Supplementary Information

Enzymatic reconstruction of the saframycin core scaffold defines dual Pictet-Spengler mechanisms

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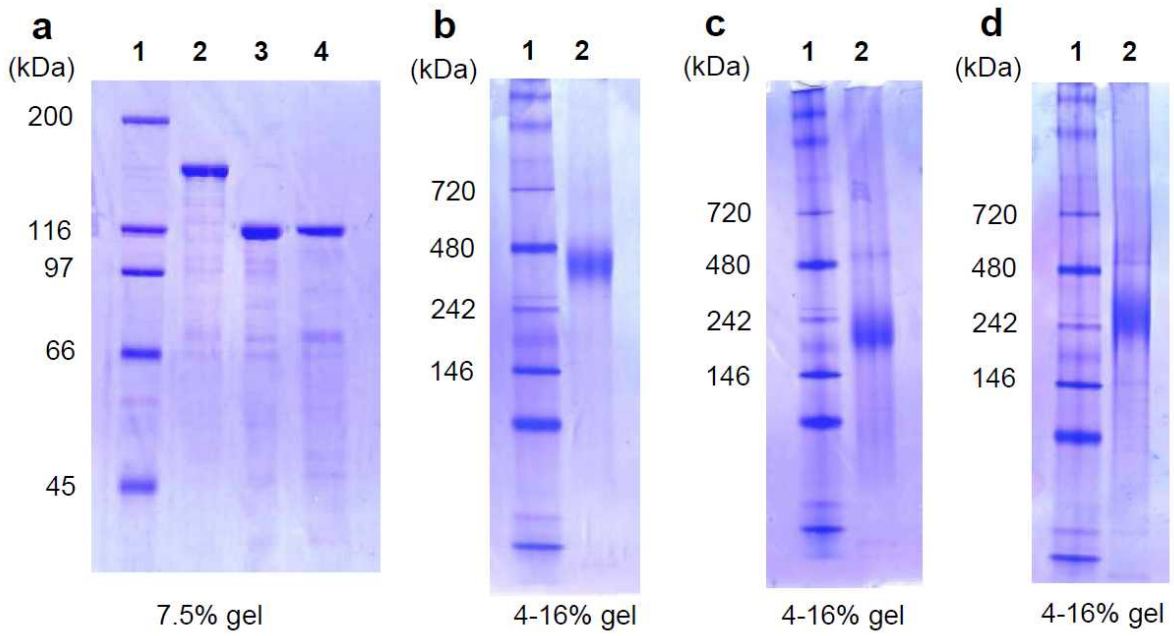
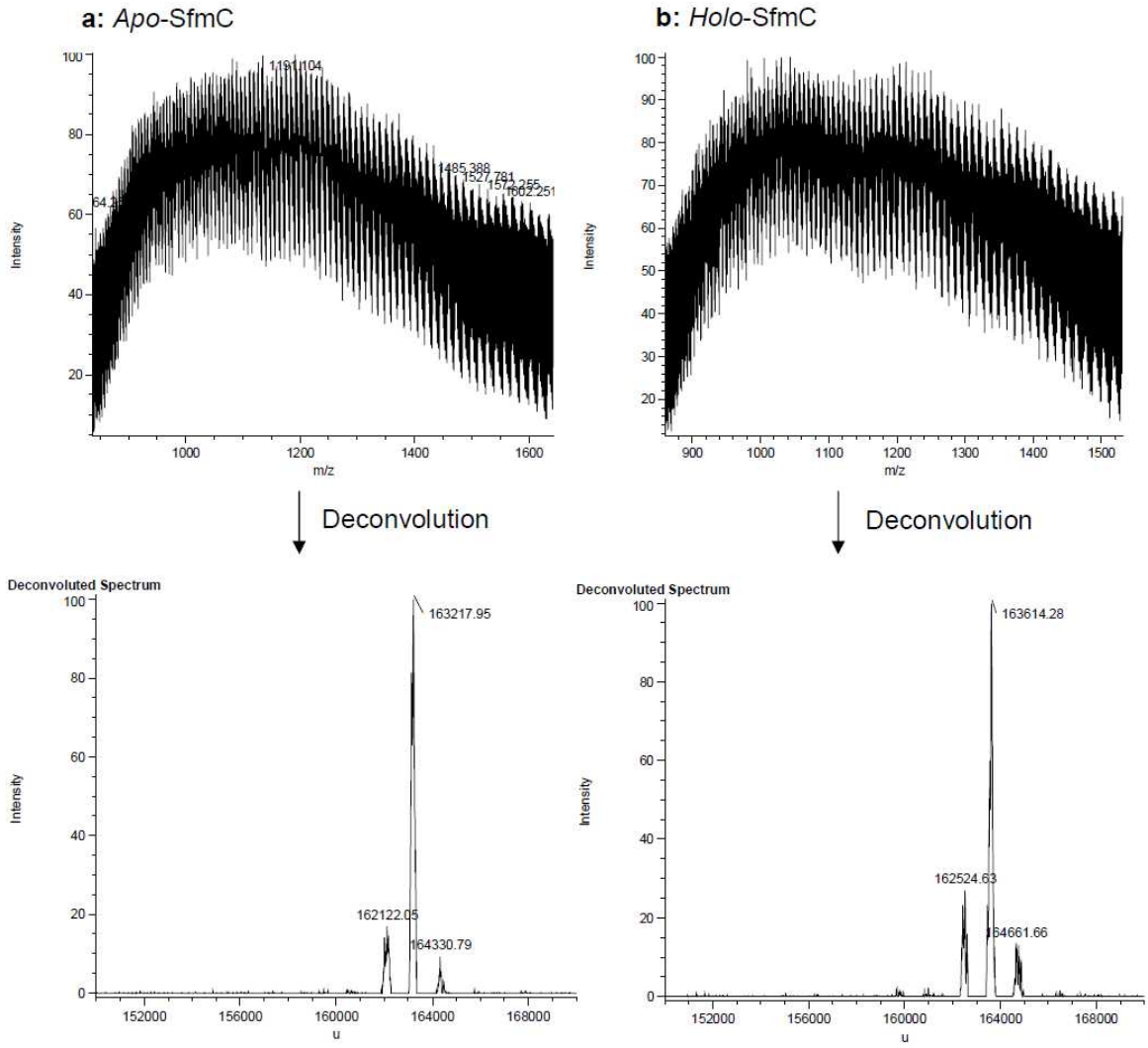
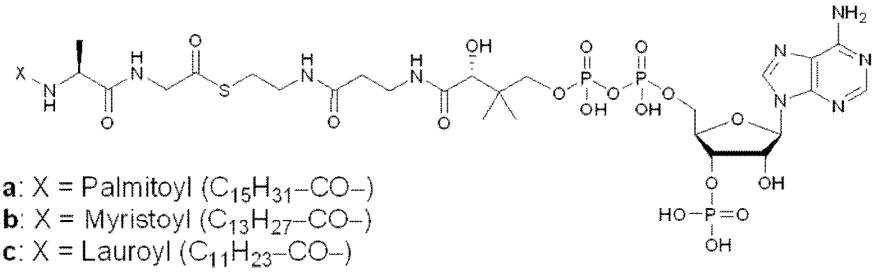
Supplementary Results



Supplementary Figure 1. SDS-PAGE and Blue-Native PAGE analysis of SfmC, A-PCP-R tridomain and C-A-PCP tridomain. (**a**) SDS-PAGE (7.5% gel): lane 1; SDS-PAGE standard, broad (Bio-Rad), lane 2; SfmC [calcd. AvMW: 163 kDa], lane 3; A-PCP-R tridomain [calcd. AvMW: 117 kDa], lane 4; C-A-PCP tridomain [calcd. AvMW: 115 kDa]. (**b**) BN-PAGE analysis (4-16% gradient gel) of SfmC: lane 1; NativeMark unstained protein standard, lane 2; native SfmC, homodimer [AvMW: 326 kDa]. (**c**) BN-PAGE analysis (4-16% gradient gel) of A-PCP-R tridomain: lane 1; NativeMark unstained protein standard, lane 2; native A-PCP-R tridomain, homodimer [AvMW: 234 kDa]. (**d**) BN-PAGE analysis (4-16% gradient gel) of C-A-PCP tridomain: lane 1; NativeMark unstained protein standard, lane 2; native C-A-PCP tridomain, homodimer [AvMW: 230 kDa].



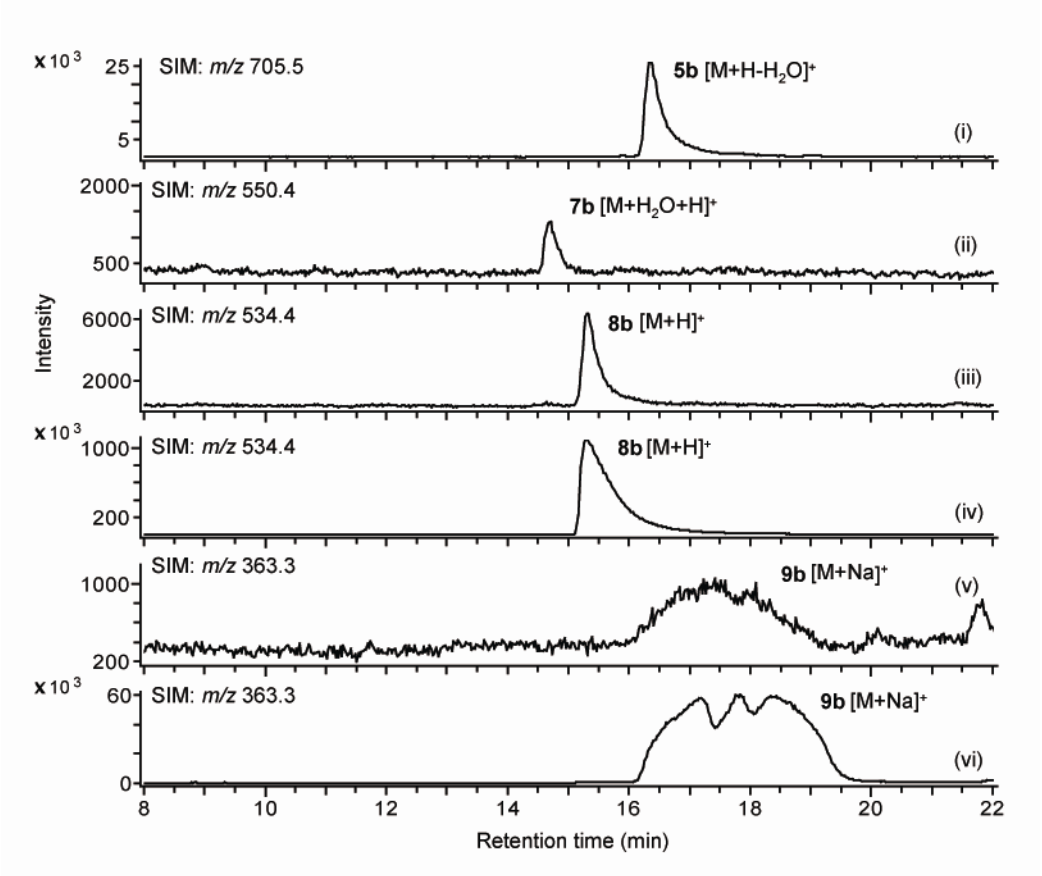
Supplementary Figure 2. ESI-MS analysis of *apo*- and *holo*-SfmC. (**a**) ESI-MS spectra of *apo*-SfmC [calcd. mass; 163175.8 Da]. (**b**) ESI-MS spectra of *holo*-SfmC [calcd. mass; 163516.1 Da].



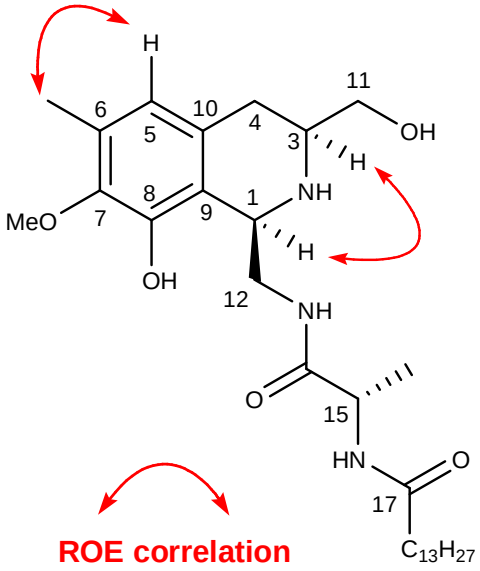
4a: X = Palmitoyl ($C_{15}H_{31}$–CO–)
4b: X = Myristoyl ($C_{13}H_{27}$–CO–)
4c: X = Lauroyl ($C_{11}H_{23}$–CO–)
4d: X = Acetyl (C_1H_3–CO–)
4e: X = H

Compound	Calculated Mass	Observed Mass
	$[M-H]^-$ [Da]	$[M-H]^-$ [Da]
4a: X = Palmitoyl ($C_{15}H_{31}$)	1132.3962	1132.3971
4b: X = Myristoyl ($C_{13}H_{27}$)	1104.3649	1104.3663
4c: X = Lauroyl ($C_{11}H_{23}$)	1076.3336	1076.3363
Compound	Calculated Mass	Observed Mass
	$[M+H]^+$ [Da]	$[M+H]^+$ [Da]
4d: X = Acetyl (C_1H_3)	938.1916	938.1873
4e: X = H	896.1811	896.1856

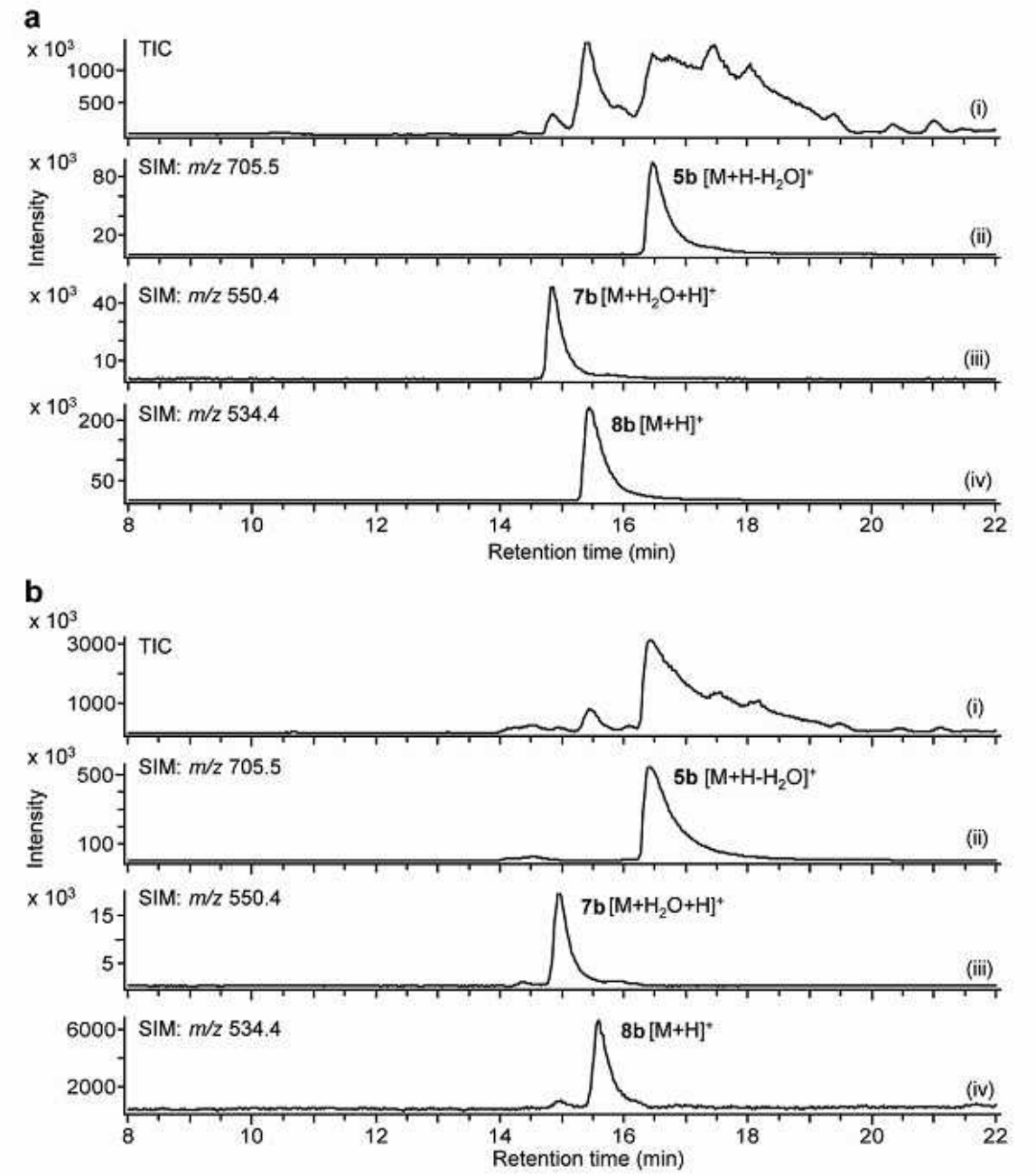
Supplementary Figure 3. Characterization of synthetic dipeptidyl-*S*-CoA thioesters **4a** - **4e**.



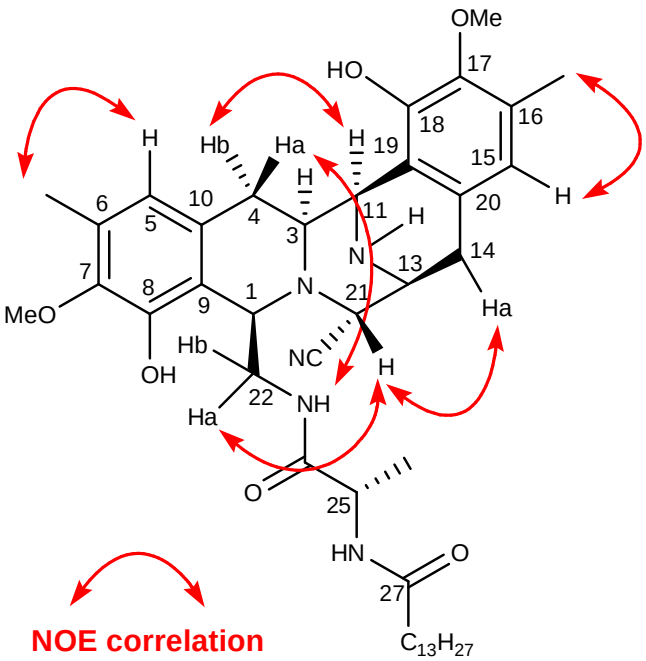
Supplementary Figure 4. SfmC-catalyzed reaction with *N*-myristoyldipeptidyl-CoA thioester **4b**. (i) Chromatogram in the SIM mode [*m/z* 705.5 ± 0.1] for detection of **5b** ($[M+H-H_2O]^+$ = 705.5). (ii) Chromatogram in the SIM mode [*m/z* 550.4 ± 0.1] for detection of **7b** ($[M+H_2O+H]^+$ = 550.4). (iii) Chromatogram in the SIM mode [*m/z* 534.4 ± 0.1] for detection of **8b** ($[M+H]^+$ = 534.4). (iv) Synthetic standard of **8b**. (v) To detect **9b**, the reaction was performed in the absence of **2**. Chromatogram in the SIM mode [*m/z* 363.3 ± 0.1] for detection of **9b** ($[M+Na]^+$ = 363.3). (vi) Synthetic standard of **9b**. SIM: selected ion monitoring. MS/MS spectra of **5b**, **7b** and **8b** are shown in **Fig. 2**.



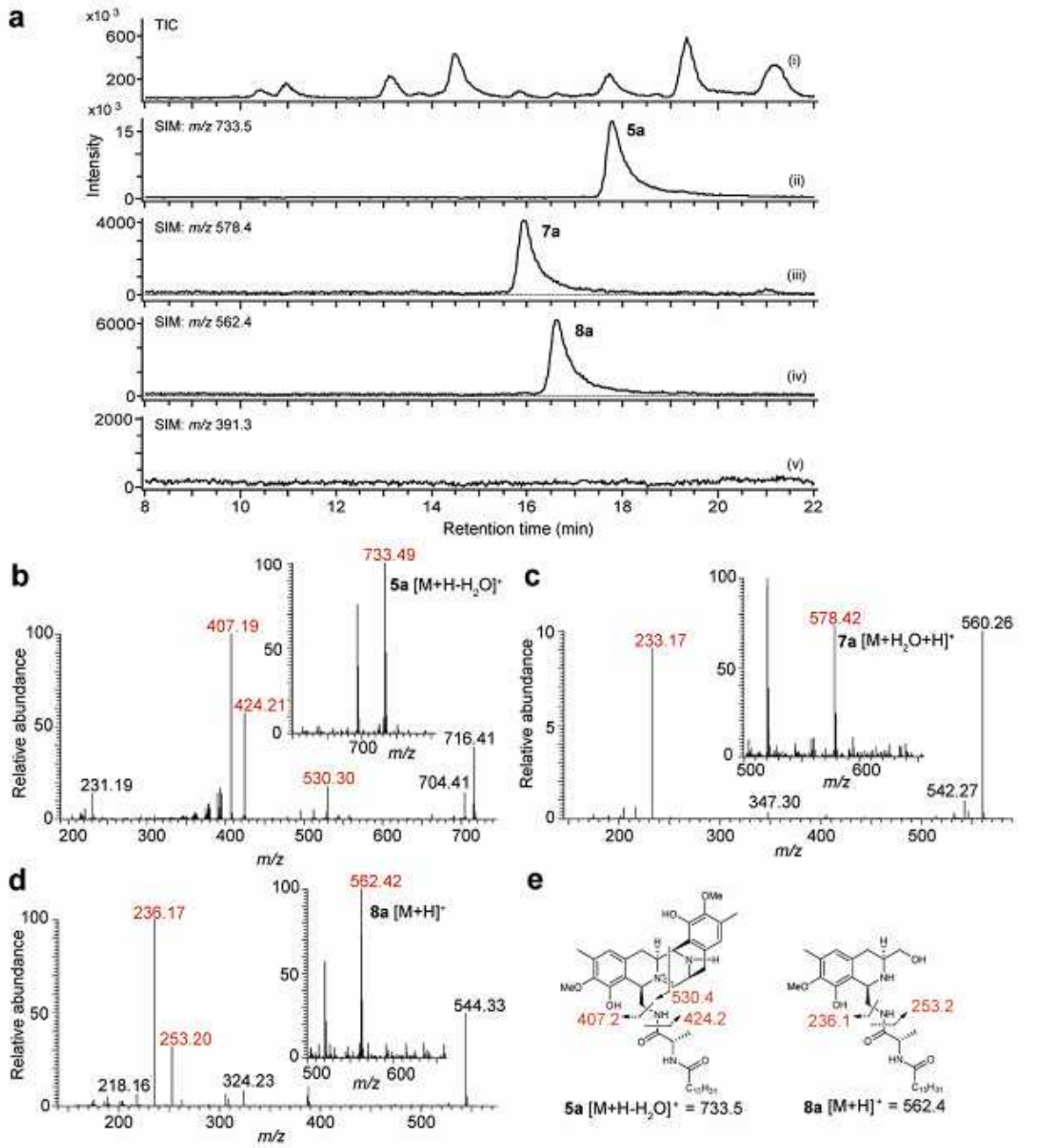
Supplementary Figure 5. Selected ROE correlations for bicyclic alcohol **8b**.



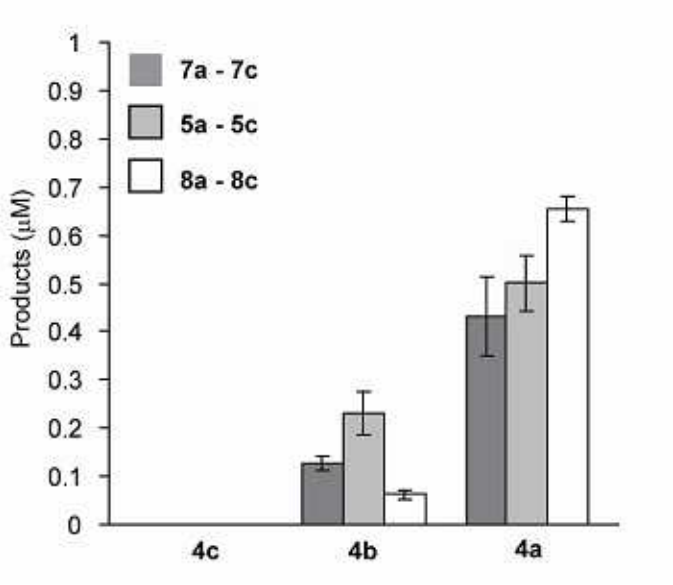
Supplementary Figure 6. HPLC-MS analysis of SfmC-catalyzed reaction with *N*-myristoyl-L-Ala-glycinal **9b**. (**a**) SfmC-catalyzed reaction in the presence of NADPH. (i) TIC of reaction mixture. (ii) HPLC-MS chromatogram in SIM mode [*m/z* 705.5 ± 0.1] for detecting pentacyclic **5b** ($[M\text{-}H_2O+H]^+$ = 705.5). (iii) HPLC-MS chromatogram in SIM mode [*m/z* 550.4 ± 0.1] for detecting bicyclic aldehyde **7b** ($[M+H_2O+H]^+$ = 550.4). (iv) HPLC-MS chromatogram in SIM mode [*m/z* 534.4 ± 0.1] for detecting bicyclic alcohol **8b** ($[M+H]^+$ = 534.4). (**b**) SfmC-catalyzed reaction in the presence of NADH. (i) TIC of the reaction mixture. (ii) HPLC-MS chromatogram in SIM mode [*m/z* 705.5 ± 0.1] for detecting pentacyclic **5b**. (iii) HPLC-MS chromatogram in SIM mode [*m/z* 550.4 ± 0.1] for detecting bicyclic aldehyde **7b**. (iv) HPLC-MS chromatogram in SIM mode [*m/z* 534.4 ± 0.1] for detecting bicyclic alcohol **8b**. TIC: total ion chromatogram; SIM: selected ion monitoring.



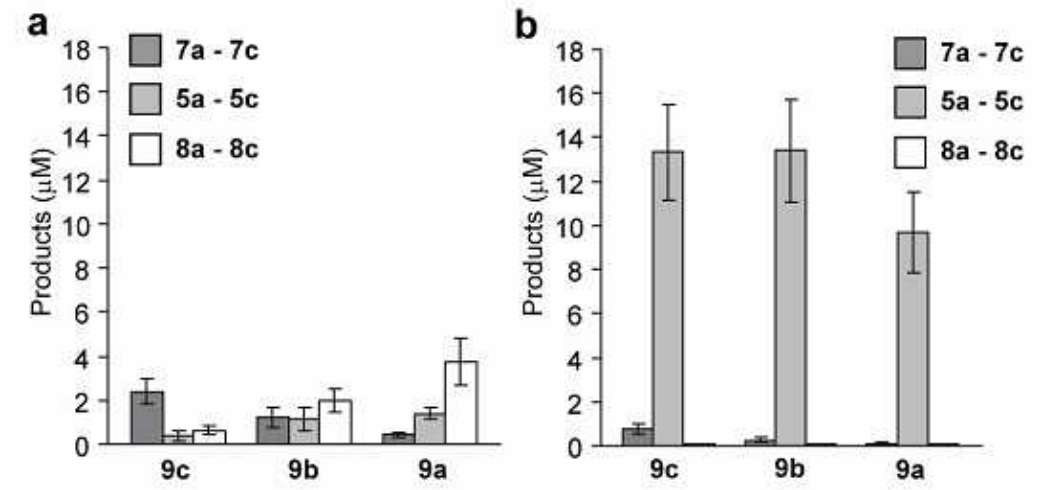
Supplementary Figure 7. Selected NOE correlations for *N*-myristoylcyanopresaframycin **6**.



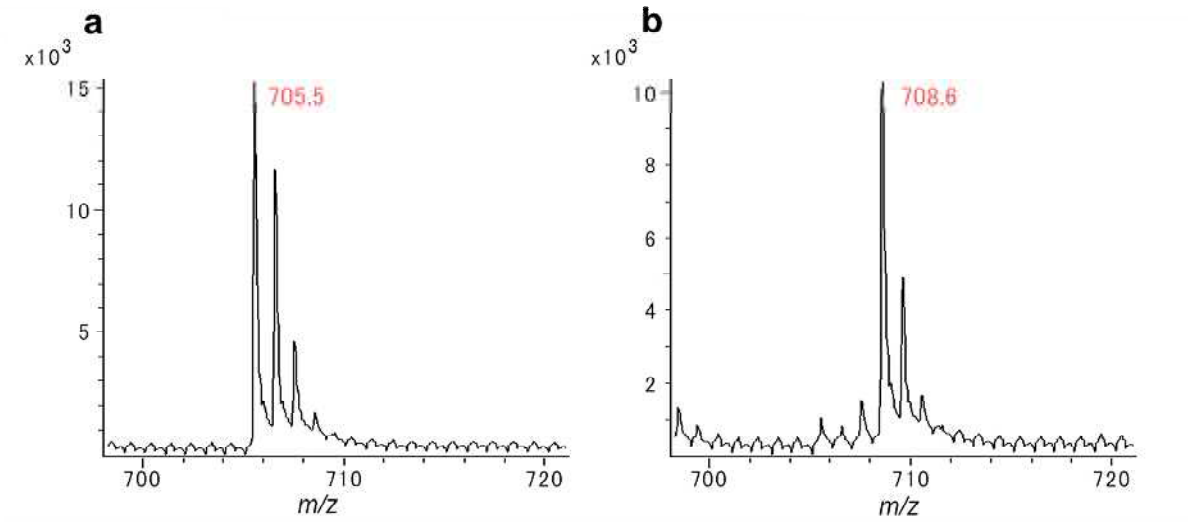
Supplementary Figure 8. SfmC-catalyzed reaction with *N*-palmitoyldipeptidyl-CoA thioester **4a**. (i) TIC of the reaction mixture. (ii) HPLC-MS chromatogram in SIM mode [*m/z* 733.5 ± 0.1] for detecting pentacyclic **5a** ($[M\text{-}H_2O+H]^+$ = 733.5). (iii) HPLC-MS chromatogram in SIM mode [*m/z* 578.4 ± 0.1] for detecting bicyclic aldehyde **7a** ($[M+H_2O+H]^+$ = 578.4). (iv) HPLC-MS chromatogram in SIM mode [*m/z* 562.4 ± 0.1] for detecting bicyclic alcohol **8a** ($[M+H]^+$ = 562.4). (iv) HPLC-MS chromatogram in SIM mode [*m/z* 391.3 ± 0.1] for detecting dipeptidyl aldehyde **9a** ($[M+Na]^+$ = 391.3). TIC: total ion chromatogram; SIM: selected ion monitoring. (**b**) ESI-MS (inset) and ESI-MS/MS spectra of **5a**. (**c**) ESI-MS (inset) and ESI-MS/MS spectra of the bicyclic aldehyde **7a**. (**d**) ESI-MS (inset) and ESI-MS/MS spectra of the bicyclic alcohol **8a**. (**e**) Structures of **5a** and **8a** with proposed MS/MS fragmentations.



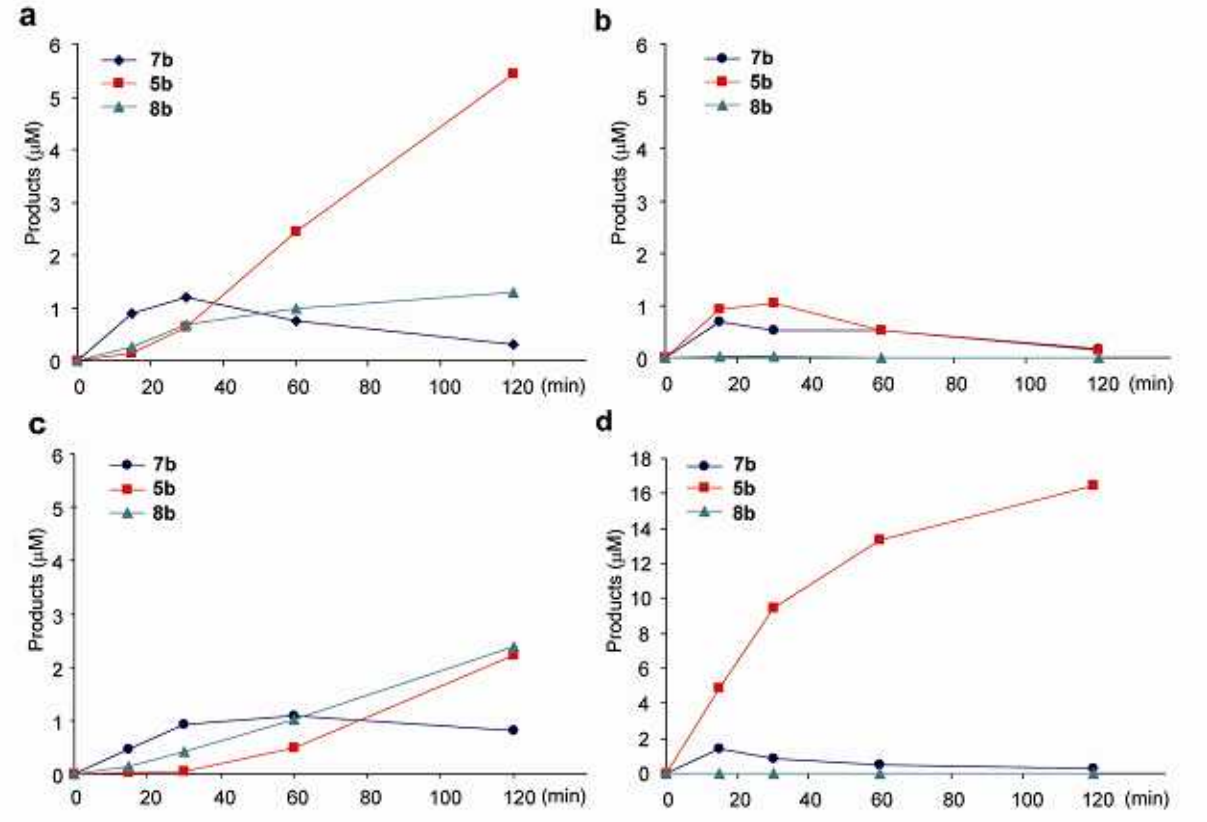
Supplementary Figure 9. The yields of products formed upon 2 h-incubation of 2 μM *holo*-SfmC with 100 μM various fatty acyl dipeptidyl-CoA substrates **4a-4c** in the presence of 3 mM NADPH. The error bars represent s.d. of the mean of three independent experiments. The product formation using **4c** was only a trace of amount, so the yield could not be calculated.



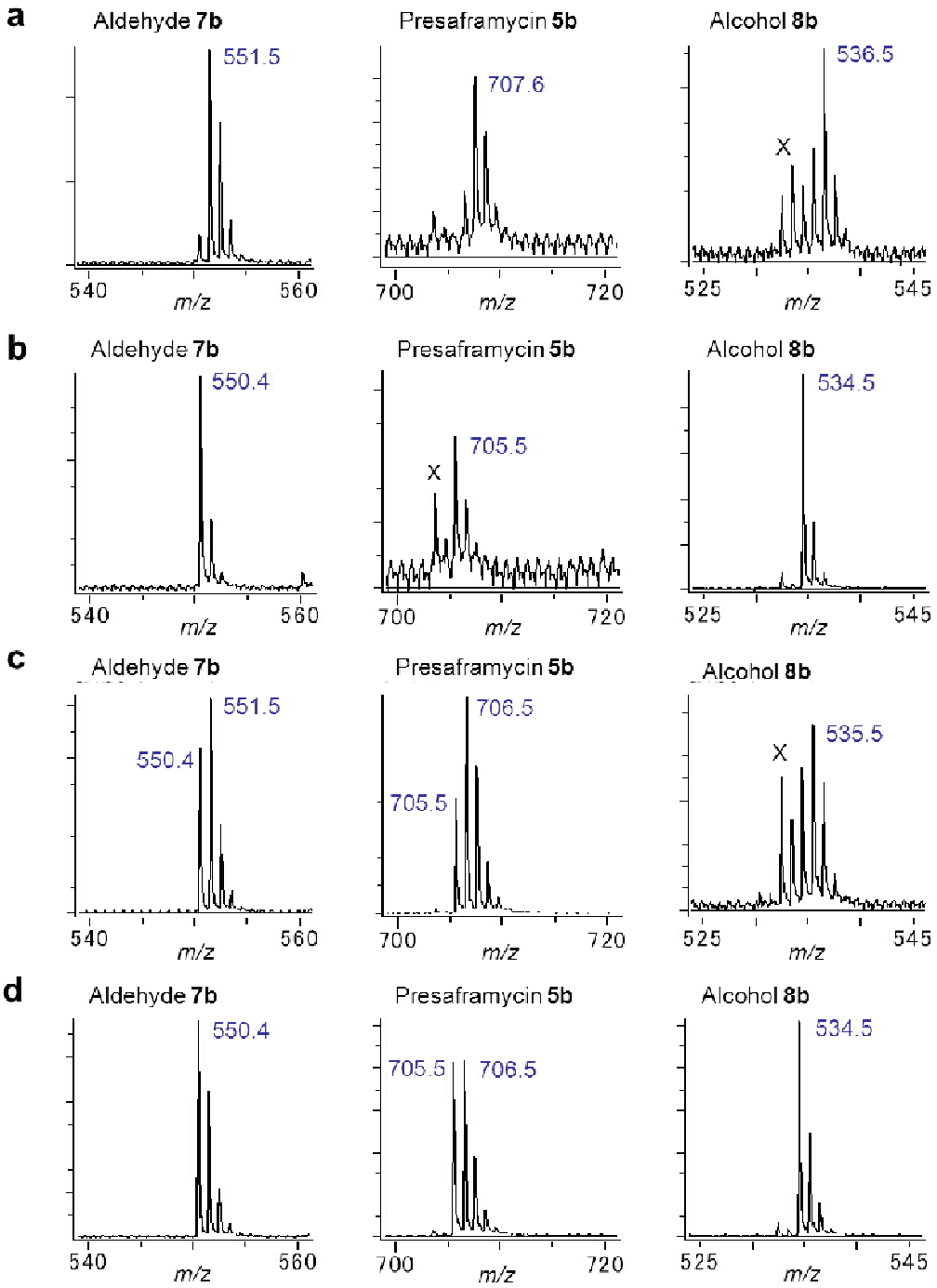
Supplementary Figure 10. The yields of products in the reaction with the dipeptidylaldehydes **9a**, **9b** and **9c**. (**a**) The yields of products formed upon 2 h-incubation of 2 μM *holo*-SfmC with 100 μM lauroyldipeptidylaldehyde **9c** or myristoyldipeptidylaldehyde **9b** or palmitoyldipeptideylaldehyde **9a** in the presence of 3 mM NADPH. (**b**) The yields of products formed upon 2 h-incubation of 2 μM *holo*-SfmC with 100 μM various aldehydes **9a – 9c** in the presence of 3 mM NADH. The error bars represent s.d. of the mean of three independent experiments.



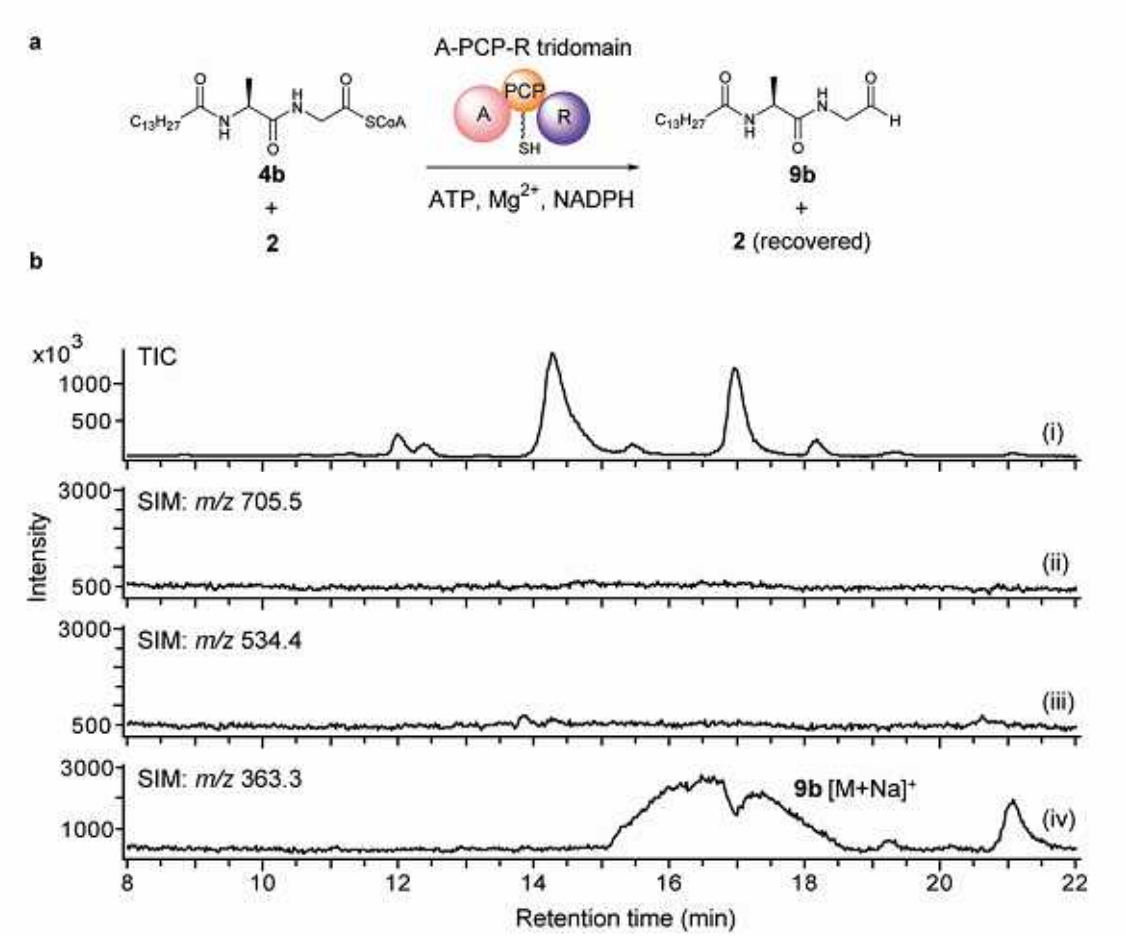
Supplementary Figure 11. SfmC-catalyzed reaction with *N*-myristoyldipeptidyl-CoA thioester **4b** in the presence of 4*S*- or 4*R*- deuterium-labeled NADPH. Mass spectra of **5b** are shown. (**a**) *R*-(4-^{2}H)NADPH, ESI-MS: *m/z* 705.5 (calcd. *m/z* 705.5), **5b** $[M+H-H_2O]^+$. (**b**) *S*-(4-^{2}H)NADPH, ESI-MS: *m/z* 708.6 (calcd. *m/z* 708.5), deuterium-labeled **5b** $[M+H-H_2O]^+$.



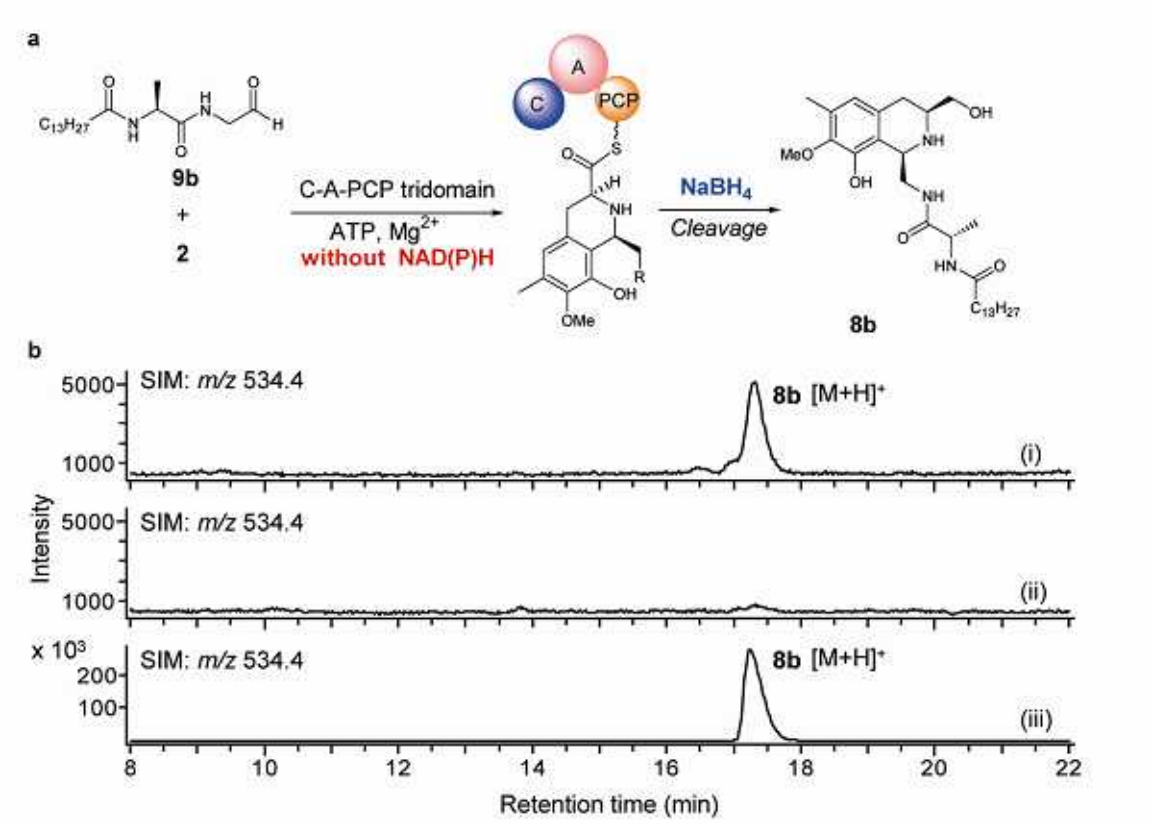
Supplementary Figure 12. SfmC-time course analyses with the *N*-myristoyldipeptidylaldehyde **9b**. (**a**) Time course of the SfmC-reaction products formation from 100 μM **9b** and 250 μM **2** by 2 μM *holo*-SfmC in the presence of 200 μM NADPH. (**b**) Time course of the SfmC-reaction products formation from 100 μM **9b** and 250 μM **2** by 2 μM *holo*-SfmC in the presence of 200 μM NADH (**c**) Time course of the SfmC-reaction products formation from 100 μM **9b** and 250 μM **2** by 2 μM *holo*-SfmC in the presence of 2 mM NADPH. (**d**) Time course of the SfmC-reaction products formation from 100 μM **9b** and 250 μM **2** by 2 μM *holo*-SfmC in the presence of 2 mM NADH. The yields were calculated as an average of two independent experiments.



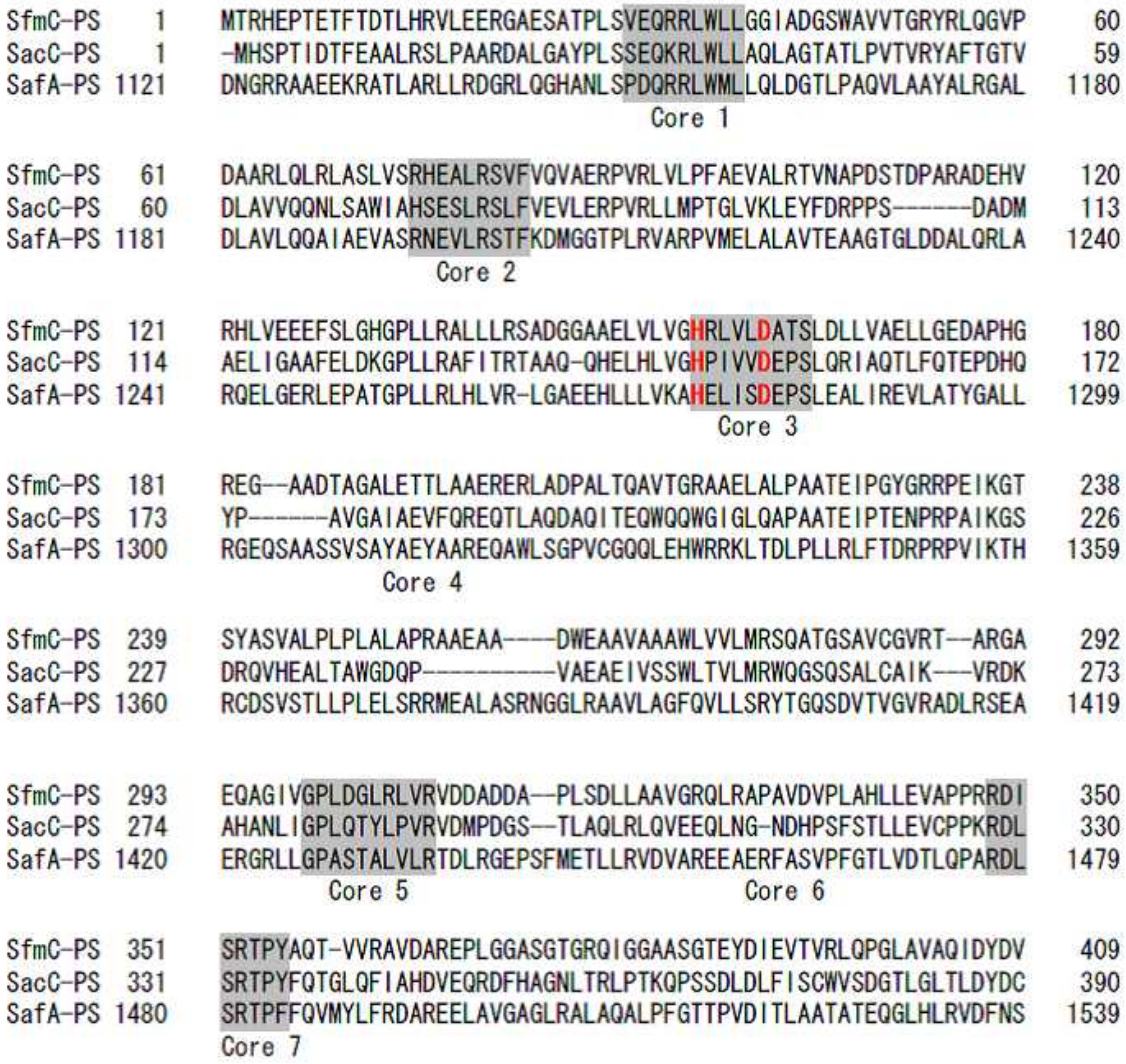
Supplementary Figure 13. Deuterium-incorporation into the products **5b**, **7b** and **8b** formed upon incubation of 2 μM *holo*-SfmC with *N*-myristoyldipeptidylaldehyde **9b** in the presence of the low (200 μM) or high (2 mM) concentrations of labeled- or non-labeled NADPH and NADH as a 1:1 mixture, either of which is labeled with deuterium. Mass spectra of pentacyclic **5b** ($[M+H-H_2O]^+$ = *m/z* 705.5), the bicyclic aldehyde **7b** ($[M+H_2O+H]^+$ = *m/z* 550.4) and the bicyclic alcohol **8b** ($[M+H]^+$ = *m/z* 534.4) are shown. (**a**) 100 μM *S*-(4-^{2}H)NADPH and 100 μM non-labeled NADH. (**b**) 100 μM non-labeled NADPH and 100 μM *S*-(4-^{2}H) NADH. (**c**) 1 mM *S*-(4-^{2}H)NADPH and 1 mM non-labeled NADH. (**d**) 1 mM non-labeled NADPH and 1 mM *S*-(4-^{2}H) NADH.



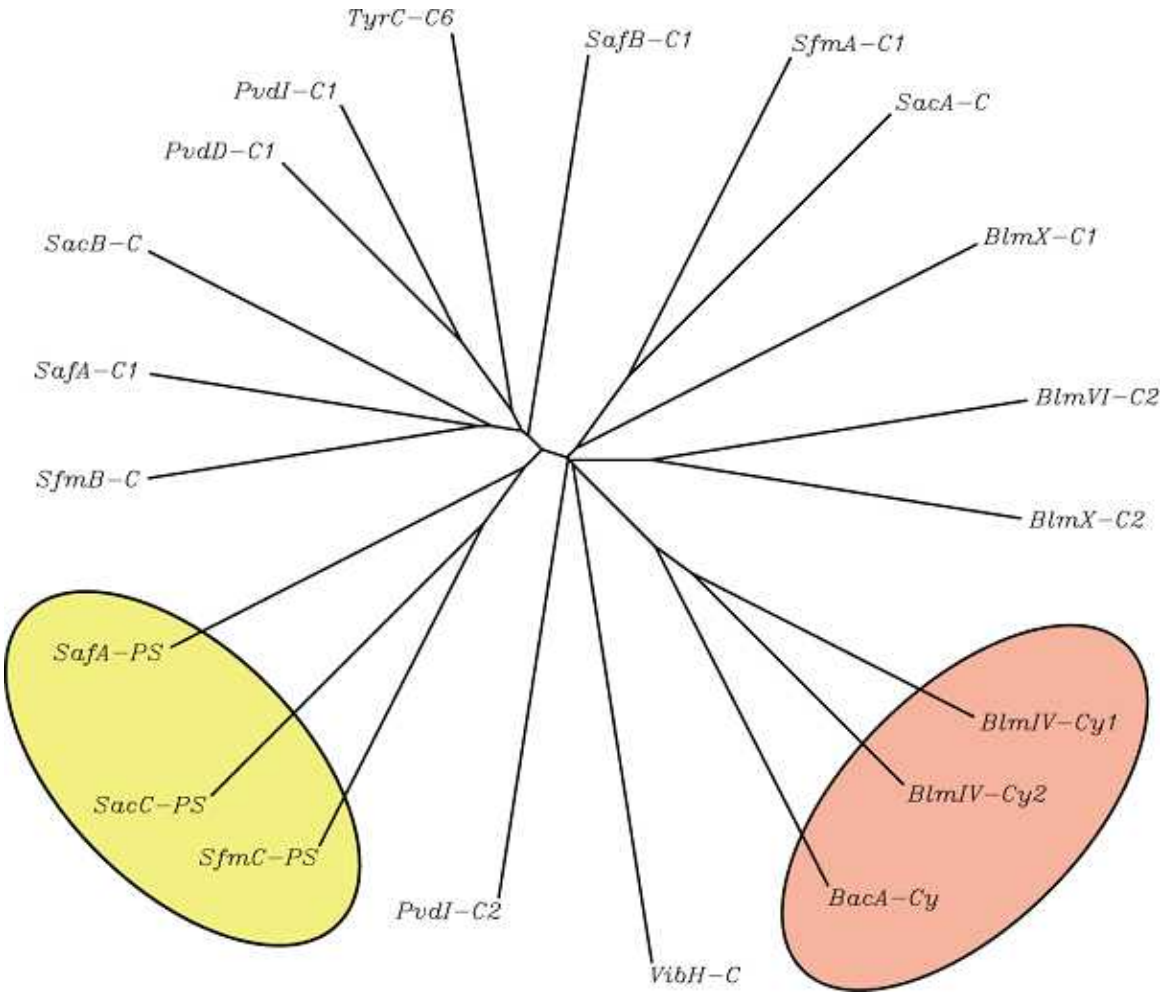
Supplementary Figure 14. A-PCP-R tridomain-catalyzed reaction with *N*-myristoyldipeptidyl-CoA **4b** and **2**. (**a**) Schematic representation of the reaction using A-PCP-R tridomain. (**b**) LC-MS analyses of the reaction. (i) TIC of reaction mixture. (ii) HPLC-MS chromatogram in SIM mode [*m/z* 705.5 ± 0.1] for detecting pentacyclic **5b** ($[M\text{-}H_2O+H]^+$ = 705.5). (iii) HPLC-MS chromatogram in SIM mode [*m/z* 534.4 ± 0.1] for detecting bicyclic alcohol **8b** ($[M+H]^+$ = 534.4). (iv) HPLC-MS chromatogram in SIM mode [*m/z* 363.3 ± 0.1] for detecting dipeptidyl aldehyde **9b** ($[M+Na]^+$ = 363.3). TIC: total ion chromatogram; SIM: selected ion monitoring.



Supplementary Figure 15. Characterization of the intermediate bound on the PCP-domain of C-A-PCP tridomain or A-PCP-R tridomain. (**a**) Schematic representation of the reaction using C-A-PCP tridomain and release of **8b** by thioester-reduction with $NaBH_4$. (**b**) LC-MS analyses of the reaction. (i) HPLC-MS chromatogram in SIM mode [*m/z* 534.4 ± 0.1] obtained by $NaBH_4$-reduction of C-A-PCP tridomain incubated in the absence of NAD(P)H. (ii) HPLC-MS chromatogram in SIM mode [*m/z* 534.4 ± 0.1] obtained by $NaBH_4$-reduction of A-PCP-R tridomain incubated in the absence of NAD(P)H. (iii) HPLC-MS chromatogram in SIM mode [*m/z* 534.4 ± 0.1] for the synthetic standard of bicyclic alcohol **8b**. TIC: total ion chromatogram; SIM: selected ion monitoring.



Supplementary Figure 16. Sequence alignment and conserved amino acid residues of the PS domains. The SfmC PS-domain is compared to the PS-domain from the safracin synthetase SacC from *Pseudomonas fluorescens* A2-2 (32% sequence identity) and the PS-domain from the saframycin Mx1 synthetase SafA from *Myxococcus xanthus* (28% sequence identity). The core consensus sequences for the C-domain are indicated by gray boxes. The characteristic HxxxxD motifs of core 3 for the PS-domain are represented with bold and red letters.



Supplementary Figure 17. Unrooted N-J tree comparing C-domains, Cy-domains and PS-domains. In addition to three PS-domains and three Cy-domains, typical C-domains are employed from various NRPS-modules. Sfm (*Streptomyces lavendulae*, saframycin A), Sac (*Pseudomonas fluorescens* A2-2, safracin B), Saf (*Myxococcus xanthus*, saframycin Mx1), Blm (*S. verticillus*, bleomycin), Pvd (*P. aeruginosa*, pyoverdine), Tyr (*Bacillus brevis*, tyrocidine), Vib (*Vibrio cholerae*, vibriobactin), Bac (*Bacillus licheniformis* ATCC 10716, bacitracin A)

Supplementary Table 1 ^{1}H- and ^{13}C-NMR and ROESY data of bicyclic alcohol **8b**.

Proton	δ_H (ppm)	Multiplicity	*J* (Hz)	δ_C (ppm)	ROESY Correlation
1-H	4.73	d	6.6	53.5	3-H
2-NH					
3-H	3.10-3.07	m		55.2	1-H
4-Ha	2.80	dd	15.0, 12.6	30.3	5-H, 11-Ha, 11-Hb
4-Hb	2.53	dd	15.0, 2.3		5-H, 11-Ha, 11-Hb
5-H	6.46	s		122.0	4-Ha, 4-Hb, 6-Me
6				129.8	
7				144.2	
8				146.3	
9				117.3	
10				130.8	
11-Ha	3.88	dd	12.1, 2.3	64.1	4-Ha, 4-Hb
11-Hb	3.70	dd	12.1, 6.7		4-Ha, 4-Hb
12-Ha	4.11	d	13.4	42.9	1-H, 12-Hb, 13-H
12-Hb	3.64-3.59	m			1-H, 12-Ha, 13-H
13-NH	8.09	brs			12-Ha, 12-Hb, 15-H
14-H				174.5	
15-H	4.34	dq	6.8, 4.9	49.5	13-H, 16-H
16-NH	6.91	brd	4.9		15-H, 18-H
17				174.4	
18-H	2.19	t	7.7	36.4	16-H
19-H	1.56	m		25.5	
20-29-H	1.30-1.21	m		31.9, 30.3, 29.7-29.3, 22.7	
30-H	0.87	t	7.0	14.1	
6-Me	2.22	s		15.6	5-H, 7-OMe
7-OMe	3.73	s		60.6	6-Me
15-Me	1.30-1.21	m		17.9	

Supplementary Table 2 ^{1}H- and ^{13}C-NMR and NOESY data of *N*-myristoyl-cyanopresaframycin (**6**).

Proton	δ_H (ppm)	Multiplicity	*J* (Hz)	δ_C (ppm)	NOESY Correlation
1-H	4.11	s		57.17	22-Ha, 22-Hb
2	–				
3-H	3.27	dt	12.0/2.4	56.70	11-H, 4-Hb
4-Ha	2.13	dd	15.6/12.0	31.97	4-Hb, 23-H
4-Hb	2.83	dd	15.6/2.4		3-H, 4-Hb, 11-H, 5-H
5-H	6.42	s		120.91	6-Me, 4-Hb
6	–			117.09	
7	–			143.39	
8	–			144.74	
9	–			131.53	
10	–			128.91	
11-H	4.39	d	2.4	49.19	3-H, 4-Hb
12-NH					
13-H	3.71	m		49.46	14-Ha, 21-H
14-Ha	3.15	dd	18.0/8.4	31.16	13-H, 14-Hb, 15-H
14-Hb	2.76	d	18.0		14-Ha, 15-H
15-H	6.52	s		121.18	16-Me, 14-Hb
16	–			121.09	
17	–			142.88	
18	–			144.98	
19	–			131.15	
20	–			129.09	
21-H	4.04	d	1.8	59.53	14-Hb, 22-Ha
22-Ha	3.23	dt	13.8/4.8	41.40	1-H, 21-H, 22-Hb
22-Hb	3.72	m			1-H, 22-Ha
23-NH	5.30	t	4.8		4-Ha, 22-Ha, 25-H, 25-Me
24	–			171.64	
25-H	3.71-3.67	m		48.14	23-H, 25-Me
26-NH	5.96	d	7.2		25-Me, 28-H
27	–			172.02	
28-H	2.04	t	7.8	36.46	29-H
29-H	1.50	m		25.35	
30-H	1.29	m		22.46	
31-39-H	1.28-1.20	m		31.69, 29.45-29.07	
40-H	0.88	t	7.2	13.89	
6-Me	2.22	s		15.44	5-H, 7-OMe
7-OMe	3.75	s		60.53	6-Me
16-Me	2.27	s		15.57	15-H, 17-OMe
17-OMe	3.79	s		60.58	16-Me
21-CN	–			117.55	
25-Me	0.91	d	7.2	18.66	23-H, 25-H, 26-H

Supplementary Methods

Materials and general information.

Protected amino acids, HBTU, HOBt and wang resin (1.1 mol/g, 100-200 mesh, 1% DVB) were purchased from Watanabe Chemical Industries. Lauric acid (purity of > 98%) was obtained from Sigma-Aldrich. Myristic acid (purity of > 98%) and palmitic acid (purity of > 95%) were purchased from Wako Pure Chemical Industries. Coenzyme A trilithium salt (purity of > 95%) was purchased from Sigma-Aldrich and coenzyme A trisodium salt (purity of > 90%) was purchased from Wako Pure Chemical Industries. Dess-Martin periodinane was synthesized according to literature procedure.[1] Other chemical reagents were obtained from Wako Pure Chemical Industries. NMR spectra were recorded on JEOL ECP-300 (^{1}H/300 MHz, ^{13}C/75 MHz) and JEOL ECA-600 (^{1}H/600 MHz, ^{13}C/150 MHz) spectrometers. Chemical Shifts are reported in δ (ppm) using chloroform as an internal standard of δ 7.26 (^{1}H NMR) and δ 77.0 (^{13}C NMR). TLC was carried out on glass sheets pre-coated with silica-gel 60 (F254, Merck) and spots were charred with PMS solution (2.4% 12 Molybdo (IV) phosphoric acid in the mixture of 1 : 3.3 : 66.6 of 85% phosphoric acid : 95% sulfonic acid : H_2O) or ninhydrin. Mass spectra were recorded on JEOL JMS-T100CS (ESI) spectrometer. MS/MS analysis was performed using LTQ-Orbitrap XL (Thermo Scientific). Optical rotations were recorded on a JASCO DIP-360 polarimeter. All reactions were carried out under an inert nitrogen atmosphere. Flash column chromatography was performed using Kanto Silica Gel 60N.

Genomic library construction and DNA manipulations.

A DNA library covering the entire genome of *S. lavendulae* was constructed using pECBAC1 vector. Probes used were prepared using the PCR DIG probe synthesis kit (Roche Diagnostics). Colony and DNA hybridizations were performed using the conventional

techniques. Plasmids were routinely isolated from DH5α using QIAprep Spin Miniprep columns (Qiagen). All PCR reactions were carried out with *PfuUltra* High-Fidelity DNA polymerase (Stratagene) using Eppendorf Mastercycler ep gradient S thermal cycler. To isolate the plasmid carrying the entire biosynthetic gene cluster for **1**, a set of primers (sfmA4F: 5'–GCCTATCTCATCTATACCTCGGGCACCACGGGCCGCCCC–3' and sfmA4R: 5'–GGGTGTCGATGGTGGACACGTGGTGGACGGCCTTGAGGCG–3') were designed for preparing an A-domain-specific probe.

Construction of *sfmC* expression vectors.

Plasmid pKW734, based on pET21c (Novagen), was prepared to overexpress *sfmC* using the T7 expression system. The *Nde*I–*Eco*RI fragment of *sfmC* cloned into pKW423[2] was prepared by PCR using a set of primers (sfmCF: 5'–CCTGGAGGTTTCCATATGACCCGGCACGAGCCCACCGAGACGTTCACCG–3' and sfmCR: 5'–CGGTGTCGGCCGGTGCCGTGAATTCTCCTCCAGCGTGCTGCTCGGC–3').

Construction of *sfmC* mutants expression vectors.

To prepare the C-domain deficient mutant, A-PCP-R tridomain, and the R-domain deficient mutant, C-A-PCP tridomain, the region containing genes were amplified by PCR from pKW734 using KOD DNA polymerase (Toyobo) with the following oligonucleotides: for A-PCP-R tridomain, Fw primer; 5'-AACTTGCCATATGACCGTCGTCCCGCTGC-3', Rv primer; 5'-AAAGAATTCTCGCTCCTCCAGCGTGC-3' and for C-A-PCP tridomain, Fw primer; 5'-AATTGCCATATGACCCGGCACGAGCCCACC-3', Rv primer; 5'-AAGAATTCTCAGCCGAGGACGCCGGCGAGG-3'. Each amplified *A-PCP-R tridomain* and *C-A-PCP tridomain* fragment was subcloned into the *Nde*I/*Eco*RI site of pET28b (Novagen) using Ligation high Ver.2 (Toyobo). The accuracy of the DNA sequences of the coding region of the plasmids prepared here was confirmed by DNA sequencing.

Gene expression and enzyme preparation.

Expression plasmids were transformed into *E. coli* BL21(DE3), and the cells were grown in LB medium supplemented with either 100 μg/mL ampicilin for SfmC or 50 μg/mL kanamycin for A-PCP-R tridomain and C-A-PCP tridomain. Two 500 mL Erlenmeyer flasks containing 250 mL LB medium were inoculated with the 2.5 mL overnight cultures. Cells were grown at 37 °C until an OD_{600} reached 0.4-0.6 and then induced with 200 μM IPTG. After the additional incubation at 15 °C for 12 h, the cells were harvested by centrifugation at 3,000 x *g* for 10 min, and the cell pellets were resuspended in the disruption buffer [50 mM NaH_2PO_4-NaOH (pH 7.5), 0.2 M NaCl, 5 mM imidazole, 10 μg/mL DNase, 20% glycerol] and disrupted by two passages through French Press at 12,000 psi. After clarified by centrifugation at 20,000 x *g* for 30 min, the supernatant was added into Ni-NTA resin (1.5 mL, Qiagen) filled in a column. The resin was washed with 10 column volumes of 40 mM imidazole in the disruption buffer, and the protein was eluted with 200 mM imidazole in the buffer. The pooled fraction was desalted with two PD-10 desalting columns (GE Healthcare) equilibrated with the storage buffer [50 mM NaH_2PO_4-NaOH (pH 7.5), 50 mM NaCl, 30% glycerol]. The final protein concentrations were estimated using the Bradford assay for the BSA standard. The molecular weights of the proteins were estimated by SDS-PAGE analysis. Further, the native complexes of these proteins were analyzed using blue native polyacrylamide gel electrophoresis (BN-PAGE, Invitrogen) according to the recommended procedure.

***In vitro* 4'-phosphopantetheinylation of *apo*-SfmC, *apo*-C-A-PCP tridomain and *apo*-A-PCP-R tridomain.**

A reaction mixture (50 μL) containing either 20 μM *apo*-SfmC or *apo*-A-PCP-R tridomain, 100 μM coenzyme A, 10 mM $MgCl_2$, 1.0 μM recombinant *Bacillus subtilis* 4'-phosphopantetheinyl transferase (PPase) Sfp[3] in the storage buffer (pH 7.5) were

incubated at rt for 30 min. The reaction mixtures were then quenched by adding 0.5 μL of formic acid. The resulting reaction mixtures were directly analyzed by LC-ESI-MS (LC: Agilent 1100 system, MS: JEOL, JMS-T100LP AccuTOF LC-plus) with a Protein-R column (Nacalai tesque, 4.6 x 150 mm, 300 Å, 5 μm) under the following conditions: 0–30 min, linear gradient 30–65% CH_3CN/0.1% formic acid in H_2O/0.1% formic acid at a flow rate of 0.7 mL/min at 30 °C. Measurements were carried out three times for each protein, and their average molecular weights were calculated as follows;

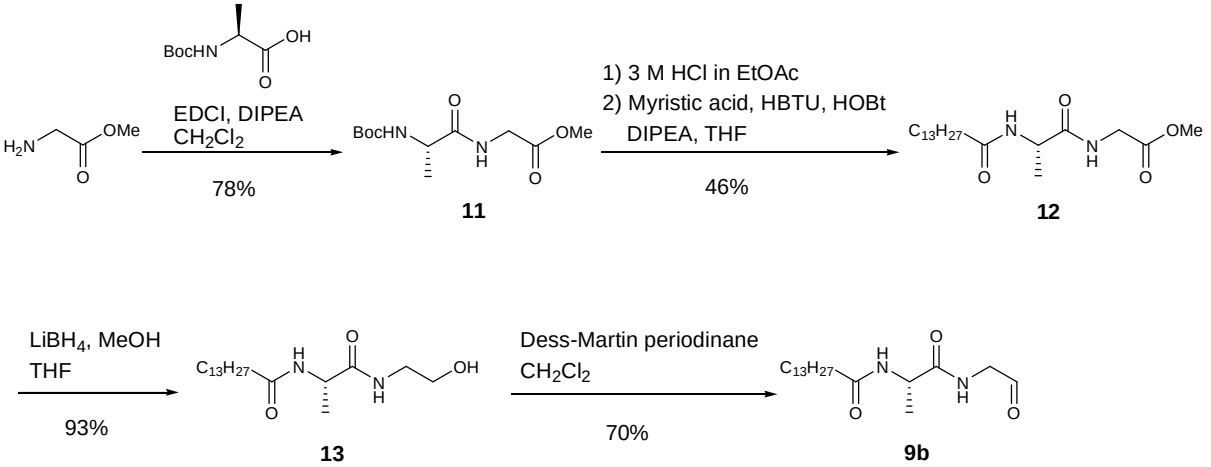
apo-SfmC: calcd. mass; 163175.8 Da, observed mass; 163218.9 ± 2.4 Da (error of < 0.03%). *holo*-SfmC: calcd. mass; 163516.1 Da, observed mass; 163618.3 ± 9.4 Da (error of < 0.06%). *apo*-A-PCP-R tridomain: calcd. mass; 117390.6 Da, observed mass; 117492.6 ± 2.4 Da (error of < 0.09%). *holo*-A-PCP-R tridomain: calcd. mass; 117730.9 Da, observed mass; 117829.6 ± 3.0 Da (error of < 0.09%). *apo*-C-A-PCP tridomain: calcd. mass; 115084.7 Da, observed mass; 115156.6 ± 32.6 Da (error of < 0.06%). *holo*-C-A-PCP tridomain: calcd. mass; 115425.0 Da, observed mass; 115560.8 ± 12.3 Da (error of < 0.12%).

Synthesis of dipeptidyl-*S*-CoA substrates 4a - 4e.

Peptides were synthesized manually based on a stepwise solid-phase protocol as reported previously.[4] Fmoc group of *N*-Fmoc-Gly supported on wang resin was removed using 20% (v/v) piperidine in DMF (2 x 10 min). Peptide couplings were carried out for 30 min in DMF using a 5 eq. (over resin loading) of *N*-Fmoc-L-Ala preliminarily activated with HBTU (5 equiv), HOBt (5 eq.) and *i*-Pr_2NEt (10 eq.). After the Fmoc group of L-Ala residue was removed, fatty acyl group was connected with the amino group as described above. Polymer-bound peptide chains were cleaved by exposure to TFA/H_2O (95:5) for 2 h at rt and the solvent was then removed by azeotropic distillation with toluene, affording the crude fatty acyldipeptides. To prepare the dipeptidyl-*S*-CoA thioesters **4a - 4e**, condensations of the

dipeptides with coenzyme A were carried out as follows: To a solution of the peptide with a *C*-terminal carboxylic acid in dry THF (0.026 M), 1.5 eq. of carbonyldiimidazole (CDI) was added and stirred at rt for 1.5 h. Coenzyme A trisodium salt (1 eq.) dissolved in 0.2 M KH_2PO_4-KOH buffer (pH 7.0) was subsequently added to the mixture. After stirring at rt for 1 h, the resulting reaction mixtures were directly purified by high-performance liquid chromatography (HPLC) on a LC-10AD vp HPLC system (Shimadzu) with a Wakosil-II 5C18 column (Wako, 10.0 x 250 mm, 5 μm) under the following conditions: **4a** and **4b**; 0–30 min, linear gradient 20–90% CH_3CN /0.1% TFA in H_2O/0.1% TFA at a flow rate of 3.0 mL/min at rt, **4c**; 0–30 min, linear gradient 30–80% CH_3CN/0.1% TFA in H_2O/0.1% TFA at a flow rate of 3.0 mL/min at rt, **4d**; 0–30 min, linear gradient 5–40% CH_3CN /0.1% TFA in H_2O/0.1% TFA at a flow rate of 3.0 mL/min at rt. The fractions containing the desired dipeptidyl-*S*-CoA thioesters were lyophilized to dryness and then dissolved in DMSO for the enzyme assay, and the resulting solutions were stored at -20 °C. Preparation of **4e** was commenced with condensation of Boc-L-Ala-Gly-OH by treatment of coenzyme A trilithium salt (1 eq.), PyBOP (1 eq.) and K_2CO_3 (4 eq.) in THF/H_2O (1:1) at rt for 1 h. Removal of the Boc group with 1 mL of TFA/H_2O (95:5) solution and subsequent evaporation of the excess TFA *in vacuo* produced the crude thioester, which was purified by HPLC under the following conditions: 0–30 min, linear gradient 5–30% CH_3CN /0.1% TFA in H_2O/0.1% TFA at a flow rate of 3.0 mL/min at rt. The identities of the dipeptidyl-*S*-CoA thioesters (**4a** - **4e**) were verified by ESI-MS (JEOL, JMS-T100LP AccuTOF LC-plus).

Supplementary Scheme 1



Synthesis of *N*-myristoyl-L-Ala-glycinal (9b).

***N*-Boc-L-Ala-Gly-OMe (11).** A solution of *N*-Boc-L-Ala (1.57 g, 8.30 mmol) and Gly-OMe·HCl (868 mg, 6.90 mmol) in CH_2Cl_2 (23 mL) was treated with *i*-Pr_2NEt (2.86 mL, 16.6 mmol) and EDCI (1.59 g, 8.30 mmol) at 0 ºC. After being stirred at rt for 15 h, the reaction mixture was diluted with EtOAc (500 mL) and washed with 0.5 M HCl, saturated $NaHCO_3$, and brine. After being dried over Na_2SO_4, the solvent was evaporated *in vacuo*. The residue was purified by silica-gel column chromatography (hexane/EtOAc = 2:1–1:1) to yield 1.40 g of *N*-Boc-L-Ala-Gly-OMe **11** (2.18 mmol, 78%). $[\alpha]_D^{28}$ -23.1 (*c* 1.0, $CHCl_3$); ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 6.68 (brs, 1H), 4.98 (brs, 1H), 4.25-4.19 (m, 1H), 4.05 (dd, 2H, *J* = 2.8, 5.3 Hz), 3.75 (s, 3H), 1.45 (s, 9H), 1.38 (d, 3H, *J* = 7.1 Hz); ^{13}C-NMR ($CDCl_3$, 75 MHz) δ 172.9, 170.1, 155.5, 80.2, 52.3, 49.9, 41.1, 28.3, 18.2; HRMS(ESI$^+$) calcd. for $C_{11}H_{20}N_2NaO_5$ $[M+Na]^+$ 283.1264, found 283.1242.

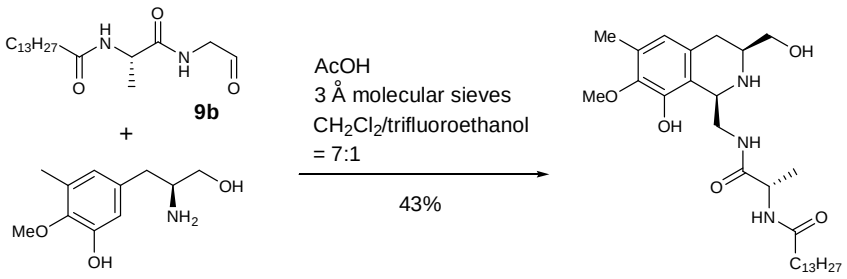
***N*-myristoyl-L-Ala-Gly-OMe (12).** A mixture of *N*-Boc-L-Ala-Gly-OMe **11** (449 mg, 1.72 mmol) and 3 M HCl in EtOAc (10 mL) was stirred at rt for 30 min. The volatiles were removed under reduced pressure. The residual HCl was further azeotropically removed with diethylether to give a hydrochloric salt of NH_2-L-Ala-Gly-OMe. A solution of myristic acid (393 mg, 1.89 mmol) and HOBt (290 mg, 1.89 mmol) in THF (18 mL) was treated with

i-Pr_2NEt (660 μL, 3.78 mmol) and HBTU (719 mg, 1.89 mmol) at rt. After being stirred for 5 min, the reaction mixture was added to the crude NH_2-L-Ala-Gly-OMe dissolved in DMF (1 mL). After stirring at rt for 1 h, the reaction mixture was concentrated and then diluted with $CHCl_3$ (1 mL). The crude material was directly purified by silica-gel column chromatography ($CHCl_3$ only, $CHCl_3$/MeOH = 200:1) to yield 294 mg of *N*-myristoyl-L-Ala-Gly-OMe **12** (0.68 mmol, 46%). $[\alpha]_D^{27}$ -44.0 (*c* 1.0, $CHCl_3$); ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 6.70 (brs, 1H), 6.00 (d, 1H, *J* = 6.6 Hz), 4.55 (dq, 1H, *J* = 7.1, 6.6 Hz), 4.03 (d, 2H, *J* = 5.4 Hz), 3.75 (s, 3H), 2.20 (t, 2H, *J* = 7.7 Hz), 1.67-1.58 (m, 2H), 1.39 (d, 3H, *J* = 7.1 Hz), 1.33-1.22 (m, 20H), 0.88 (t, 3H, *J* = 6.7 Hz); ^{13}C-NMR ($CDCl_3$, 75 MHz) δ 173.3, 172.5, 170.0, 52.4, 48.4, 41.2, 36.6, 31.9, 29.7-29.2, 25.5, 22.7, 17.9, 14.1; HRMS(ESI$^+$) calcd. for $C_{20}H_{38}N_2NaO_4$ $[M+Na]^+$ 371.2910, found 371.2868.

***N*-myristoyl-L-Ala-glycinol (13).** $LiBH_4$ (52 mg, 2.37 mmol) and MeOH (96 μL, 2.37 mmol) were added to the solution of *N*-myristoyl-L-Ala-Gly-OMe **12** (294 mg, 7.90 x 10^{-1} mmol) in THF (8 mL) and stirred at 0 ºC for 10 min, then further agitated at rt for 2 h. The reaction was quenched with MeOH (5 mL) and H_2O (5 mL). The reaction mixture was extracted with $CHCl_3$ (50 mL x 3) and the combined organic phase was washed with 0.5 M HCl and brine. After being dried over Na_2SO_4, the solvent was evaporated *in vacuo*. The concentrated material was purified by short silica-gel column chromatography ($CHCl_3$/MeOH = 5:1) to yield 251 mg of *N*-myristoyl-L-Ala-glycinol **13** (7.33 x 10^{-1} mmol, 93%). $[\alpha]_D^{26}$ -33.4 (*c* 0.20, $CHCl_3$); ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 6.95 (brs, 1H), 6.27 (d, 1H, *J* = 7.1 Hz), 4.49 (dq, 1H, *J* = 7.1, 6.9 Hz), 3.71 (t, 2H, *J* = 5.0 Hz), 3.52-3.32 (m, 2H), 2.20 (t, 2H, *J* = 7.6 Hz), 1.68-1.56 (m, 2H), 1.39 (d, 3H, *J* = 6.9 Hz), 1.25 (m, 20H), 0.88 (t, 3H, *J* = 6.7 Hz); ^{13}C-NMR ($CDCl_3$, 75 MHz) δ 173.2, 172.7, 61.5, 48.5, 42.1, 36.2, 31.5, 29.2-28.8, 25.2, 22.3, 17.9, 13.7; HRMS(ESI$^+$) calcd. for $C_{19}H_{39}N_2O_3$ $[M+H]^+$ 343.2961, found 343.2943.

***N*-myristoyl-L-Ala-glycinal (9b).** Dess-Martin periodinane (445 mg, 1.05 mmol) was added to the solution of *N*-myristoyl-L-Ala-glycinol **13** (120 mg, 3.50 x 10^{-1} mmol) in dry CH_2Cl_2 (7 mL). After sitrring at rt for 1 h, the mixture was diluted with Et_2O (5 mL). The excess Dess-Martin periodinane was reduced with $Na_2S_2O_3$ (1.0 g) in saturated $NaHCO_3$ (5 mL). The resulting mixture was extracted with $CHCl_3$ (40 mL x 3), and the combined organic phase was washed with H_2O (10 mL) and brine (20 mL). After being dried over Na_2SO_4, the solvent was evaporated *in vacuo*. The crude material was purified by silica-gel column chromatography ($CHCl_3$ only, $CHCl_3$/MeOH = 100:1-50:1-10:1) to yield 83.2 mg of *N*-myristoyl-L-Ala-glycinal **9b** (2.44 x 10^{-1} mmol, 70%). $[\alpha]_D^{25}$ -29.3 (*c* 0.62, $CHCl_3$); ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 9.61 (s, 1H), 7.40 (brs, 1H), 6.39 (d, 1H, *J* = 7.5 Hz), 4.63 (dq, 1H, *J* = 7.5, 7.1 Hz), 4.13 (d, 2H, *J* = 5.2 Hz), 2.19 (t, 2H, *J* = 7.5 Hz), 1.63-1.54 (m, 2H), 1.39 (d, 3H, *J* = 7.1 Hz), 1.28-1.18 (m, 20H), 0.86 (t, 3H, *J* = 6.6 Hz); ^{13}C-NMR ($CDCl_3$, 75 MHz) δ 196.5, 173.5, 173.1, 50.0, 48.4, 36.5, 31.9, 29.6-29.2, 25.6, 22.7, 18.4, 18.3, 14.1; HRMS(ESI$^+$) calcd. for $C_{19}H_{37}N_2O_3$ $[M+H]^+$ 341.2804, found 341.2772.

Supplementary Scheme 2



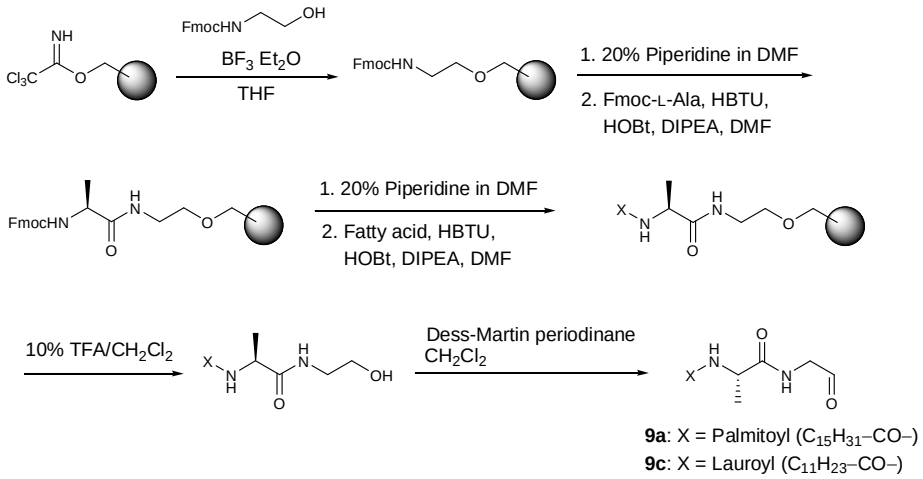
Bicyclic alcohol **8b**

Synthesis of bicyclic tetrahydroisoquinoline alcohol 8b.

A mixture of *N*-myristoyl-L-Ala-glycinal **9b** (33.3 mg, 9.7 x 10^{-2} mmol), L-3-hydroxy-5-methyl-*O*-methyltyrosinol (20.6 mg, 8.07 x 10^{-2} mmol) synthesized according to literature procedures,[5, 6] and 3 Å molecular sieves (40 mg) in CH_2Cl_2/trifluoroethanol (7:1, v/v, 400 μL) was degassed with liq. N_2. After the addition of AcOH (12 μL, 2.0 mmol), the

reaction mixture was stirred at rt for 16 h.[7] Then reaction mixture was filtrated with cerite and concentrated *in vacuo*. The crude mixture was directly purified by HPLC on a LC-10AD vp HPLC system (Shimadzu) with a Wakosil-II 5C18 column (Wako Pure Chemical Industries, 10.0 x 250 mm, 5 μm) under the following conditions: 0–30 min, linear gradient 40–65% CH_3CN/0.05% formic acid in H_2O/0.05% formic acid at a flow rate of 4.0 mL/min at 35 °C. The desired cyclic product **8b** (18.5 mg) with 1,3-*syn* stereochemical relationship between either site of nitrogen was isolated as a major product in 43% yield. The product **8b** was fully characterized including a series of 2D-NMR analyses. $[\alpha]_D^{27}$ -80.7 (*c* 0.85, $CHCl_3$); ^{1}H-NMR ($CDCl_3$, 600 MHz) δ 8.09 (brs, 1H), 6.91 (brd, 1H, *J* = 4.9 Hz), 6.46 (s, 1H), 4.73 (d, 1H, *J* = 6.5 Hz), 4.34 (dq, 1H, *J* = 4.9, 6.8 Hz), 4.11 (d, 2H, *J* = 13.4 Hz), 3.88 (dd, 1H, *J* = 2.3, 12.1 Hz), 3.73, (s, 3H), 3.70 (dd, 1H, *J* = 6.7, 12.1 Hz), 3.64-3.59 (m, 1H), 3.10-3.07 (m, 1H), 2.80 (dd, 1H, 12.6, 15.0 Hz), 2.53 (dd, 1H, *J* = 15.0, 2.3 Hz), 2.22 (s, 3H), 2.19 (t, 3H, *J* = 7.7 Hz), 1.58-1.53 (m, 2H), 1.30-1.21 (m, 23H), 0.87 (t, 3H, *J* = 7.0 Hz); ^{13}C-NMR ($CDCl_3$, 150 MHz) δ 174.5, 174.4, 169.7, 146.3, 144.2, 130.8, 129.8, 122.0, 117.3, 64.1, 60.6, 55.2, 53.5, 49.5, 42.9, 36.4, 31.9, 30.3, 29.7-29.3, 25.5, 22.7, 17.9, 15.6, 14.1; HRMS(ESI^+) calcd. for $C_{30}H_{52}N_3O_5$ $[M+H]^+$ 534.3901, found 534.3908.

Supplementary Scheme 3



9a: X = Palmitoyl ($C_{15}H_{31}$–CO–)
9c: X = Lauroyl ($C_{11}H_{23}$–CO–)

Solid-phase synthesis of *N*-palmitoyl-L-Ala-glycinal (9a) and *N*-lauroyl-L-Ala-glycinal (9c) using trichloroacetimidate linker.

Solid-phase peptide syntheses for preparation of the fatty acid-connected dipeptidylalcohols were carried out according to the reported procedure.[8] Trichloroacetimidate wang resin[9] washed with dry CH_2Cl_2 and dry THF was mixed with *N*-Fmoc-glycinol[10] dissolved in dry THF. The resulting solution was added with boron trifluoride diethylether (final concentration of 25 mM) and gently agitated for 1 h at rt. The reaction was quenched with MeOH and incubated for 5 min, the resin was then washed with THF, MeOH and CH_2Cl_2. The amount of the resin-loading Fmoc-protected aminoalcohol was estimated by measure of the absorbance (301 nm) of the *N*-(9-fluorenylmethyl)-piperidine adduct (ε = 7800 $M^{-1}cm^{-1}$) liberated from the resin by treatment of 50% piperidine in DMF. Fmoc group of the glycinol supported on wang resin through ether-linkage was removed using 20% (v/v) piperidine in DMF (2 x 10 min). Peptide couplings were carried out for 30 min in DMF using a 5 eq. (over resin loading) of *N*-Fmoc-L-Ala preliminarily activated with HBTU (5 eq.), HOBt (5 eq.) and *i*-Pr_2NEt (10 eq.). After the Fmoc group of L-Ala residue was removed, a fatty acyl group was connected with the amino group in the same procedure described above. Polymer-bound peptide chains were cleaved by exposure to 10% TFA in CH_2Cl_2 and the solvent was then removed by azeotropic distillation with toluene affording the crude fatty acyldipeptidylalcohols, which were confirmed by ESI-MS. Conversion of the alcohol to the corresponding aldehyde was performed with Dess-Martin periodinane. Dess-Martin periodinane was added to the solution of the crude palmitoyldipeptidylalcohol (52.1 mg) in dry CH_2Cl_2 (2.8 mL). After sitrring at rt for 40 min, the mixture was diluted with Et_2O (3 mL). The excess Dess-Martin periodinane was reduced with $Na_2S_2O_3$ (0.4 g) in saturated $NaHCO_3$ (2 mL) at 0 °C for 10min. The resulting mixture was extracted with $CHCl_3$ (10 mL x 2), and the combined organic phase was washed with brine (5 mL). After being dried over Na_2SO_4, the solvent was evaporated *in vacuo*. The crude materials were purified by

silica-gel column chromatography ($CHCl_3$/MeOH = 200:1-100:1-50:1) to yield 11.8 mg of *N*-palmitoyl-L-Ala-glycinal **9a**. *N*-lauroyl-L-Ala-glycinal **9c** was obtained as a yield of 10.8 mg with the same procedure.

***N*-palmitoyl-L-Ala-glycinal (9a).** $[\alpha]_D^{26}$ -28.2 (*c* 0.55, $CHCl_3$); ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 9.63 (s, 1H), 7.15 (brs, 1H), 6.21 (d, 1H, *J* = 7.2 Hz), 4.60 (dq, 1H, *J* = 7.5, 7.1 Hz), 4.13 (d, 2H, *J* = 5.0 Hz), 2.19 (t, 2H, *J* = 7.6 Hz), 1.65-1.54 (m, 2H), 1.40 (d, 3H, *J* = 7.1 Hz), 1.30-1.18 (m, 24H), 0.86 (t, 3H, *J* = 6.8 Hz); ^{13}C-NMR ($CDCl_3$, 75 MHz) δ 196.2, 173.5, 172.9, 50.1, 48.5, 36.5, 31.9, 29.7-29.2, 25.6, 22.7, 18.4, 18.1, 14.1; HRMS(ESI$^+$) calcd. for $C_{19}H_{37}N_2O_3$ $[M+H]^+$ 369.3112, found 369.3128.

***N*-lauroyl-L-Ala-glycinal (9c).** $[\alpha]_D^{26}$ -31.5 (*c* 0.50, $CHCl_3$); ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 9.63 (s, 1H), 7.11 (brs, 1H), 6.16 (d, 1H, *J* = 7.4 Hz), 4.60 (dq, 1H, *J* = 7.5, 7.1 Hz), 4.16 (d, 2H, *J* = 5.1 Hz), 2.21 (t, 2H, *J* = 7.6 Hz), 1.62-1.54 (m, 2H), 1.39 (d, 3H, *J* = 7.1 Hz), 1.28-1.18 (m, 16H), 0.86 (t, 3H, *J* = 6.6 Hz); ^{13}C-NMR ($CDCl_3$, 75 MHz) δ 196.2, 173.5, 172.9, 50.1, 48.5, 36.5, 31.9, 29.6-29.2, 25.5, 22.7, 18.1, 14.1; HRMS(ESI$^+$) calcd. for $C_{19}H_{37}N_2O_3$ $[M+H]^+$ 313.2485, found 313.2507.

SfmC-catalyzed reaction employing the dipeptidyl-*S*-CoA 4a - 4e and *N*-myristoyl-Ala-glycinal 9b.

The enzymatic reactions were performed in 200 μL reaction mixtures of 50 mM KH_2PO_4-KOH (pH 7.0) containing 200 mM dipeptidyl-*S*-CoA, 500 μM tyrosine derivative **2**, 2 mM ATP, 10 mM $MgCl_2$, 10 μM $MnCl_2$ and 2 μM *holo*-SfmC. *Holo*-SfmC was prepared from 8 μM *apo*-SfmC by treatment with 50 μM coenzyme A, 1 μM Sfp, 10 mM $MgCl_2$ at rt for 10 min. The reactions were initiated by adding 3 mM NAD(P)H. After the incubation at

30 ºC for 2 h, 10% formic acid (20 μL) and CH_3CN (180 μL) were successively added to the reaction mixtures, and then insoluble materials were removed by centrifugation. The resulting supernatants were analyzed by LC-ESI-MS (LC: Agilent 1100 system, MS: JEOL, JMS-T100LP AccuTOF LC-plus) with a Zorbax Eclipse XDB-C18 column (Agilent, 2.1, 5 μm) under the following conditions: 0–20 min, linear gradient 30–80% CH_3CN/0.1% formic acid in H_2O/0.1% formic acid at a flow rate of 0.2 mL/min at 30 °C. The enzymatic reactions using *N*-myristoyl-L-Ala-glycinal **9b** were carried out under the same conditions as described above. Similarly, the conversion of *N*-myristoyldipeptidyl-*S*-CoA substrate **4b** with C-domain-deletion mutant of SfmC, *holo*-A-PCP-R tridomain, was also performed according to the identical protocol.

Large-scale enzymatic reaction to prepare *N*-myristoyl-cyanopresaframycin (6) for NMR analysis.

The large-scale enzymatic reaction was performed in 50 mL reaction mixture of 50 mM KH_2PO_4-KOH (pH 7.0) containing 200 μM **9b**, 500 μM **2**, 2 mM ATP, 10 mM $MgCl_2$, 10 μM $MnCl_2$ and 2 μM *holo*-SfmC. *Holo*-SfmC was prepared from 8 μM *apo*-SfmC by treatment with 50 μM coenzyme A, 1 μM Sfp, 10 mM $MgCl_2$ at rt for 10 min. The reaction was initiated by adding 2 mM NADH and incubated at 30 ºC for 2.5 h. Then, 100 mM KCN (0.5 mL) was added, and the resulting mixture was stirred at rt for 5 min. To remove the enzyme, the mixture was added MeOH (150 mL) and subsequently centrifuged at 5000 x *g* for 15 min. The obtained supernatant was evaporated *in vacuo*. The remaining solution was extracted with $CHCl_3$ (100 mL x 3), and the combined organic phase was washed with brine, dried over Na_2SO_4 and evaporated in the reduced pressure. The resultant material was diluted with MeOH/H_2O (4:1, 1 mL) and purified twice by high-performance liquid chromatography (HPLC) on a LC-10AD vp HPLC system (Shimadzu) with a Wakosil-II 5C18 column (Wako,

10.0 x 250 mm, 5 μm) under the following conditions: 0–30 min, linear gradient 40–75% CH_3CN/0.05% formic acid in H_2O/0.05% formic acid at a flow rate of 3.0 mL/min at 35 ºC. The fraction containing the desired product was lyophilized to dryness to yield **6** (1.6 mg, 22% yield) as well as **5b** (0.4 mg, 6% yield). The isolated cyanoadduct **6** was analyzed by a series of NMR measurements including ^{1}H-NMR, ^{13}C-NMR, COSY, HSQC, HMBC and NOESY. $[\alpha]_D^{28}$ -21.0 (*c* 0.08, $CHCl_3$); ^{1}H-NMR ($CDCl_3$, 600 MHz) δ 6.52 (s, 1H), 6.42 (s, 1H), 5.96 (d, 1H, *J* = 7.2 Hz), 5.30 (t, 1H, *J* = 4.8 Hz), 4.38 (d, 1H, *J* = 2.4 Hz), 4.11 (s, 1H), 4.04 (d, 1H, *J* = 1.8 Hz), 3.79 (s, 3H), 3.75 (s, 3H), 3.74-3.70 (m, 2H), 3.71-3.67 (m, 1H), 3.27 (dt, 1H, *J* = 2.4, 12.0 Hz), 3.23 (dt, 1H, *J* = 4.8, 13.8 Hz), 3.15, (dd, 1H, *J* = 8.4, 18.0 Hz), 2.83 (dd, 1H, *J* = 2.4, 15.6 Hz), 2.76 (d, 1H, *J* = 18.0 Hz), 2.27 (s, 3H), 2.22 (s, 3H), 2.13 (dd, 1H, *J* = 12.0, 15.6 Hz), 2.04 (t, 1H, *J* = 7.8 Hz), 1.54-1.48 (m, 2H), 1.29-1.20 (m, 20H), 0.92 (d, 3H, *J* = 7.2 Hz), 0.88 (t, 3H, *J* = 7.2 Hz); ^{13}C-NMR ($CDCl_3$, 150 MHz) δ 172.0, 171.6, 145.0, 144.7, 143.4, 142.9, 131.5, 131.2, 129.1, 128.9, 121.2, 121.1, 120.9, 117.5, 117.1, 60.6, 60.5, 59.5, 57.2, 56.7, 49.5, 49.2, 48.1, 41.4, 36.5, 32.0, 31.7, 31.2, 29.5-29.0, 25.4, 22.5, 18.7, 15.6, 15.4, 13.9; HRMS(ESI$^+$) calcd. for $C_{42}H_{62}N_5O_6$ $[M+H]^+$ 732.4695, found 732.4716.

Enzymatic reaction with *S*-(4-^{2}H)NADPH and *R*-(4-^{2}H)NADPH.

S-(4-^{2}H)NADPH and *R*-(4-^{2}H)NADPH were prepared according to reported procedures.[11, 12] The reactions were conducted under the same conditions as described above using *N*-myristoyl-Ala-Gly-*S*-CoA **4b** and either 3 mM *S*-(4-^{2}H)NADPH or *R*-(4-^{2}H)NADPH. The resulting mixtures were directly analyzed by LC-ESI-MS.

SfmC-time course analyses with *N*-myristoyldipeptidylaldehyde 9b.

The enzymatic reactions for the time course analyses in the presence of the different concentration (200 μM or 2 mM) of NADPH or NADH were performed in 100 μL reaction mixtures of 50 mM KH_2PO_4-KOH (pH 7.0) containing 100 μM dipeptidylaldehyde **9b**, 250

μM **2**, 2 mM ATP, 10 mM $MgCl_2$, 10 μM $MnCl_2$ and 2 μM *holo*-SfmC. *Holo*-SfmC was prepared from 8 μM *apo*-SfmC by treatment with 50 μM coenzyme A, 1 μM Sfp, 10 mM $MgCl_2$ at rt for 10 min. The reactions were initiated by adding 200 μM or 2 mM NAD(P)H. At the various time points (15, 30, 60, 120 min), each aliquot of the reaction mixture was quenched with CH_3CN (80 μL) and 10% formic acid (20 μL) and stored at -80 °C until it would be subjected to LCMS analysis. The yields of the reaction products were determined as the ionization efficiency of the compounds is equivalent, and the synthetic bicyclic alcohol **8b** was used as a standard for calculation. The yields at the each time point are collected by the mean of two independent reactions.

Competition experiments with the mixture of labeled and non-labeled cofactors.

SfmC-catalyzed reaction with *N*-myristoyldipeptidylaldehyde **9b** in the presence of the low (200 μM) or high (2 mM) concentrations of NADPH and NADH as a 1:1 mixture, either of which are labeled with deuterium. The enzymatic reactions were performed in 100 μL reaction mixtures of 50 mM KH_2PO_4-KOH (pH 7.0) containing 200 μM dipeptidylaldehyde **9b**, 500 μM tyrosine derivative **2**, 2 mM ATP, 10 mM $MgCl_2$, 10 μM $MnCl_2$ and 2 μM *holo*-SfmC. *Holo*-SfmC was prepared from 8 μM *apo*-SfmC by treatment with 50 μM coenzyme A, 1 μM Sfp, 10 mM $MgCl_2$ at rt for 10 min. The reactions were initiated by adding the 1:1 mixtures of NAD(P)H and deuterium-labeled NAD(P)H at the low (200 μM) or high (2 mM) concentration. After the incubation at 30 ºC for 2 h, 10% formic acid (20 μL) and CH_3CN (80 μL) were successively added to the reaction mixtures. The resulting mixtures were analyzed by LC-ESI-MS. *S*-(4-^{2}H)NADPH and *S*-(4-^{2}H)NADH were prepared according to reported procedures using the glucose dehydrogenase from *Bacillus megaterium* and D-(1-^{2}H)-glucose.[11, 12] Before employing deuterium-labeled NAD(P)H for the SfmC reaction, the glucose dehydrogenase was removed using the microcon centrifugal device (Millipore).

The yields of products in the reaction with CoA thioesters (4a, 4b and 4c) and with dipeptidylaldehydes (9a, 9b and 9c).

The enzymatic reactions with the fatty acyldipeptidyl-CoAs **4a** - **4c** and with the fatty acyldipeptidylaldehydes **9a** – **9c** were performed in 100 μL reaction mixtures of 50 mM KH_2PO_4-KOH (pH 7.0) containing 100 μM substrate, 250 μM tyrosine derivative **2**, 2 mM ATP, 10 mM $MgCl_2$, 10 μM $MnCl_2$ and 2 μM *holo*-SfmC. *Holo*-SfmC was prepared from 8 μM *apo*-SfmC by treatment with 50 μM coenzyme A, 1 μM Sfp, 10 mM $MgCl_2$ at rt for 10 min. The reactions were initiated by adding 3 mM NAD(P)H. After the incubation at 30 ºC for 2 h, 10% formic acid (20 μL) and CH_3CN (80 μL) were successively added to the reaction mixtures. The resulting mixtures were analyzed by LC-ESI-MS. The yields of the reaction products were determined as the ionization efficiency of the compounds is equivalent, and the synthetic bicyclic alcohol **8b** was used as a standard for calculation. The error bars represent s.d. of the mean of three independent experiments.

Detection of the intermediate bound to PCP-domain.

The enzymatic reactions were performed in 500 μL reaction mixtures containing 200 μM *N*-myristoyl-Ala-glycinal **9b**, 500 μM tyrosine derivative **2**, 2 mM ATP, 10 mM $MgCl_2$, 10 μM $MnCl_2$, 50 mM KH_2PO_4-KOH (pH 6.0) and 4 μM *holo*-C-A-PCP tridomain or *holo*-A-PCP-R tridomain. *Holo*-enzymes were prepared from 8 μM *apo*-enzymes by treatment with 50 μM coenzyme A, 1 μM Sfp, 10 mM $MgCl_2$ at rt for 10 min. After incubation at 30 ºC for 1h, EtOH (500 μL) was added to the mixture, and then the resulting protein was collected by centrifugation. The precipitant was successively washed with EtOH/H_2O = 1:1 (1 mL) and EtOH (1 mL) and then suspended with THF/MeOH/H_2O = 3:1:0.1 (0.4 mL). To liberate the intermediate bound to PCP, the suspension was added excess $NaBH_4$ (10 mg) and incubated at rt for 30 min. After centrifugation, the supernatant was separated and concentrated *in vacuo*. The remaining $NaBH_4$ was quenched with 10% formic

acid (100 μL) and CH_3CN (50 μL). The supernatant was analyzed by LC-ESI-MS (LC: Agilent 1100 system, MS: JEOL, JMS-T100LP AccuTOF LC-plus) with a Zorbax Eclipse XDB-C18 column (Agilent, 2.1 x 50 mm, 5 μm) under the following conditions: 0–20 min, linear gradient 30–80% CH_3CN /0.1% formic acid in H_2O/0.1% formic acid at a flow rate of 0.2 mL/min at 30 °C.

Supplementary References

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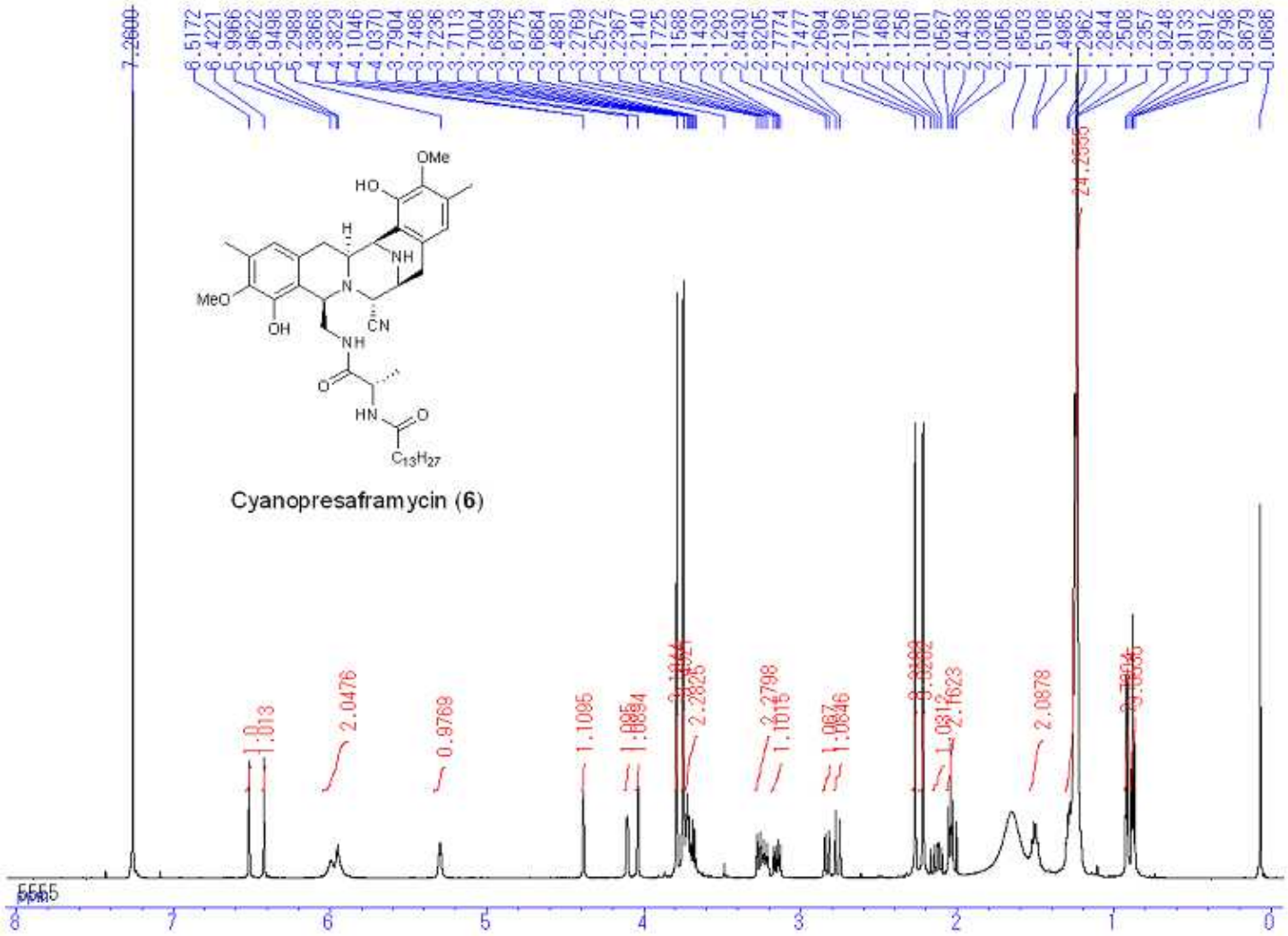
Copies of 1D and 2D NMR spectra

^{1}H NMR spectrum of *N*-myristoyl-cyanopresaframycin (6) in $CDCl_3$ (600 MHz)

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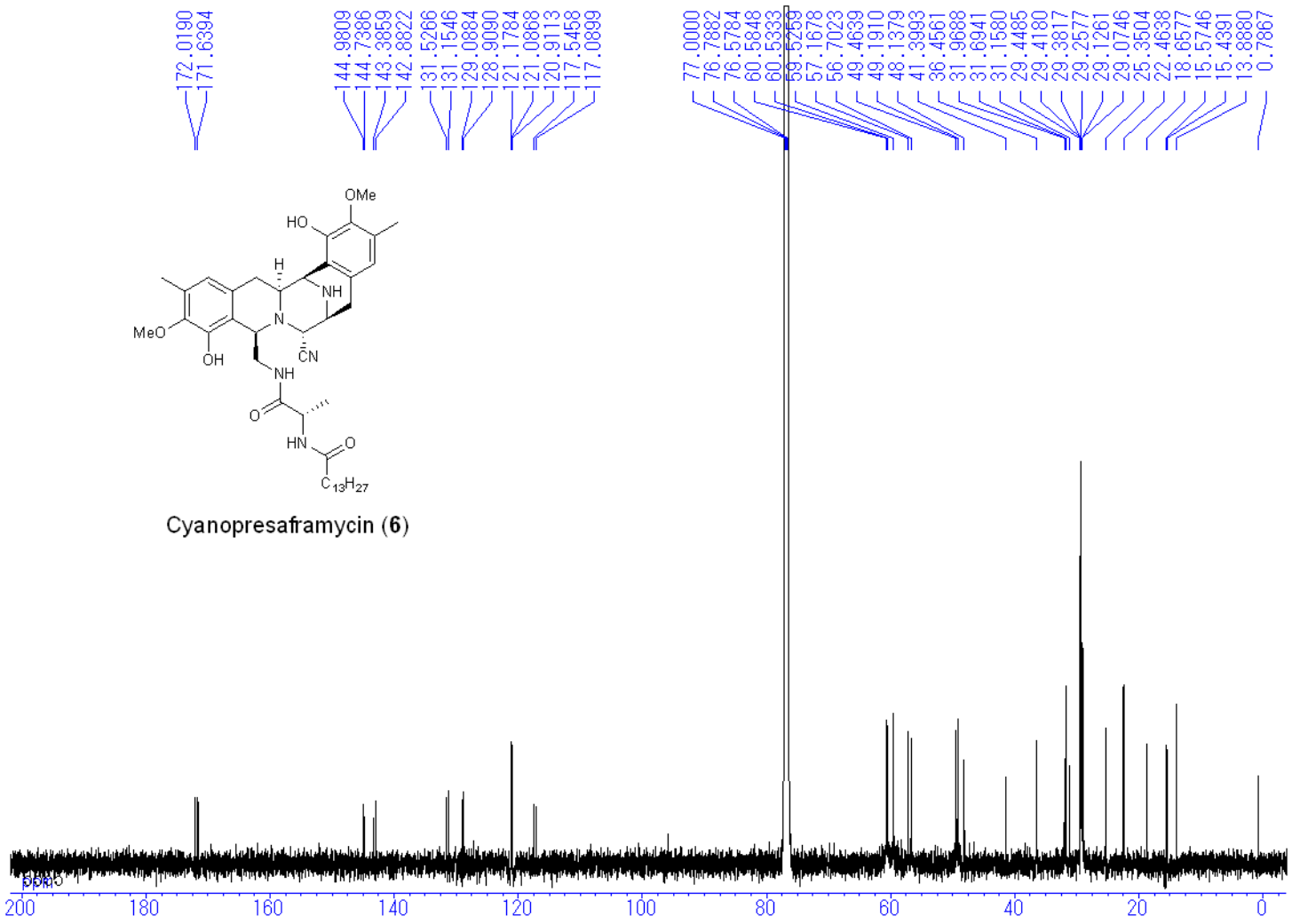


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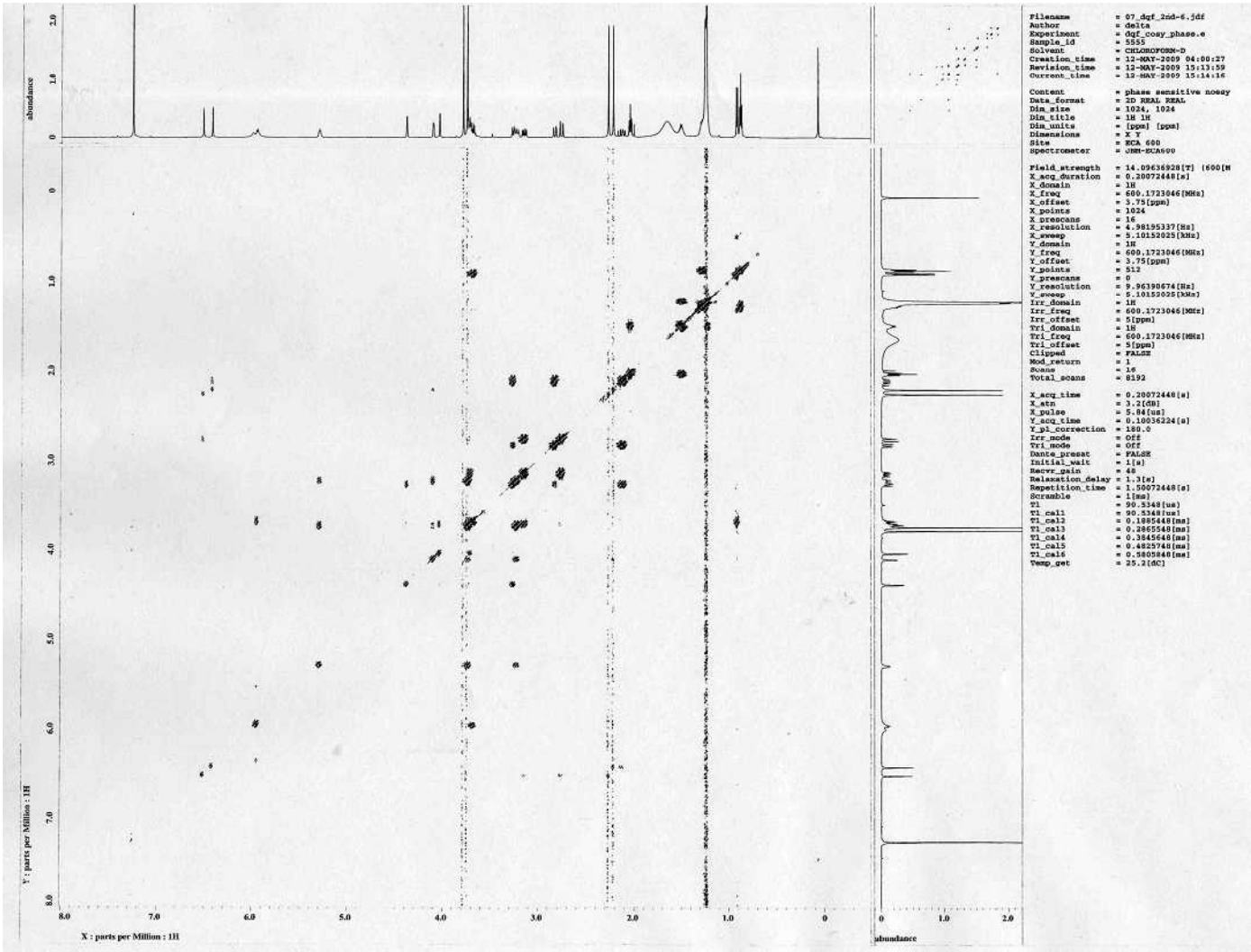

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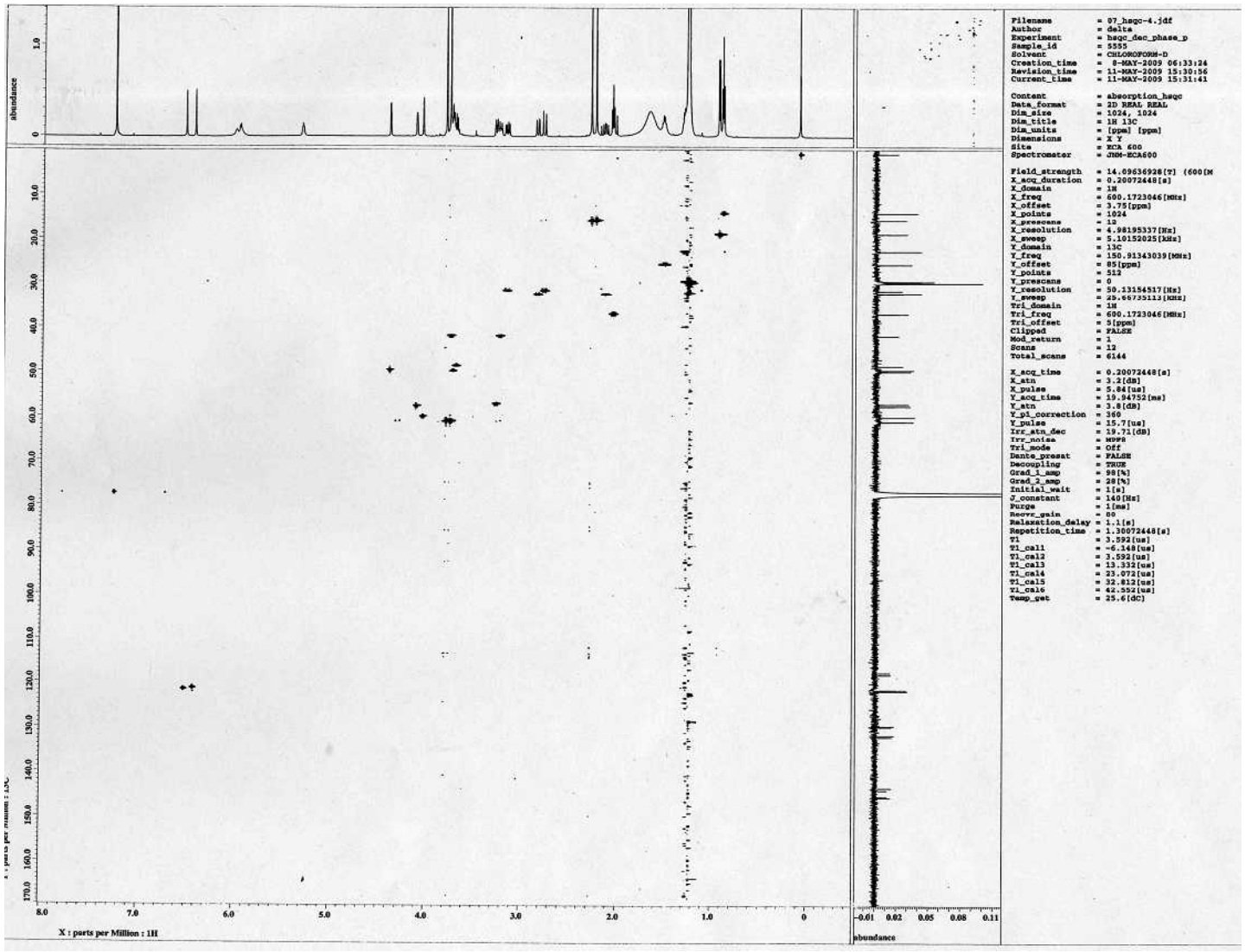

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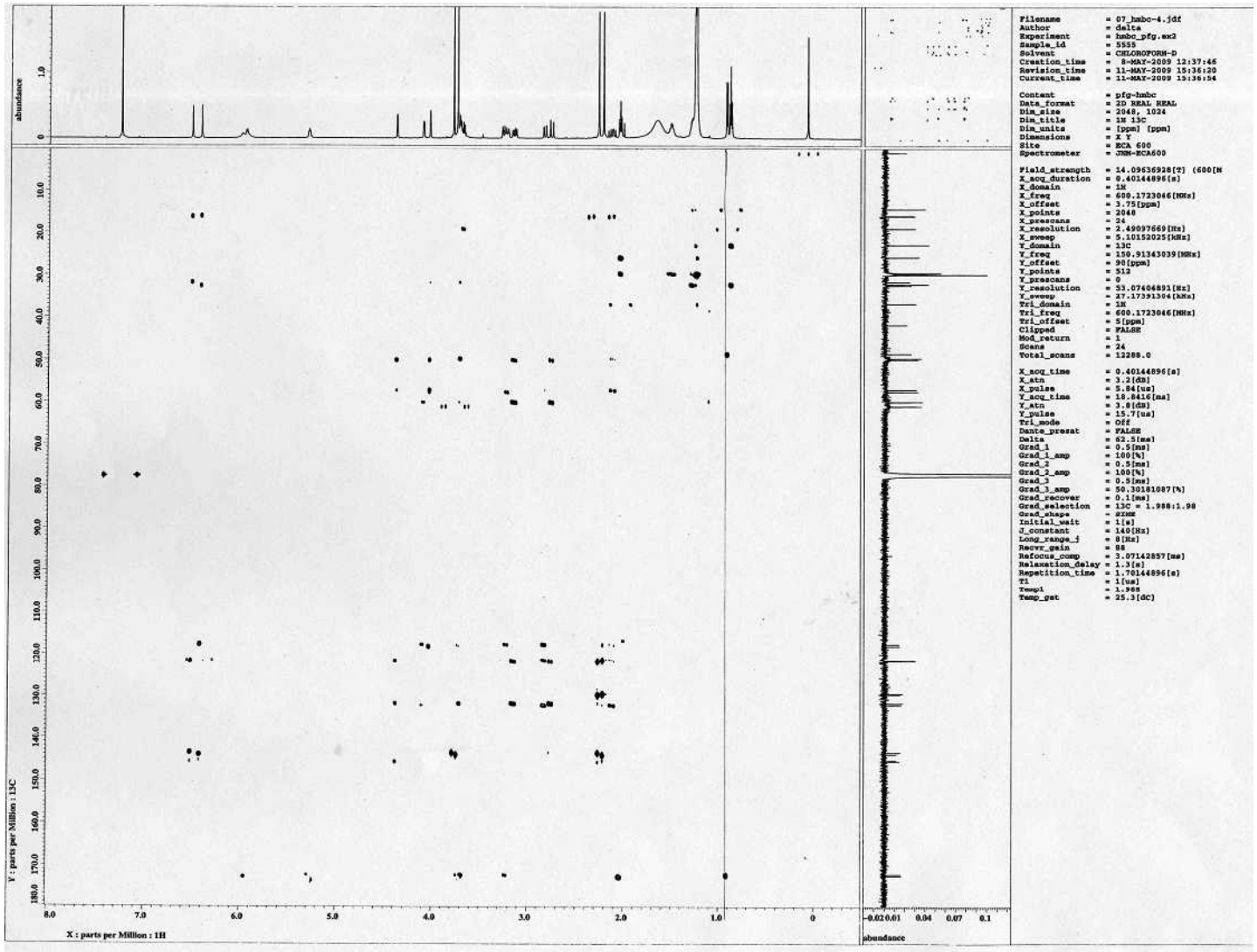

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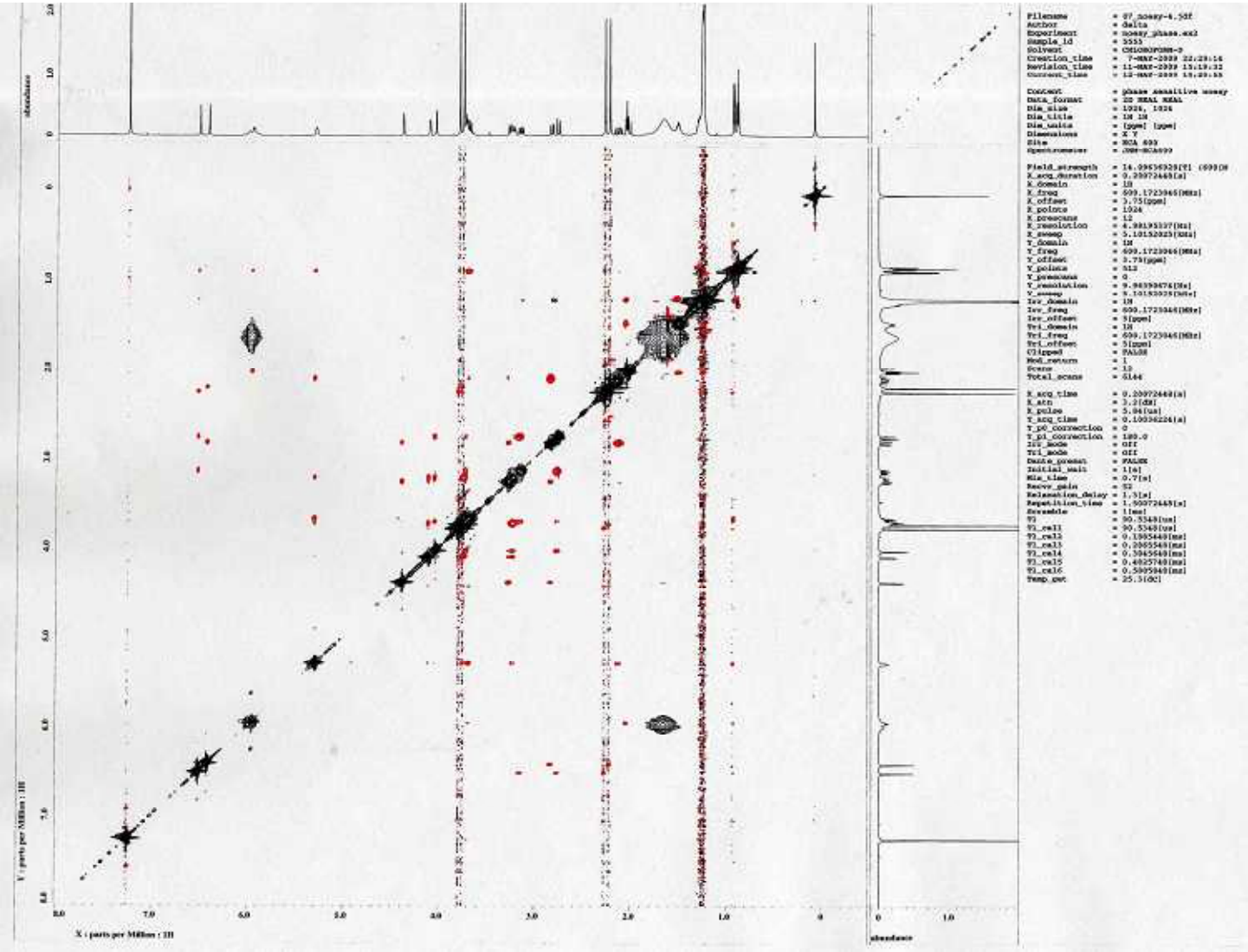

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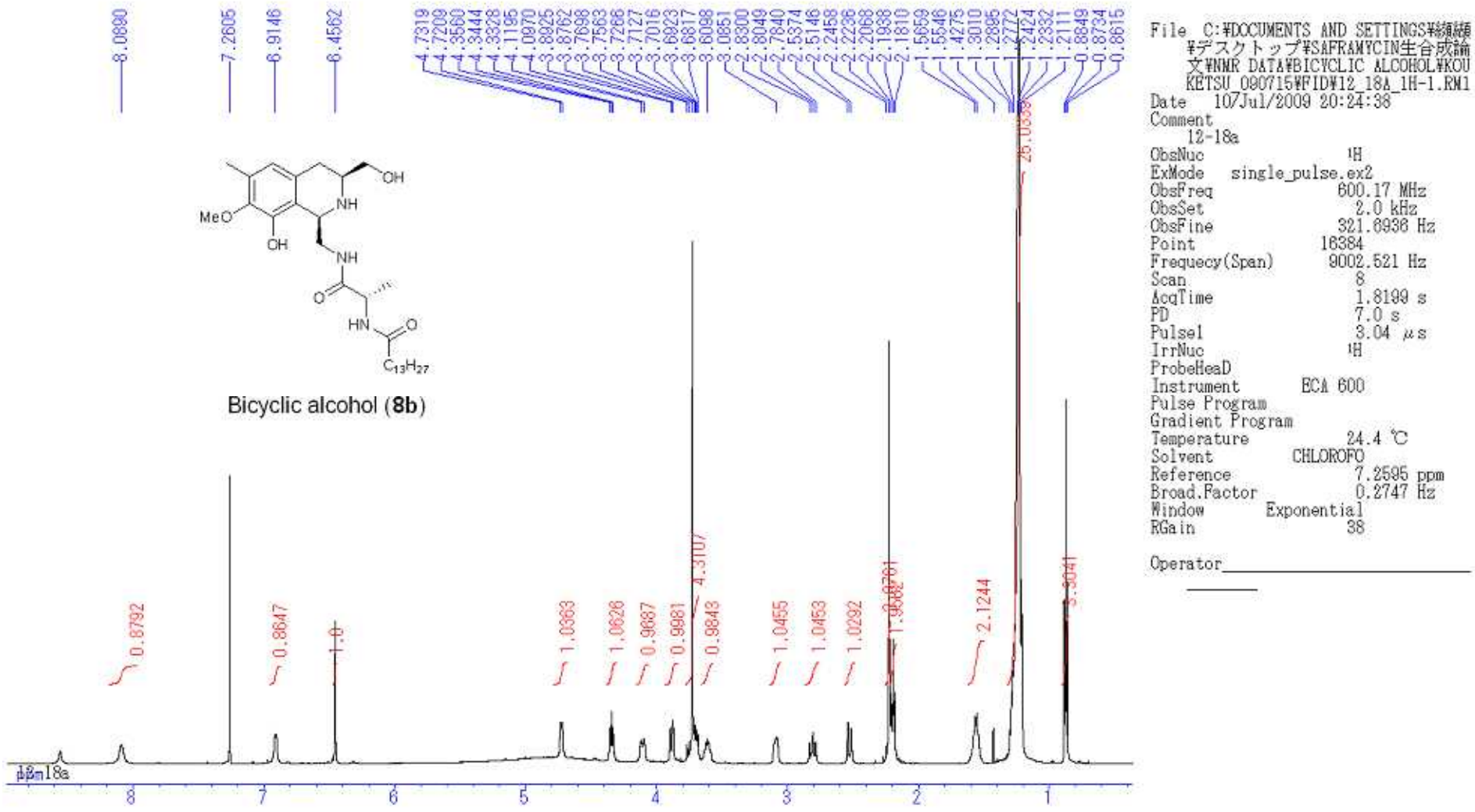

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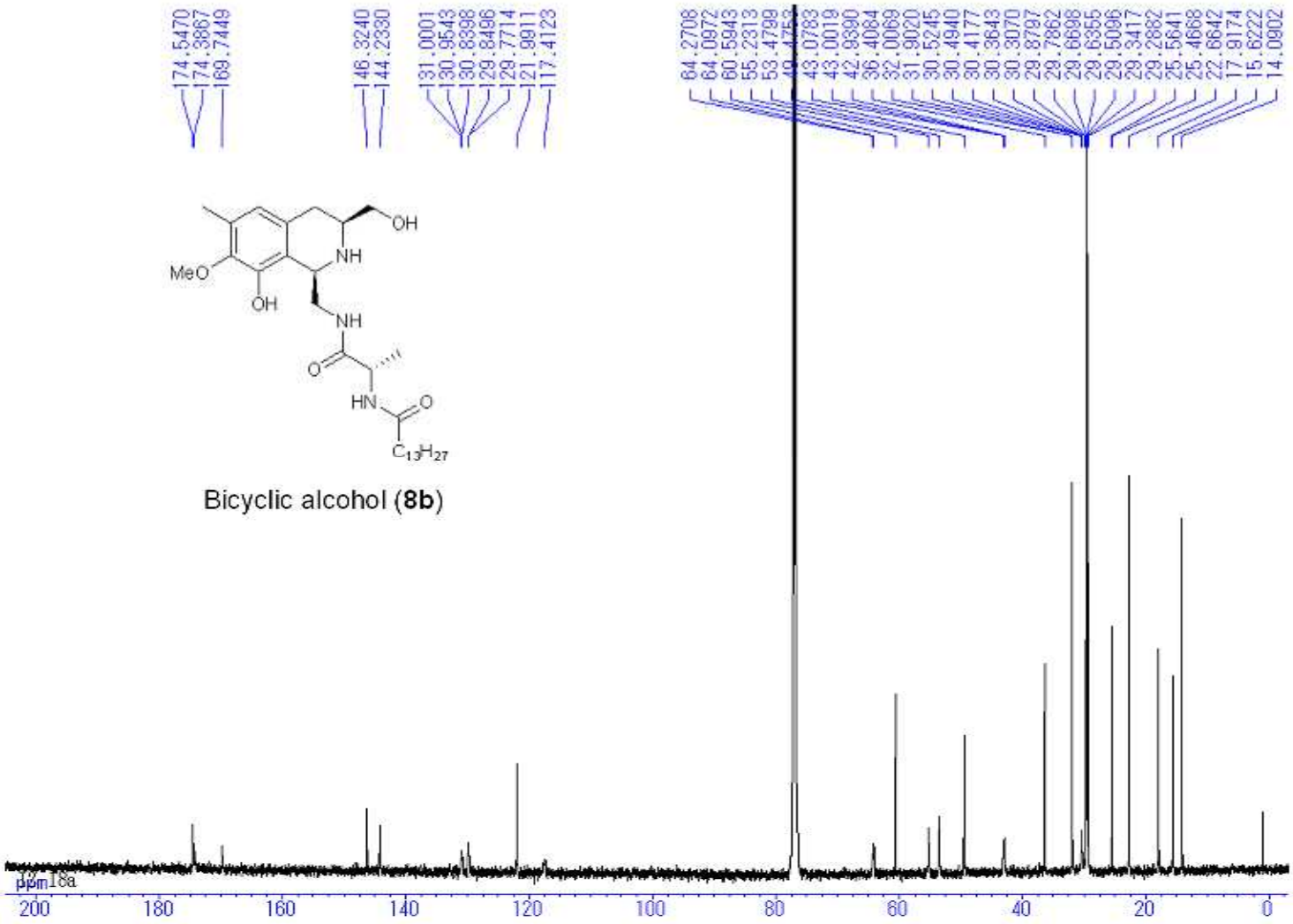

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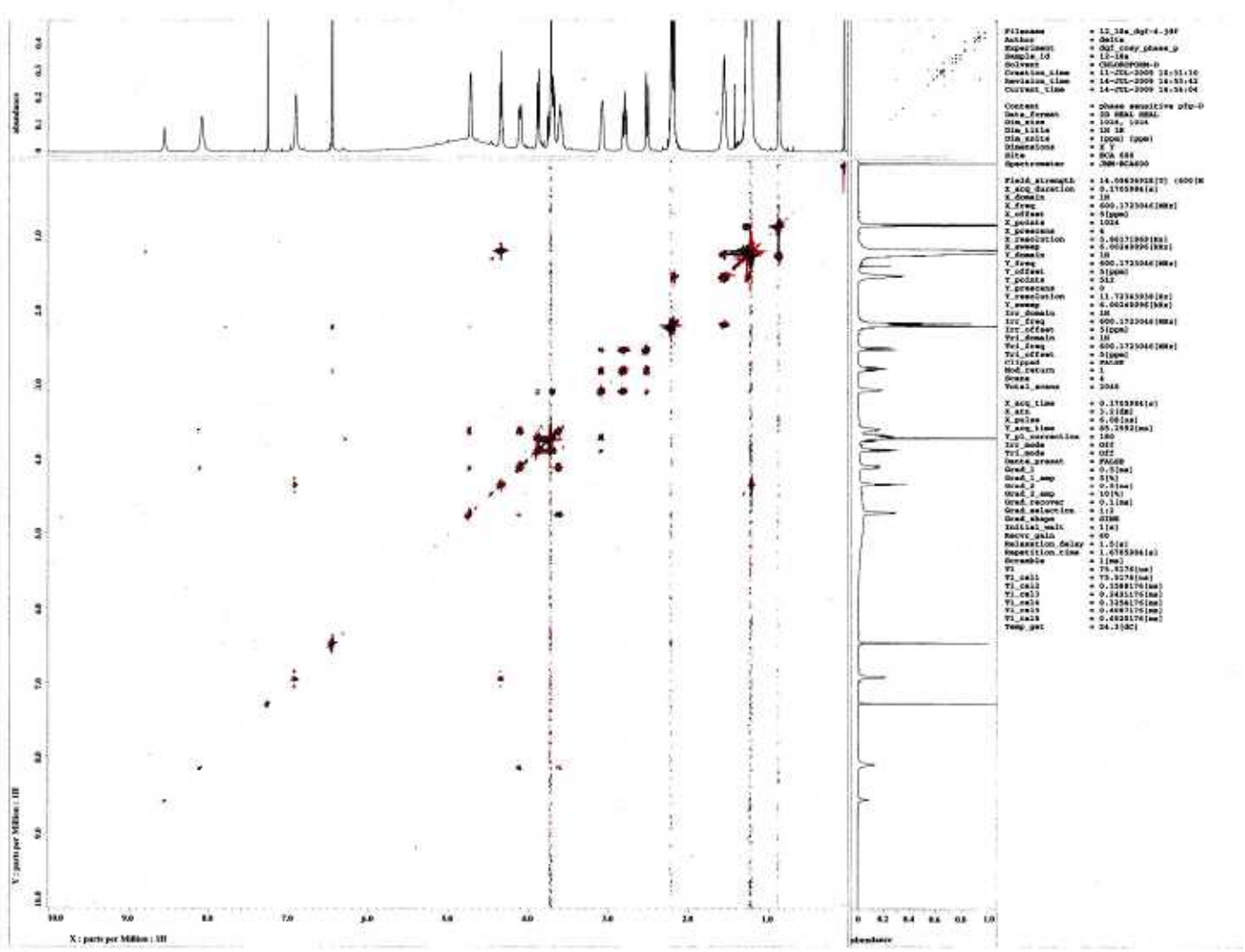
COSY spectrum of bicyclic alcohol (8b) in $CDCl_3$ (600 MHz)



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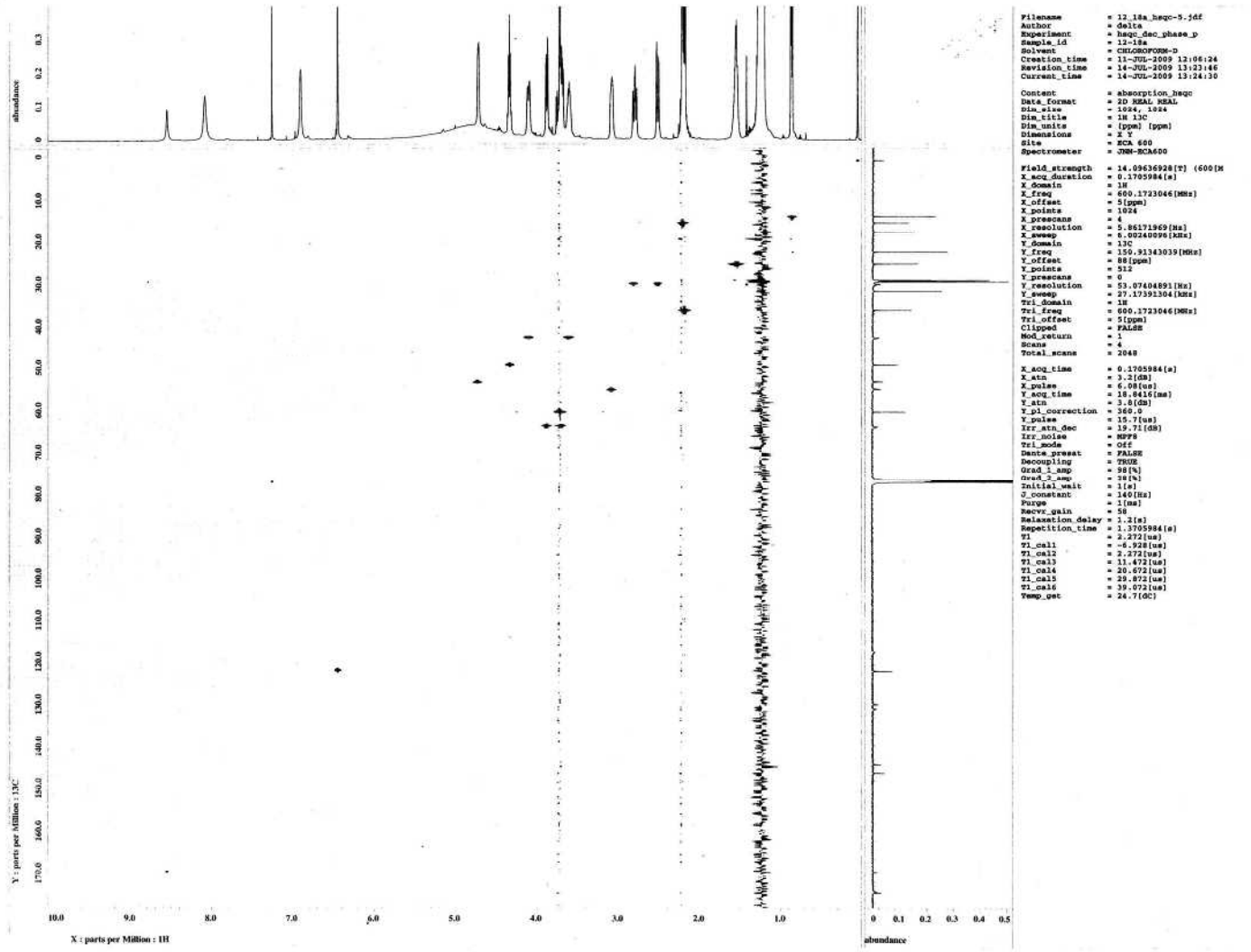

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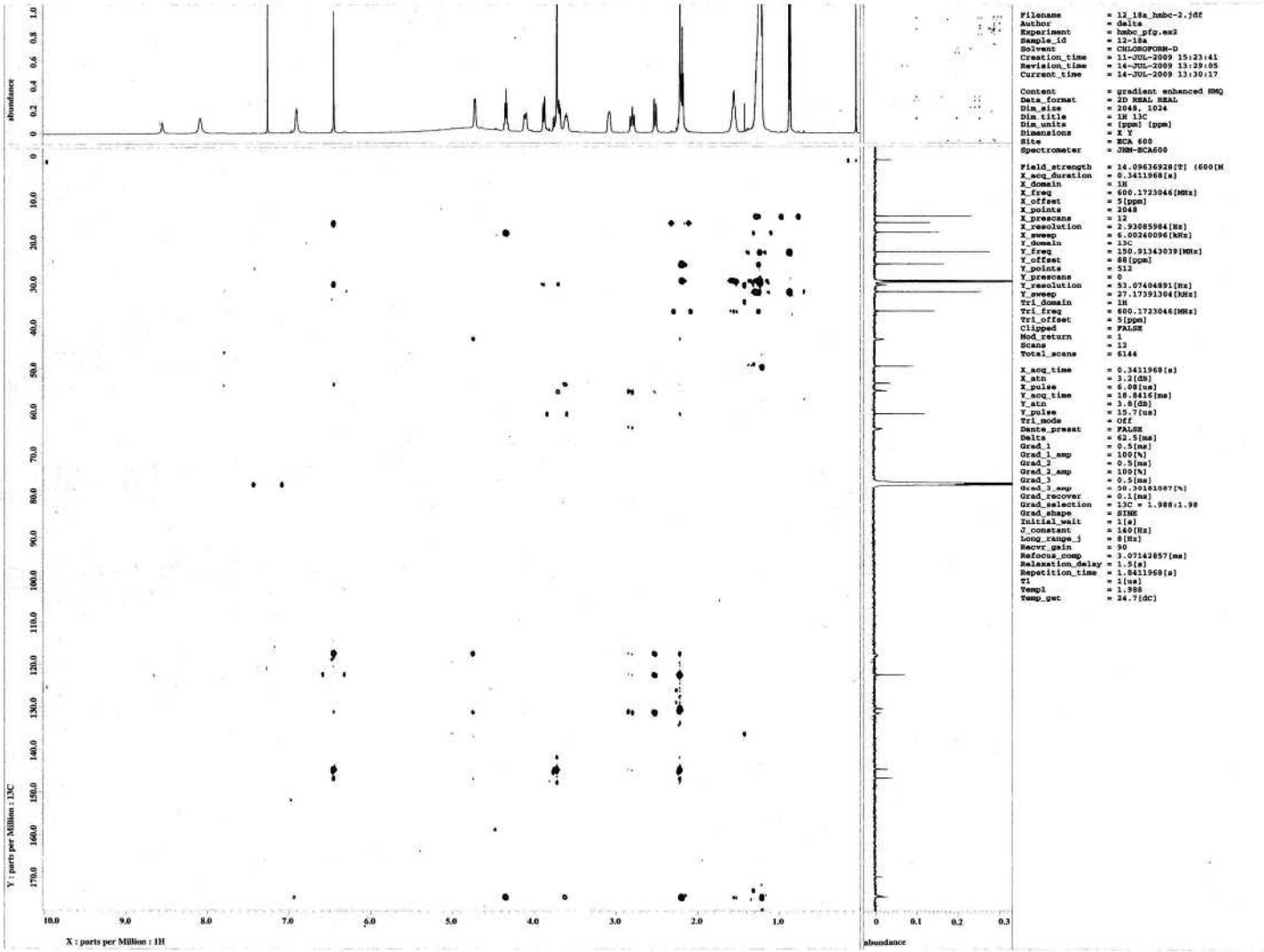

ROESY spectrum of bicyclic alcohol (8b) in $CDCl_3$ (600 MHz)