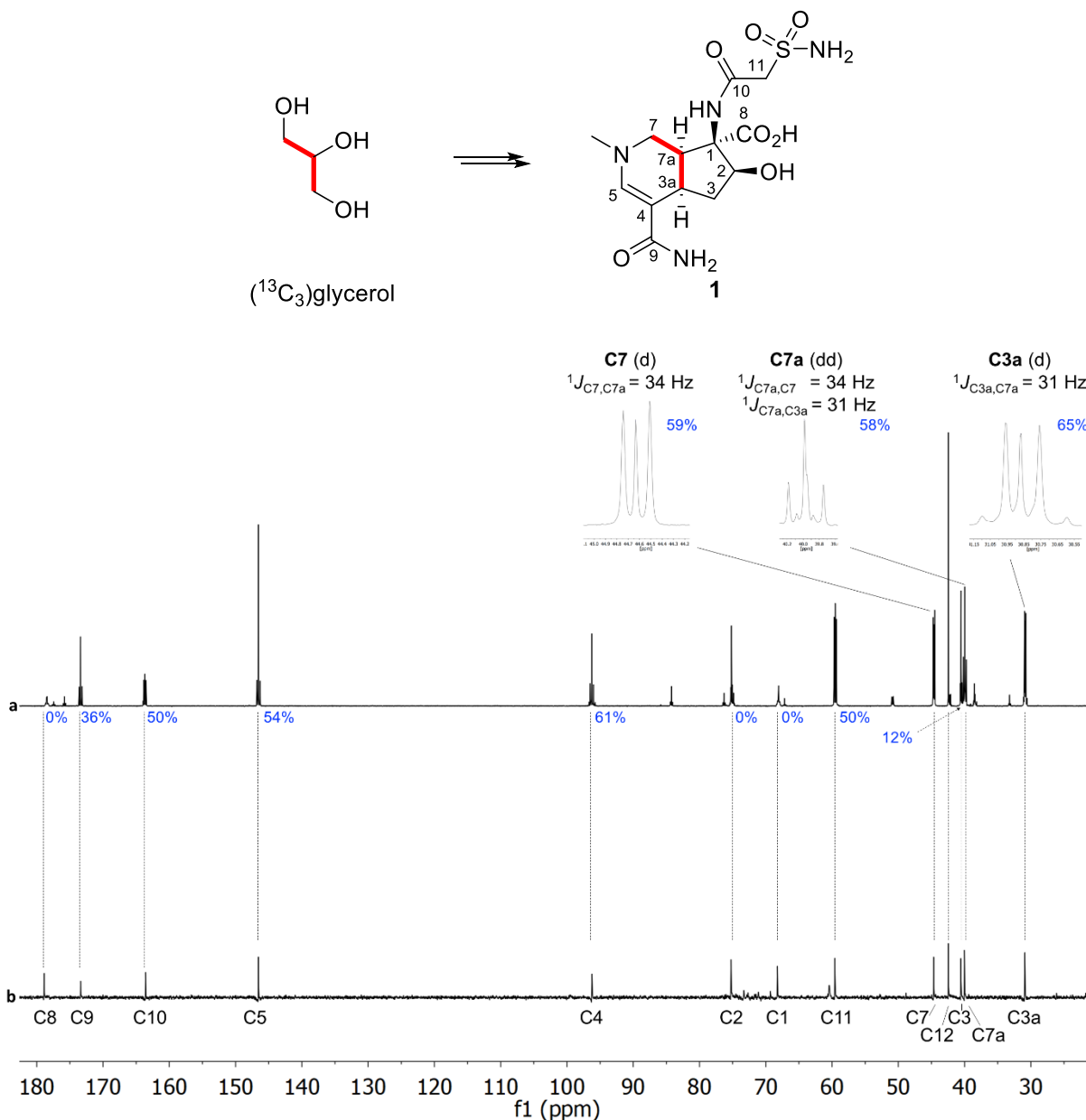
Gene	Amino acid (base pairs)	Closest protein homologue	ID	SI	Proposed function
<i>sbzA</i>	1033(3102)	Q5YYW9 (<i>Nocardia farcinica</i> IFM 10152)	57%	70%	Isoleucine-tRNA synthetase
<i>sbzB</i>	532(1599)	Q9R9J0 (<i>Bacillus subtilis</i>)	36%	53%	AMP-binding enzyme
<i>sbzC</i>	346(1041)	Q47LA0 (<i>Thermobifida fusca</i> YX)	41%	61%	GNAT family enzyme
<i>sbzD</i>	334(1005)	Q643C8 (<i>Streptomyces hygroscopicus</i>)	40%	56%	Methyltransferase MppJ like
<i>sbzE</i>	260(783)	P54458 (<i>Bacillus subtilis</i> subsp. <i>subtilis</i> str. 168)	27%	48%	Methyltransferase
<i>sbzF</i>	320(963)	E8NCH3 (<i>Microbacterium testaceum</i> StLB037)	30%	45%	F420-dependent glucose-6-phosphate dehydrogenase
<i>sbzG</i>	85(258)	Q89VT6 (<i>Bradyrhizobium diazoefficiens</i> USDA 110)	39%	63%	Peptidyl carrier protein
<i>sbzH</i>	334(1005)	P05149 (<i>Acinetobacter calcoaceticus</i>)	35%	53%	Aldose 1-epimerase
<i>sbzI</i>	188(567)	C7IZ16 (<i>Oryza sativa</i> Japonica Group)	28%	37%	GNAT family enzyme
<i>sbzJ</i>	464(1395)	Q6D6Y7 (<i>Pectobacterium atrosepticum</i> SCRI1043)	39%	52%	Gamma-aminobutyraldehyde dehydrogenase
<i>sbzK</i>	93(282)	A7ZA76 (<i>Bacillus velezensis</i> FZB42)	36%	49%	Peptidyl carrier protein
<i>sbzL</i>	559(1680)	O07610 (<i>Bacillus subtilis</i> subsp. <i>subtilis</i> str. 168)	28%	43%	AMP-binding enzyme
<i>sbzM</i>	228(687)	A1WGK0 (<i>Verminephrobacter eiseniae</i> EF01-2)	35%	45%	Cupin
<i>sbzN</i>	385(1158)	Q8TZ14 (<i>Methanopyrus kandleri</i> AV19)	27%	41%	Sugar isomerase
<i>sbzO</i>	268(807)	Q58515 (<i>Methanocaldococcus jannaschii</i> DSM 2661)	27%	51%	BtpA
<i>sbzP</i>	523(1572)	Q0S962 (<i>Rhodococcus jostii</i> RHA1)	34%	56%	Aspartate aminotransferase
<i>sbzQ</i>	304(915)	Q94FY7 (<i>Arabidopsis thaliana</i>)	38%	54%	α -KG Dioxygenase
<i>sbzR</i>	409(1230)	O34546 (<i>Bacillus subtilis</i> subsp. <i>subtilis</i> str.168)	24%	36%	MFS transporter



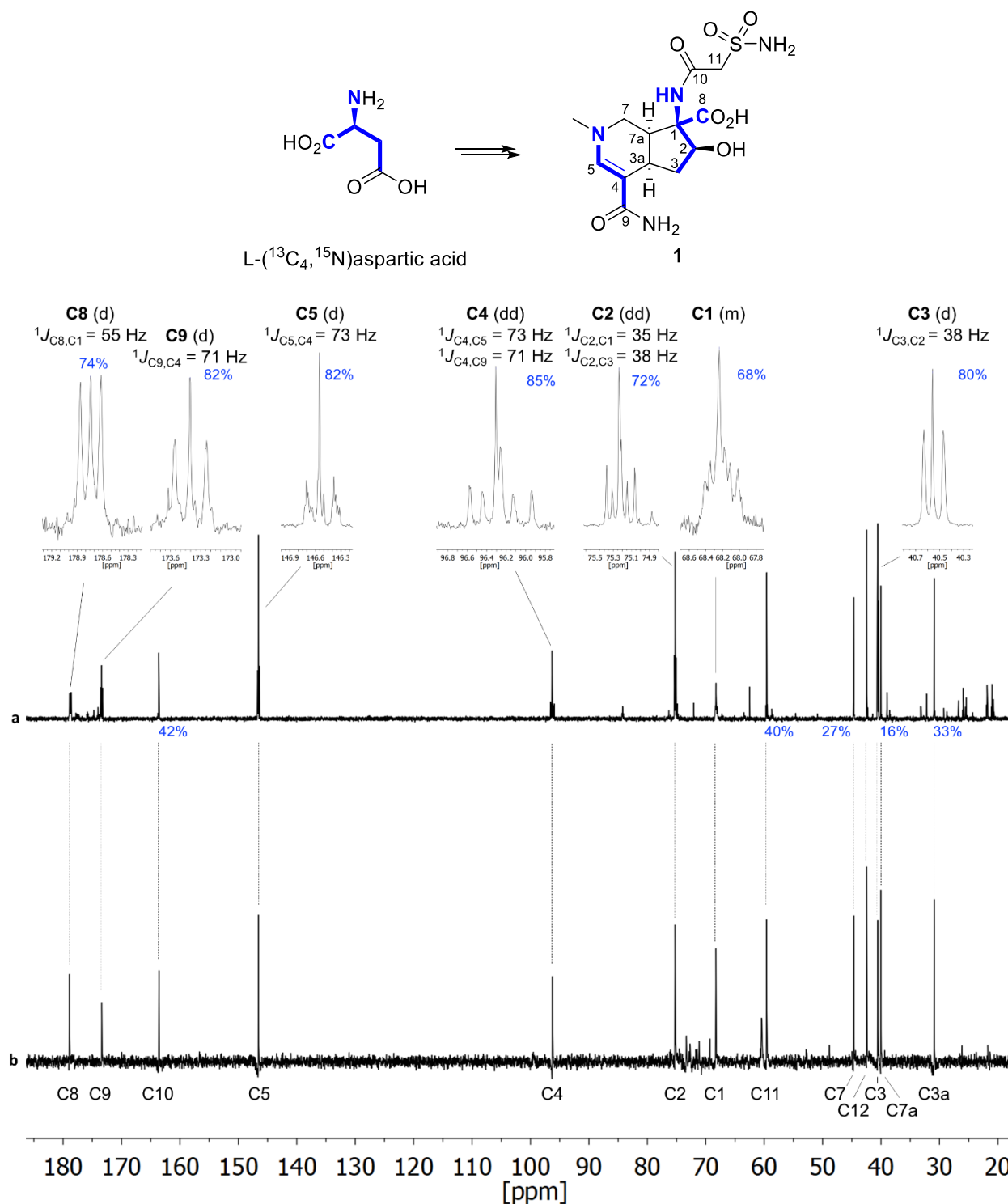
Supplementary Figure 1 The function of *SbzABCDGIJLM* as previously reported¹.

Supplementary Note 1: Results of Isotope Feeding Experiments

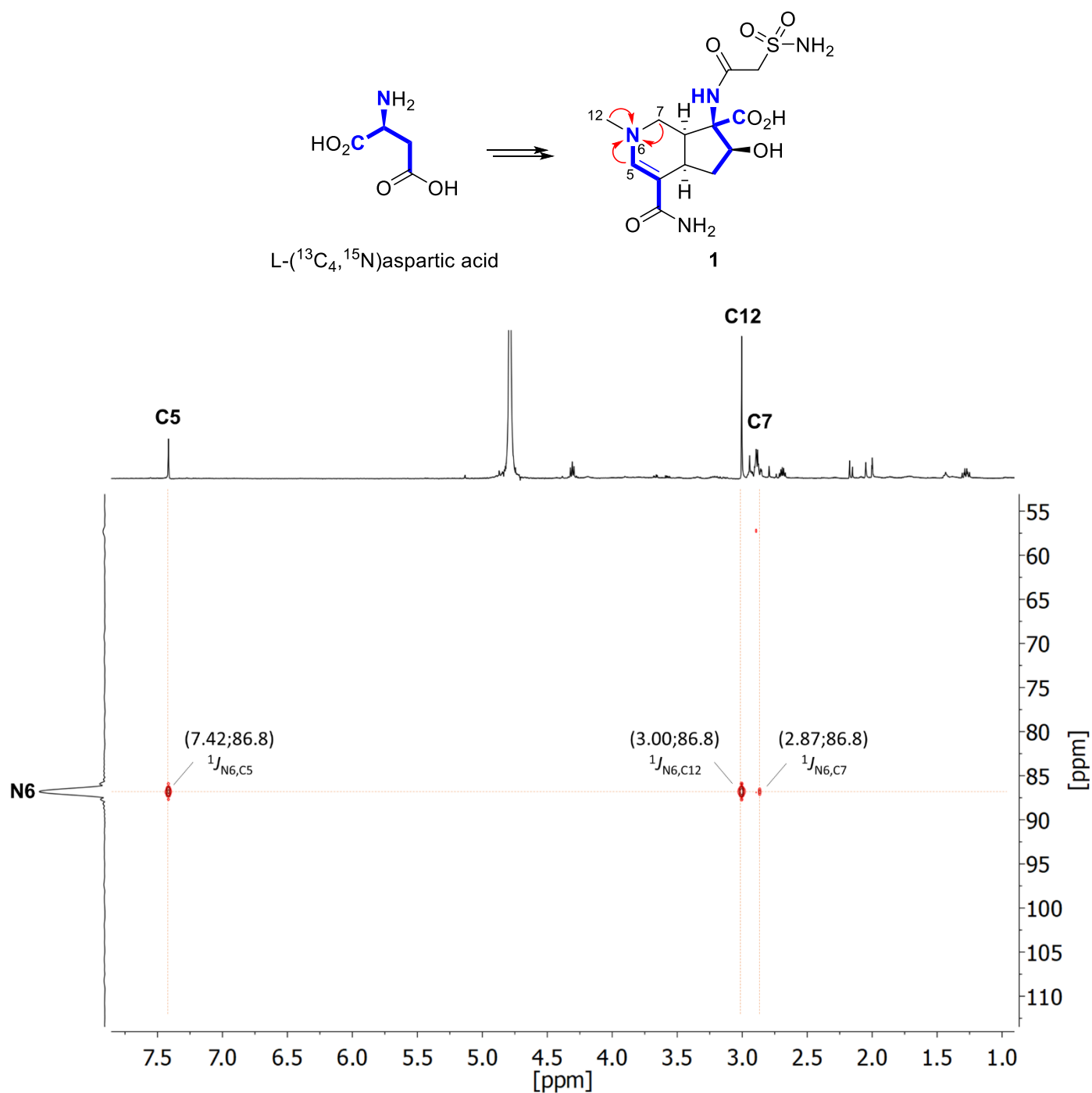
In order to gain insight into the cryptic biosynthetic origin of **1**, we conducted feeding experiments with isotopically enriched ($^{13}\text{C}_3$)glycerol and L-($^{13}\text{C}_4, ^{15}\text{N}$)aspartic acid, utilizing the previously constructed heterologous expression strain *S. lividans/sbz1*¹. The label incorporation into **1** was traced by ^{13}C -NMR and $^1\text{H}, ^{15}\text{N}$ -HMBC NMR analysis. Whereas feeding of (^{13}C)glycerol led to incorporation into positions C7, C7a and C3a (Supplementary Fig.2), enriched carbon signals after feeding of L-($^{13}\text{C}_4, ^{15}\text{N}$)aspartic acid were found for C5, C4, C9, as well as C8, C1, C2 and C3 (Supplementary Fig.3). The nitrogen atom in the tetrahydropyridine ring was shown to metabolically originate from aspartic acid (Supplementary Fig.4).



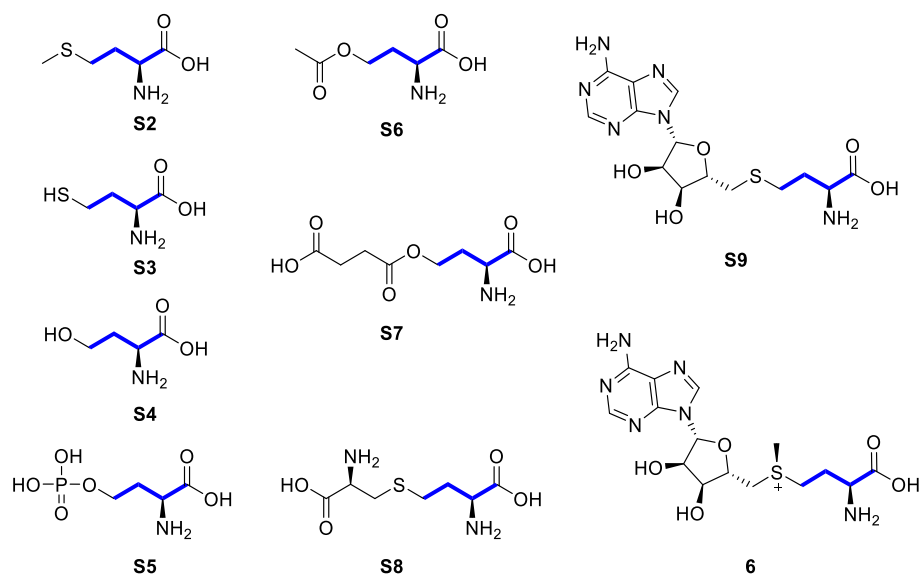
Supplementary Figure 2 Comparison of ^{13}C -NMR spectra for unlabelled altemicidin (**1**) (b) and altemicidin obtained after feeding of ($^{13}\text{C}_3$)glycerol (a). Selected signals are highlighted and relative incorporation rates into each position are given and have been referenced against C12 signal.



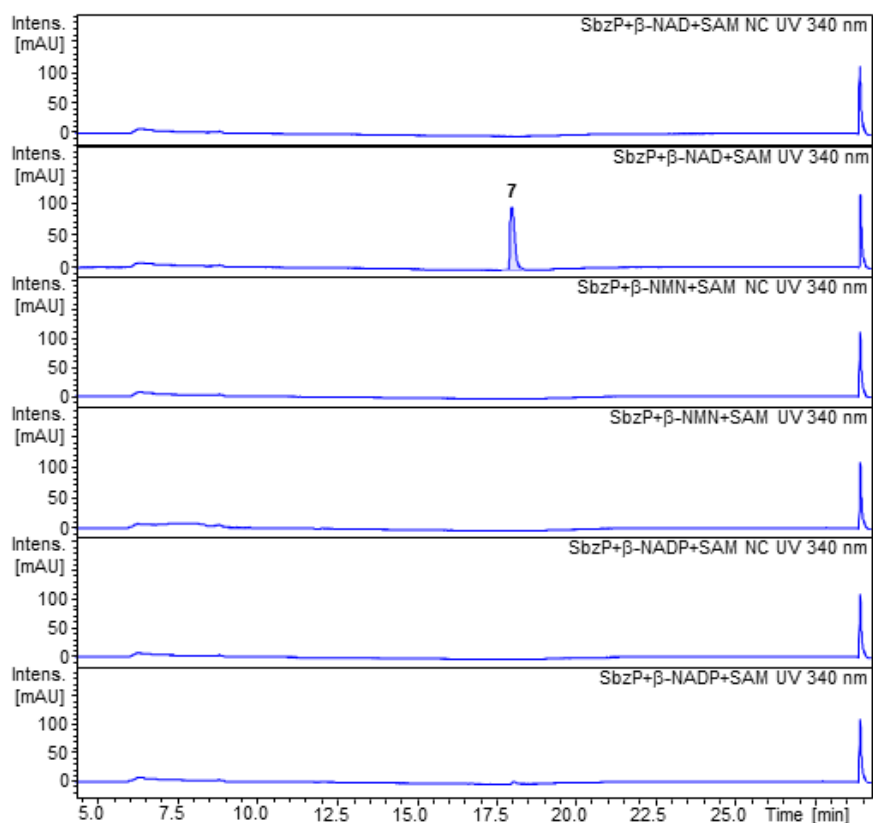
Supplementary Figure 3 Comparison of ^{13}C -NMR spectra for unlabelled altemicidin (**1**) (b) and altemicidin obtained after feeding of L-($^{13}\text{C}_4$, ^{15}N)aspartic acid (a). Selected signals are highlighted and relative incorporation rates into each position are given and have been referenced against C12 signal. Note that signals for C1 and C5 are part of higher order spin systems involving $^1J_{\text{C},\text{N}}$ coupling. Furthermore, note that for C4 ($^1J_{\text{C},\text{C}} = 73$ Hz) and C2 ($^1J_{\text{C},\text{C}} = 38$ Hz) additional doublets are present, indicating the presence of isotopologs in lower concentrations.



Supplementary Figure 4 ^1H , ^{15}N -HMBC spectrum of **1** after feeding of L-($^{13}\text{C}_4$, ^{15}N)aspartic acid and assigned ^1H , ^{15}N -HMBC correlations (single-headed arrows).



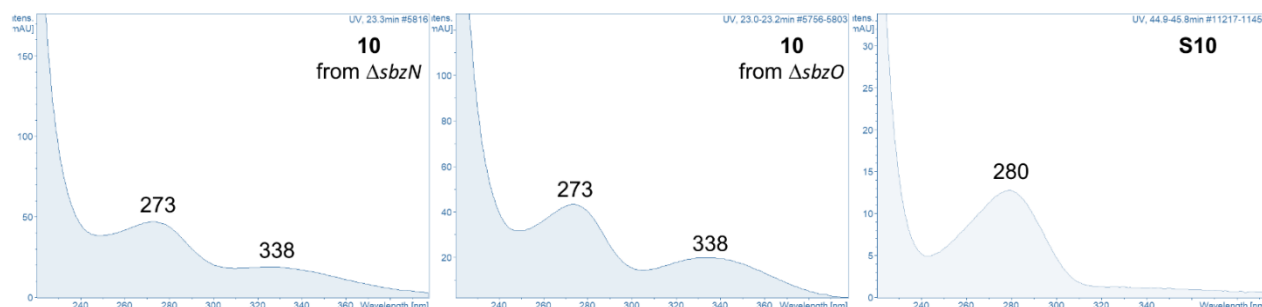
Supplementary Figure 5 Structures of screened amino acid substrates for SbzP reaction: methionine (**S2**), homocysteine (**S3**), homoserine (**S4**), O-phospho-L-homoserine (**S5**), O-acetyl-L-homoserine (**S6**), O-succinyl-L-homoserine (**S7**), L-cystathionine (**S8**), S-(5'-adenosyl)-L-homocysteine (**S9**) and S-adenosyl methionine (**6**). **S2** to **S9** were not accepted by SbzP.



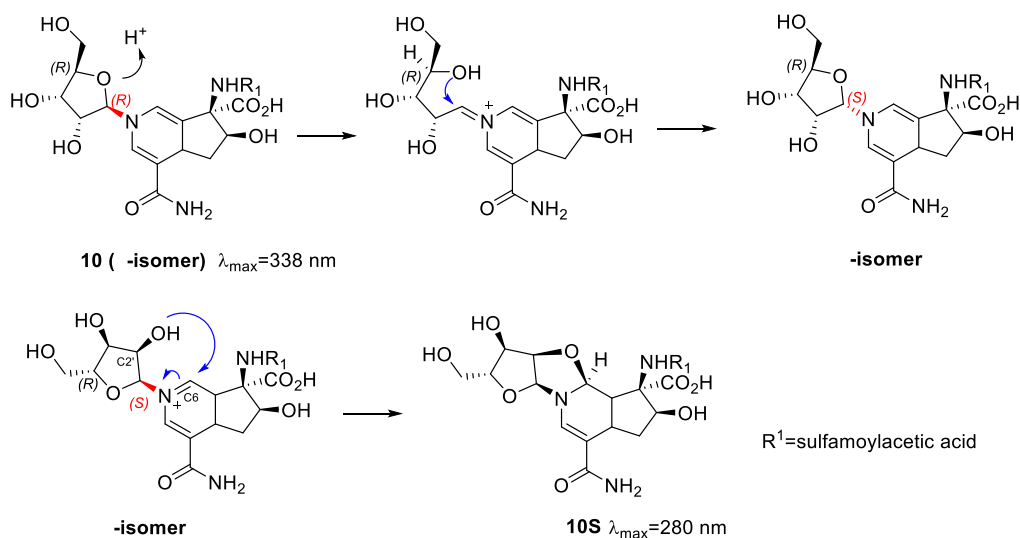
Supplementary Figure 6 Result of substrate screening for SbzM with SAM (**6**) in combination with β-NAD (**5**), β-NMN or β-NADP. Note that trace amounts of **7** are also detected in the SbzM+SAM+β-NADP reaction, which arises from small quantities of β-NAD present in commercial β-NADP. UV trace (340 nm) of LC-MS analysis is shown.

Supplementary Note 2: Identification of $\Delta sbzN$ and $\Delta sbzO$ Metabolite 10

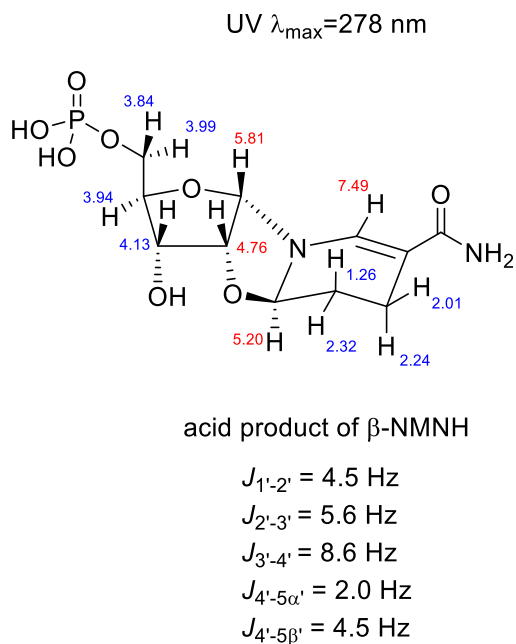
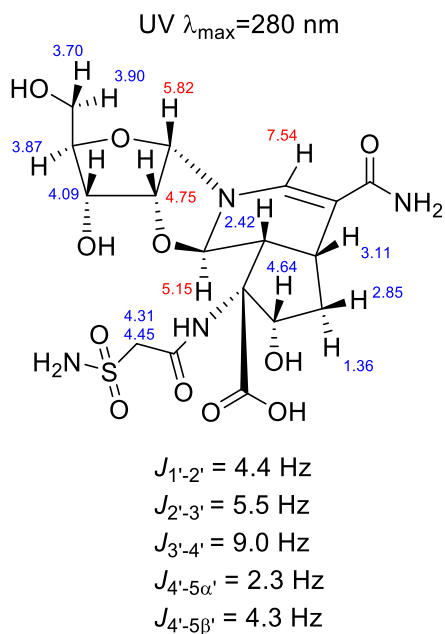
Deletion of *sbzN* and *sbzO* led to accumulation of an identical metabolite (**10**) judging from a shared mass, retention time, ESI fragmentation pattern and UV absorption profile. During the purification process, compound **10** ($\lambda_{\text{max}}=338$ nm) was found to be unstable and specific formation of a new compound (**S10**) with identical *m/z* but different UV absorption ($\lambda_{\text{max}}=280$ nm) appeared (Supplementary Fig.7). Incubation of the crude extract of $\Delta sbzO$ culture at pH4 overnight at 30°C led to complete conversion of **10** to **S10**, which could be successfully purified and structurally characterized by NMR data analysis. The structure was identified as shown in Supplementary Fig.8 and we propose that an intramolecular, acid-catalyzed cyclization between the ribosyl hydroxy function at C2' and C7 of **10** occurred. Similar reactions are well known for dihydropyridine compounds such as β -NADH and β -NMNH (reduced form of β -NMN) and proceed via epimerization of the glycosidic bond, enamine/imine isomerization, and intramolecular nucleophilic attack³. The UV spectral changes, as well as the NMR properties of **S10** are in line with reported values for cyclo-nicotinamide derivatives (Supplementary Fig.9)³.



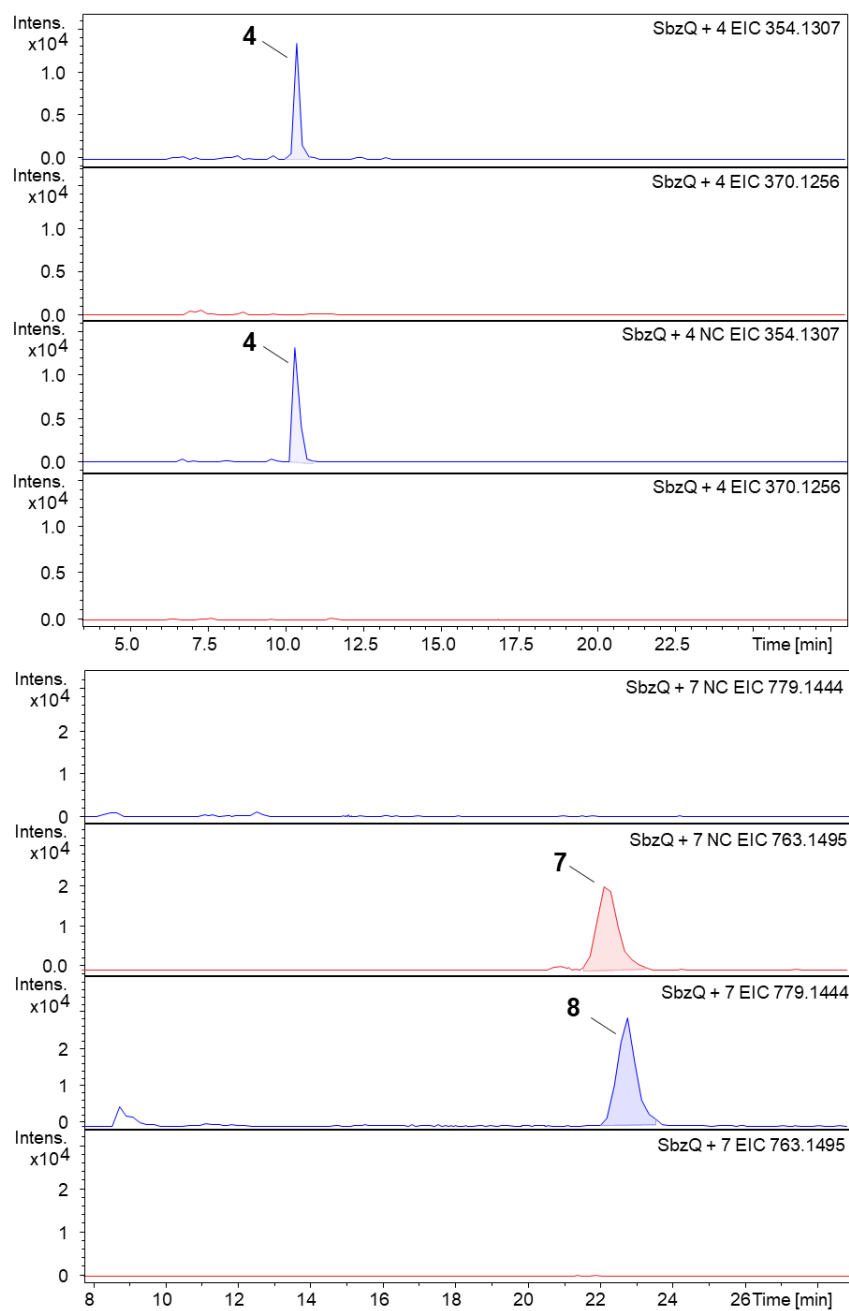
Supplementary Figure 7 Comparison of UV absorption spectra of compound **10** (accumulated in $\Delta sbzN$ or $\Delta sbzO$ culture extracts), and isomerized product **S10**.



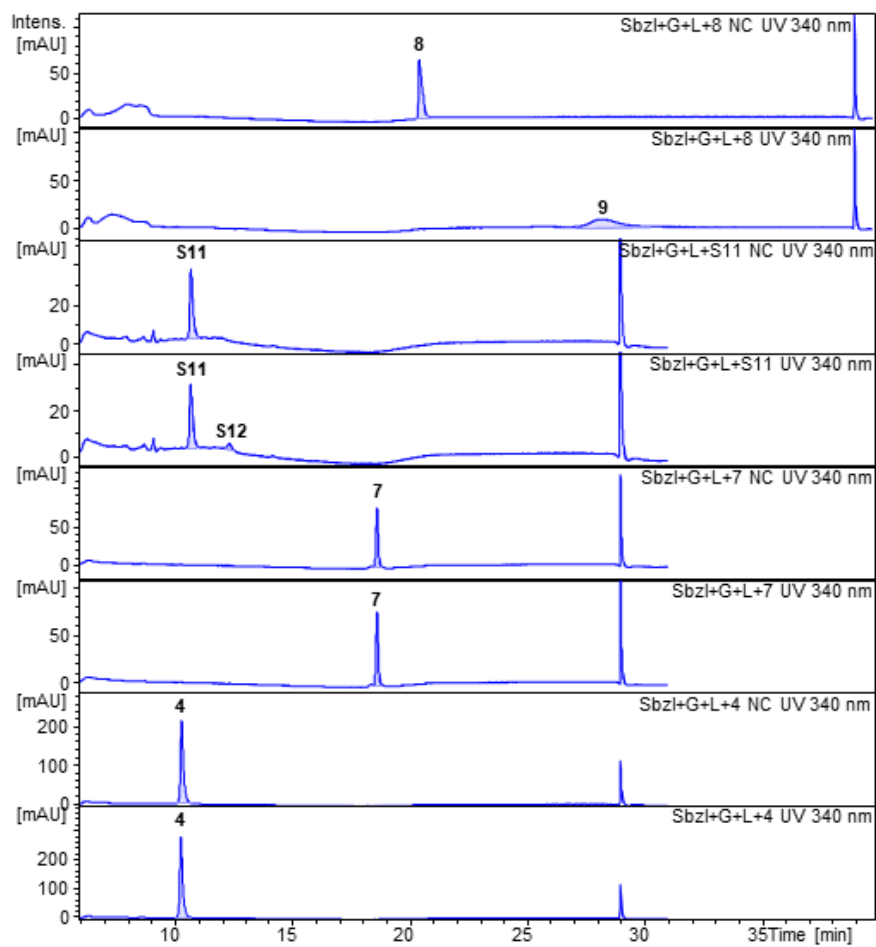
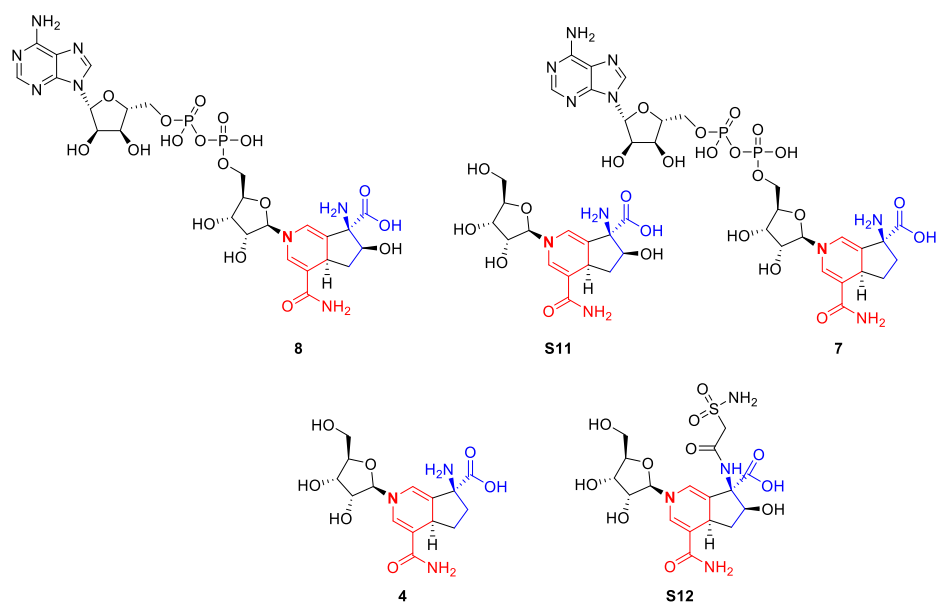
Supplementary Figure 8 Proposed acid-catalyzed conversion of **10** to **S10**.



Supplementary Figure 9 Comparison of the ^1H -NMR data for **S10** to the reported acid product of β -NMNH. The large coupling constant of $J_{3'-4'}$ is diagnostic for a 150° - 160° dihedral angle (α -3' *endo* conformation).



Supplementary Figure 10 Result of SbzQ reaction with mononucleoside **4** and dinucleotide analog **7**. Extracted ion chromatograms (EIC) of substrates and expected products are shown.

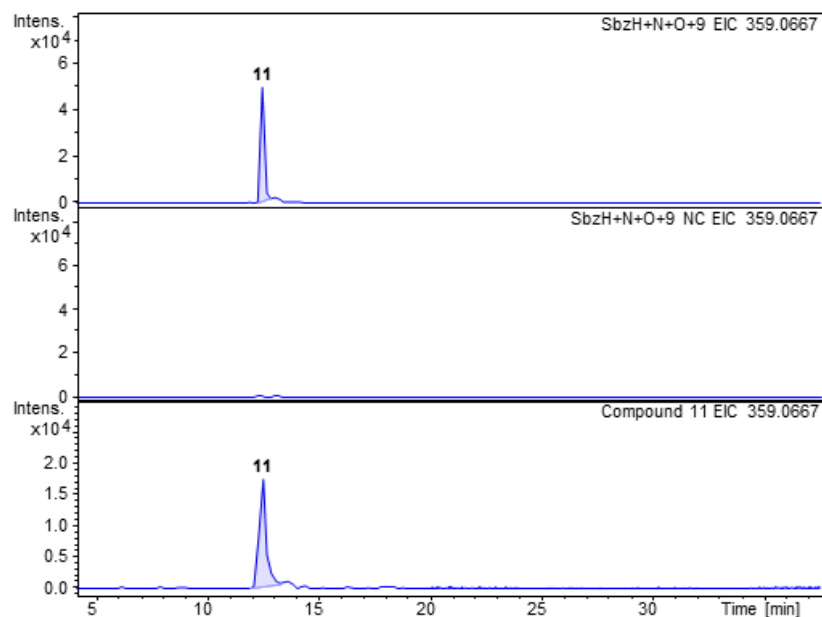


Supplementary Figure 11 Structures of tested substrates **8**, **S11**, **7**, and **4** with Sbzl+SbzG+SbzL, and proposed structure of **S12** (m/z 491.1100). LC-MS chromatograms (UV trace at 340 nm) of *in vitro* reactions are shown.

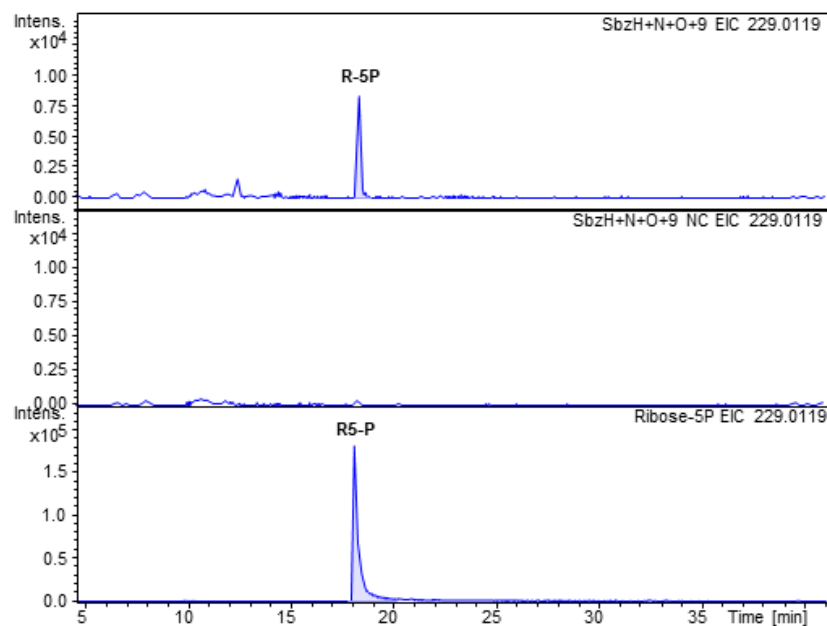
Supplementary Note 3: Deadenosylribosylation reaction

Mechanistically, the glycosidic bond cleavage can be explained by a nucleophilic substitution reaction at C1' and in line with this mechanism, the $\Delta sbzF$ compound (UV λ_{max} =275 nm) lacks the dihydropyridine-specific UV absorption of 338 nm (Supplementary Fig.14). Therefore, the reaction is proposed to proceed *via* enamine/imine isomerization, generating an intermediary iminium cation, which facilitates a nucleophilic substitution reaction with H₂O, while generating the conjugated imine **11** (Extended Data Fig.2g). Single protein incubations of SbzH, SbzN, or SbzO with **9** did not result in any specific intermediate formation, suggesting an intimate interplay between the three enzymes, possibly by protein-protein interactions. Bioinformatically, SbzN exhibits moderate homology (29% identity) to the C-terminal isomerase domain of glutamine-fructose-6-phosphate aminotransferase, a ubiquitous protein that converts D-fructose-6-phosphate to D-glucosamine-6-phosphate⁴. SbzO on the other hand showed no significant homology to proteins with known function. A PFAM analysis suggested that SbzO might be related to BtpA, a protein family proposed to play a role in stabilization of cyanobacterial photosystem I by protein-protein interactions⁵. However, no catalytic function has been reported for this scarcely investigated protein family before. SbzH on the other hand exhibits high homology to galactose mutarotase⁶, a sugar isomerase. The mechanism of the deadenosylribosylation reaction and the distinct protein functions of SbzH, SbzN and SbzO are on-going in our laboratory.

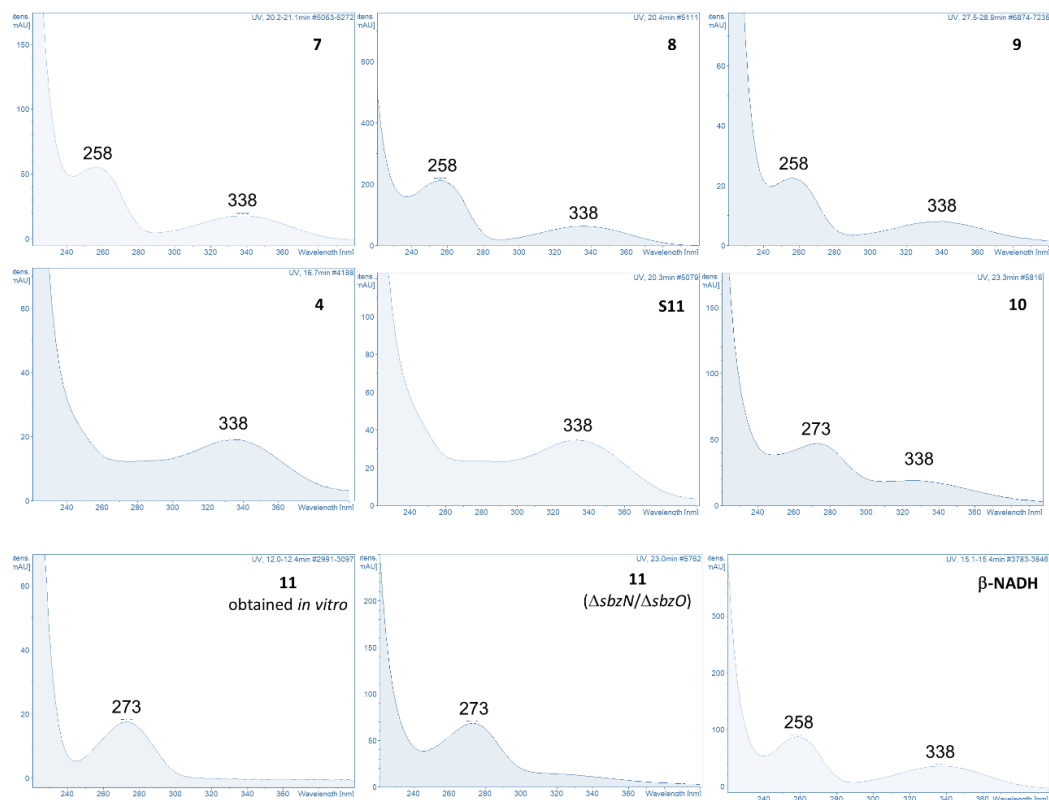
The conversion of **9** to **11**, proposed to proceed *via* enamine/imine isomerization is supported by the absence of a UV absorption maximum at 338 nm, characteristic for the dihydropyridine structural motif (Supplementary Fig.14). However, **11** was found to be unstable and despite several attempts, we were unable to support the proposed structure by NMR data analysis.



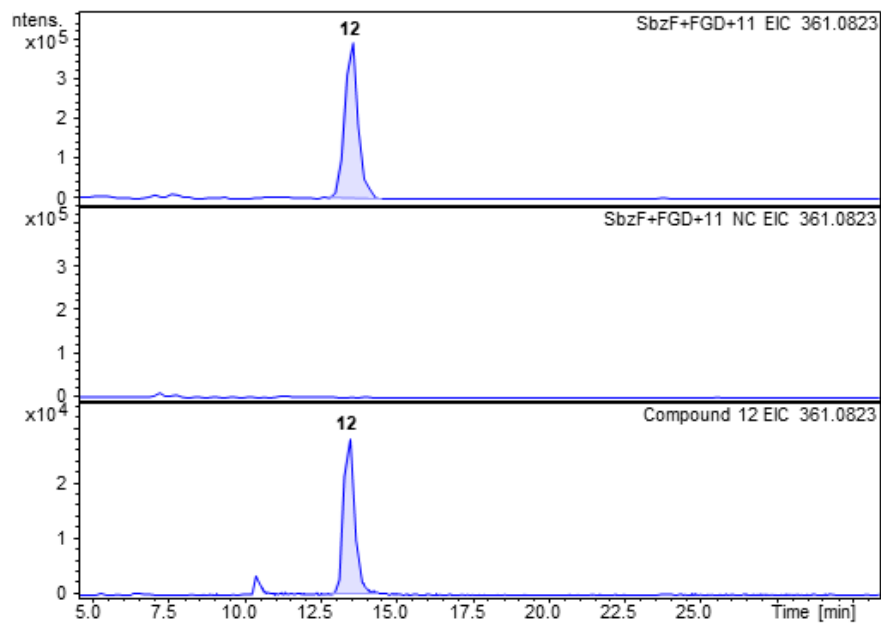
Supplementary Figure 12 Identification of **11** as the product of SbzN+SbzO+SbzH + **9** enzyme reaction. Extracted ion chromatograms (EIC) for m/z 359.0667 is shown.



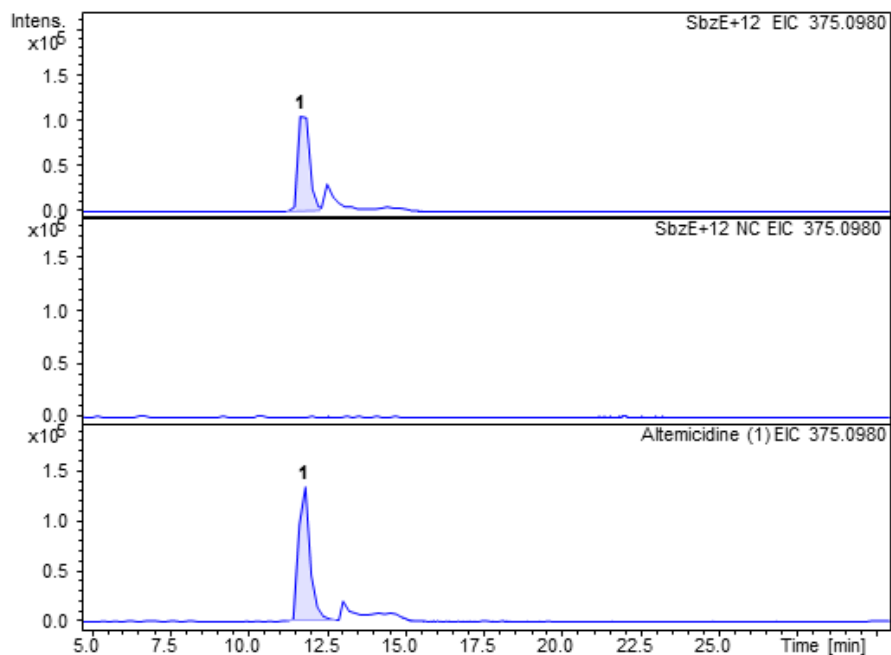
Supplementary Figure 13 Identification of ribose-5-phosphate (R5-P) as the byproduct of the Sbz+SbzO+SbzH catalyzed deadenylation. Extracted ion chromatograms (EIC) for m/z 229.0119 is shown. The 2nd chromatogram indicates the negative control without enzyme, and the 3rd chromatogram indicates the authentic standard.



Supplementary Figure 14 Comparison of UV absorption spectra of dihydropyridine metabolites 7, 8, 9, 4, S12, 10, *in vitro* obtained product 11, $\Delta sbzN/\Delta sbzO$ metabolite 11, and commercial β -NADH are shown.



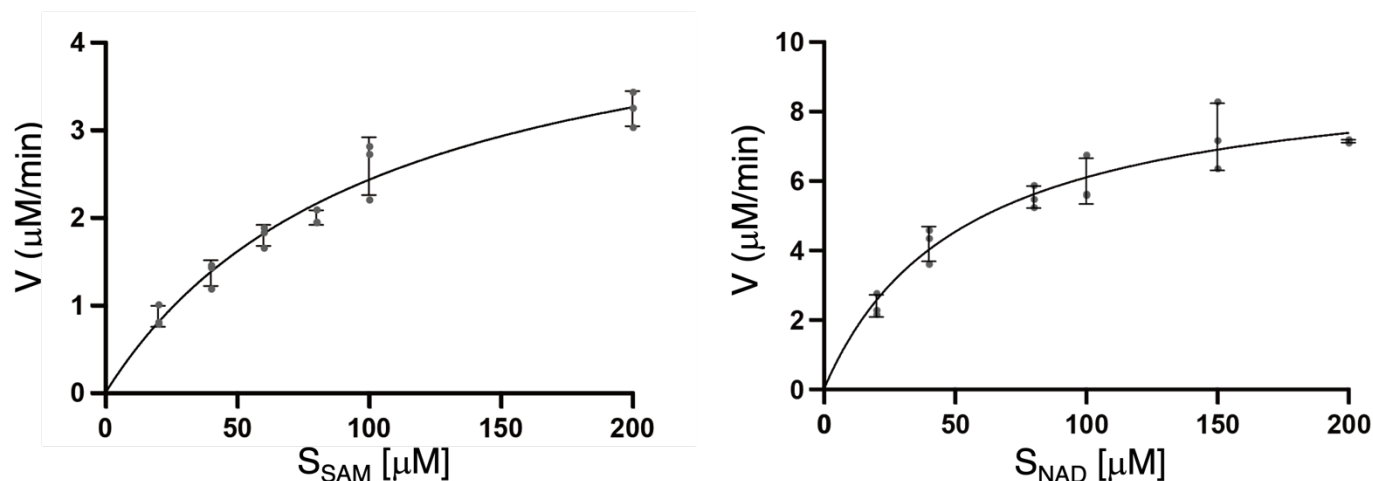
Supplementary Figure 15 Identification of **12** as the product of SbzF + **11** enzyme reaction. Extracted ion chromatograms (EIC) for m/z 361.0832 is shown. The 2nd chromatogram indicates the negative control without enzyme, and the 3rd chromatogram indicates the authentic standard.



Supplementary Figure 16 Identification of **1** as the product of SbzE + **12** enzyme reaction. Extracted ion chromatograms (EIC) for m/z 375.0980 is shown. The 2nd chromatogram indicates the negative control without enzyme, and the 3rd chromatogram indicates the authentic standard.

Supplementary Note 4: Steady-State Kinetics and Inhibitor studies

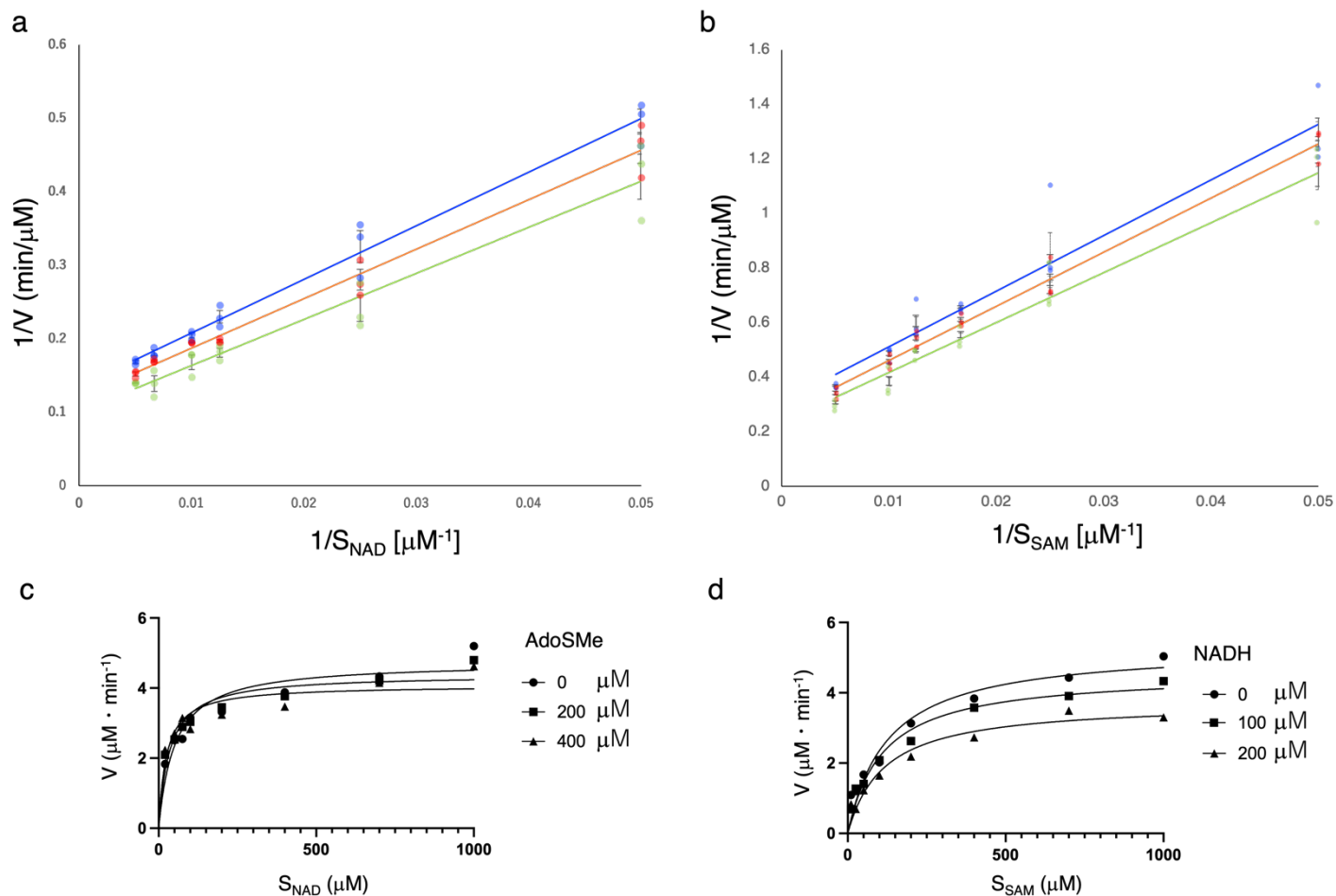
The plots of initial velocity were fitted to the equation $Y = V_{\max} * X / (K_m + X)$, and kinetic parameters were calculated accordingly (Supplementary Fig.17).



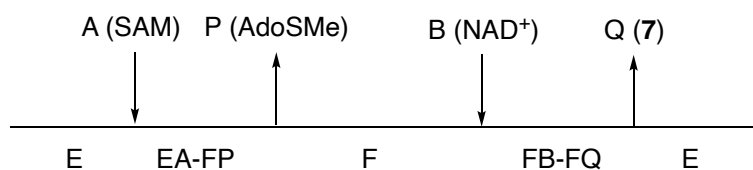
	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($\text{min}^{-1}/\mu\text{M}$)
SAM	103 ± 16	1.12 ± 0.09	2.27×10^{-1}
β -NAD	53.1 ± 9.1	2.12 ± 0.12	4.00×10^{-2}

Supplementary Figure 17 The non-linear regression of PsePQ reaction. Assay 1: 4.4 μM PsePQ, β -NAD 1.0 mM, and SAM 20, 40, 60, 80, 100, 200 μM in 20 min at 28 °C. Assay 2: 4.4 μM PsePQ, SAM 1.0 mM, and β -NAD 20, 40, 80, 100, 150, 200 μM in 10 min at 28 °C. Error bar indicates s.d. of three independent replicates.

PsePQ was also assayed with variable concentrations of β -NAD and fixed concentrations of SAM (Supplementary Fig.18a), as well as variable concentrations of SAM and fixed concentrations of β -NAD (Supplementary Fig.18b). In both cases, a series of parallel lines were observed in the reciprocal initial velocity plot. This confirms the steady-state mechanism as being Ping-Pong Bi-Bi (Supplementary Fig.19)⁷, and similar results were also reported for PLP-dependent aminotransferases⁸⁻⁹. In addition, inhibitor assays with AdoSMe (cleavage product of SAM, corresponding to product P) and β -NAD as the variable substrate, as well as assays with inhibitor β -NADH [product B (an analog for **7** as product Q) and SAM as the variable substrate, did not show the characteristic pattern for competitive inhibition (Supplementary Fig.18c, d), thereby ruling out a bi-substrate sequential mechanism⁷.

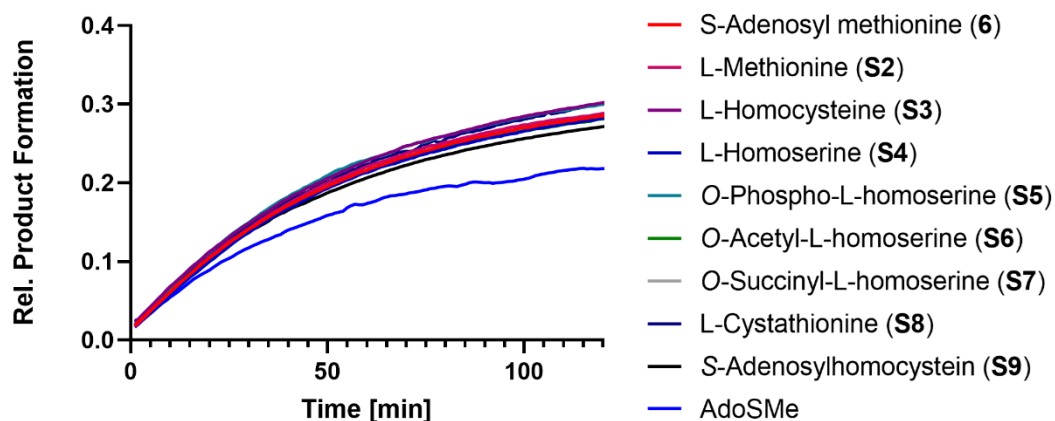


Supplementary Figure 18. The obtained plots of initial velocity patterns showing characteristics for Ping-Pong Bi-Bi mechanism. **a**, β -NAD as the variable substrate (20, 40, 80, 100, 150, 200 μM) and fixed SAM concentrations of 400 (blue), 700 (orange), and 1000 μM (green) ($n=3$). **b**, SAM as the variable substrate (20, 40, 60, 80, 100, 200 μM) and fixed β -NAD concentrations of 500 (blue), 800 (orange), and 1000 μM (green). Error bar indicates s.d. of three independent replicates. **c**, β -NAD as the variable substrate (20, 50, 75, 100, 200, 400, 700, 1000 μM) and fixed SAM concentrations of 1000 μM in presence of 0 μM , 200 μM , or 400 μM of AdoSMe ($n=2$). **d**, SAM as the variable substrate (10, 25, 50, 100, 200, 400, 700, 1000 μM) and fixed β -NAD concentrations of 1000 μM in presence of 0 μM , 100 μM , or 200 μM of β -NADH ($n=2$).

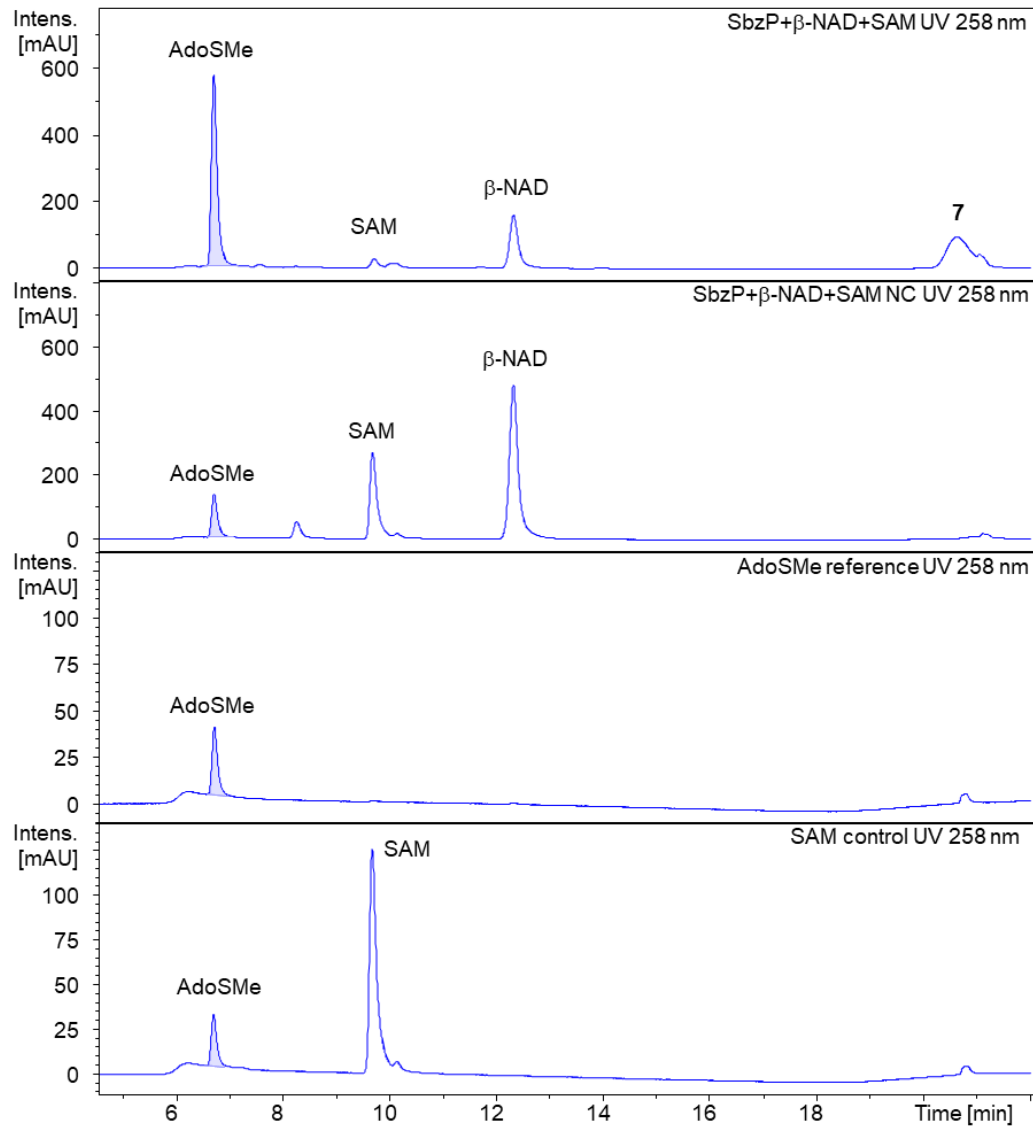


Supplementary Figure 19. Schematic representation of the proposed Ping-Pong Bi-Bi mechanism for SbzP and homologs.

Amino acid substrates with $C\gamma$ leaving group (compounds **S2** – **S9**) were also evaluated as putative inhibitors of SbzP homolog PsePQ. Only AdoSMe was found to exhibit inhibitory activity (Supplementary Fig.20).



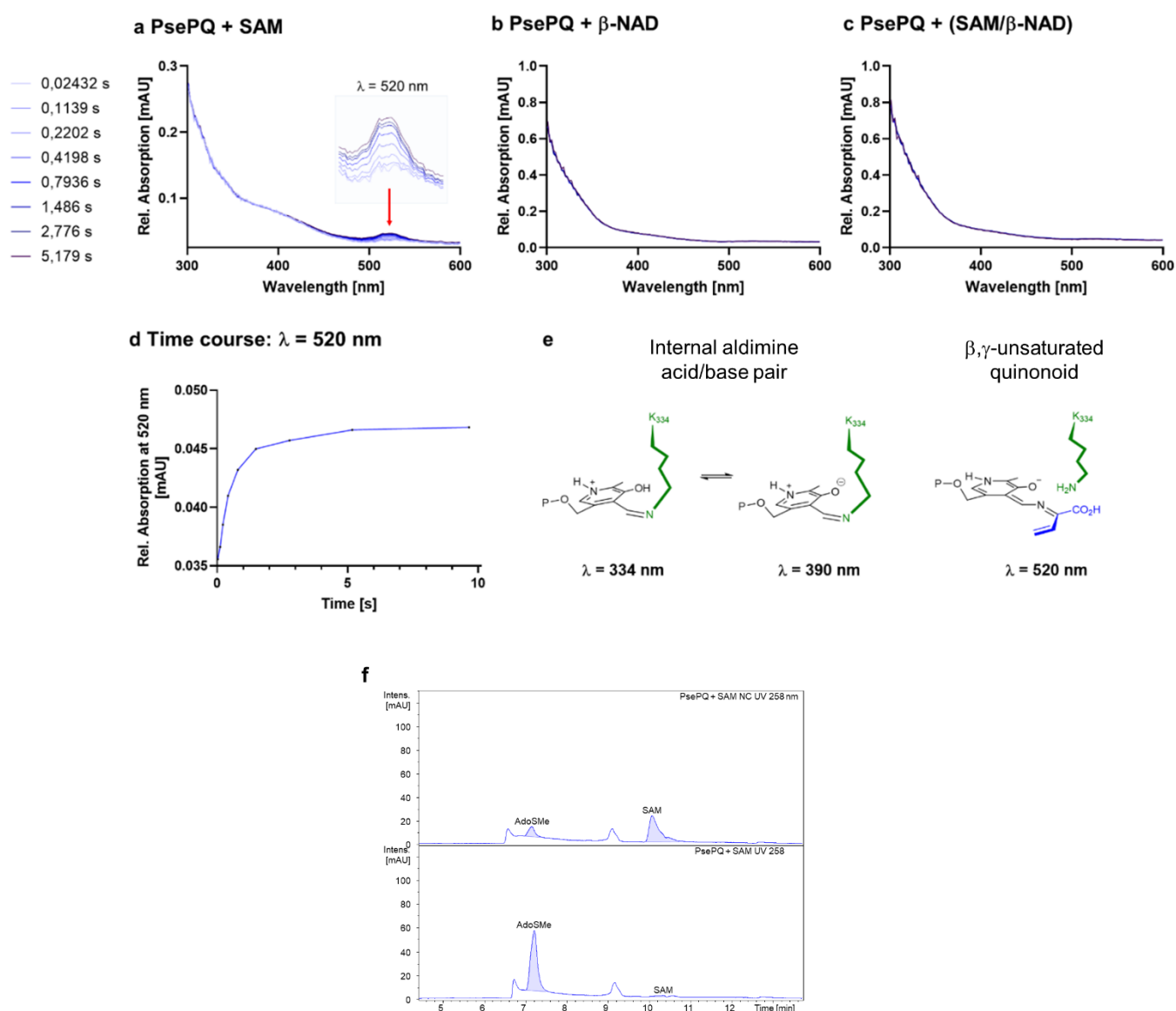
Supplementary Figure 20 Analysis of inhibitory activity of **S2** – **S9**. The formation of enzyme product **7** was monitored over time by tracing the specific UV absorption at 338 nm.



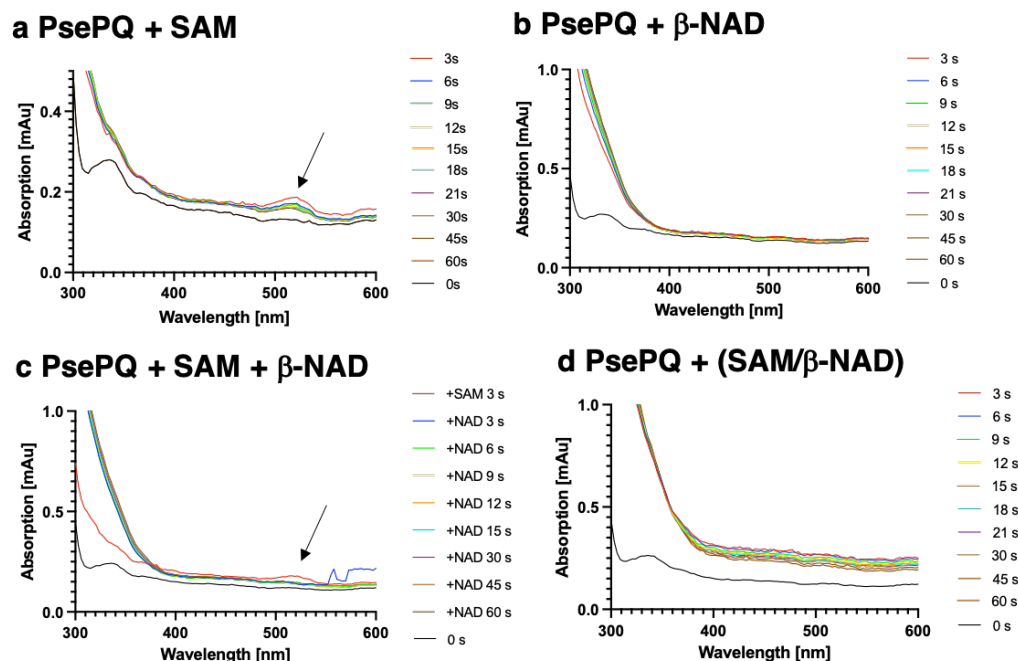
Supplementary Figure 21 Detection of byproduct AdoSMe in the SbzM reaction. UV trace at 258 nm of LC-MS analysis is shown. Note that AdoSMe is also present in small amounts in the negative control, arising from chemical degradation of SAM (compare trace 4, incubation of SAM under identical conditions). NC indicates the negative control reaction without enzyme.

Supplementary Note 5: Photospectroscopic investigations

To gain further insights into the SbzP mechanism, time-resolved photospectroscopic investigations were conducted, utilizing a stopped-flow apparatus. Spectral changes were recorded after mixing of enzyme with a) SAM, b) β -NAD, and c) SAM and β -NAD. Whereas mixing of enzyme with β -NAD (Supplementary Fig.22b), and β -NAD and SAM (Supplementary Fig.22c) did not result in any characteristic spectral changes, addition of only SAM led to the formation of a distinct absorption band at 520 nm (Fig.2, Supplementary Fig.22a). The long wavelength peak is proposed to correspond to the β,γ -unsaturated quinonoid, which accumulates in the absence of β -NAD in the enzyme active site (Fig.2). Simultaneous formation of AdoSMe was confirmed by LC-MS analysis (Supplementary Fig.22f). Furthermore, the UV absorption band at 520 nm (formed after addition of SAM to enzyme) was shown to abolish after subsequent addition of β -NAD (Supplementary Fig.23), further supporting intermediary formation of the proposed β,γ -unsaturated quinonoid species during SbzP/PsePQ catalysis.



Supplementary Figure 22 a-c, Results of stopped-flow experiments. **d**, Detection of 520 nm absorption over time. **e**, Proposed structures of spectroscopically observed PLP species¹⁰⁻¹⁵. **f**, Detection of AdoSMe by LC-MS.

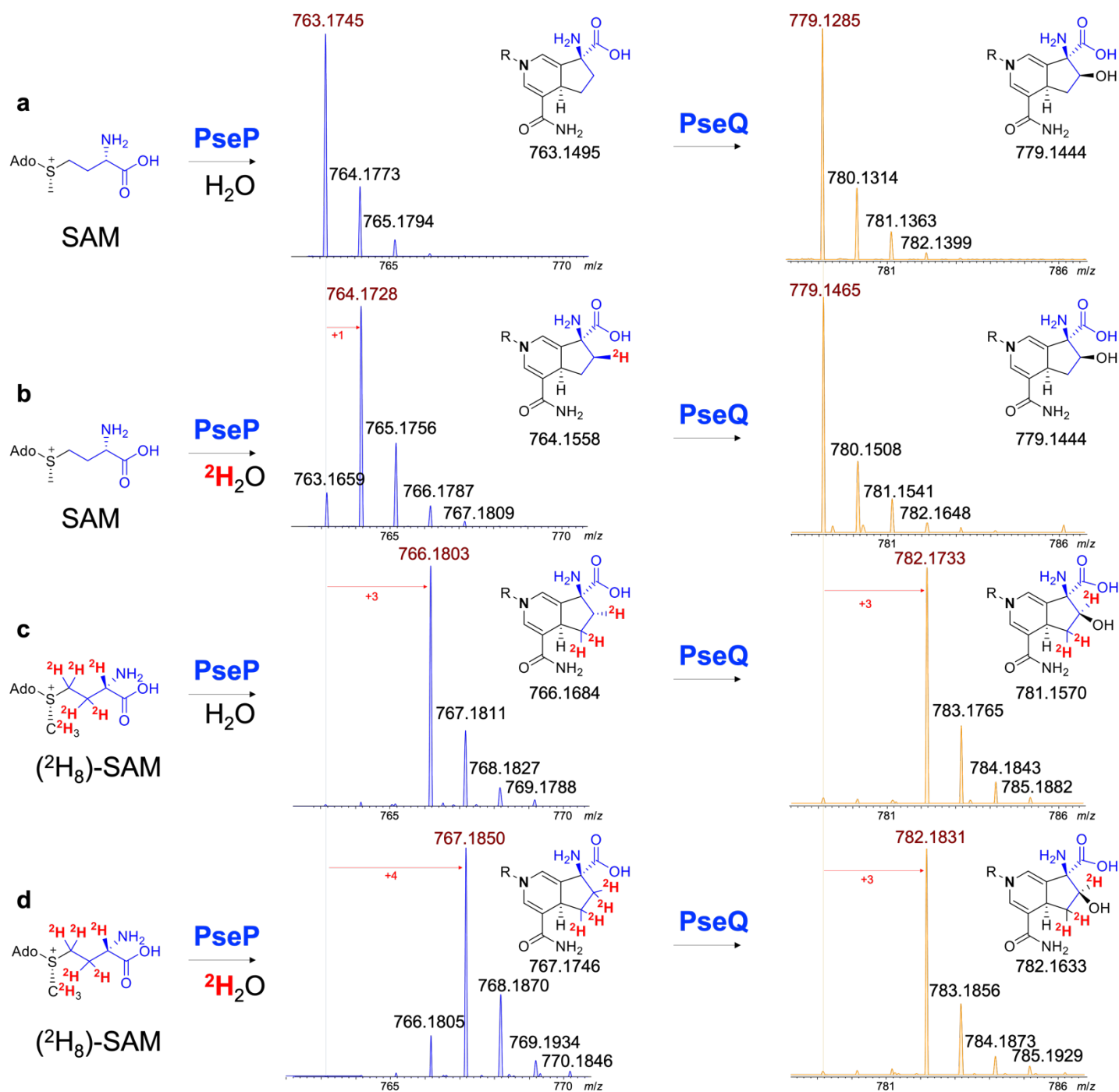


Supplementary Figure 23 Time course of UV spectral changes of PsePQ after mixing with substrates. **a**, PsePQ (27 μ M) mixed with 5 mM SAM. **b**, PsePQ mixed with 5 mM β -NAD. **c**, PsePQ mixed with 5 mM SAM followed by addition of 5 mM β -NAD. **d**, PsePQ mixed with the solution of 5 mM SAM/ β -NAD. Spectra were recorded with a Cary 60 UV-Vis spectrophotometer (Agilent Technologies, Santa Clara, CA, USA).

Supplementary Note 6: Isotopic labelling experiments

($^2\text{H}_8$)-SAM was enzymatically prepared from commercial (2,3,3,4,4, *methyl*- $^2\text{H}_8$)-L-methionine. Unlabelled SAM and the prepared isotopolog were utilized as substrates in combination with β -NAD for the reaction with SbzP homolog PsePQ in H_2O or $^2\text{H}_2\text{O}$. Incubation of unlabelled SAM and β -NAD in $^2\text{H}_2\text{O}$ led to incorporation of one ^2H , as indicated by a mass shift of +1 compared to the reaction in H_2O (Supplementary Fig.24, trace i and ii). Utilization of ($^2\text{H}_8$)-SAM in H_2O on the other hand, resulted in a mass shift of +3, indicating loss of two ^2H -labels in the transferred methionine portion of SAM at position $\text{C}\alpha$ and presumably $\text{C}\beta$ (Supplementary Fig.24, trace iii). The complementary experiment in $^2\text{H}_2\text{O}$, led to an expected mass shift of +4 ((Supplementary Fig.24, trace iv). To gain further insights into the position of the introduced or lost ^2H -label, the obtained isotopologs of **7** were further subjected to the PsePQ-mediated oxidation at C2 by addition of α -ketoglutarate to obtain the corresponding isotopologs of **8**. The results indicate selective loss of the ^2H -label introduced into **7** by incubation in $^2\text{H}_2\text{O}$ (trace ii and iv), whereas hydroxylation of the ($^2\text{H}_8$)-SAM obtained product in H_2O , did not show any loss of ^2H -label (trace iii).

The results support the proposed elimination of one proton at $\text{C}\beta$, during the formation of the β,γ -unsaturated quinonoid, as well as the occurrence of a reprotonation at $\text{C}\beta$, from intermediate I to the geminal amine/imine species (Fig.2). Assuming that the PsePQ-mediated hydroxylation at C2 proceeds with retention of configuration (H-abstraction from the same face as hydroxylation), the reprotonation step at $\text{C}\beta$ can be assigned as pro-S selective from the *Si*-face of intermediate I, which was further supported by NMR studies (please refer to Supplementary Note 7 for further details).

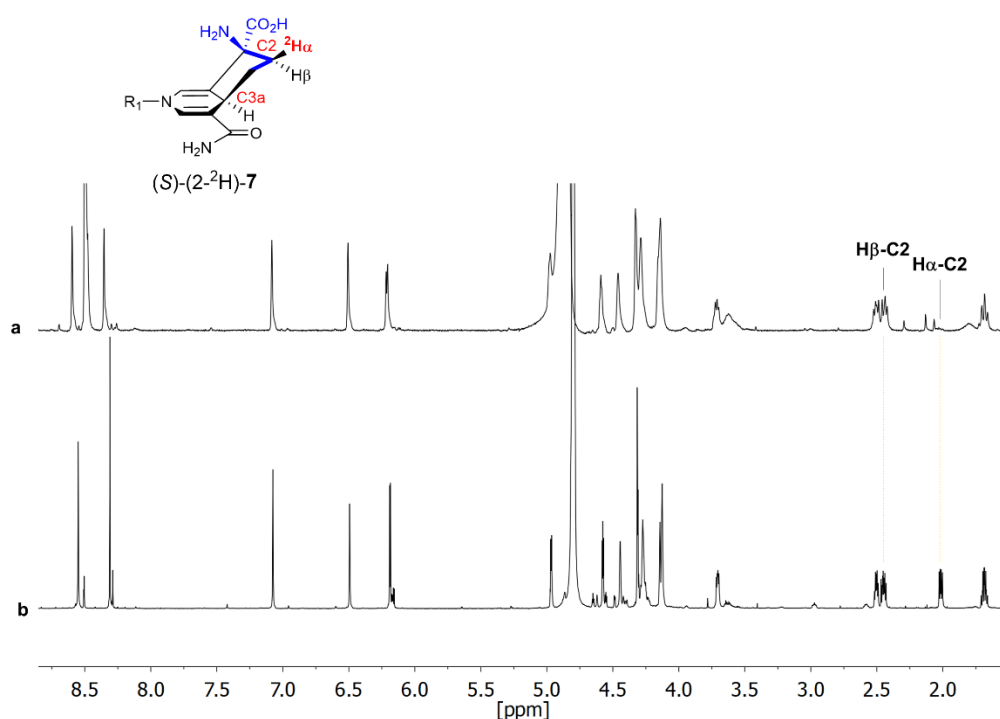


Supplementary Figure 24 MS data of **7**, obtained from incubation of PsePQ with a, β -NAD and SAM in H₂O (control), b, β -NAD and SAM in ²H₂O, c, β -NAD and (2H₈)-SAM in ²H₂O, and d, β -NAD and (2H₈)-SAM in ²H₂O. The obtained enzyme products were further subjected to PsePQ-mediated hydroxylation at C2. Label retention or loss was traced by MS data analysis of obtained isotopologs of **8**. R=ADP-ribose.

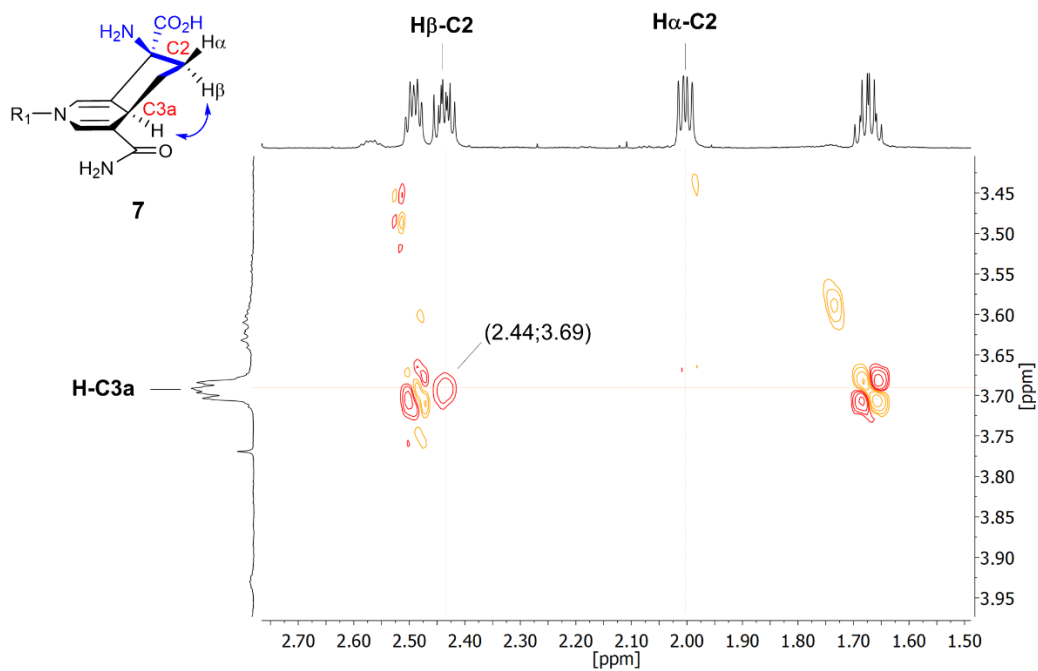
Supplementary Note 7: Stereospecificity of C β reprotonation during SbzP catalysis.

To follow the stereospecificity of the reprotonation step at C β of proposed intermediate I, the PsePQ reaction was conducted in $^2\text{H}_2\text{O}$ and the resulting product was isolated from preparative scale enzyme reactions. Analysis of the ^1H -NMR spectrum showed that the signal for H α -C2 is reduced by 96% (integration of peak area), indicating the formation of (S)-(2- ^2H)-7 (Supplementary Fig.25). Assignment of the diastereotopic methylene protons at C2 is based on a clear NOESY correlation between H-C3a and H β -C2, while no correlation is present for H-C3a and H α -C2 (Supplementary Fig.26).

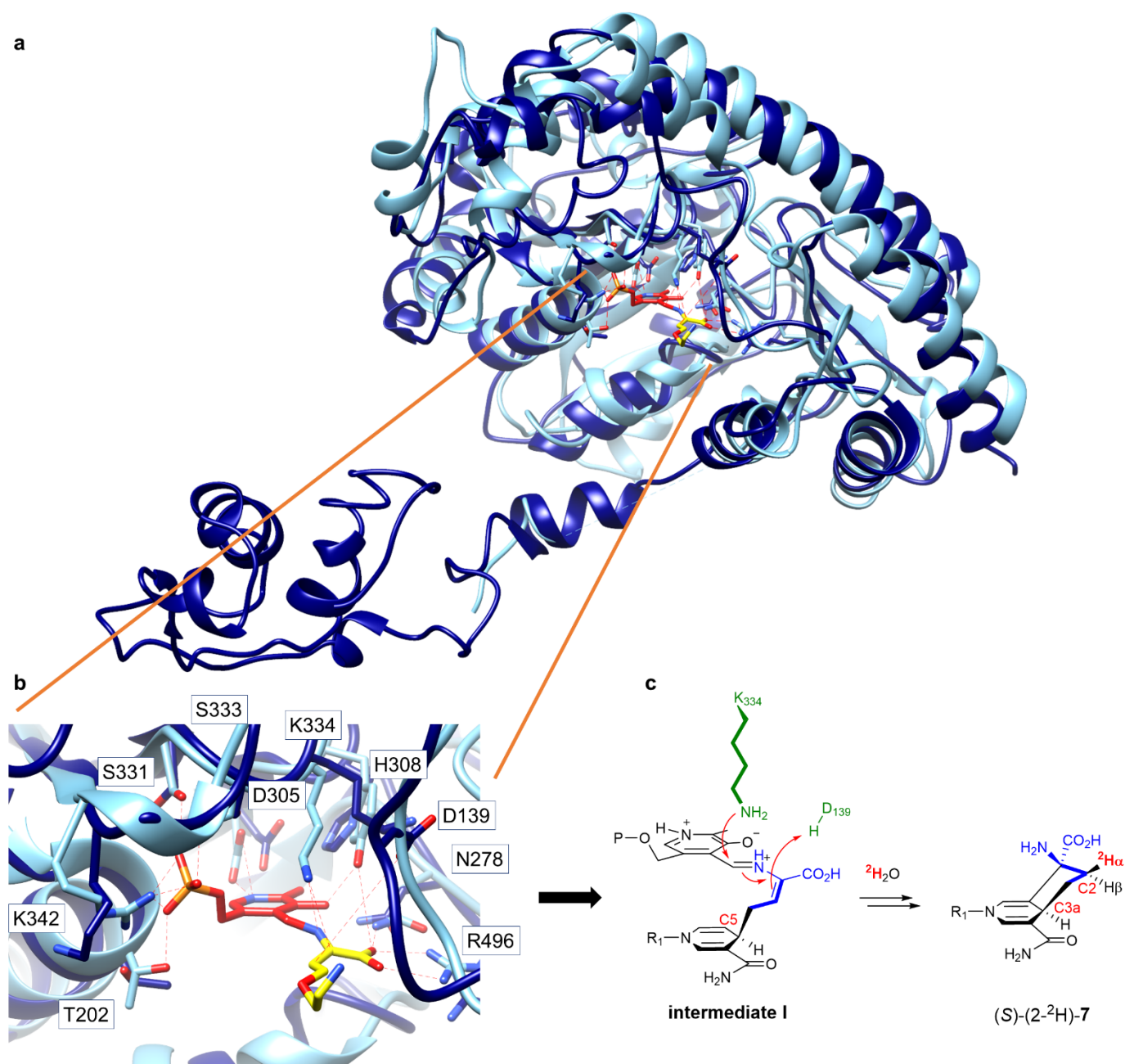
The labelling result suggests that the reprotonation occurs from the *Si*-face of intermediate I (Fig.2). To identify a possible catalytic acidic residue, a homology model of SbzP was constructed and superimposed with the protein structure of SAM-utilizing ACCS¹⁴ with inhibitor aminoethoxyvinylglycine (AVG) bound to PLP (PDB code: 1M7Y) (Supplementary Fig.27a). Overall, the homology model of SbzP aligned well with ACCS and amino acids involved in PLP and SAM binding were found to be conserved (Supplementary Fig.27b). Based on investigation of the active site space, β -NAD likely binds to the lower face of the PLP-AVG plane (resembling the β,γ -unsaturated quinonoid), as indicated in Supplementary Fig.27c. To explain the stereochemical outcome of the reaction, the catalytic acidic residue is expected to be located on the opposite site of the PLP-AVG plane. In close proximity to C β of AVG, a flexible loop is located, which harbors an aspartic acid residue, conserved in all members of the SbzP-like family (compare alignment Supplementary Fig.29). Point mutation of the aspartic acid residue led to 85% abolished enzyme activity, suggesting a possible involvement in the reprotonation step (Supplementary Fig.27c).



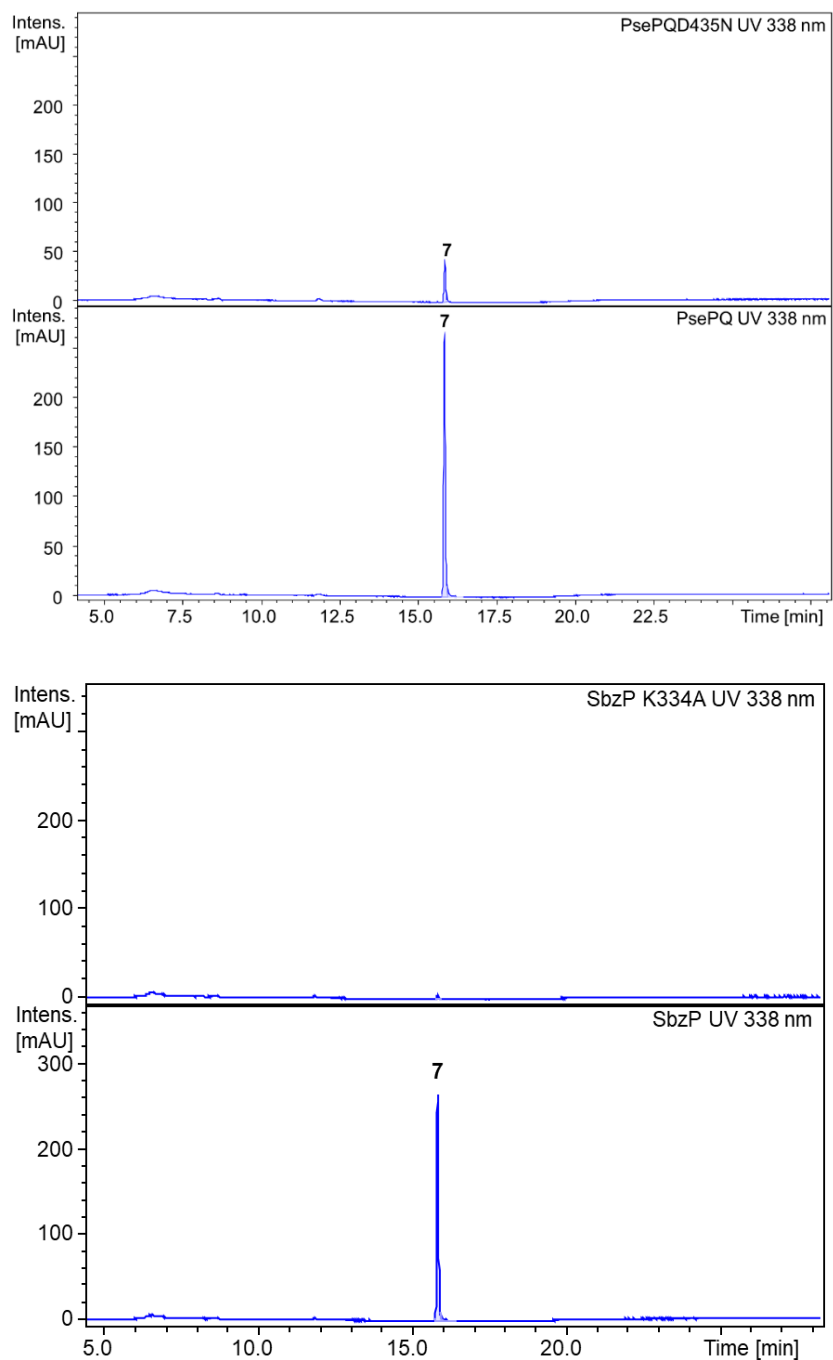
Supplementary Figure 25 Analysis of ^2H -incorporation into 7 during PsePQ catalysis in $^2\text{H}_2\text{O}$. Trace a shows the ^1H -NMR spectrum of 7 obtained in $^2\text{H}_2\text{O}$, while trace b shows the ^1H -NMR spectrum of unlabelled 7. Relevant signals are highlighted.



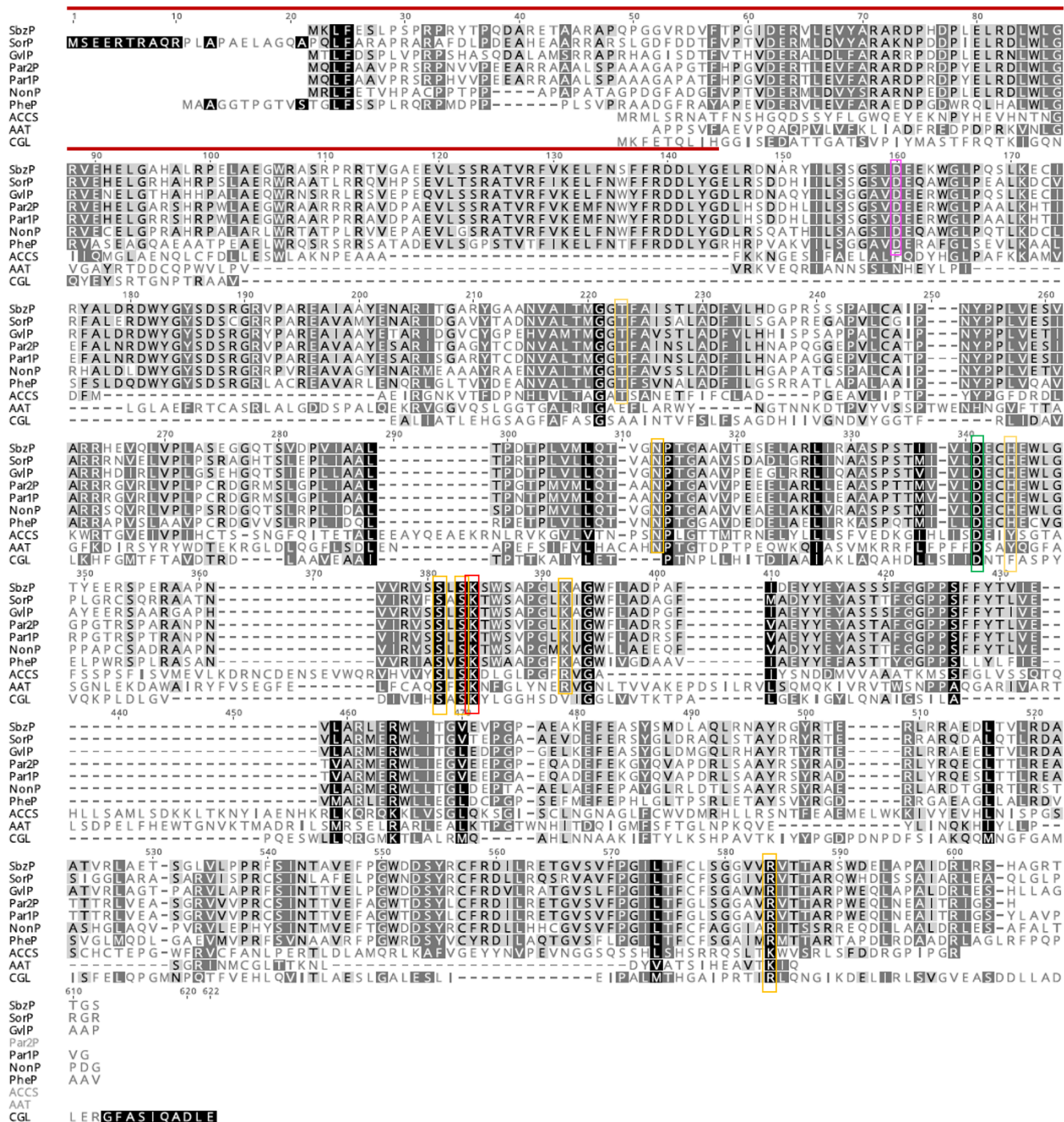
Supplementary Figure 26 NOESY correlation of H-C3a and $\text{H}_\alpha\text{-C}2$ and $\text{H}_\beta\text{-C}2$ for unlabeled **7**.



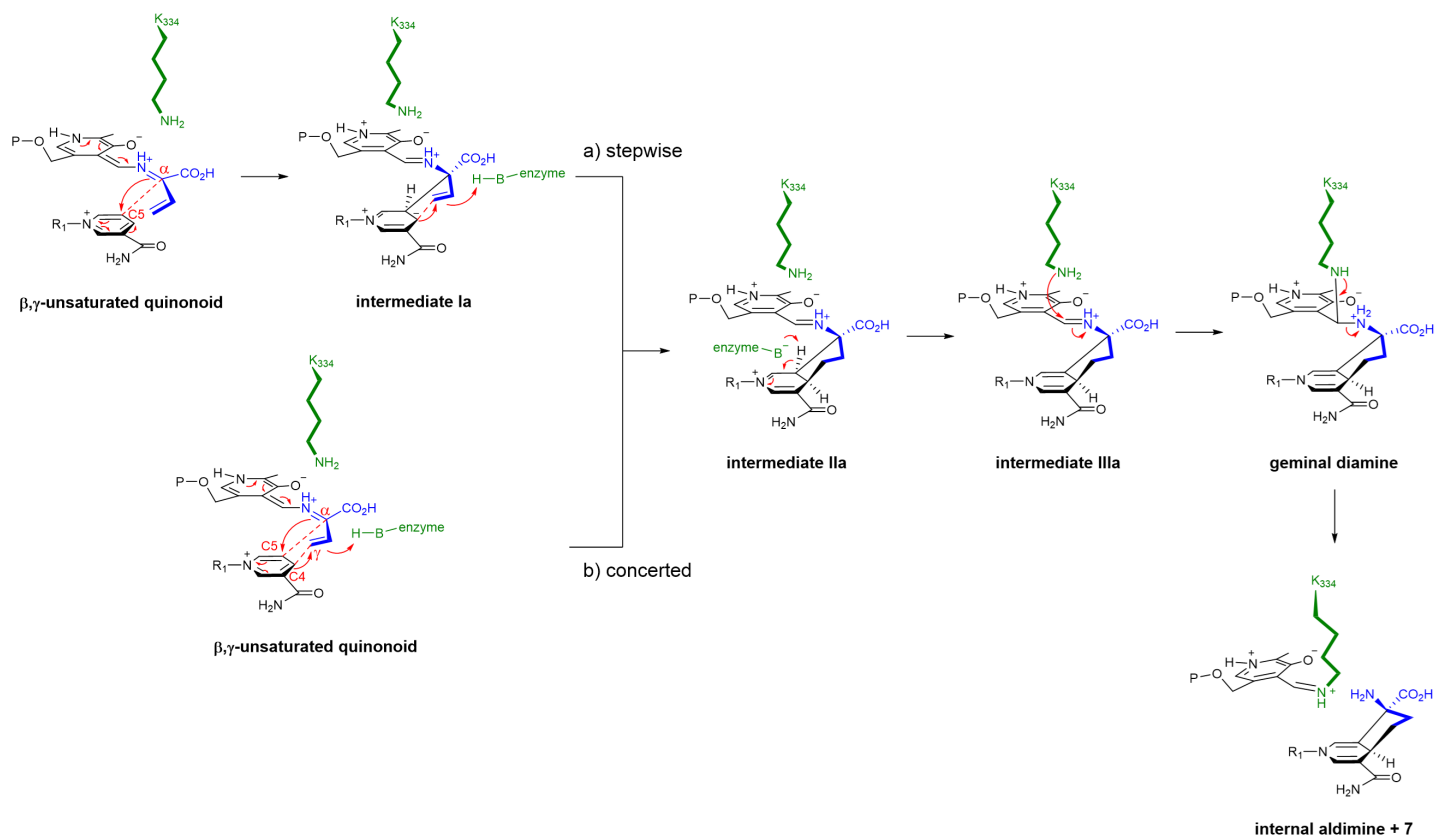
Supplementary Figure 27 **a**, Homology model of SbzP superimposed with ACCS (PDB code: 1m7y) and PLP-bound inhibitor AVG. **b**, close up view on active site. Archetypical fold type I PLP and amino acid substrate binding residues are highlighted and were found to be completely conserved. Numbers refer to SbzP protein sequence. Proposed catalytic acid D139 positioned on a flexible loop is shown. **c**, proposed mechanistic model and stereospecific outcome of reprotonation step. SbzP model is shown in navy blue, ACCS in cyan, PLP in red, and AVG in yellow.



Supplementary Figure 28 Activity of PsePQ_D435N and SbzP K334A mutants. UV trace at 338 nm of LC-MS analysis is shown. D435 in PsePQ is equivalent to D139 in SbzP.



Supplementary Figure 29 Protein sequence alignment of SbzP (BBG06565) and functionally confirmed homologs (SorP (CAN98403), NonP (SDH71014), Par2P (WP_064726415), Par1P (WP_114529778), GlvP (WP_083103041), PheP (WP_156466939)), AAT (aspartate aminotransferase, 1AJS), ACCS (1-aminocyclopropane-1-carboxylate synthase, 1B8G), and CGL (cystathionine γ -lyase, 6LE4). Active site lysine residue (K334) is highlighted in red, the acidic amino acid residue (D139) mutated in this study (Supplementary Fig.27, 28) in pink, conserved aspartic acid that coordinates pyridinium nitrogen in DECH motif in green, and the other conserved amino acid residues for PLP binding in AAT-I superfamily¹⁵ in yellow, respectively. Note that SbzP and homologs exhibit a histidine substitution at position 308 (instead of tyrosine) and lysine at position 341 (instead of arginine) (AA position number as in SbzP). The 100 amino acid long N-terminal region is highlighted in brown. The alignment was calculated with MAFFT algorithm (Version 7).



Supplementary Figure 30 Alternative mechanistic models for SbzP reaction from β,γ -unsaturated quinonoid to internal aldimine and enzyme product **7**.

Supplementary Note 8: Novelty of SbzP-like Protein Family

As outlined in the main text, SbzP catalyzes an unprecedented [3+2]-annulation reaction *via* the proposed reaction mechanism depicted in Fig.2. Here, a summary of the literature, which respect to the I. novelty of reaction specificity, and II. novelty of substrate specificity is given. A comparative phylogenetic analysis for all discussed proteins is shown in Supplementary Fig.36.

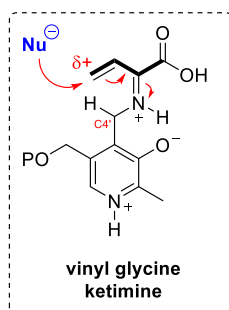
I. Novelty of reaction specificity

Known enzymes that catalyze alkylation at C γ :

The recently identified enzyme CndF represents the first biochemically characterized example of a C γ alkylating, PLP-dependent enzyme (Supplementary Fig.32a) and utilizes O-acetyl-L-homoserine and acetoacetate¹⁶. The mechanism involves initial substrate binding (external aldimine), deprotonation at C α (quinonoid intermediate), protonation at C4' (ketimine), and elimination of the C γ -leaving group (OAc) to form the typical vinyl glycine ketimine species. This conjugated ketimine is a Michael acceptor with electrophilic character at C γ , prone to nucleophilic attack (1,4-addition). In case of CndF catalysis, acetoacetate with carbanionic character at C2 is utilized. Subsequent enamine/imine isomerization, deprotonation at C4', reprotonation at C α , and transaldimination yields the C γ -substituted product and regenerates the internal aldimine. The overall catalysis can be described as β,γ -elimination/nucleophilic C γ -addition reaction.

Two further enzymes have been reported to catalyze C γ alkylation reactions: Fub7¹⁷ and FlvA¹⁸ (Supplementary data Fig.32b, c). Both enzymes also utilize O-acetyl-L-homoserine and proceed via the same mechanism. However, instead of acetoacetate, carbon nucleophiles hexanal (Fub7) and dimethyl pyruvate (FlvA) are utilized.

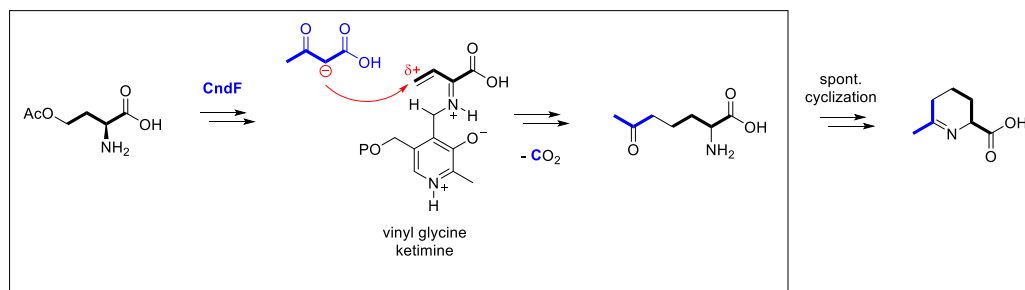
The key intermediate in these PLP-mediated transformations is a vinyl glycine ketimine, generated by protonation of C4'.



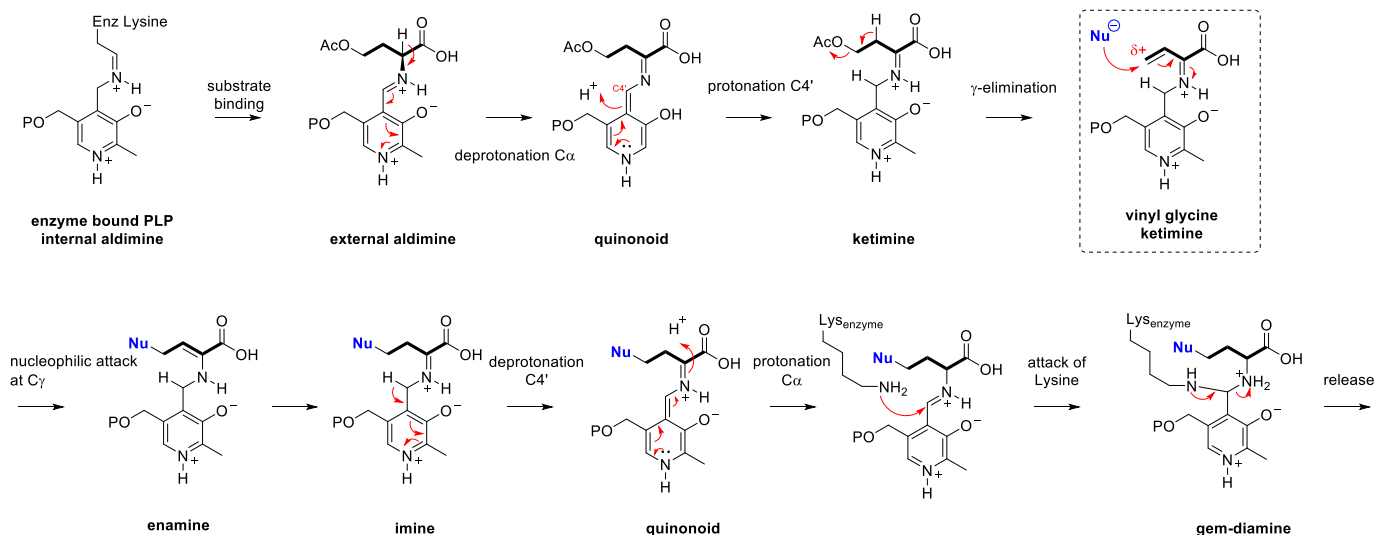
The β,γ -elimination/nucleophilic C γ -addition *via* a vinyl glycine ketimine intermediate is also known from other enzymes, such as LolC¹⁹⁻²¹, Mur24²², O-acetyl-L-homoserine sulfhydrylase (AHS)²³ (primary metabolism) and cystathionine- γ -synthase (MetB)²⁴ (primary metabolism) (Supplementary Fig.33). Instead of an alkyl nucleophile, heteroatom nucleophiles are utilized (amine-N for LolC and Mur24 (C-N bond formation), and thiol-S for AHS and MetB (C-S bond formation)).

These mechanistically related group of enzymes is phylogenetically related and clustered in the cystathionine- γ -synthase¹⁵ family of proteins (Supplementary Fig.36).

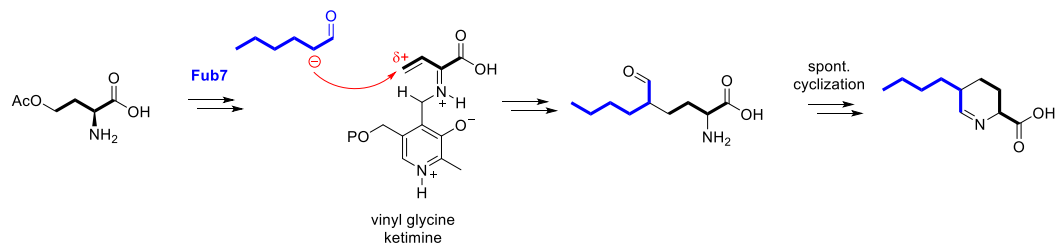
A.) CndF, vinyl glycine ketimine attacked by C-nucleophile, C-C bond formation, citrinadin pathway (fungi)



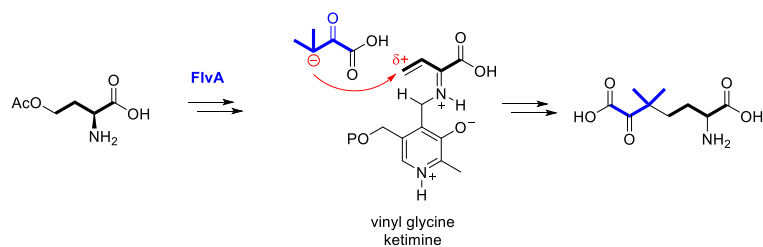
General Mechanism



B.) Fub7, vinyl glycine ketimine attacked by C-nucleophile, C-C bond formation, fusaric acid pathway (fungi)



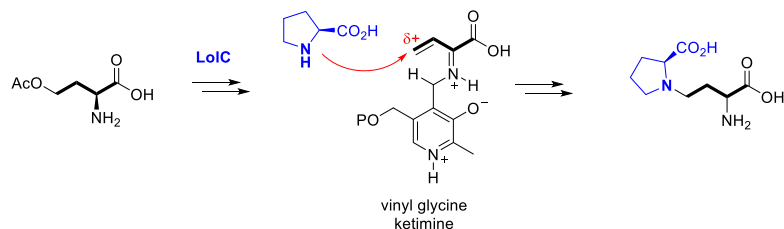
C.) FlvA, vinyl glycine ketimine attacked by C-nucleophile, C-C bond formation, flavuonidine pathway (fungi)



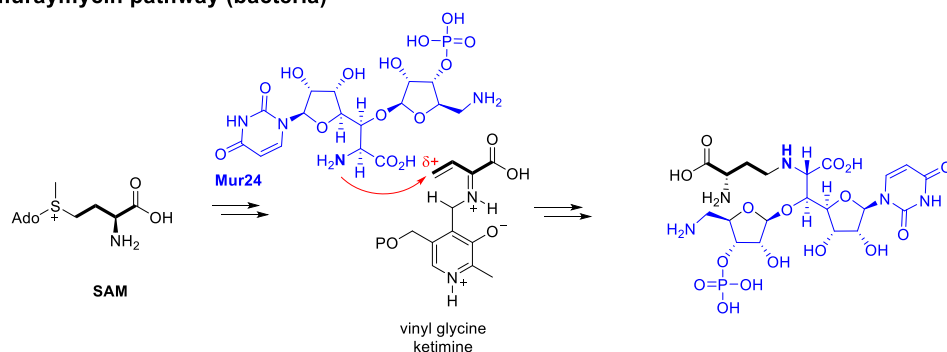
Supplementary Figure 32 Reported C γ alkylating enzymes and general reaction mechanism of the γ -elimination/electrophilic γ -addition with C nucleophiles.

A.) LoIC, vinyl glycine ketimine attacked by N-nucleophile, C-N bond formation, loline pathway (plant)

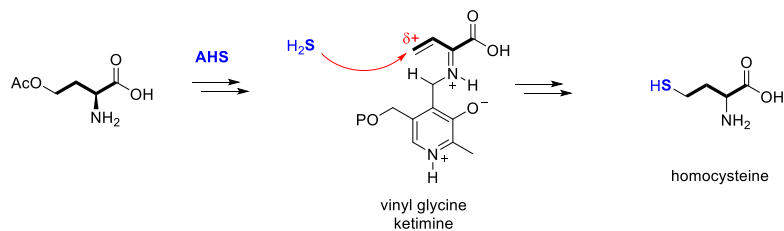
Note: no biochemical evidence



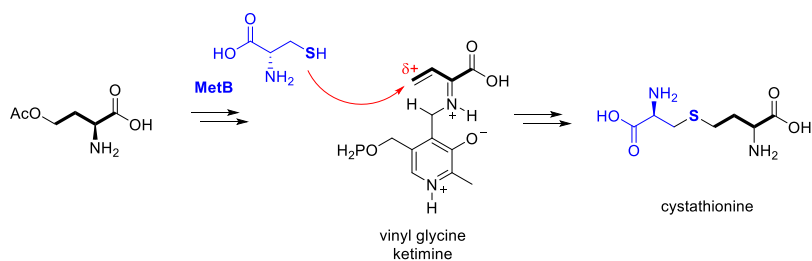
B.) Mur24, vinyl glycine ketimine attacked by N-nucleophile, C-N bond formation, muraymycin pathway (bacteria)



C.) O-Acetyl-L-homoserine sulfhydrylase (primary metabolism), vinyl glycine ketimine attacked by S-nucleophile, C-S bond formation



D.) Cystathionine- γ -synthase (primary metabolism), vinyl glycine ketimine attacked by S-nucleophile, C-S bond formation

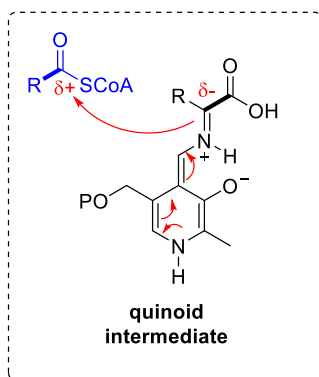


Supplementary Figure 33 Reported examples of γ -elimination/nucleophilic γ -addition (= γ -substitution) with X nucleophiles.

Known enzymes that catalyze alkylation at C α :

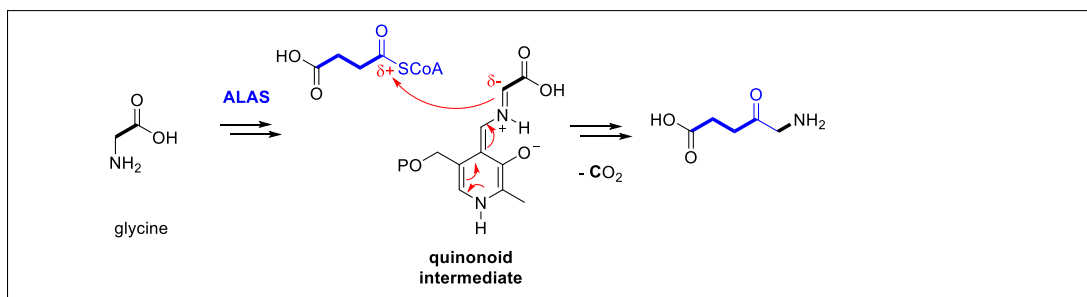
PLP-mediated C α alkylation reactions proceed *via* Claisen-like reactions with acyl-CoA esters (Supplementary Fig.34). This enzyme family is known as α -oxoamine synthase family. Prototypical examples from primary metabolism are 5-aminolevulinic acid synthase (ALAS)²⁸ and 8-amino7-oxononanoate synthase (AONS)²⁹. The general reaction mechanism involves formation of the external aldimine and subsequent deprotonation at C α to yield a quinonoid species, with nucleophilic character at C α . Subsequent addition to an acyl-CoA ester in a Claisen type reaction, decarboxylation at C α , and final reprotonation at C α , yields the product and internal aldimine.

The key intermediate is a conjugated quinonoid with nucleophilic character at C α .

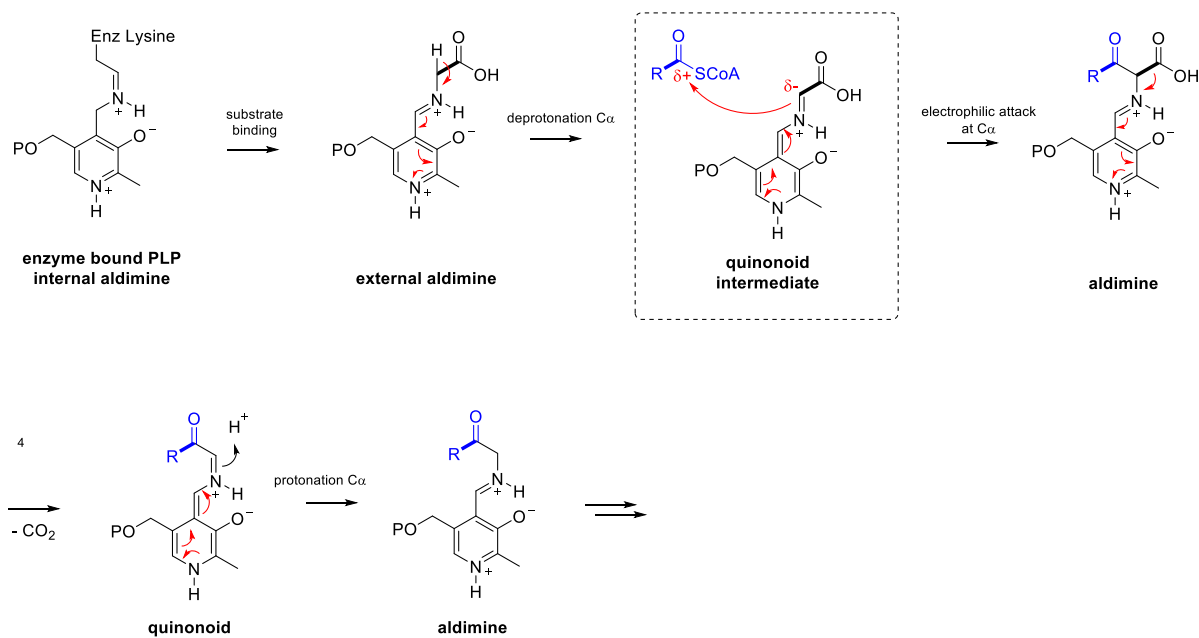


The C α -alkylating family of PLP enzymes forms a distinct clade in the phylogenetic analysis (Supplementary Fig.36).

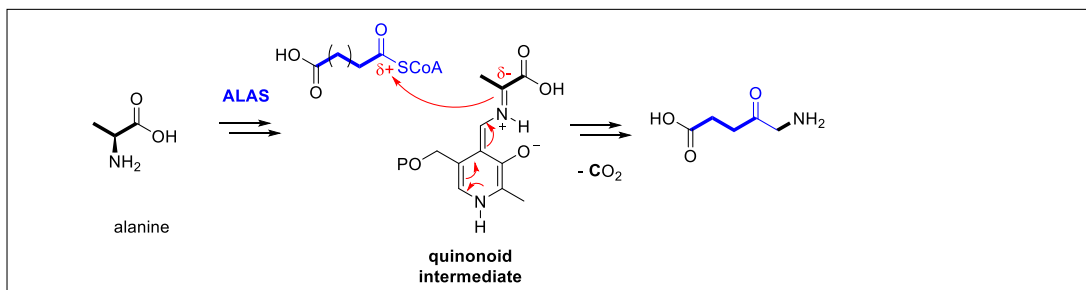
1.) **5-Aminolevulinate synthase (ALAS)** (primary metabolism), C-C bond formation, heme biosynthesis



General mechanism



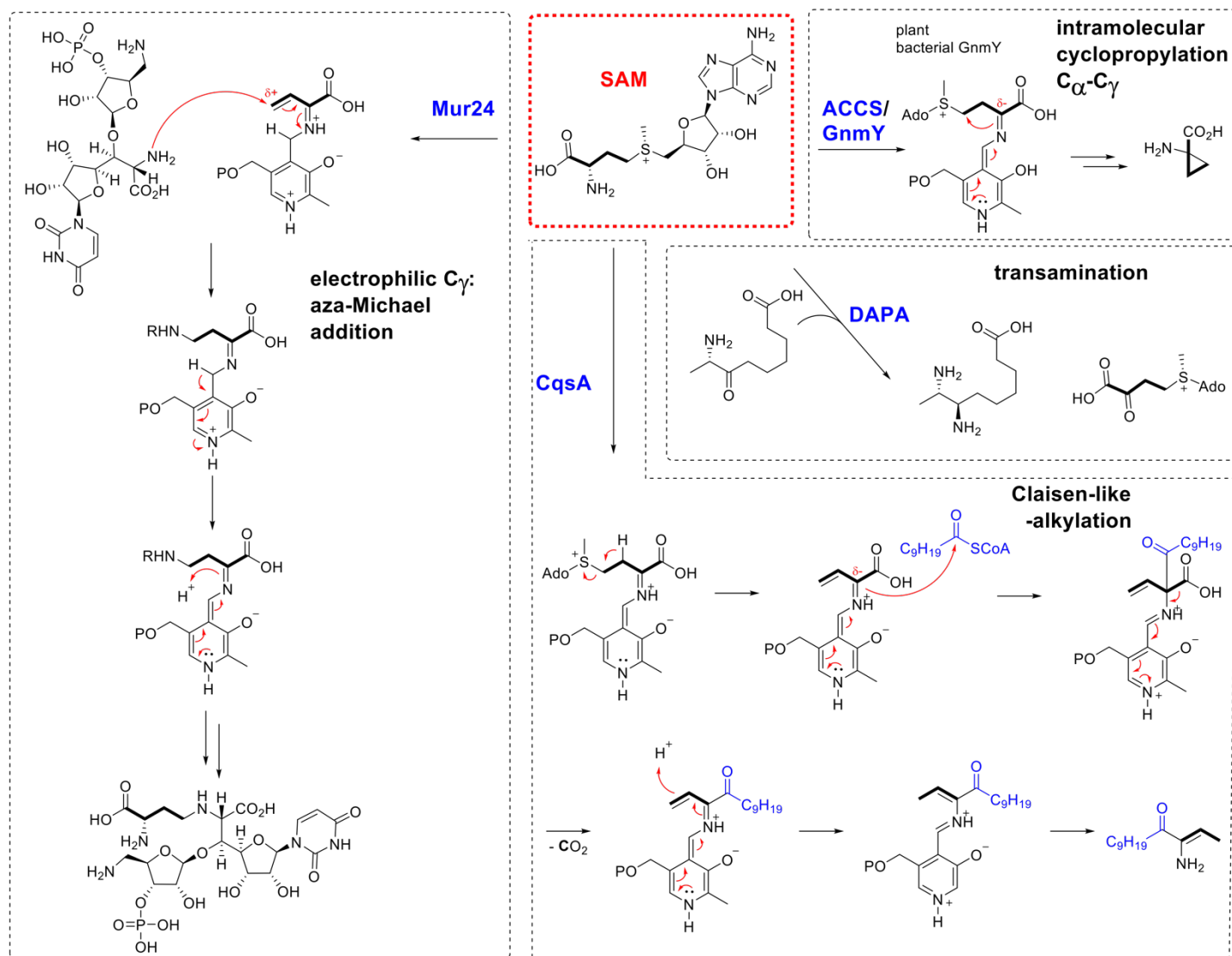
2.) **8-Amino-7-oxononanoate synthase (AONS)** (primary metabolism), C-C bond formation, biotin metabolism



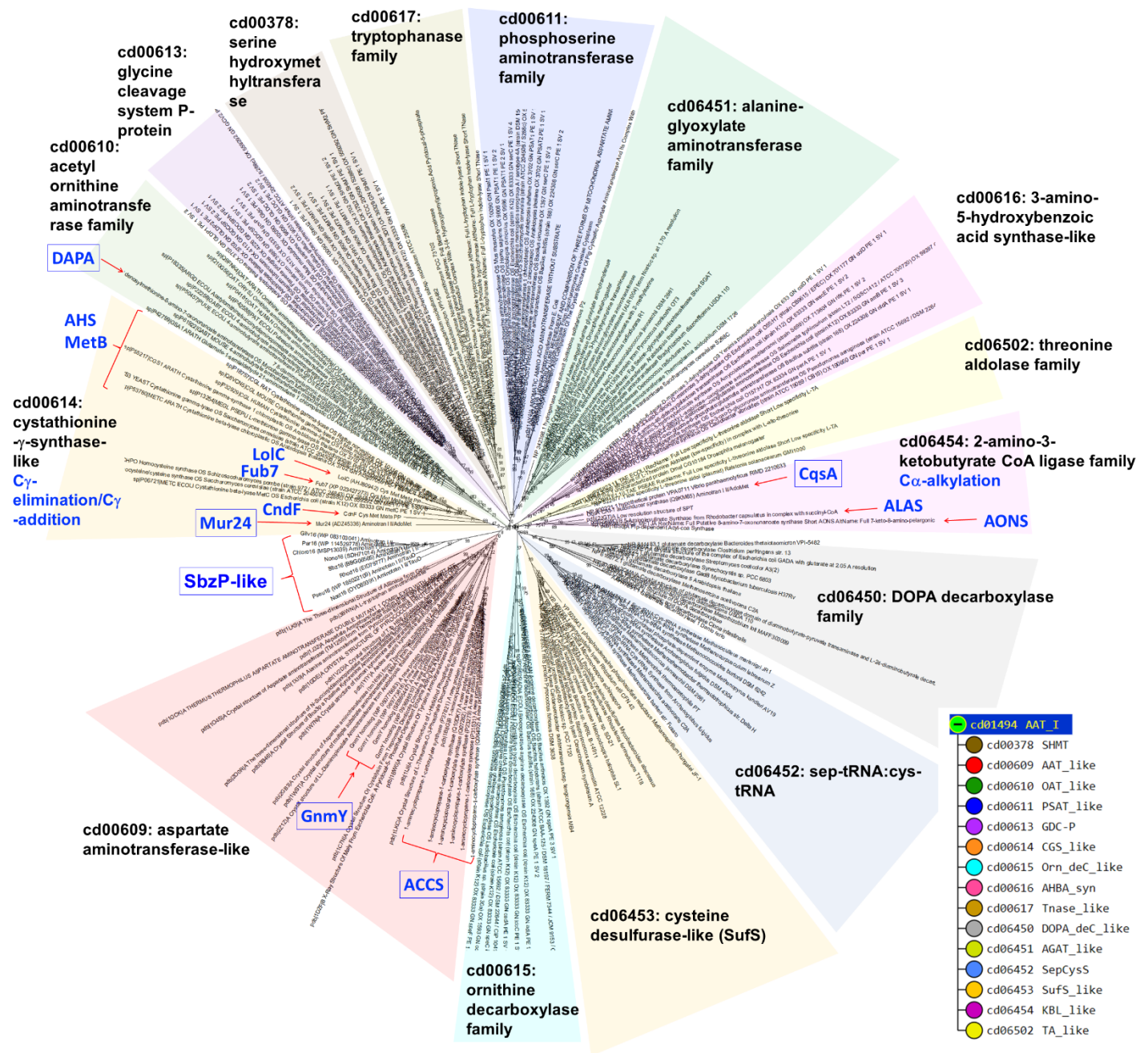
Supplementary Figure 34 Reported examples of C_α-alkylating PLP enzymes and general mechanism.

II. Novelty of Substrate Specificity:

Whereas the utilization of β -NAD is unprecedented, a few PLP enzymes have been reported that utilize SAM: 1-aminocyclopropane-1-carboxylate synthase (plant (ACCS)²⁷ and bacteria (GnmY)²⁸), adenosylmethionine-8-amino-7-oxononanoate aminotransferase (DAPA)²⁹, CqsA³⁰, and Mur24²² (Supplementary Fig.35). The SAM-utilizing enzymes are not clustered in the phylogenetic analysis, but distributed in mechanistically related protein family clades, such as cystathionine- γ -synthase family for Mur24, 2-amino-3-ketobutyrate CoA ligase family for CqsA, acetyl ornithine aminotransferase family for DAPA and amino transferase family for ACCS and GnmY (Supplementary Fig.35).



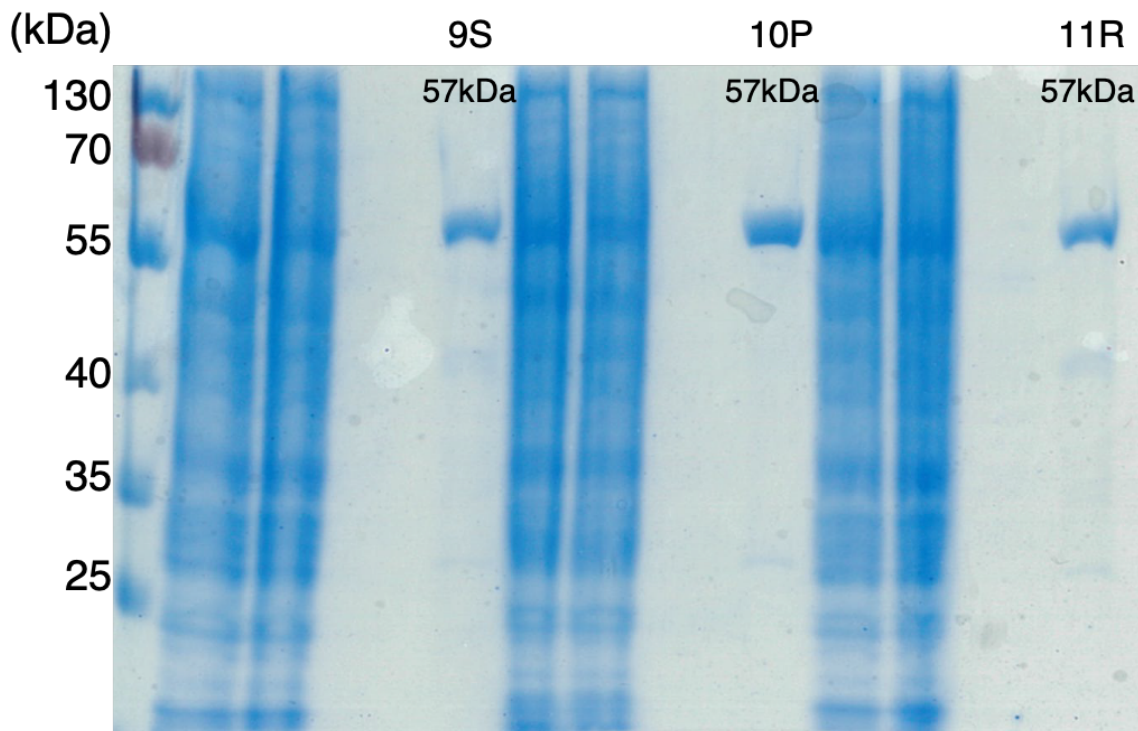
Supplementary Figure 35 SAM-utilizing PLP-dependent enzymes.



Supplementary Figure 36 Phylogenetic analysis of SbzP and related PLP enzymes. Representative protein sequences of AAT-I subfamilies were downloaded from the conserved domain database (<https://www.ncbi.nlm.nih.gov/cdd/>) and are highlighted in colors (see legend). SAM-utilizing enzymes are highlighted in blue boxes. The tree was constructed with MUSCLE alignment and Maximum Likelihood algorithm using MEGA 7.0.18.

Supplementary Note 9. Truncation of SbzP N-terminal domain

Truncated variants of SbzP were prepared (9S, 10P, 11R, Q17, R25, R33, R38, V47, and L60). The shortest truncations, which could successfully be expressed and purified were 9S, 10P, and 11R (Supplementary Fig. 37) and all longer truncations led to insoluble protein aggregates. However, the variants 9S, 10P and 11R, were found to be inactive and did not yield compound **7** in *in vitro* enzyme assays, nor accumulated compound **6** *in vivo*.



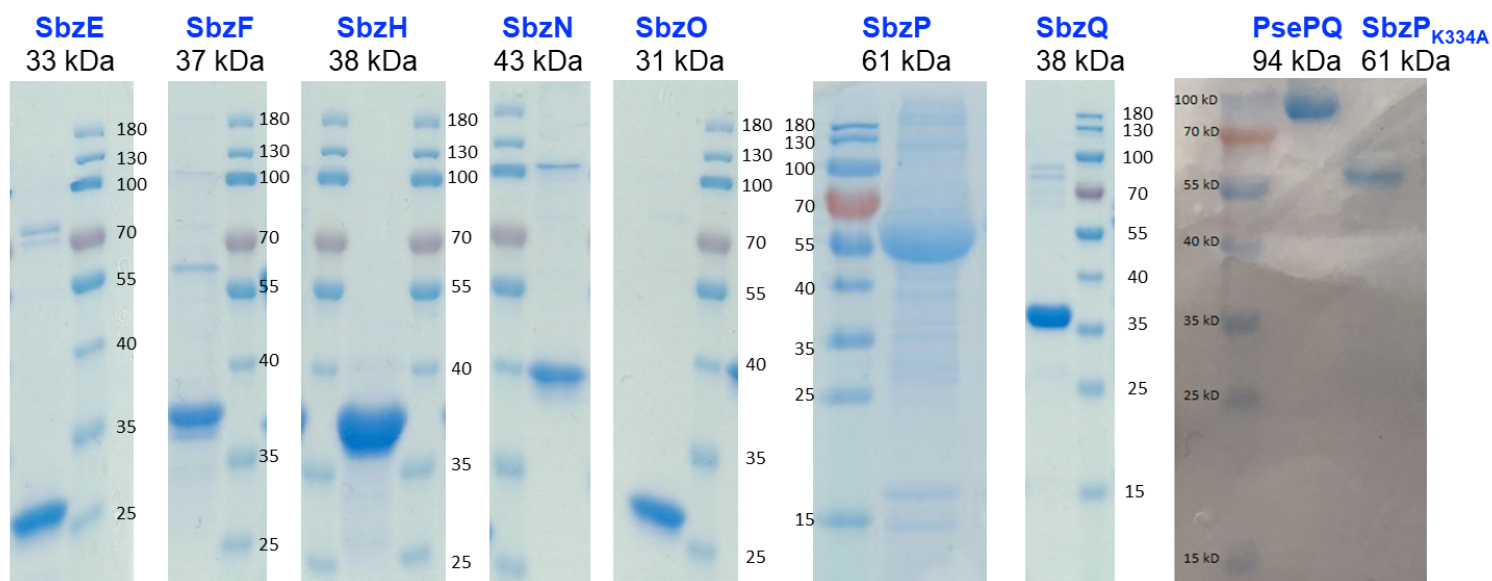
Supplementary Figure 37 SDS-PAGE of purified SbzP variants (truncation site: 9S, 10P, and 11R).

This result was repeated twice with independent experiments.

2. Supplementary Data

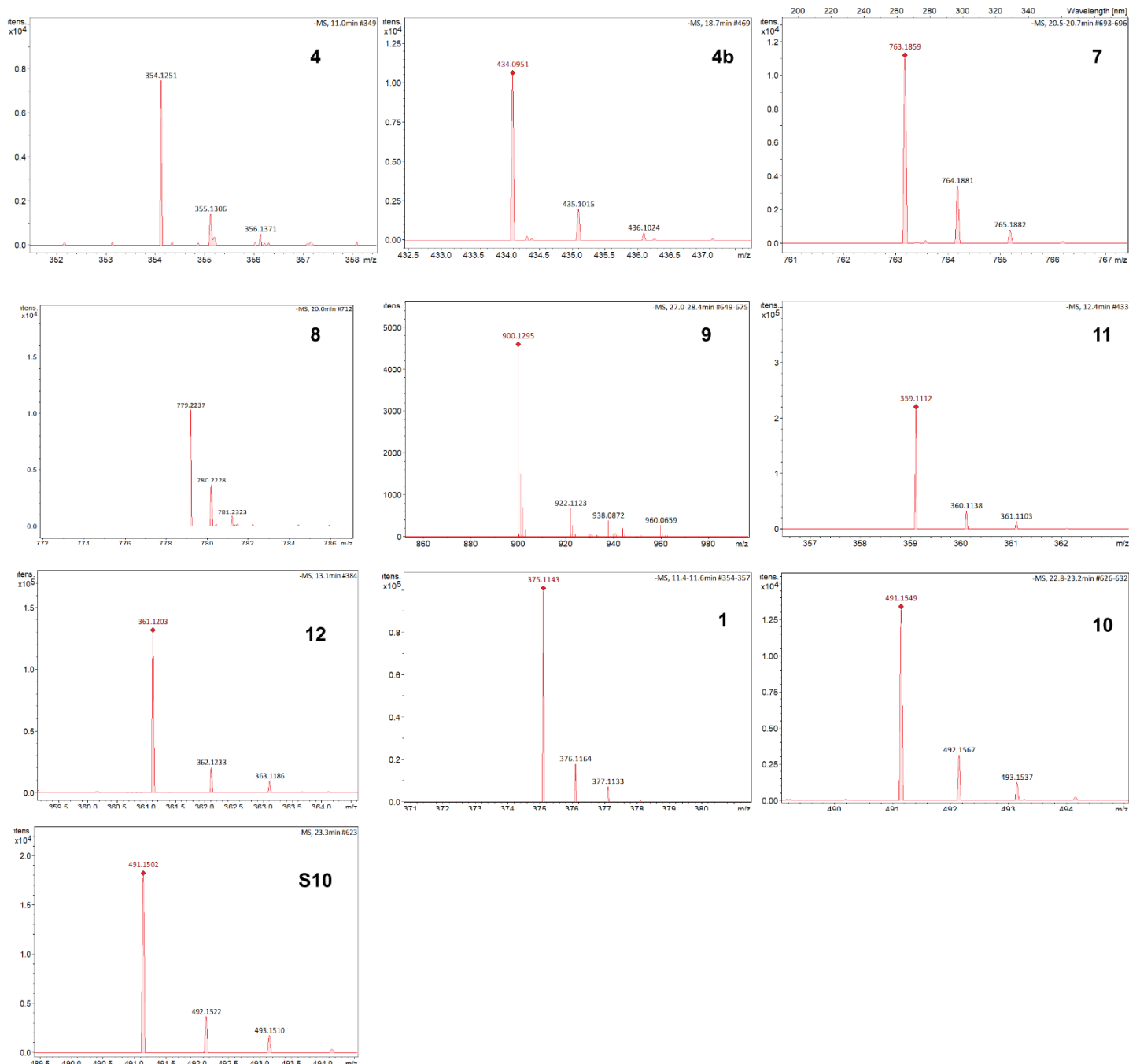
Supplementary Table 2 SbzP homologs functionally characterized in this study.

Protein and strain	Bacterium type	Gene source	Acc. No.
SorP (<i>Sorangium cellulosum</i> So ce56)	Gram-negative, myxobacterium	from gDNA	CAN98403
NonP (<i>Nonomurae jiangxiensis</i>)	Gram-positive, actinomycete	from gDNA	SDH71014
Par1P (<i>Streptomyces parvulus</i>)	Gram-positive, streptomycete	from gDNA	WP_114529778
Par2P (<i>Streptomyces parvulus</i>)	Gram-positive, streptomycete	from gDNA	WP_064726415
GlvP (<i>Streptomyces gilvosporeus</i>)	Gram-positive, streptomycete	from gDNA	WP_083103041
NosP (<i>Nostoc</i> sp. 'Peltigera membranacea cyanobiont' 210A)	Gram-negative, cyanobacterium	syn. gene	OYD89391
PsePQ (<i>Pseudomonas fluorescens</i>)	Gram-negative, proteobacteria	syn. gene	WP_185022109
PheP (<i>Phenylobacterium</i> sp. CCH9-H3)	Gram-negative, Caulobacteraceae	syn. gene	WP_156466939



Supplementary Figure 39 SDS-PAGE analysis of recombinant proteins.

This result was repeated twice with independent experiments.



Supplementary Figure 40 ESI-MS spectra of compounds reported in this study. All spectra were acquired in negative ionization mode.

HR-MS data of the isolated compounds.

Compound **4**: HR-ESI-MS m/z $[M-H]^-$ 354.1307 (calc. 354.1307, $C_{15}H_{20}N_3O_7^-$, 0 ppm)

Compound **7**: HR-ESI-MS m/z $[M-H]^-$ 763.1489 (calc. 763.1495, $C_{25}H_{33}N_8O_{16}P_2^-$, -0.79 ppm)

Compound **8**: HR-ESI-MS m/z $[M-H]^-$ 779.1449 (calc. 779.1444, $C_{25}H_{33}N_8O_{17}P_2^-$, 0.64 ppm)

Compound **S10**: HR-ESI-MS m/z $[M-H]^-$ 491.1088 (calc. 491.1090, $C_{17}H_{23}N_4O_{11}SO^-$, -0.41 ppm)

Compound **12**: HR-ESI-MS m/z $[M-H]^-$ 361.0815 (calc. 361.0823, $C_{12}H_{17}N_4O_7S^-$, -2.22 ppm)

Compound **S11**: HR-ESI-MS m/z $[M-H]^-$ 370.1249 (calc. 370.1256, $C_{15}H_{20}N_3O_8^-$, -1.89 ppm)

Supplementary Table 3 Bacterial strains, vectors and primers used in this study.

Strains	Description	Source
<i>Streptomyces lividans</i> TK21	Heterologous expression host	John Innes Centre
<i>S. lividans</i> /sbz1	Heterologous expression	Ref 1
<i>Streptomyces</i> sp. NCIMB40513	SB-203208 producer	GSK
<i>Streptomyces parvulus</i> NBRC 13193	Par1P and Par2P producer	NBRC
<i>Streptomyces gilvosporeus</i>	GlvP producer	A gift from Dr. Guagxiang Cao
<i>Nonomuraea jiangxiensis</i> JCM18570	NonP producer	JCM
<i>Sorangium cellulosum</i> So Ce56 DSM53796	SorP producer	DSMZ
<i>E. coli</i> LOBSTR harboring pBB528, pBB541, and SbzF-pET28a	SbzF expression host	In this work
Vectors	Description	Source
pET28a	Vector for protein expression in <i>E. coli</i>	Novagen
pQTEv	Vector for protein expression in <i>E. coli</i>	Addgene
pHSA81	Vector for protein expression in <i>S. lividans</i>	Dr. M. Kobayashi
pZH1	Vector for heterologous expression in <i>S. lividans</i> comprising ϕ BT1, <i>aac(3)IV</i> and <i>ermE</i> promotor based on pUC19	Ref 1
pZH2	Vector for heterologous expression in <i>S. lividans</i> comprising ϕ C31, <i>tsr</i> and <i>ermE</i> promotor based on pUC19	Ref 1
pTNT	Vector for heterologous expression in <i>S. lividans</i> comprising two replication origin for <i>E. coli</i> and <i>Streptomyces</i> , <i>kam</i> and <i>tip</i> promotor	Ref 2
pZH1-sbzR-F	Vectors for gene deletion of <i>sbzE</i>	In this study
pZH1-sbzR-P	Vectors for gene deletion of <i>sbzQ</i>	Ref 1
pZH2-sbzN-G		This study
pET28a-sbzE	Vector for protein expression in <i>E. coli</i> .	This study
pET28a-sbzF	Vector for protein expression in <i>E. coli</i> .	This study
pET28a-sbzH	Vector for protein expression in <i>E. coli</i> .	This study
pET28a-sbzN	Vector for protein expression in <i>E. coli</i> .	This study
pET28a-sbzO	Vector for protein expression in <i>E. coli</i> .	This study
pET28a-sbzP	Vector for protein expression in <i>E. coli</i> .	This study
pET28a-sbzQ	Vector for protein expression in <i>E. coli</i> .	This study
pET28a-sbzQ _{mel}	Vector for protein expression in <i>E. coli</i> .	This study
pQTEv-Par1P	Vector for protein expression in <i>E. coli</i> .	This study
pQTEv-Par2P	Vector for protein expression in <i>E. coli</i> .	This study
pQTEv-GlvP	Vector for protein expression in <i>E. coli</i> .	This study
pQTEv-NonP	Vector for protein expression in <i>E. coli</i> .	This study
pQTEv-SorP	Vector for protein expression in <i>E. coli</i> .	This study
pQTEv-PheP	Vector for protein expression in <i>E. coli</i> .	This study
pQTEv-NosP	Vector for protein expression in <i>E. coli</i> .	This study
pQTEv-PsePQ	Vector for protein expression in <i>E. coli</i> .	This study
pQTEv-PsePQD435N	Vector for protein expression in <i>E. coli</i> .	This study
pHSA81-sbzP	Vector for protein expression in <i>S. lividans</i> .	This study
pHSA81-PsePQ	Vector for protein expression in <i>S. lividans</i> .	This study
pHSA81-PsePQD435N	Vector for protein expression in <i>S. lividans</i> .	This study
pHSA81-NonP	Vector for protein expression in <i>S. lividans</i> .	This study

pHSA81-GlvP	Vector for protein expression in <i>S. lividans</i> .	This study
pHSA81-Par2P	Vector for protein expression in <i>S. lividans</i> .	This study
pHSA81-Par1P	Vector for protein expression in <i>S. lividans</i> .	This study
pHSA81-SorP	Vector for protein expression in <i>S. lividans</i> .	This study
pHSA81-PheP	Vector for protein expression in <i>S. lividans</i> .	This study
pHSA81-NosP	Vector for protein expression in <i>S. lividans</i> .	This study
pTNT-SbzQP	Vector for heterologous expression in <i>S. lividans</i> .	This study
Primers	Sequence (5' → 3')	Description
Primer I	AGGAGGACCcatatgCTGAACCGAATTCTCCCCGCTTC	Primers for pZH1-sbzR-F
Primer II	gctgaacgccggcgaggtgtgggtc	
Primer III	tcgccggcggttcagctcgccggccagttccagaaccgc	
Primer IV	ccacgccgcgtgtgccgtgcagatgg	
Primer V	gcacacgcggcggtgtaggagtcgccgtctcccacgatg	
Primer VI	tgagagtgcaccataGGCCGTGGTGTGGATGAACCGGCGAC	
Primer VII	catatggtgcactctcagtacaatc	Primers to amplify the linear vector of pZH1
Primer VIII	GGTCTCCTGTGGAGTGGTTCTG	
NdeI-Orf16F	cacaggaggaccatgaaactcttgaatcccttccgtc	Primers for gene deletion of <i>sbzO</i>
NdeI-Orf7R	tgagagtgcaccataTCAGCCCGACTGGTGGCGGCACAG	
pTNT-SbzQ-f	AAGGGAGCGGACATATGGAACAGCGATCCCCACCTC	Primers for <i>sbzQP</i> heterologous expression
pTNT-SbzP-r	GGTCTGCCCaagctTCATGGGTCAGCTCCCCGTCGTG	
pQtevSbzP_F	ttattttcagggatccATGAACTCTTGAATCCCTTCCG	Primers for K332A mutation on <i>sbzP</i>
SbzPK334A_R	CAGGACgcGGAGAGGCTGGAGAC	Primers for K332A mutation on <i>sbzP</i>
SbzPK334A_F	CCTCTCCgcGTCTGGTCGGCGCC	
pQtevSbzP_R	tcagctaattaagcttCAGCTCCCCGTCGTG	Primers for <i>sbzP9S</i> Expression
pQtevSbzP9S_BamHI-f	ttattttcagggatccTCTCCGCGTCCGCGGTACACCC	Primers for <i>sbzP10P</i> Expression
pQtevSbzP10P_BamHI-f	ttattttcagggatccCCGCGTCCGCGGTACACCCCGCAG	Primers for <i>sbzP11R</i> Expression
pQtevSbzP11R_BamHI-f	ttattttcagggatccCGTCCGCGGTACACCCCGCAGG	Primers for <i>sbzP17Q</i> Expression
pQtevSbzP17Q_BamHI-f	TTTATTTTCAGGGATCCGACGCACGCGAGACCGCCGCGCGC	Primers for <i>sbzP25R</i> Expression
pQtevSbzP25R_BamHI-f	TTTATTTTCAGGGATCCCGCGCACCGCAACCCGGTGGCGTC	Primers for <i>sbzP33R</i> Expression
pQtevSbzP33R_BamHI-f	TTTATTTTCAGGGATCCCGCGACGTCTTACACCCGGCATAG	Primers for <i>sbzP38P</i> Expression
pQtevSbzP38P_BamHI-f	TTTATTTTCAGGGATCCCCCGGCATAGACGAGCGTGTCTG	Primers for <i>sbzP47L</i> Expression
pQtevSbzP47L_BamHI-f	TTTATTTTCAGGGATCCGTGTACGCCCCGGGCACGCGACCCC	Primers for <i>sbzP60L</i> Expression
pQtevSbzP60L_BamHI-f	TTTATTTTCAGGGATCCCTGCGGGACCTGTGGCTGGGGCGG	Primers for D435N mutation on <i>PsePQ</i>
PsePQ_D435N_f	GTCCAGCGGGGCGGTCaATGAAGATGAGTATGGG	Primers for <i>sbzE</i> expression
PsePQ_D435N_r	CCCATACTCATCTTCATtGACCGCCCCGCTGGAC	
SbzE-NdeI-f	gcgcggcagccatgGGAACCTATTACTACACGGACG	Primers for <i>sbzF</i> expression
SbzE-HindIII-r	gtgcggccgcaagctTCACCCCTGTTCCAGGGGCTTGC	
SbzF-NdeI-f	cgcgcggcagccatATGCAGATCCATGGAGACGGCGTCC	Primers for <i>sbzH</i>
SbzF-HindIII-r	gtgcggccgcaagctTCATCGCTCCAGCCTCCGTACG	
SbzH-NdeI-f	gcgcggcagccatATGGCAGCGACGATCCGGCGCGAG	

SbzH-HindIII-r	gtgcggccgcaagcttCAGCGGGCCGAGAAGTGGTGGAC	expression
SbzN-NdeI-f	cgcgcggcagccatATGAGCGACGGGAACGGCCCCG	Primers for <i>sbzN</i> expression
SbzN-HindIII-r	gtgcggccgcaagctTCATGGCGTCTCTCCGTCGTGTCTG	
SbzO-NdeI-f	cgcgcggcagccatATGGACGGATGGACGGCCCCCTG	Primers for <i>sbzO</i> expression
SbzO-HindIII-r	gtgcggccgcaagctTCATCGCTCCAGCCTCCGTACG	
SbzP-NdeI-f	cgcgcggcagccatATGAAACTCTTCGAATCCCTTCCGTC	Primers for <i>sbzP</i> expression
SbzP-HindIII-r	gtgcggccgcaagcttCATGGGTCAGCTCCCCGTCTGTG	
SbzQ-NdeI-f	cgcgcggcagccatATGGAACAGCGATCCCCACCTC	Primers for <i>sbzQ</i> expression
SbzQ-HindIII-r	gtgcggccgcaagcttCTAGACGATGCGCAGACGCTTG	
Par1P-BamHI-f	ttattttcagggatccatgcagctcttcgccgcagtgcc	Primers for <i>par1P</i> expression(pQTeV)
Par1P-HindIII-r	tcagctaattaagcttcatcccacgggcaccgcgag	
Par2P-BamHI-f	ttattttcagggatccatgcagctcttcgccgcagtgcc	Primers for <i>par2P</i> expression (pQTeV)
Par2P-HindIII-r	tcagctaattaagcttcatcccacgggcaccgcgagatg	
GlvP-BamHI-f	ttattttcagggatccatgacactcttcgactcccctctg	Primers for <i>glvP</i> expression (pQTeV)
GlvP-HindIII-r	tcagctaattaagcttcacggggccgctccggcgag	
NosP-BamHI-f	ttattttcagggatccGTTGGCATTATCCAGATGGACC	Primers for <i>nosP</i> Expression (pQTeV)
NosP-HindI-r	tcagctaattaagcttaCGACTTCTGAACGACAAGAC	
PheP-BamHI-f	ttattttcagggatccATGGCGGCTGGCGGAACAC	Primers for <i>pheP</i> Expression (pQTeV)
PheP-HindI-r	tcagctaattaagcttaCACGGCTGCTGGCTGTGG	
NonP-BamHI-f	ttattttcagggatccATGCGGCTATTGAAACCGTTC	Primers for <i>nonP</i> expression (pQTeV)
NonP-HindIII-r	TCAGCTAATTAAGCtTCAGCCGTCCGGGGTCAGCGCG	
SorP-BamHI-f	ttattttcagggatccATGAGTGAGGAGCGAACCAGGG	Primers for <i>sorP</i> Expression (pQTeV)
SorP-HindIII-r	TCAGCTAATTAAGCtGGCAGCGCCTTCCGCTCAGCG	
PseQ_BamHI-f	ttattttcagggatccATGAATGGAACATTCTGTGGAGAG	Primers for <i>psePQ</i> Expression (pQTeV)
PseP_HindIII-r	TCAGCTAATTAAGCTTCAAGCATTCCCGGCCCC	
PseQ_HindIII-r	TCAGCTAATTAAGCTTACGGGTTCGTGGGGTTCAAAGC	Primers for <i>pseQ</i> expression (pQTeV)
pET28a-pHSA81Ndelf	aaaggaatgagcatatgggcagcagccatcatcatc	Primers for cloning from pET28a to pHSA81
pET28a-pHSA81HindIIIr	tagtgctagcaagctgctcgagtgcggccgcaagc	
pQTeV-pHSA81Ndelf	AAAGGAATGAGCATatgaacatcaccatcaccatcacc	Primers for cloning from pQTeV to pHSA81
pQTeV-pHSA81HindIIIr	TAGTGCTAGCAAGCTtctatcaacaggagtccaagctcagc	

The DNA sequences of synthetic genes

>*pheP*

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>*nosP*

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>*clxP*

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>psePQ

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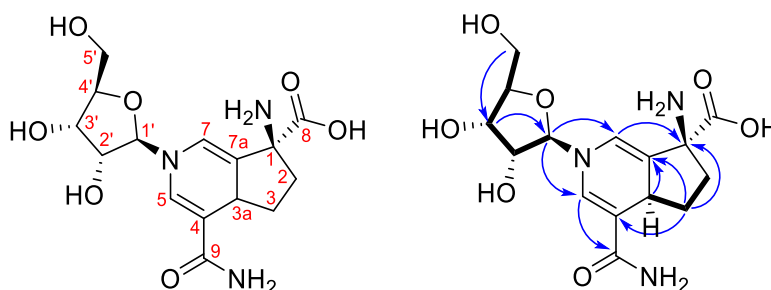
3. NMR Analysis of Isolated Compounds

Supplementary Table 4 NMR data of compound **4** (^1H :800 MHz, ^{13}C :200 MHz, D_2O).

$\text{C}^{[\text{a}]}$	type	^{13}C (δ)	^1H (δ , m, J) $^{[\text{b}]}$
1	C_q	65.1	-
2	CH_2	33.9	2.45 (ddd, $J=14.4$, 12.0, 7.4) 2.04 (dd, $J=14.4$, 7.8)
3	CH_2	30.7	2.53 (m) 1.72 (m)
3a	CH	38.0	3.72 (m)
4	C_q	106.5	-
5	CH	134.8	7.15 (s)
7	CH	123.1	6.44 (s)
7a	C_q	121.0	-
8	C_q	176.0	-
9	C_q	172.2	-
1'	CH	94.6	5.00 (d, $J=7.0$)
2'	CH	71.5	4.24 (dd, $J=7.0$, 5.6)
3'	CH	70.0	4.19 (dd, $J=5.6$, 3.0)
4'	CH	83.8	4.02 (ddd, $J=5.5$, 4.5, 3.3)
5'	CH_2	61.4	3.80 (dd, $J=12.5$, 3.3) 3.74 (dd, $J=12.5$, 4.5)

[a] Carbon numbering as shown in Supplementary Figure 29.

[b] Chemical shifts δ in ppm, multiplicity m: s=singlet, d=doublet, m=multiplet; coupling constants J are given in Hertz.



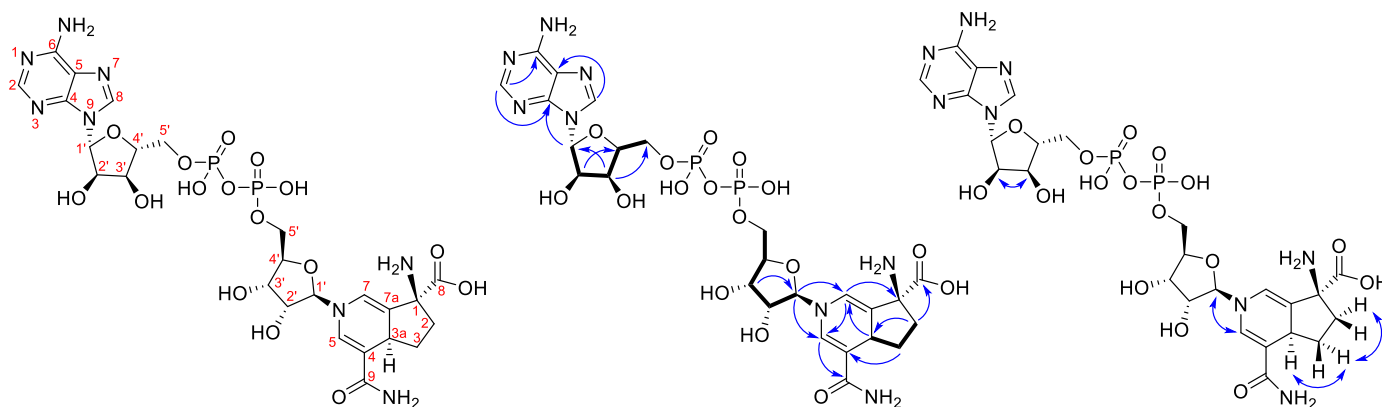
Supplementary Figure 41 Carbon numbering and elucidated structure of **4**. ^1H , ^1H -COSY correlations are indicated with bold lines, key ^1H , ^{13}C -HMBC correlations are represented by single-headed arrows. Configuration at C1' to C5' is assigned based on the measured coupling constants. Configuration at C3a was deduced from altemicidin (**1**).

Supplementary Table 5 NMR data of compound **7** (^1H :900 MHz, ^{13}C :225 MHz, D_2O).

$\text{C}^{[a]}$	type	^{13}C (δ)	^1H (δ , m, J) $^{[b]}$
1	C_q	65.1	-
2	CH_2	33.6	2.44 (ddd, $J=14.2$, 12.0, 7.5) 2.00 (dd, $J=14.2$, 8.0)
3	CH_2	30.6	2.49 (m) 1.67 (m)
3a	CH	37.9	3.69 (m)
4	C_q	106.6	-
5	CH	133.9	7.06 (s)
7	CH	123.0	6.48 (s)
7a	C_q	120.8	-
8	C_q	176.0	-
9	C_q	172.2	-
1'	CH	94.5	4.96 (d, $J=7.0$)
2'	CH	71.4	4.30 (m)
3'	CH	70.5	4.30 (m)
4'	CH	82.7	4.13 (m)
5'	CH_2	65.8	4.11 (m)
2-ade	CH	152.9	8.30 (s)
4-ade	C_q	149.2	-
5-ade	C_q	118.7	-
6-ade	C_q	155.7	-
8-ade	CH	139.9	8.54 (s)
1'-ade	CH	86.9	6.18 (d, $J=5.8$)
2'-ade	CH	74.3	4.81 (m)
3'-ade	CH	70.4	4.56 (dd, $J=4.8$, 3.7)
4'-ade	CH	83.8	4.43 (m)
5'-ade	CH_2	65.2	4.26 (m)

[a] Carbon numbering as shown in Supplementary Fig.30.

[b] Chemical shifts δ in ppm, multiplicity m: s=singlet, d=doublet, m=multiplet; coupling constants J are given in Hertz.



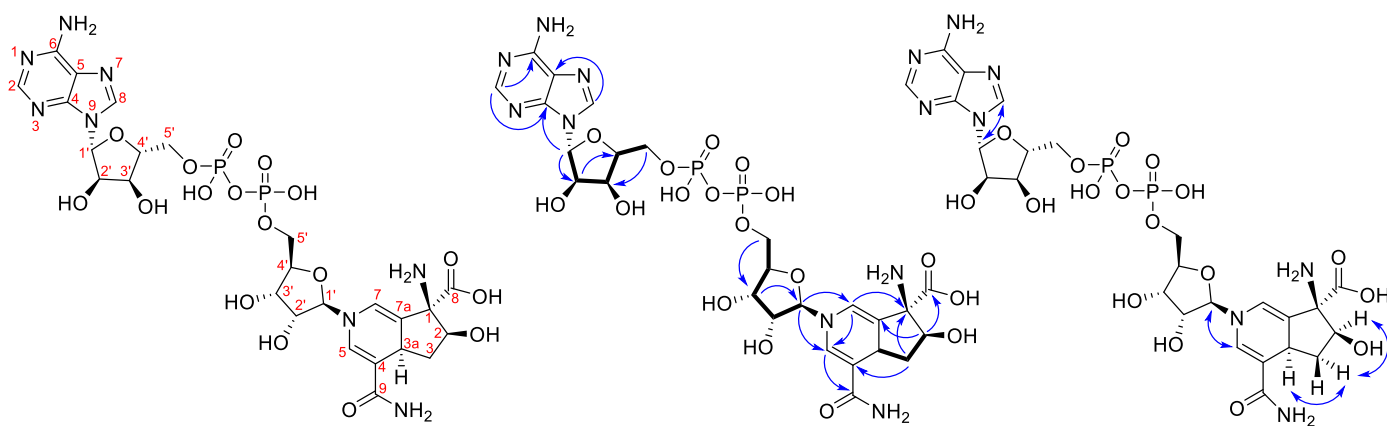
Supplementary Figure 42 Carbon numbering and elucidated structure of **7**. ^1H , ^1H -COSY correlations are indicated with bold lines, key ^1H , ^{13}C -HMBC correlations are represented by single-headed arrows and ^1H , ^1H -NOESY correlations are indicated by double headed arrows.

Supplementary Table 6 NMR data of compound **8** (^1H :900 MHz, ^{13}C :225 MHz, D_2O).

$\text{C}^{[a]}$	type	^{13}C (δ)	^1H (δ , m, J) $^{[b]}$
1	C_q	66.4	-
2	CH	73.2	4.66 (dd, $J=6.4$, 10.0)
3	CH_2	38.5	2.82 (ddd, $J=11.8$, 6.4, 6.1) 1.65 (ddd, $J=11.8$, 10.4, 10.0)
3a	CH	33.3	3.60 (m)
4	C_q	106.2	-
5	CH	133.8	7.08 (s)
7	CH	125.2	6.55 (s)
7a	C_q	118.2	-
8	C_q	175.0	-
9	C_q	171.8	-
1'	CH	94.5	4.96 (d, $J=6.7$)
2'	CH	71.5	4.30 (m)
3'	CH	70.4	4.30 (m)
4'	CH	82.8	4.14 (m)
5'	CH_2	65.7	4.12 (m)
2-ade	CH	151.4	8.34 (s)
4-ade	C_q	149.1	-
5-ade	C_q	118.7	-
6-ade	C_q	154.6	-
8-ade	CH	140.4	8.58 (s)
1'-ade	CH	87.2	6.19 (d, $J=5.7$)
2'-ade	CH	74.4	4.81 (m)
3'-ade	CH	70.5	4.57 (dd, $J=4.8$, 3.7)
4'-ade	CH	83.9	4.44 (m)
5'-ade	CH_2	65.2	4.26 (m)

[a] Carbon numbering as shown in Supplementary Fig.31.

[b] Chemical shifts δ in ppm, multiplicity m: s=singlet, d=doublet, m=multiplet; coupling constants J are given in Hertz.



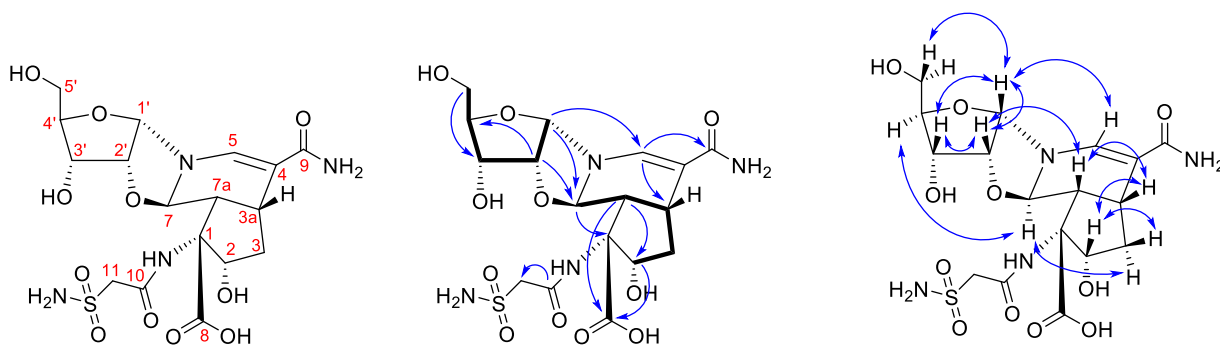
Supplementary Figure 43 Carbon numbering and elucidated structure of **8**. ^1H , ^1H -COSY correlations are indicated with bold lines, key ^1H , ^{13}C -HMBC correlations are represented by single-headed arrows and ^1H , ^1H -NOESY correlations are indicated by double headed arrows.

Supplementary Table 7 NMR data of compound **S10** (^1H :800 MHz, ^{13}C :200 MHz, D_2O).

$\text{C}^{[a]}$	type	^{13}C (δ)	^1H (δ , m, J) $^{[b]}$
1	C_q	69.9	-
2	CH	73.8	4.64 (dd, $J=7.3$, 6.0)
3	CH_2	40.7	2.85 (ddd, $J=14.1$, 7.8, 7.3) 1.36 (m)
3a	CH	32.0	3.11 (m)
4	C_q	104.5	-
5	CH	136.6	7.57 (s)
7	CH	86.7	5.15 (d, $J=8.8$)
7a	CH	46.1	2.42 (dd, $J=8.8$, 6.8)
8	C_q	177.6	-
9	C_q	172.5	-
10	C_q	163.7	-
11	CH_2	59.4	4.45 (d, $J=14.7$) 4.31 (d, $J=14.7$)
1'	CH	91.2	5.82 (d, $J=4.4$)
2'	CH	80.1	4.75 (dd, $J=5.5$, 4.4)
3'	CH	70.4	4.09 (dd, $J=9.0$, 5.5)
4'	CH	81.5	3.87 (ddd, $J=9.0$, 4.3, 2.3)
5'	CH_2	60.0	3.90 (dd, $J=12.7$, 2.3) 3.70 (dd, $J=12.7$, 4.3)

[a] Carbon numbering as shown in Supplementary Fig.32.

[b] Chemical shifts δ in ppm, multiplicity m: s=singlet, d=doublet, m=multiplet; coupling constants J are given in Hertz.



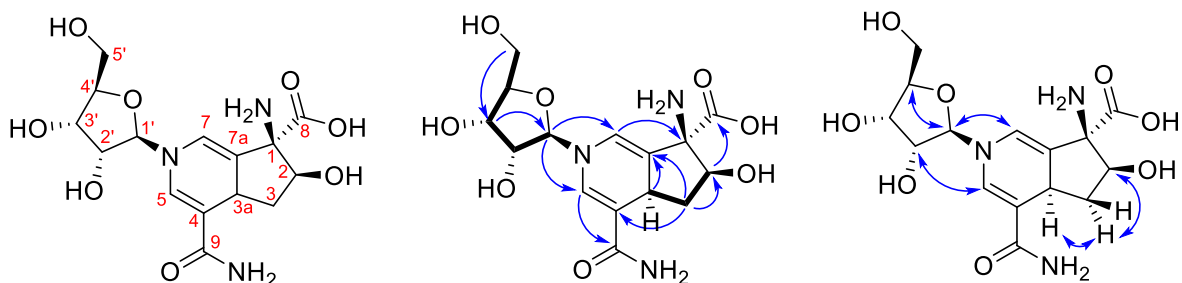
Supplementary Figure 44 Carbon numbering and elucidated structure of **S10**. ^1H , ^1H -COSY correlations are indicated with bold lines, key ^1H , ^{13}C -HMBC correlations are represented by single-headed arrows and ^1H , ^1H -NOESY correlations are indicated by double headed arrows.

Supplementary Table 8 NMR data of compound **S11** (^1H :800 MHz, ^{13}C :200 MHz, D_2O).

$\text{C}^{[a]}$	type	^{13}C (δ)	^1H (δ , m, J) $^{[b]}$
1	C_q	66.2	-
2	CH	73.2	4.69 (dd, $J=10.6$, 6.4)
3	CH_2	38.2	2.82 (ddd, $J=11.5$, 6.4, 5.9) 1.69 (ddd, $J=11.5$, 11.4, 10.6)
3a	CH	33.1	3.63 (dd, $J=11.4$, 5.9)
4	C_q	105.9	-
5	CH	134.7	7.15 (s)
7	CH	125.2	6.51 (s)
7a	C_q	118.5	-
8	C_q	174.7	-
9	C_q	171.9	-
1'	CH	94.5	5.00 (d, $J=7.0$)
2'	CH	71.7	4.24 (dd, $J=7.0$, 5.6)
3'	CH	70.0	4.19 (dd, $J=5.6$, 3.0)
4'	CH	83.9	4.03 (ddd, $J=5.5$, 4.5, 3.3)
5'	CH_2	61.4	3.81 (dd, $J=12.5$, 3.3) 3.75 (dd, $J=12.5$, 4.5)

[a] Carbon numbering as shown in Supplementary Fig.33.

[b] Chemical shifts δ in ppm, multiplicity m: s=singlet, d=doublet, m=multiplet; coupling constants J are given in Hertz.



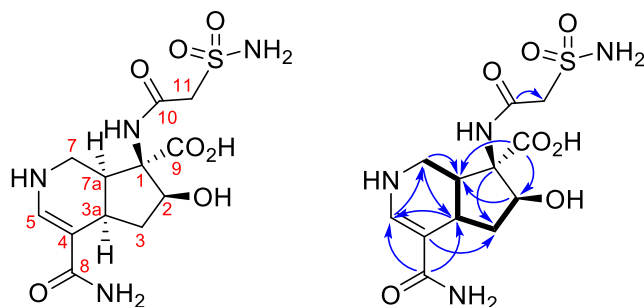
Supplementary Figure 45 Carbon numbering and elucidated structure of **S11**. ^1H , ^1H -COSY correlations are indicated with bold lines, key ^1H , ^{13}C -HMBC correlations are represented by single-headed arrows and ^1H , ^1H -NOESY correlations are indicated by double headed arrows.

Supplementary Table 9 NMR data of compound **12** (^1H :500 MHz, ^{13}C :125 MHz, D_2O).

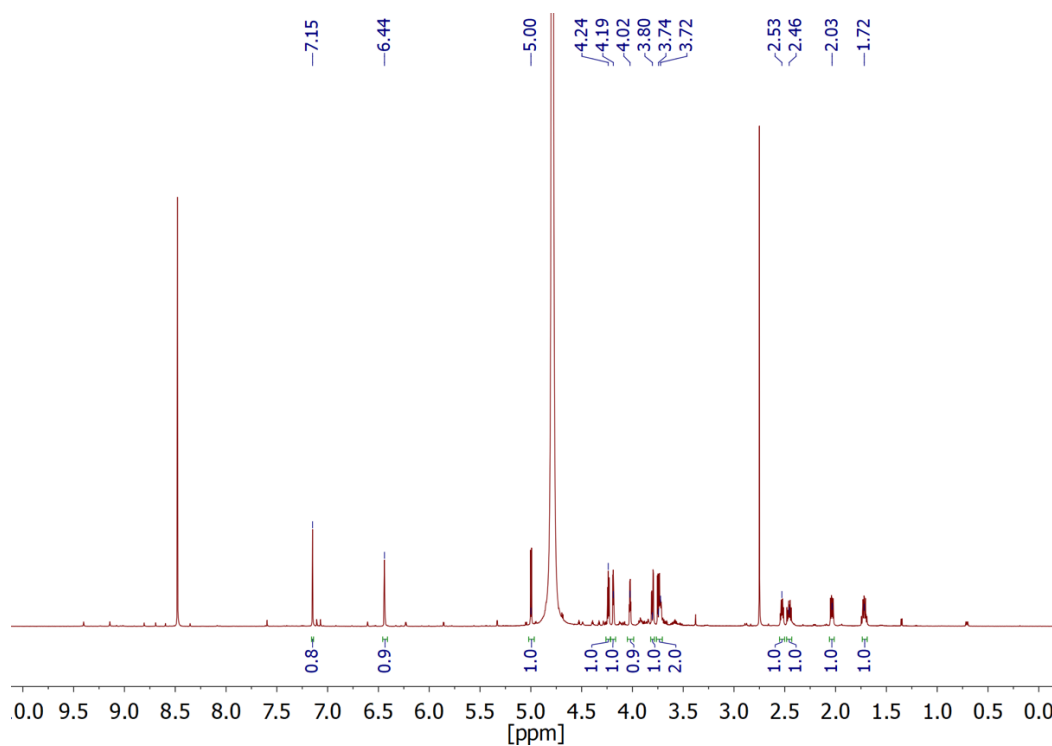
$\text{C}^{[a]}$	type	^{13}C (δ)	^1H (δ , m, J) $^{[b]}$
1	C_q	68.4	-
2	CH	75.2	4.24 (dd, $J=9.0$, 8.0)
3	CH_2	40.6	2.64 (ddd, $J=12.8$, 8.0, 6.8) 1.26 (ddd, $J=12.8$, 12.5, 9.0)
3a	CH	31.9	2.92 (ddd, $J=12.5$, 6.8, 6.0)
4	C_q	97.2	-
5	CH	143.5	7.47 (s)
7	CH_2	37.5	3.97 (dd, $J=12.5$, 5.1) 2.80 (dd, $J=12.7$, 11.9)
7a	CH	39.8	2.72 (ddd, $J=11.9$, 6.0, 5.1)
8	C_q	179.0	-
9	C_q	173.8	-
10	C_q	163.6	-
11	CH_2	59.6	4.32 (d, $J=14.4$) 4.23 (d, $J=14.4$)

[a] Carbon numbering as shown in Supplementary Fig.34.

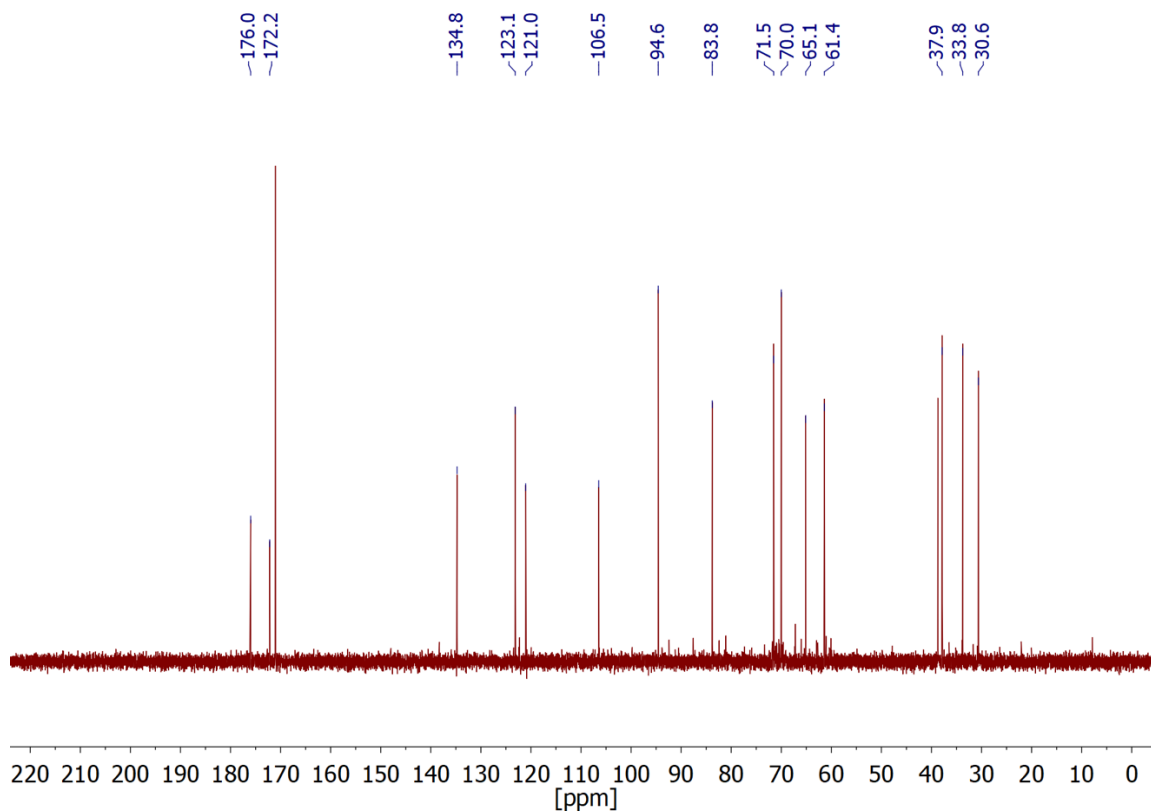
[b] Chemical shifts δ in ppm, multiplicity m: s=singlet, d=doublet, m=multiplet; coupling constants J are given in Hertz.



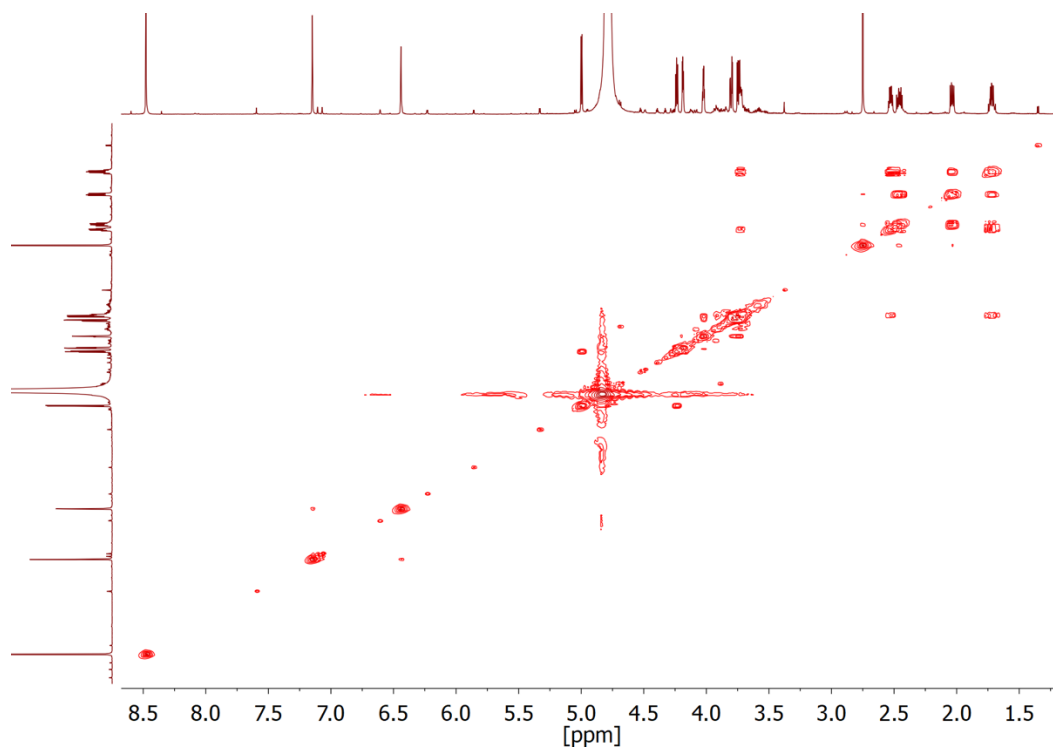
Supplementary Figure 46 Carbon numbering and elucidated structure of **12**. ^1H , ^1H -COSY correlations are indicated with bold lines, key ^1H , ^{13}C -HMBC correlations are represented by single-headed arrows.



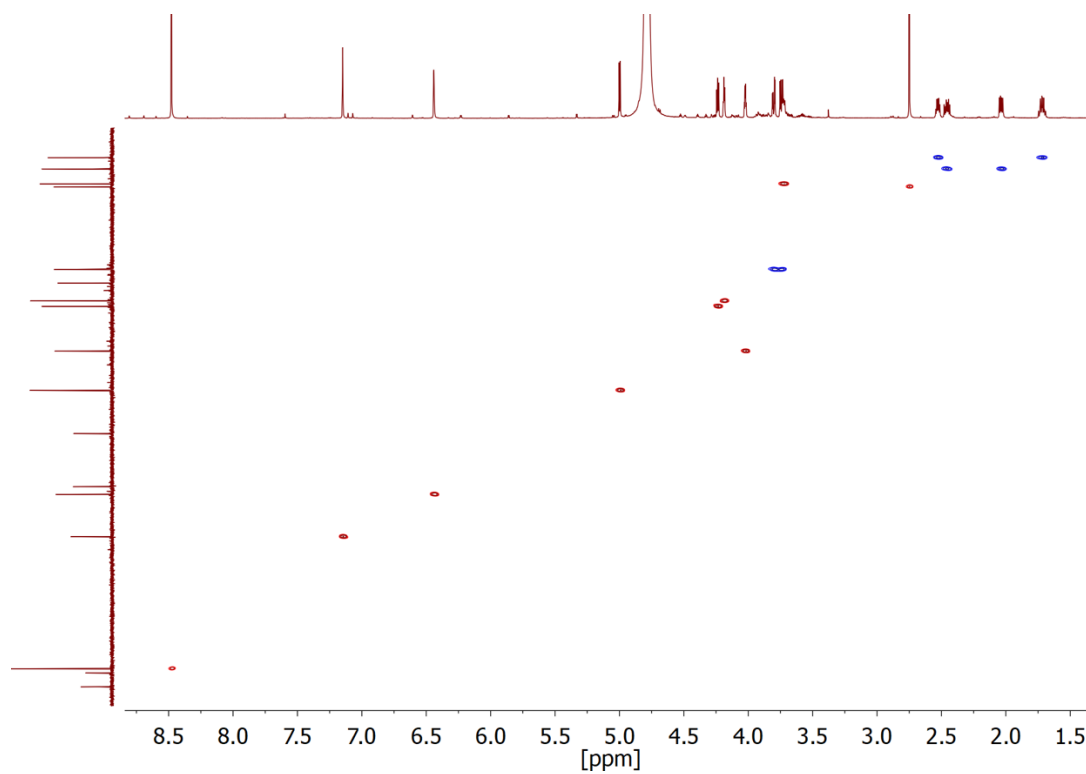
Supplementary Figure 47 ¹H-NMR spectrum of compound **4** (800 MHz, D₂O).



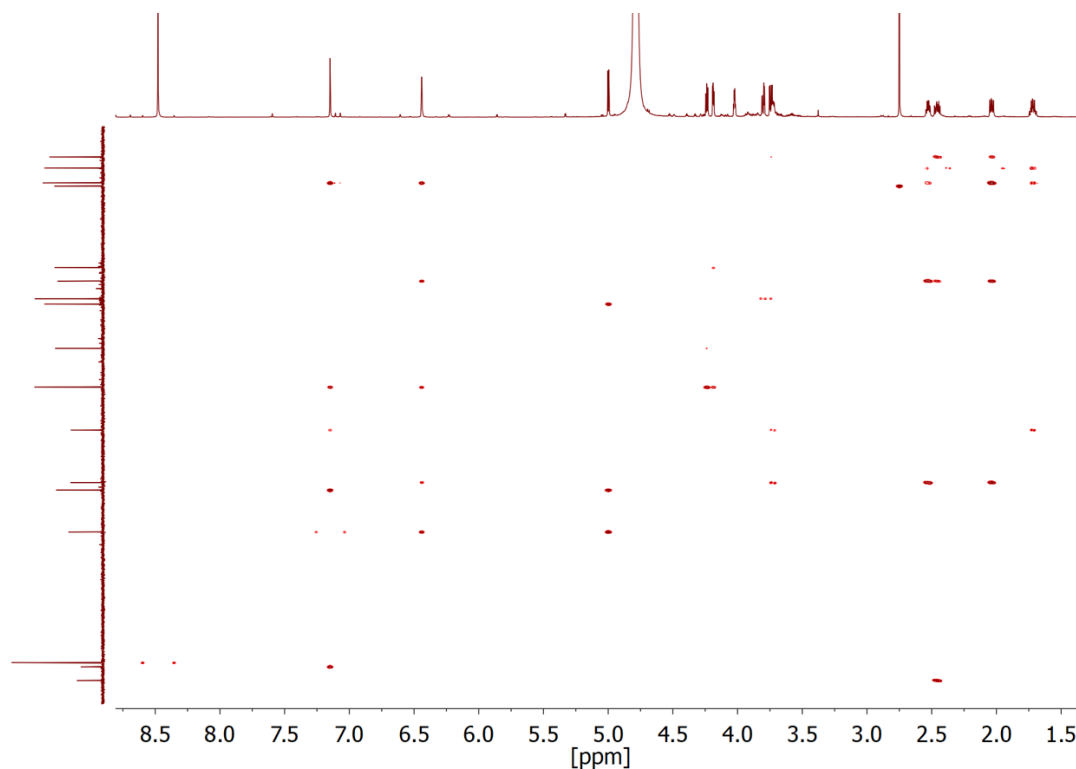
Supplementary Figure 48 ¹³C-NMR spectrum of compound **4** (200 MHz, D₂O).



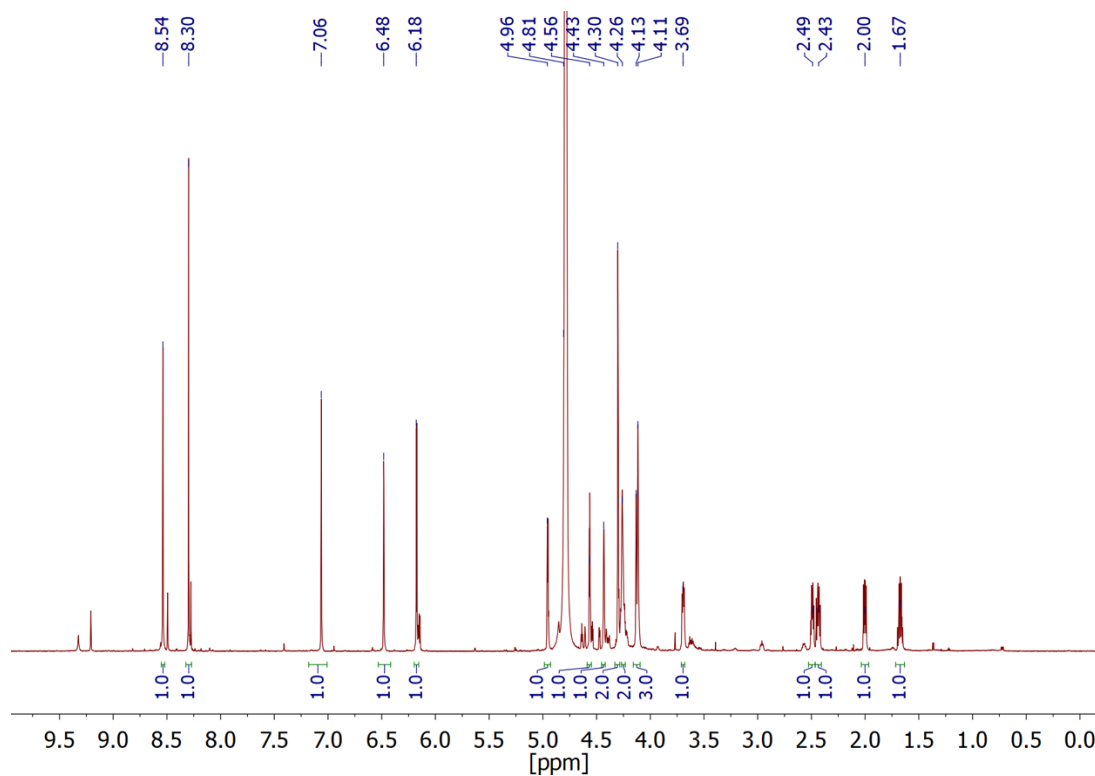
Supplementary Figure 49 ^1H , ^1H -COSY NMR spectrum of compound **4** (500 MHz, D_2O).



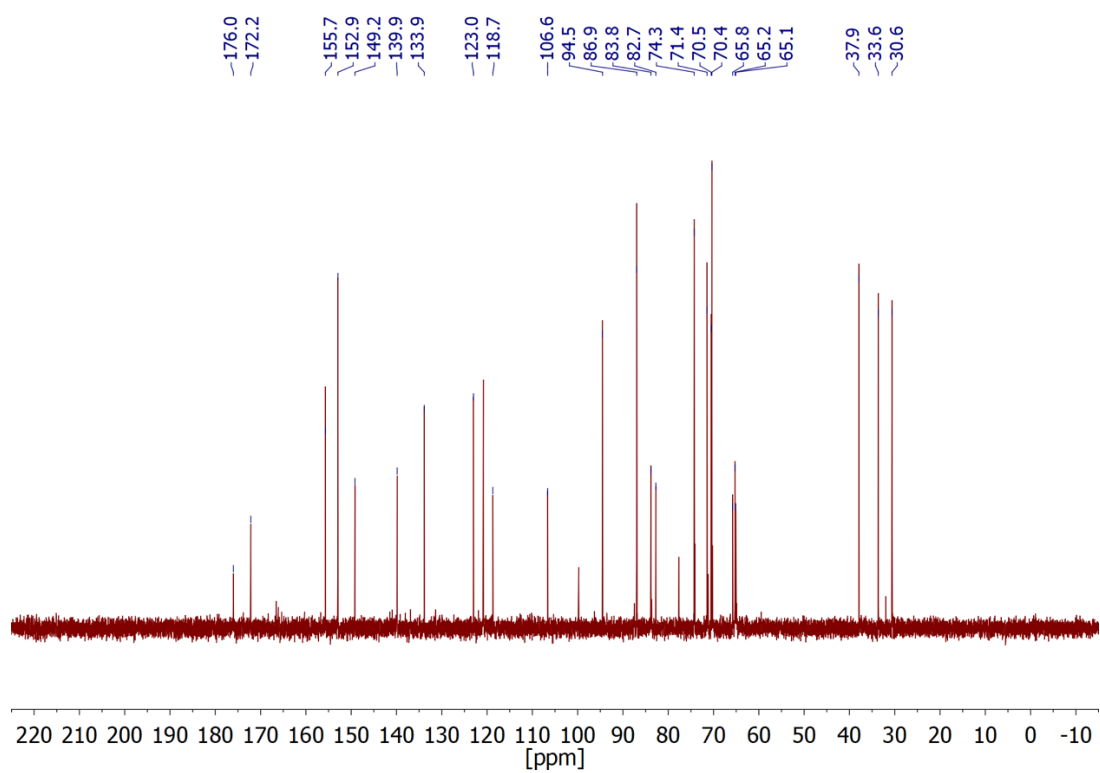
Supplementary Figure 50 ^1H , ^{13}C -HSQC spectrum of compound **4** (^1H :800 MHz, ^{13}C :200 MHz, D_2O).



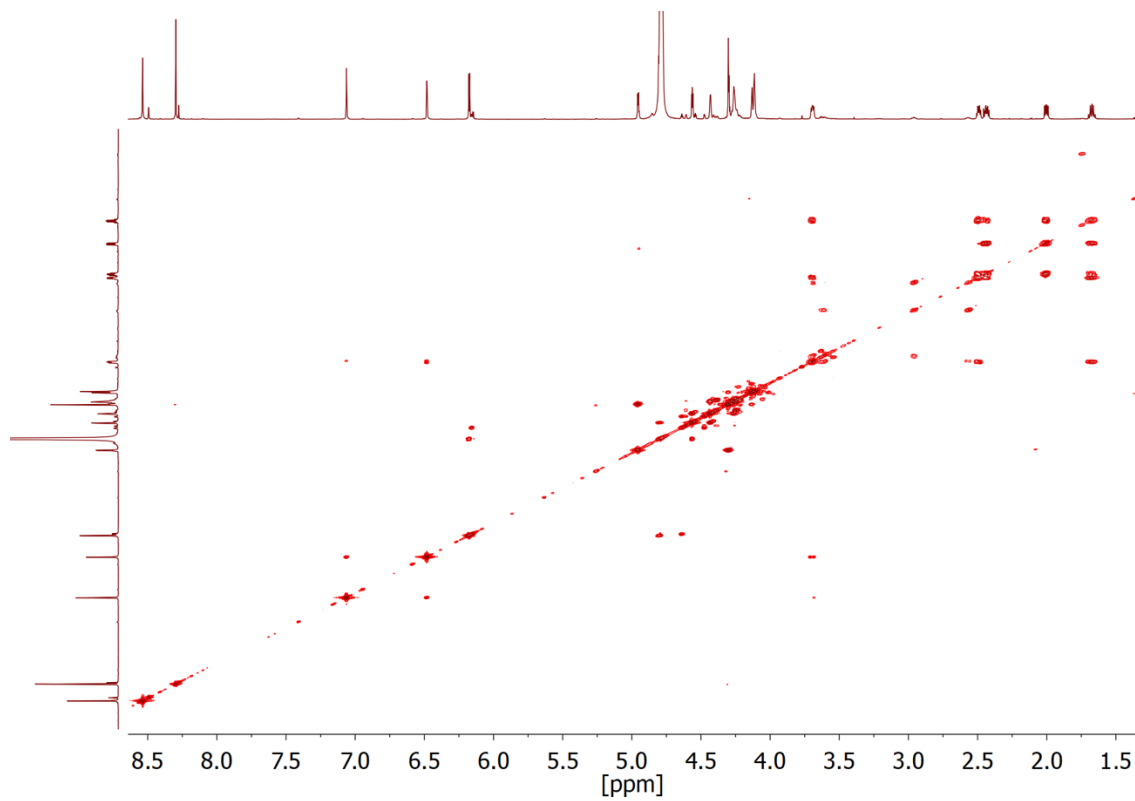
Supplementary Figure 51 ^1H , ^{13}C -HMBC spectrum of compound **4** (^1H :800 MHz, ^{13}C :200 MHz, D_2O).



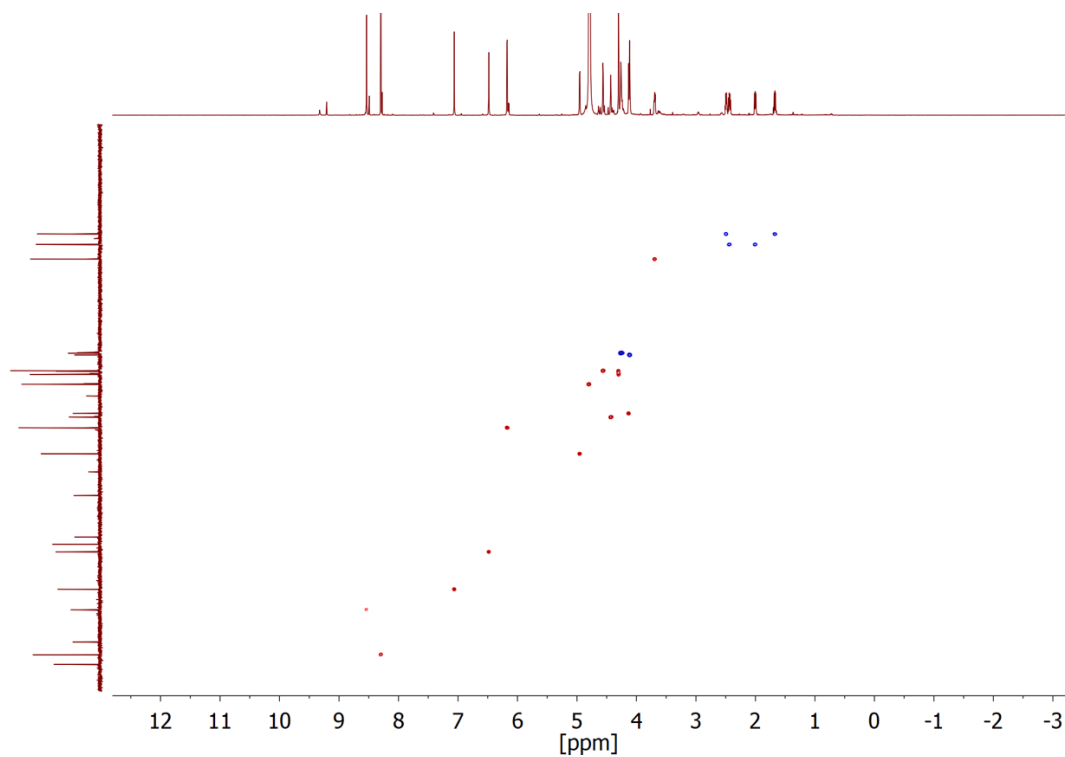
Supplementary Figure 52 ^1H -NMR spectrum of compound **7** (900 MHz, D_2O).



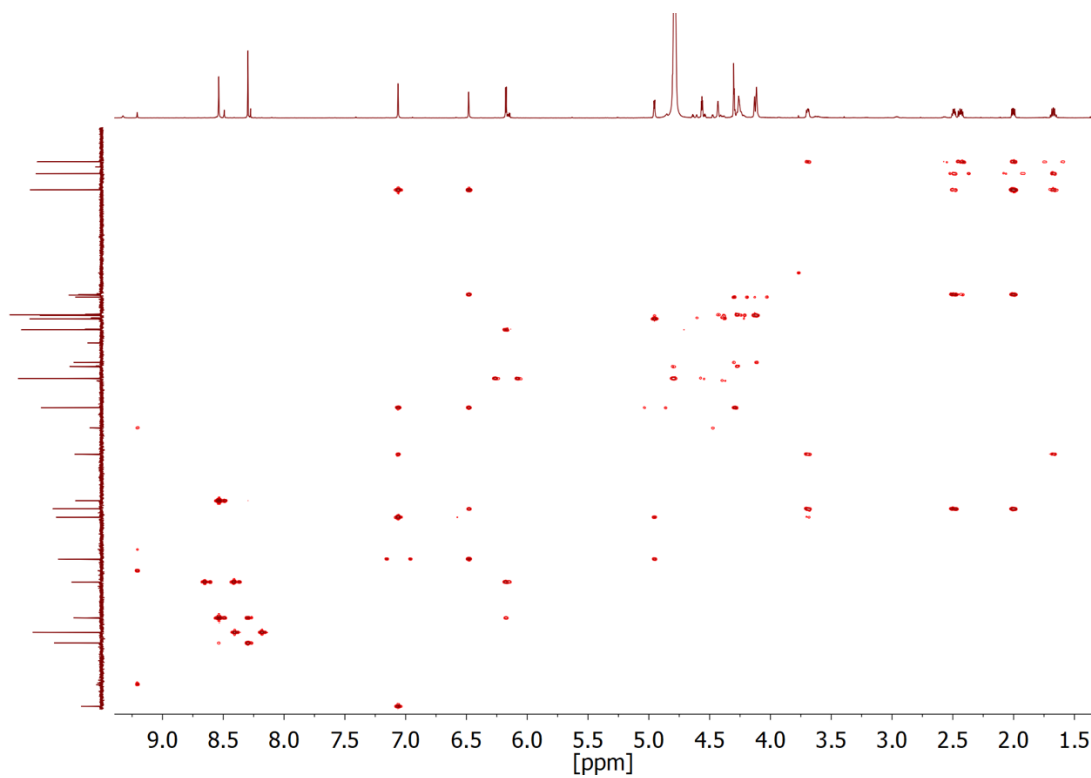
Supplementary Figure 53 ^{13}C -NMR spectrum of compound **7** (225 MHz, D_2O).



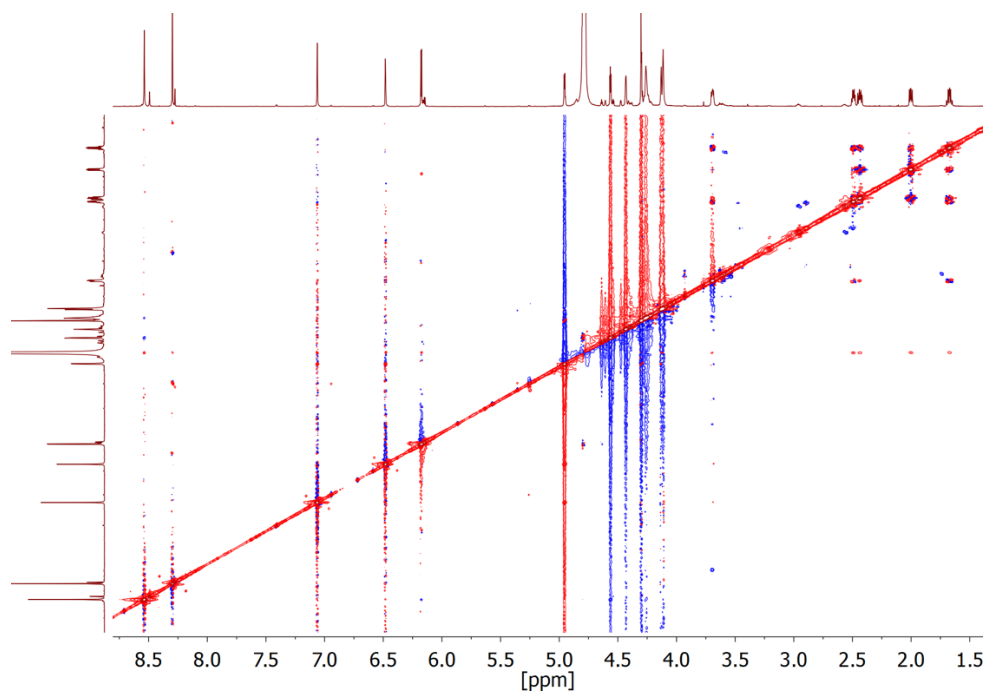
Supplementary Figure 54 ^1H , ^1H -COSY NMR spectrum of compound **7** (900 MHz, D_2O).



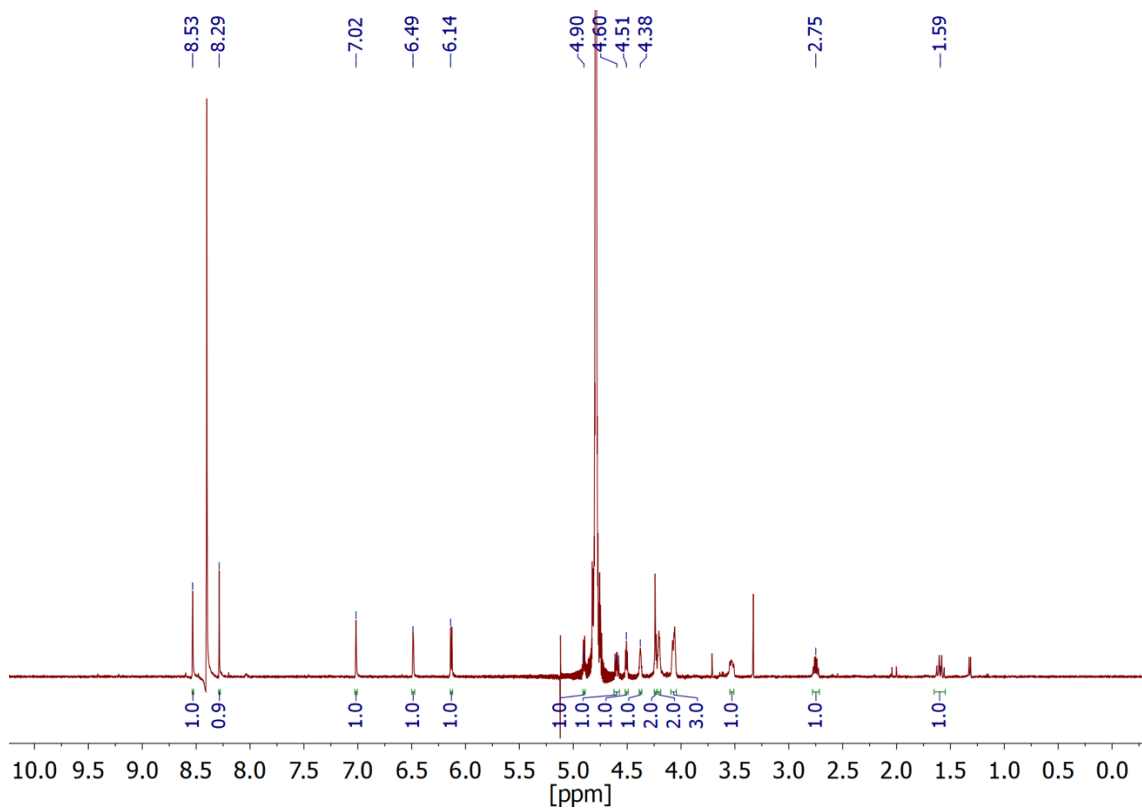
Supplementary Figure 55 ^1H , ^{13}C -HSQC NMR spectrum of compound **7** (^1H :900 MHz, ^{13}C :225 MHz, D_2O).



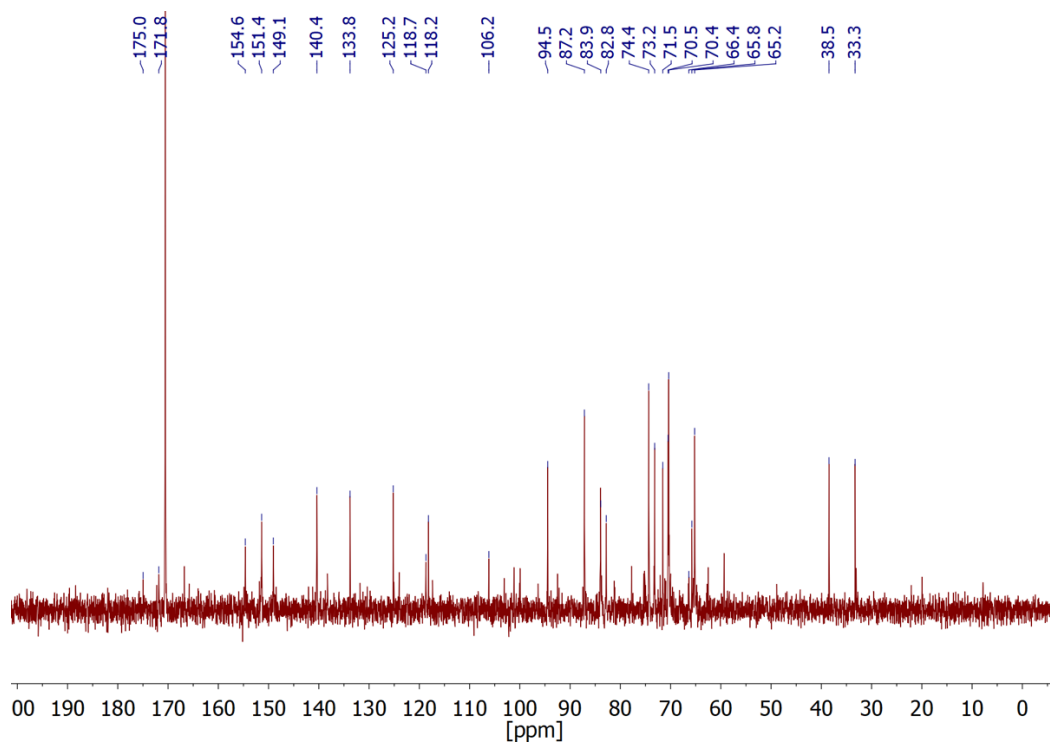
Supplementary Figure 56 ^1H , ^{13}C -HMBC NMR spectrum of compound **7** (^1H :900 MHz, ^{13}C :225 MHz, D_2O).



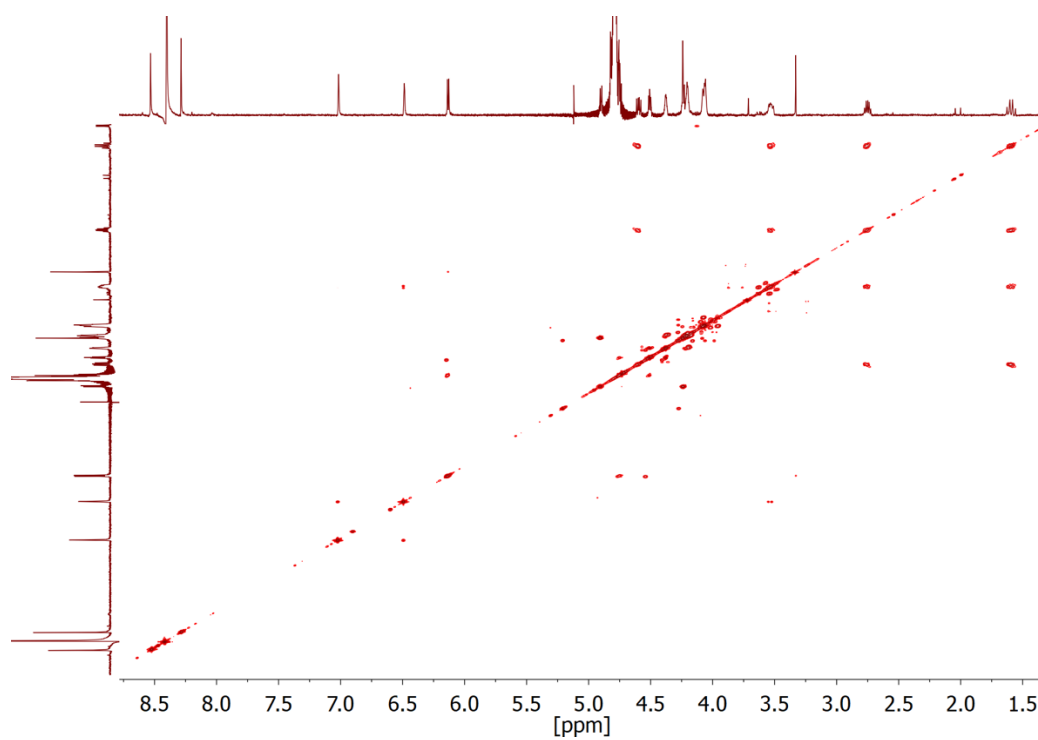
Supplementary Figure 57 ^1H , ^1H -NOESY NMR spectrum of compound **7** (900 MHz, D_2O).



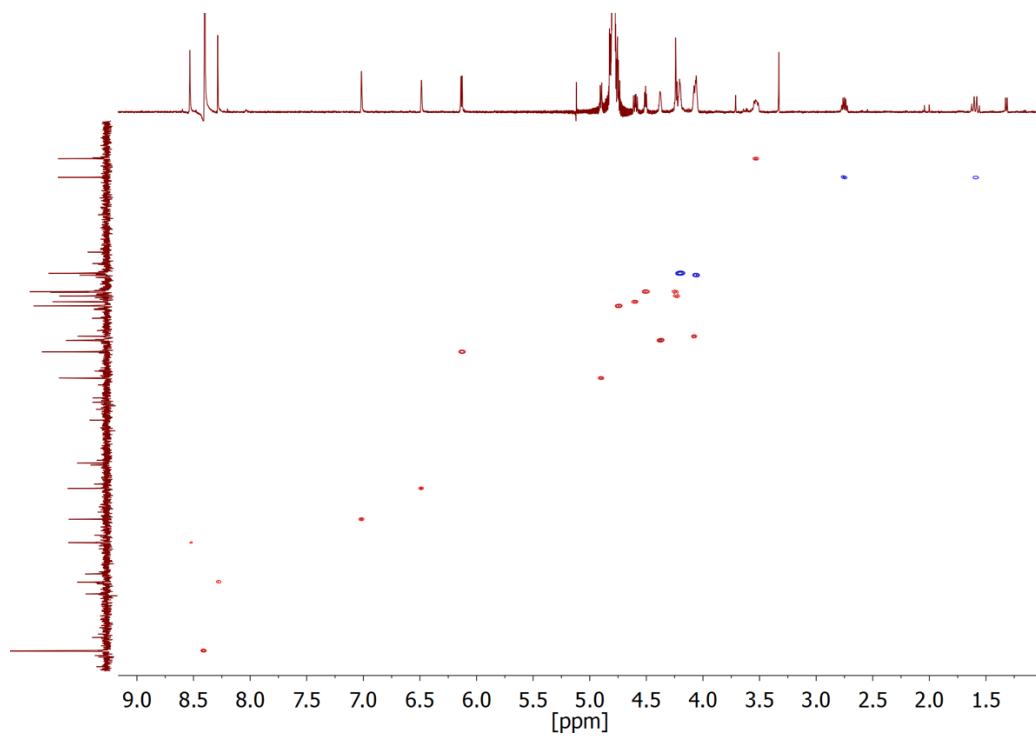
Supplementary Figure 58 ^1H -NMR spectrum of compound **8** (500 MHz, D_2O).



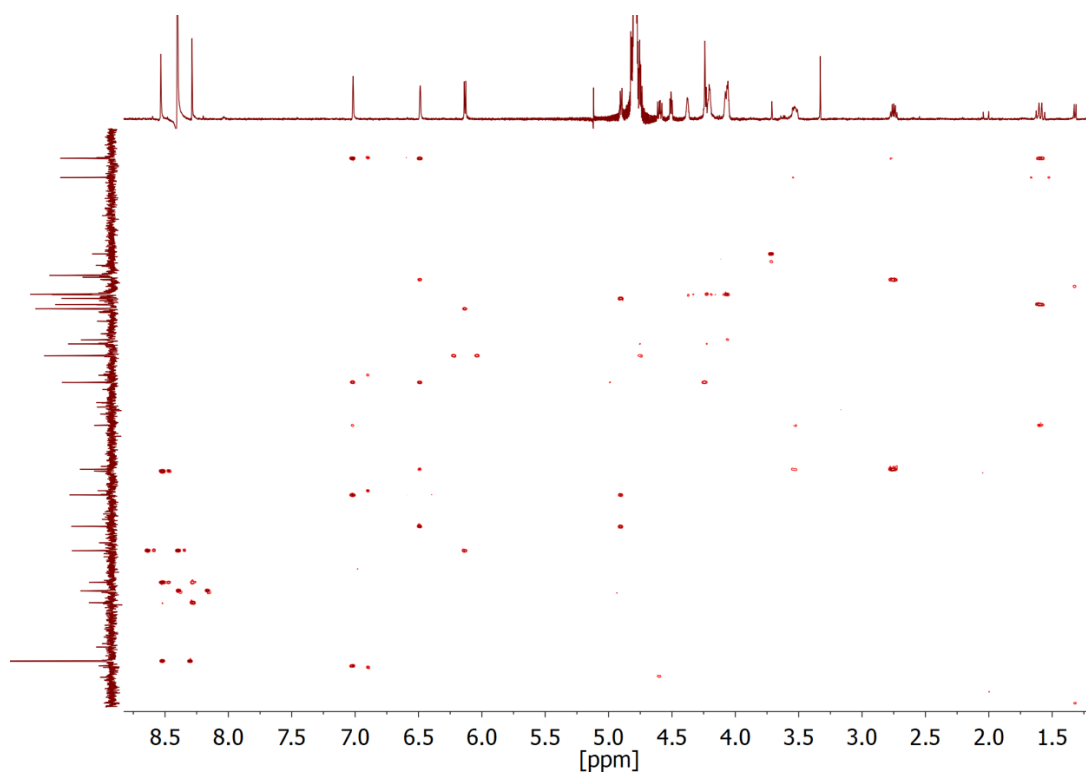
Supplementary Figure 59 ^{13}C -NMR spectrum of compound **8** (225 MHz, D_2O).



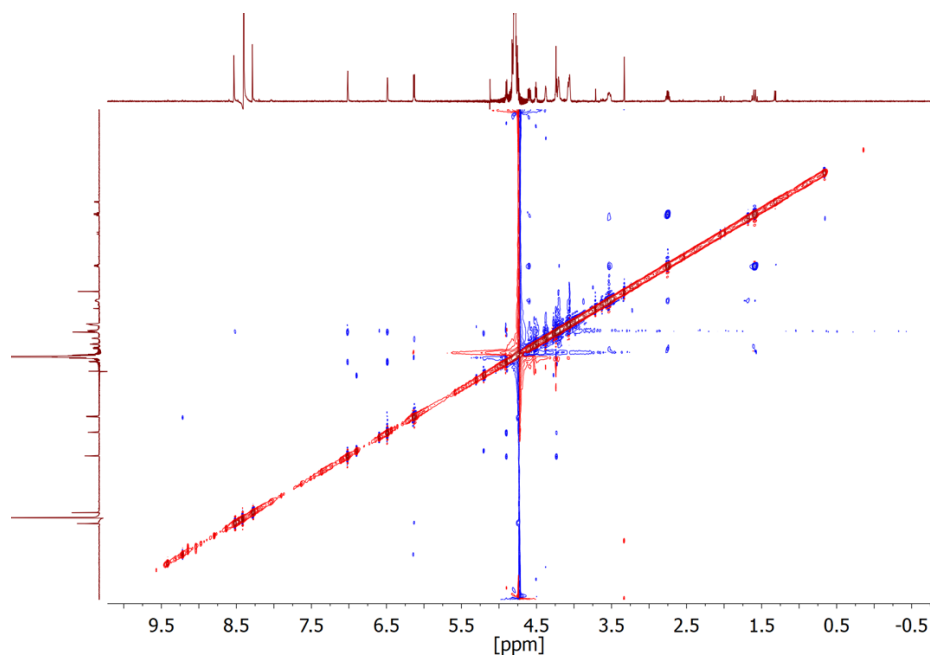
Supplementary Figure 60 ^1H , ^1H -COSY NMR spectrum of compound **8** (900 MHz, D_2O).



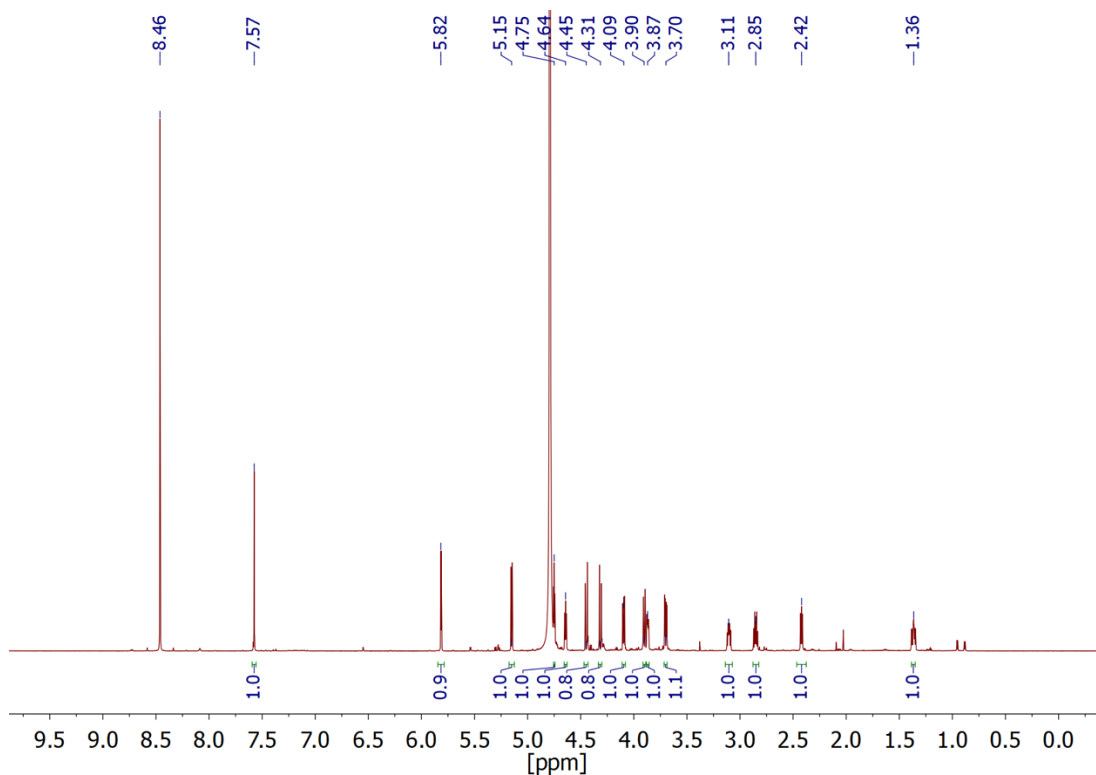
Supplementary Figure 61 ^1H , ^{13}C -HSQC NMR spectrum of compound **8** (^1H :900 MHz, ^{13}C :225 MHz, D_2O).



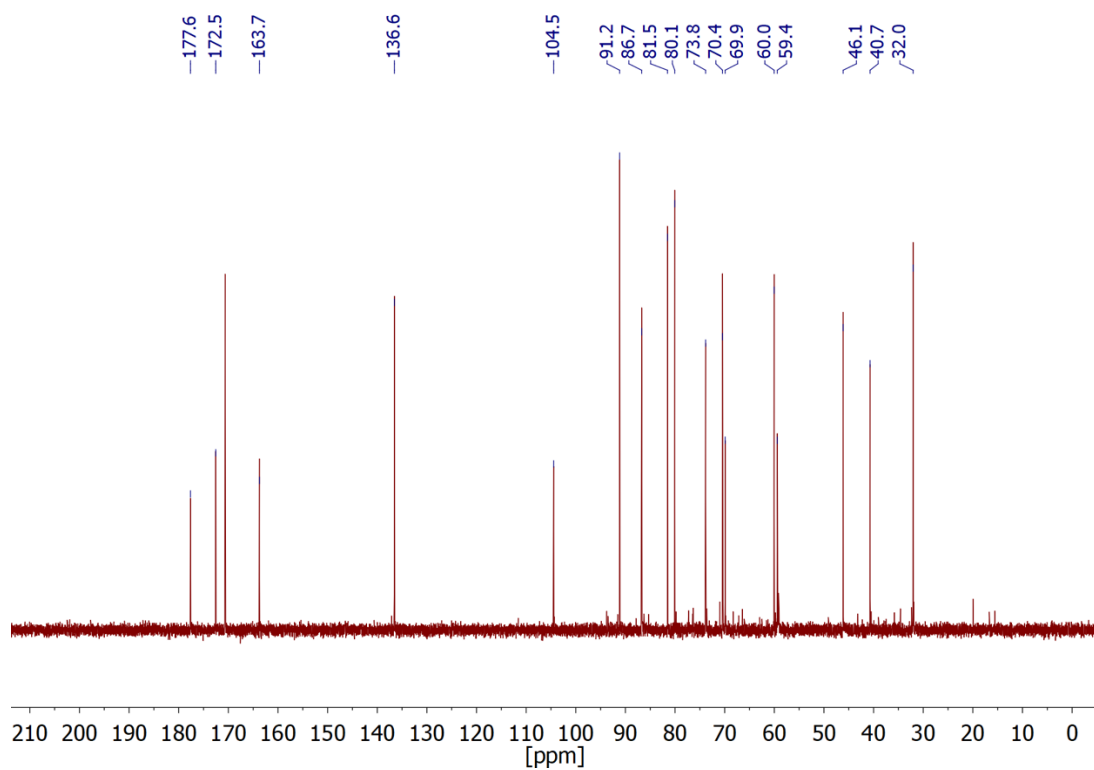
Supplementary Figure 62 ^1H , ^{13}C -HMBC NMR spectrum of compound **8** (^1H :900 MHz, ^{13}C :225 MHz, D_2O).



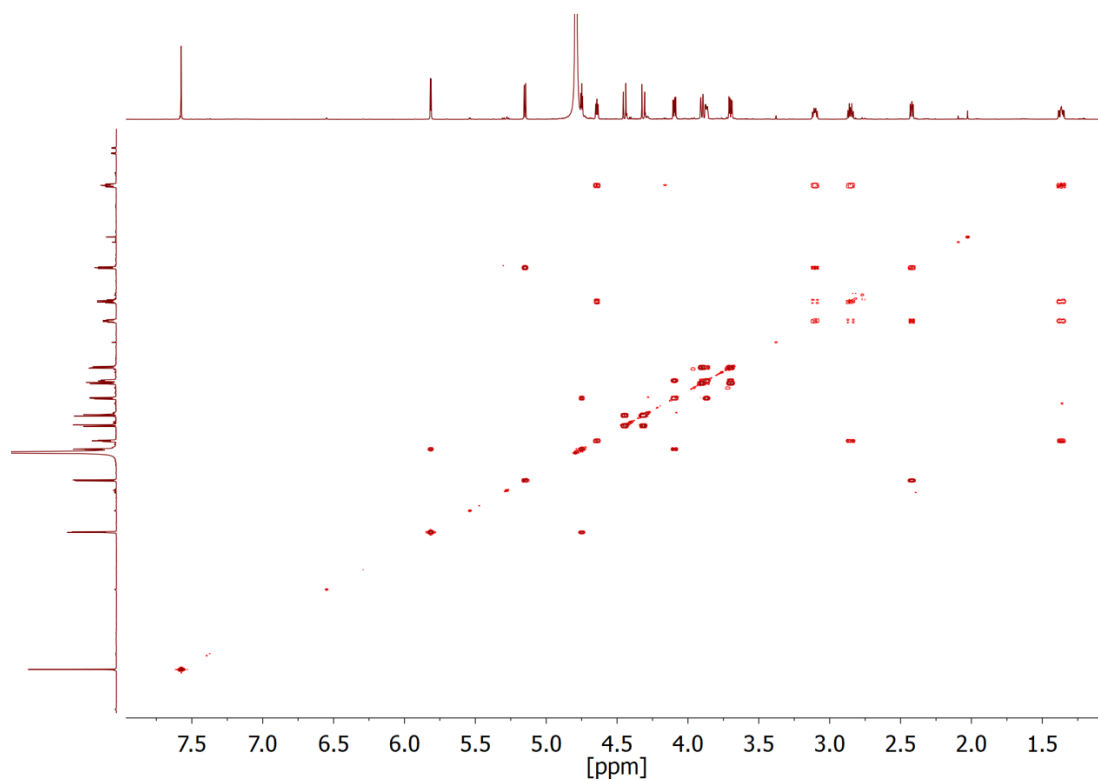
Supplementary Figure 63 $^1\text{H}, ^1\text{H}$ -ROESY spectrum of compound **8** (900 MHz, D_2O).



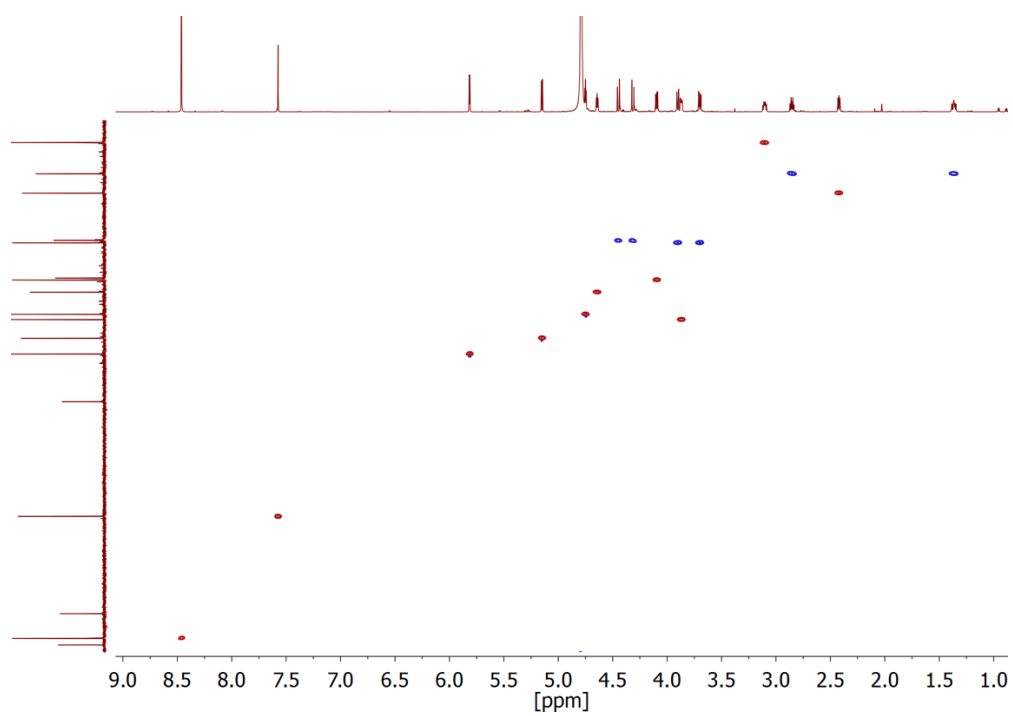
Supplementary Figure 64 ^1H -NMR spectrum of compound **S10** (800 MHz, D_2O).



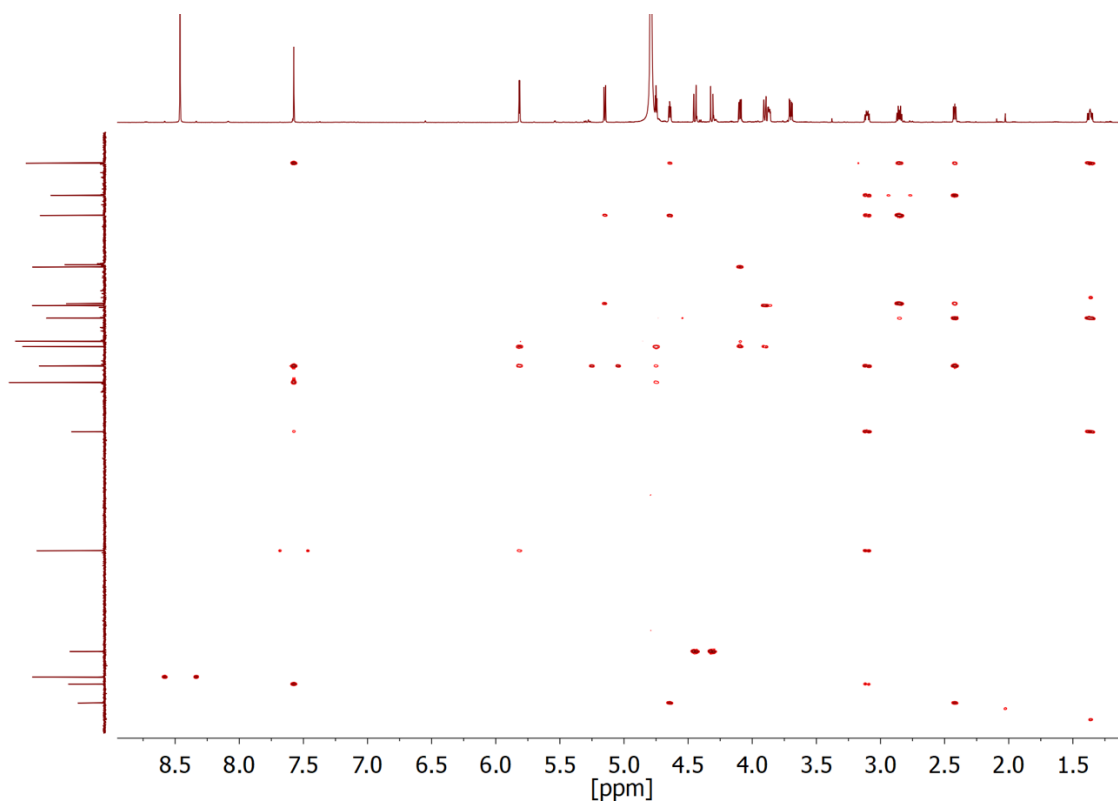
Supplementary Figure 65 ^{13}C -NMR spectrum of compound **S10** (200 MHz, D_2O).



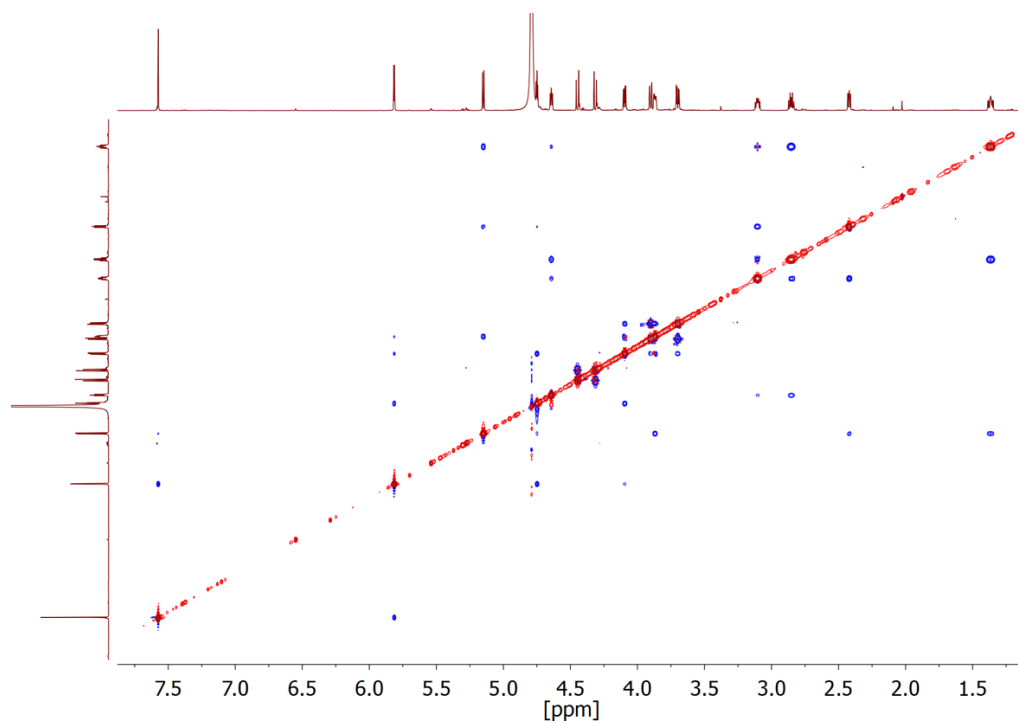
Supplementary Figure 66 ^1H , ^1H -COSY NMR spectrum of compound **S10** (800 MHz, D_2O).



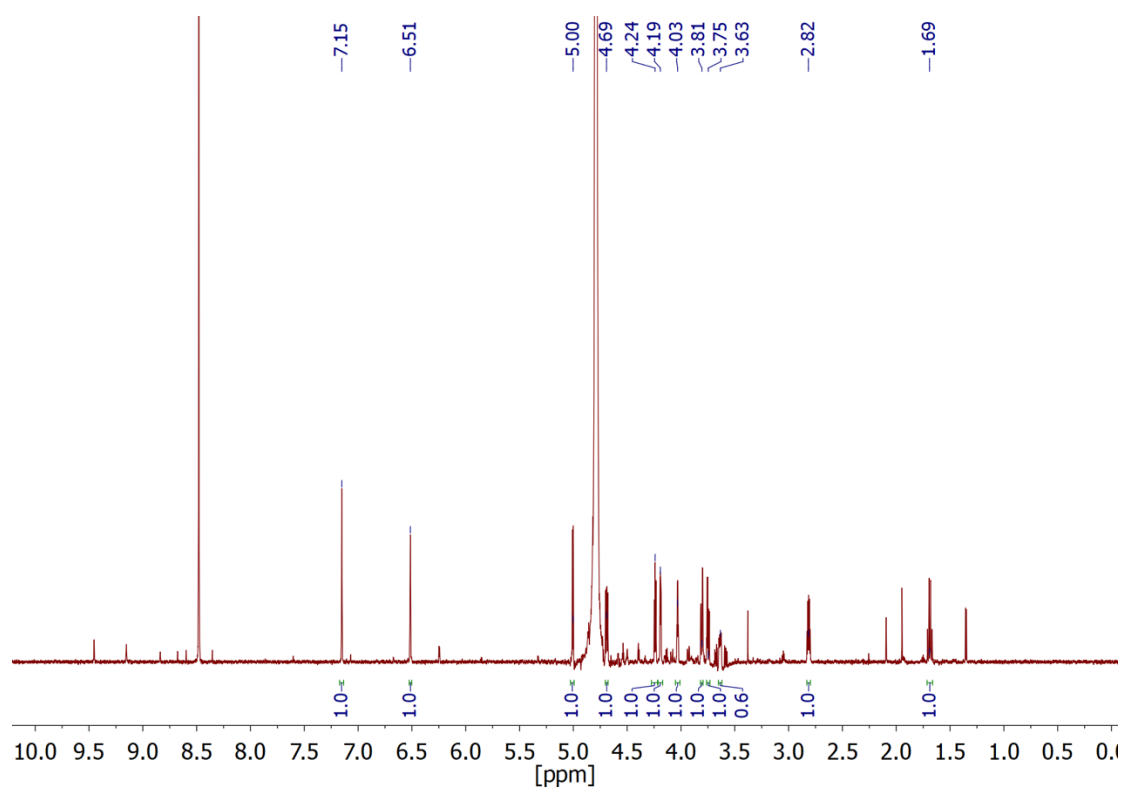
Supplementary Figure 67 ^1H , ^{13}C -HSQC NMR spectrum of compound **S10** (^1H :800 MHz, ^{13}C :200 MHz, D_2O).



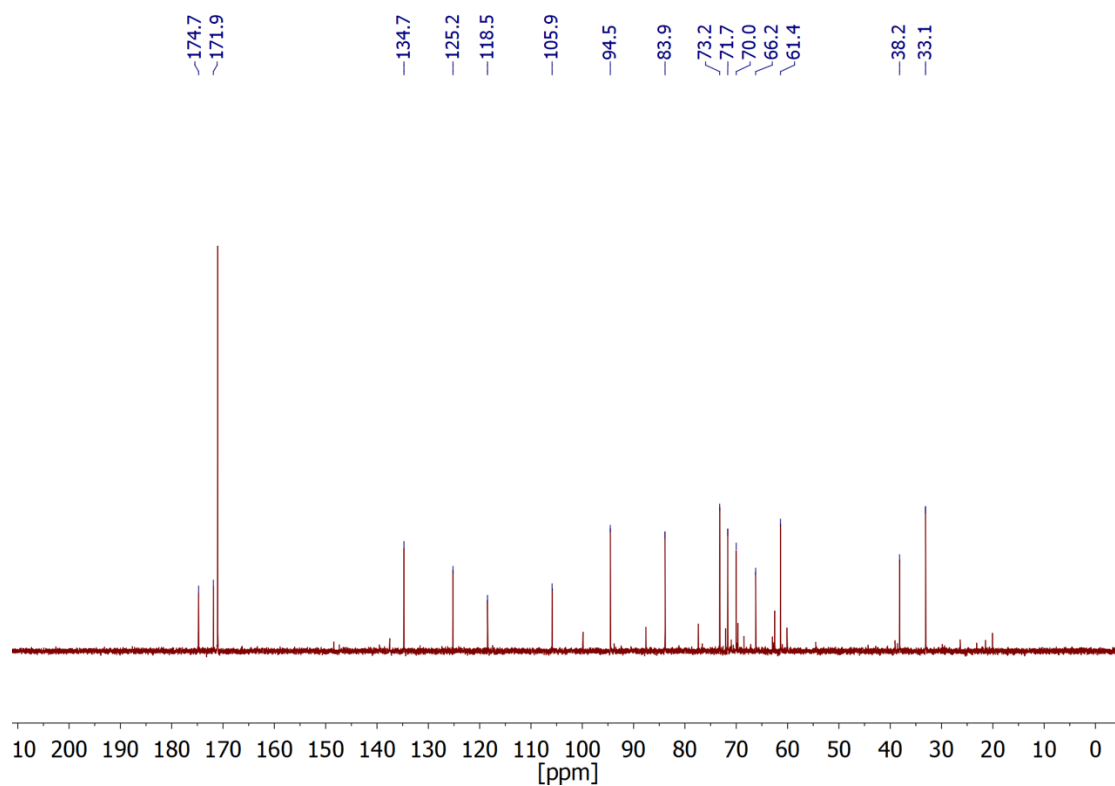
Supplementary Figure 68 ^1H , ^{13}C -HMBC NMR spectrum of compound **S10** (^1H :800 MHz, ^{13}C :200 MHz, D_2O).



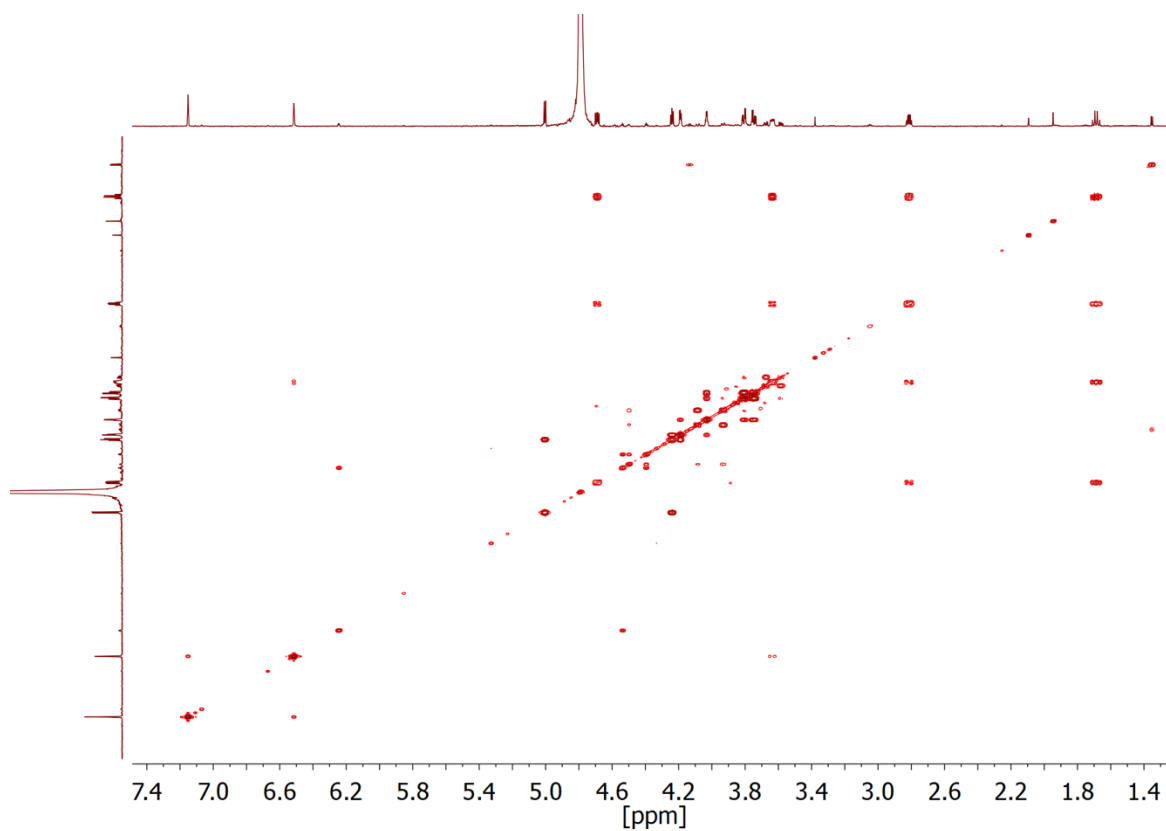
Supplementary Figure 69 ^1H , ^1H -NOESY NMR spectrum of compound **S10** (800 MHz, D_2O).



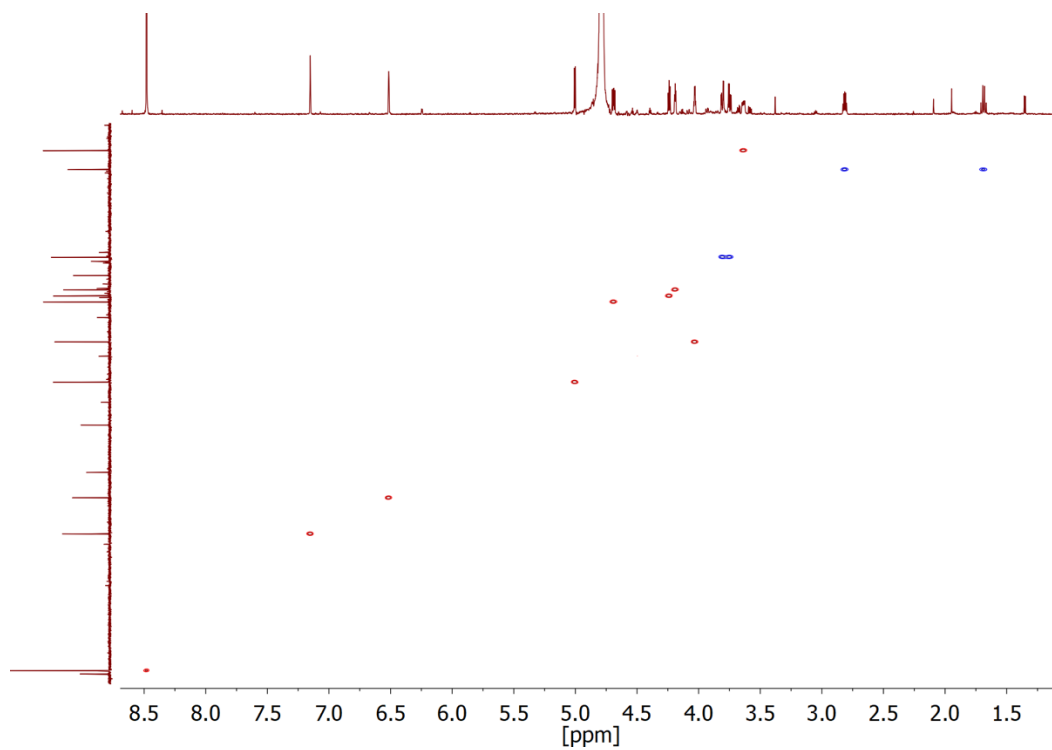
Supplementary Figure 70 ^1H -NMR spectrum of compound **S11** (800 MHz, D_2O).



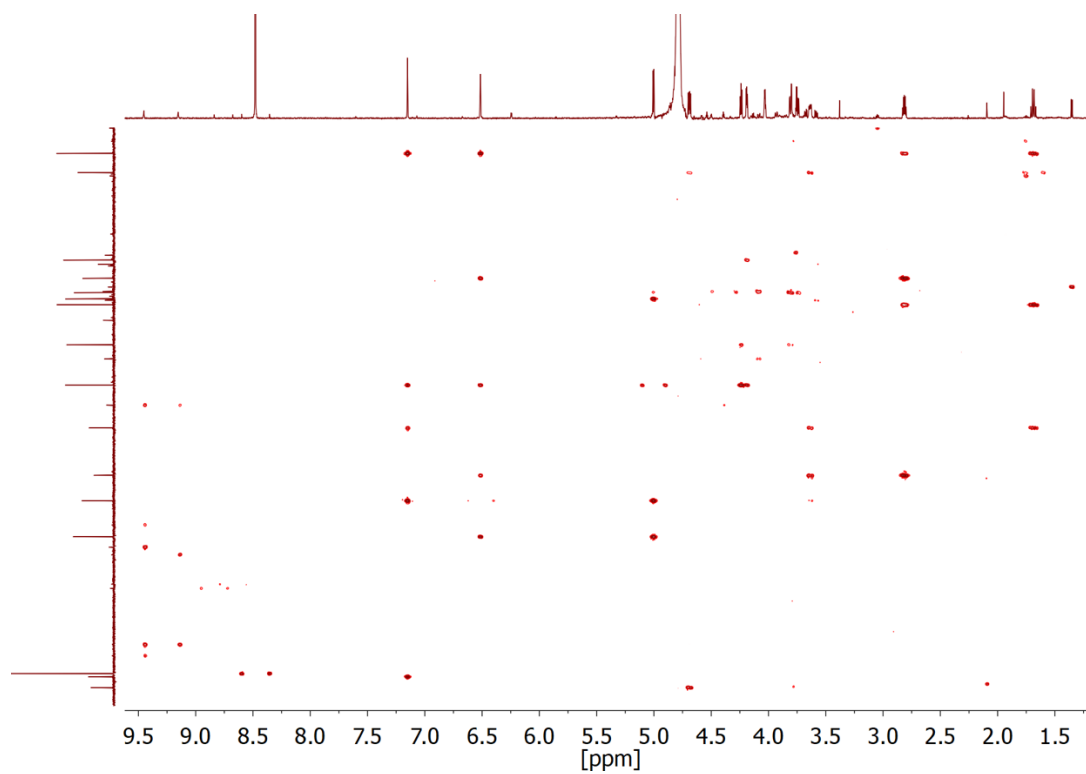
Supplementary Figure 71 ^{13}C -NMR spectrum of compound **S11** (200 MHz, D_2O).



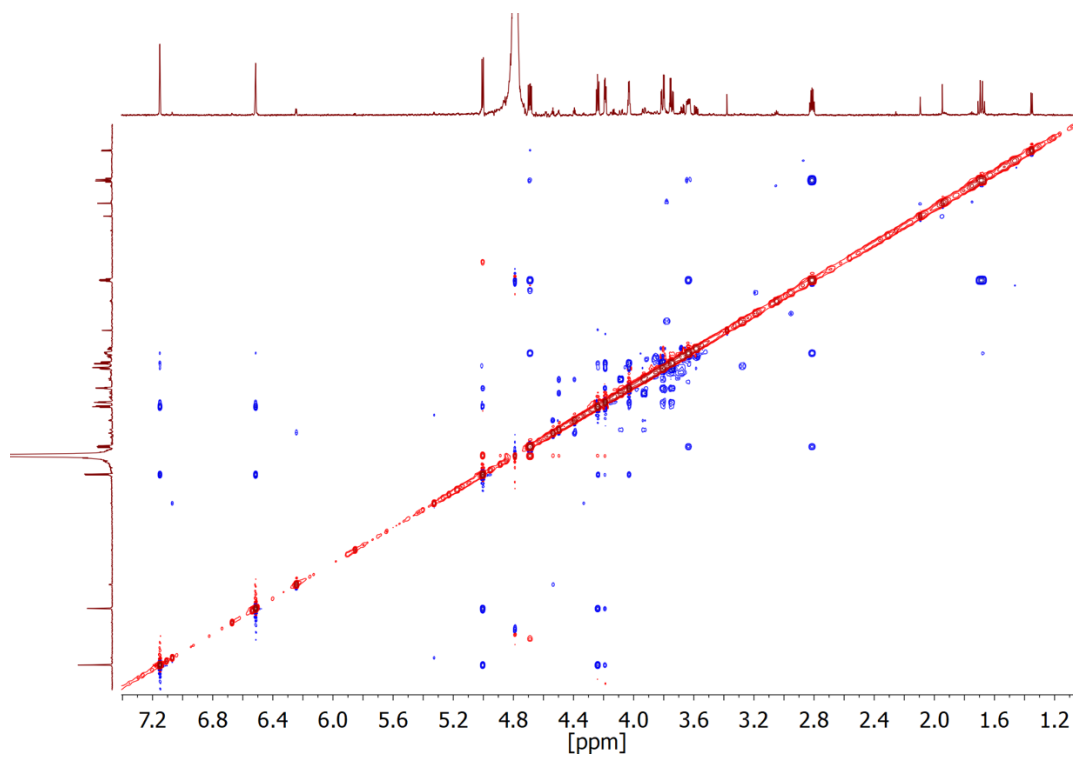
Supplementary Figure 72 $^1\text{H},^1\text{H}$ -COSY NMR spectrum of compound **S11** (800 MHz, D_2O).



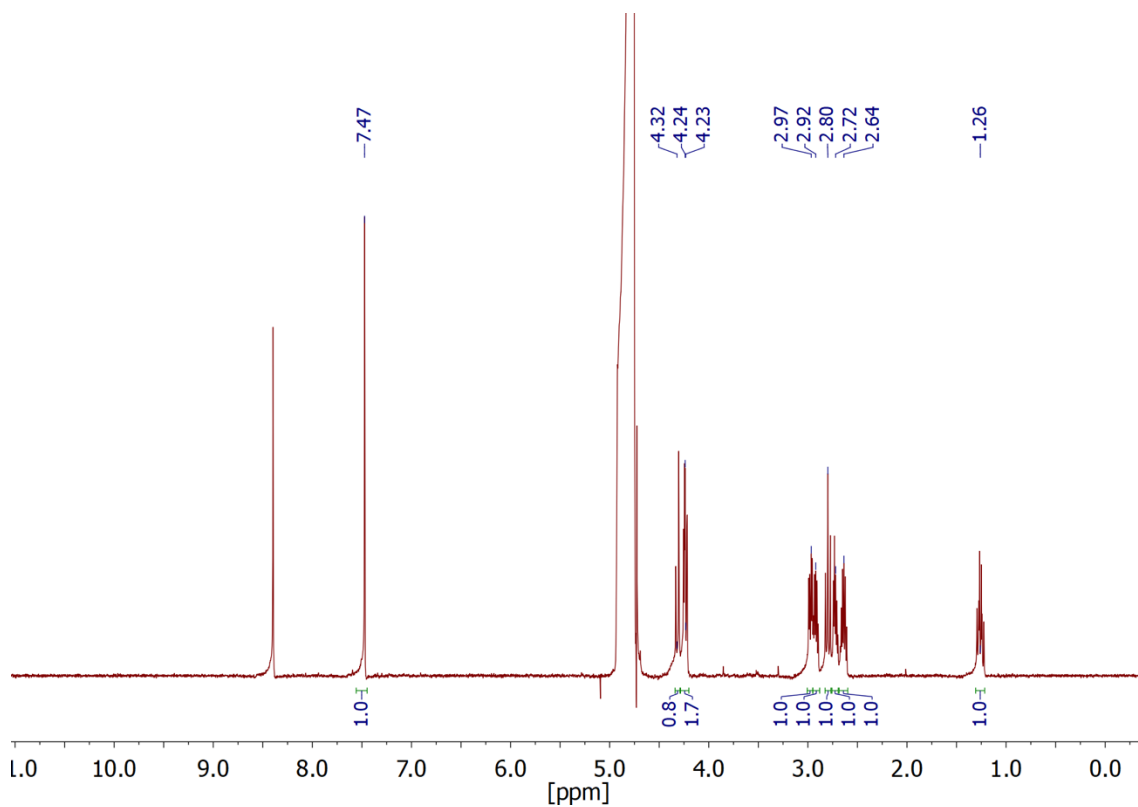
Supplementary Figure 73 ^1H , ^{13}C -HSQC NMR spectrum of compound **S11** (^1H :800 MHz, ^{13}C :200 MHz, D_2O).



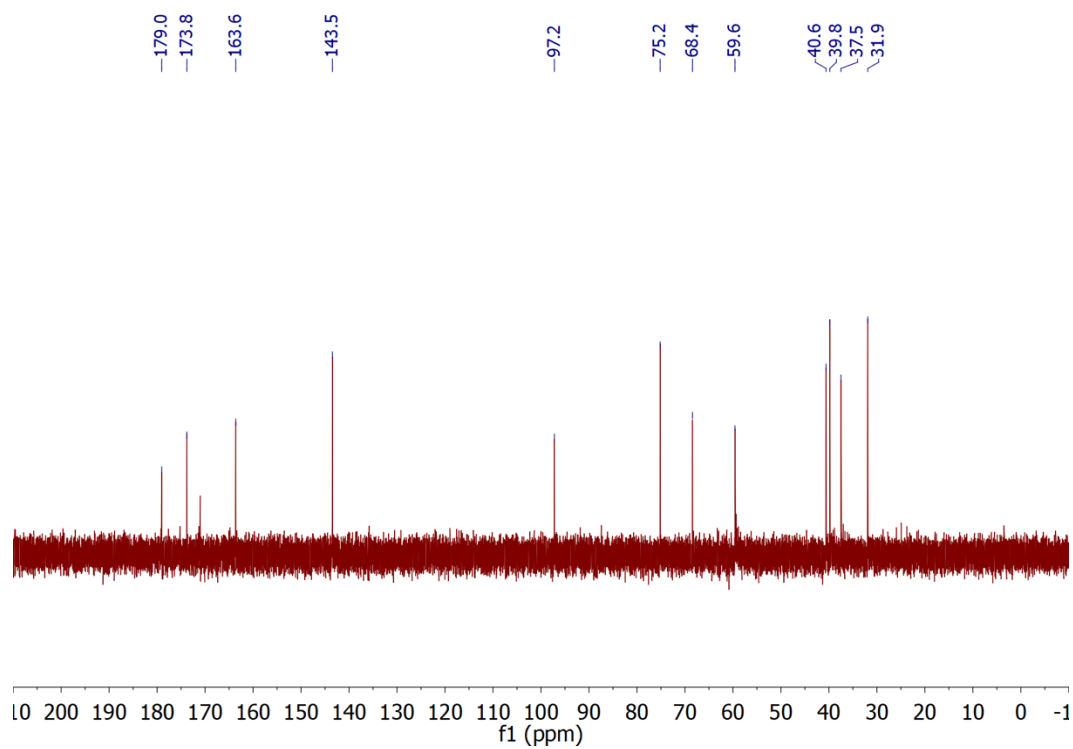
Supplementary Figure 74 ^1H , ^{13}C -HMBC NMR spectrum of compound **S11** (^1H :800 MHz, ^{13}C :200 MHz, D_2O).



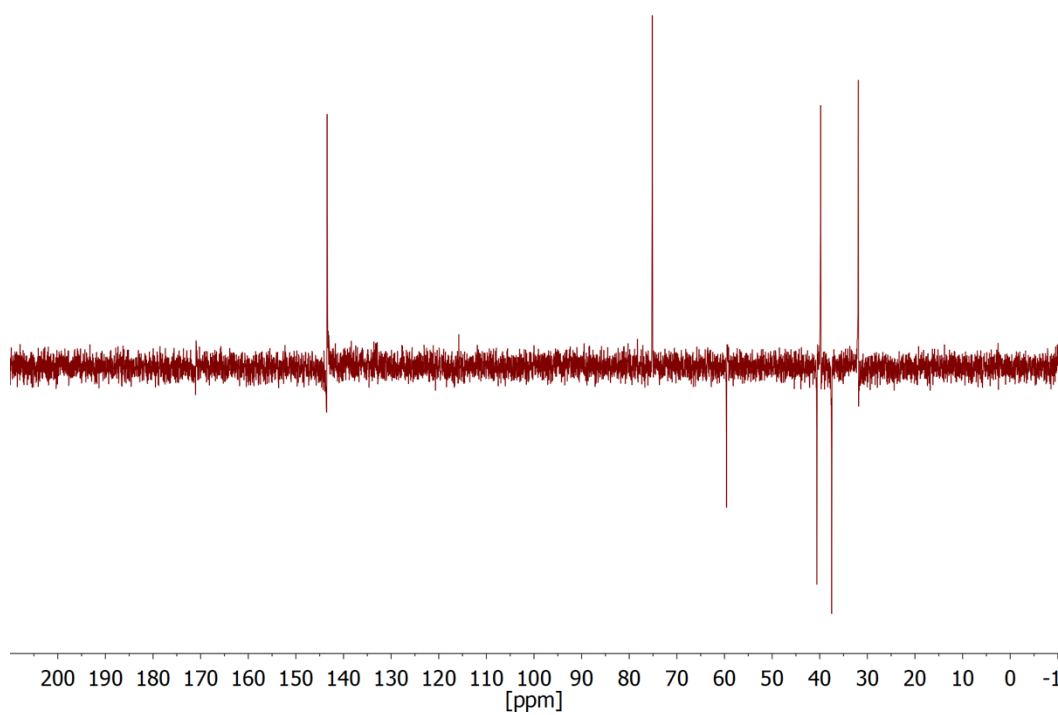
Supplementary Figure 75 $^1\text{H}, ^1\text{H}$ -NOESY spectrum of compound **S11** (800 MHz, D_2O).



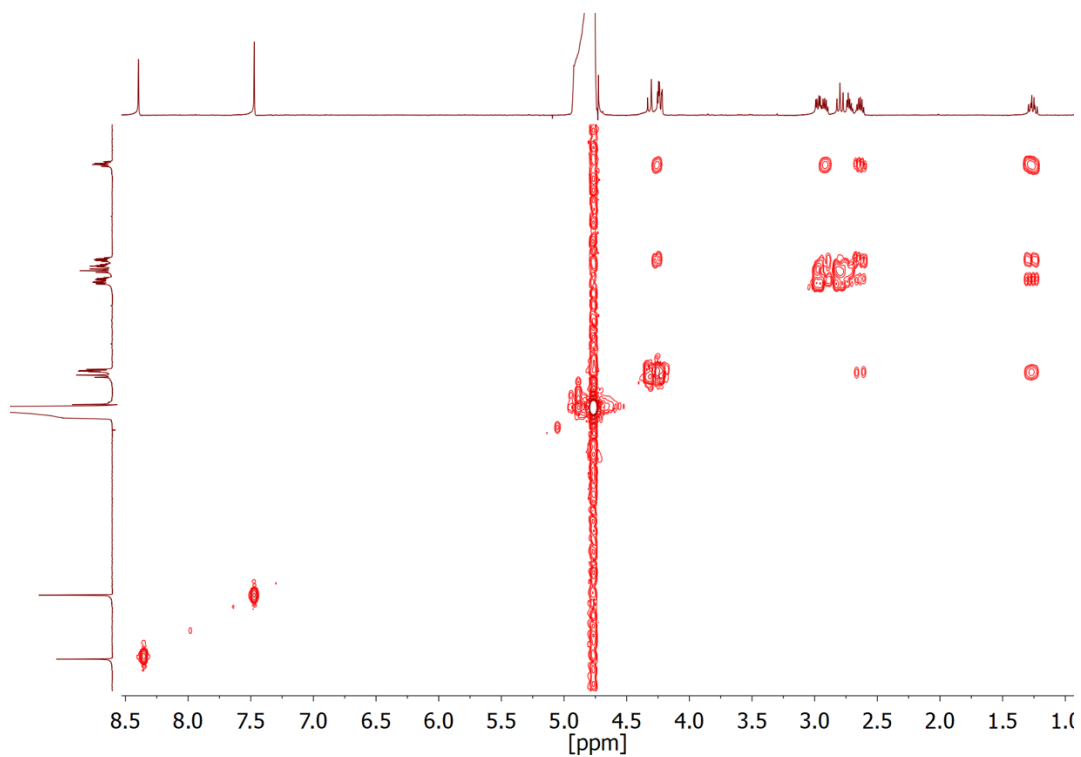
Supplementary Figure 76 ^1H -NMR spectrum of compound **12** (500 MHz, D_2O).



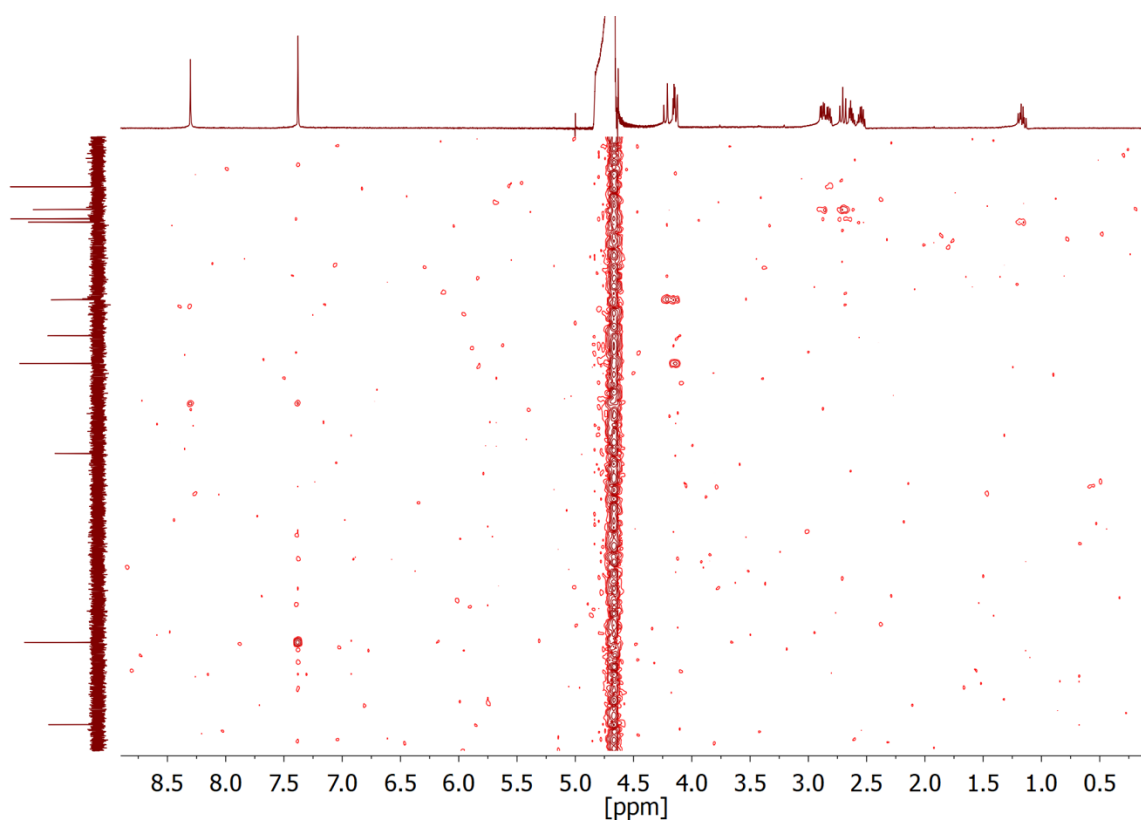
Supplementary Figure 77 ^{13}C -NMR spectrum of compound **12** (125 MHz, D_2O).



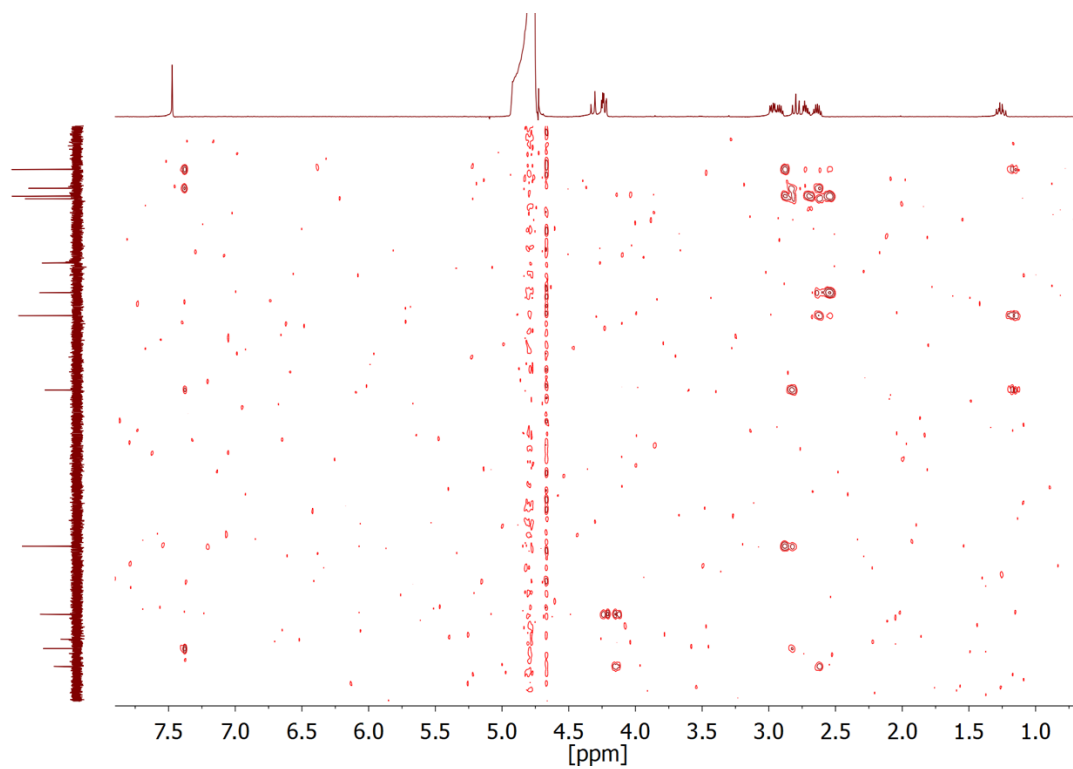
Supplementary Figure 78 ^{13}C -DEPT (145) NMR spectrum of compound **12** (125 MHz, D_2O).



Supplementary Figure 79 ^1H , ^1H -COSY NMR spectrum of compound **12** (500 MHz, D_2O).



Supplementary Figure 80 ^1H , ^{13}C -HSQC NMR spectrum of compound **12** (^1H :500 MHz, ^{13}C :125 MHz, D_2O).



Supplementary Figure 81 ^1H , ^{13}C -HMBC NMR spectrum of compound **12** (^1H :500 MHz, ^{13}C :125 MHz, D_2O).

4. Reference

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