SUPPLEMENTARY MATERIAL

Silychristin derivatives conjugated with coniferylalcohols from silymarin and their pancreatic α-amylase inhibitory activity

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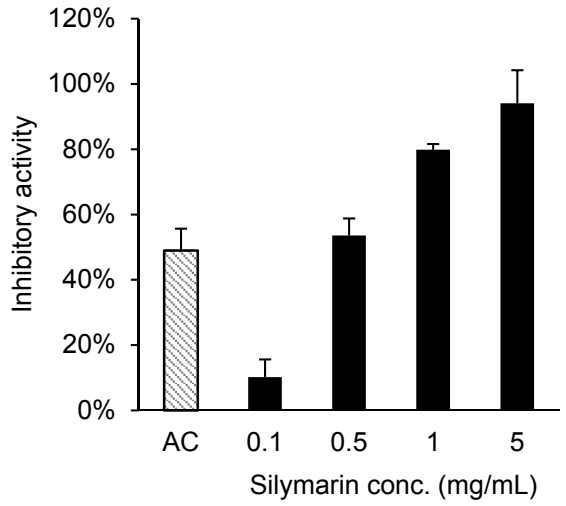


Figure S1. α-amylase inhibitory activity of silymarin. AC: acarbose (10 μM)

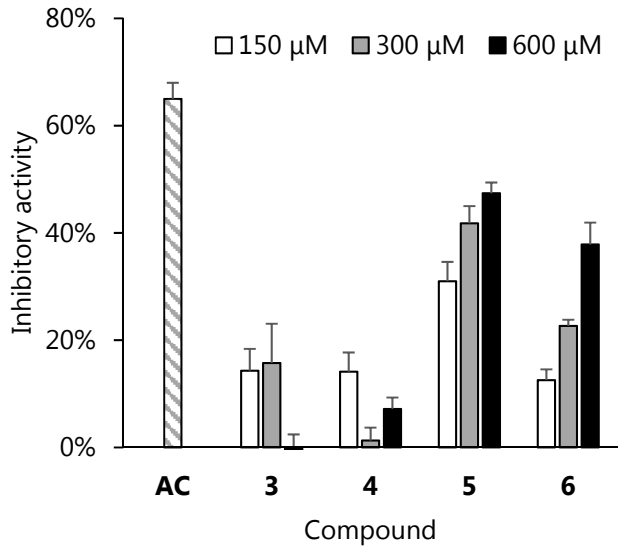


Figure S2. α-amylase inhibitory activity of isolated compounds. AC: acarbose (10 μM)

Experimental

General

Silymarin (a mixture of flavonolignans extracted from the fruits of milk thistle) was obtained from Omnica Co., Ltd. (Tokyo, Japan). Silibinin was purchased from Sigma–Aldrich Japan K. K. (Tokyo, Japan). All other chemicals were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) unless stated otherwise. Absorbance was measured using a Synergy™ MX microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). An AMX 500 system (Bruker Japan K.K., Yokohama, Japan) was used to obtain NMR spectra, and residual solvents were used as an internal standard (acetone-d_6: ^{1}H 2.04 ppm, ^{13}C 29.92 ppm; methanol-d_4: ^{1}H 3.30 ppm, ^{13}C 49.00 ppm). A LCT Premier Spectrometer (Nihon Waters K.K., Tokyo, Japan) was employed to obtain low-resolution mass spectra and an Exactive system (Thermo Fisher Scientific K.K., Tokyo, Japan) was used for HR-ESI-MS.

Measurement of α-amylase inhibitory activity

The α-amylase inhibitory activity was determined using starch azure (Sigma–Aldrich; S7629) as the substrate and porcine pancreatic α-amylase (Sigma–Aldrich; A3176) as the enzyme. Briefly, 350 μL of starch azure (4 mg/mL) suspended in Tris-HCl buffer (0.1 M, pH 6.9, 0.01 M $CaCl_2$), 100 μL of sample dissolved in water, and 50 μL of porcine pancreatic α-amylase dissolved in Tris-HCl buffer were incubated with frequent mixing for 10 min at 37°C. The reaction was stopped by addition of 50% acetic acid (50 μL) and centrifugation (1,500 × *g*, 5 min). The absorbance at 595 nm of the supernatant was measured. Absorbance of the blank (without enzyme) was subtracted and inhibitory activity was calculated compared with that of the control value. Each experiment was carried out in duplicate and repeated at least twice. Mean values are shown in the figures.

Isolation procedure

Silymarin powder (30 g) was suspended in water and extracted by ethyl acetate and then 1-butanol to obtain a layer of water (1.0 g), 1-butanol (3.73 g), and ethyl acetate (20.1 g). The ethyl-acetate layer (10.86 g) was loaded on a silica-gel column (5.0 × 25.0 cm) and eluted stepwise by

chloroform/methanol = 20/1, 10/1, 5/1, 0/1 (1.5-L each). The fractions eluted by chloroform/methanol = 5/1, 0/1 (5.84 g) were combined and purified again by silica-gel column chromatography (5.0 × 22.0 cm), and eluted stepwise by hexane/ethyl acetate = 1/2, 1/3, 1/6 and then by acetone (1.2-L each). Silychristin (**2**) was isolated from the fraction eluted by hexane/ethyl acetate = 1/2 and 1/3 using preparative thin-layer chromatography (hexane/ethyl acetate = 1/6 with 5% acetic acid) (Figure S3). The fraction eluted by acetone (AF, 1.53 g out of 1.86 g) was purified by reverse-phase column chromatography using a Cosmosil 75C_{18}-OPN column (3.0 × 22.0 cm; Nakalai Tesque, Inc., Kyoto, Japan) and eluted stepwise by methanol/water = 3/7, 5/5, 6/4, 7/3, 8/2 with 0.1% trifluoroacetic acid (TFA) (500-mL each, fraction named AF-1 to AF-5).

Fractions AF-1 and 2 were combined (410 mg) and loaded onto a Sephadex LH-20 column (1.5 × 27.0 cm; GE Healthcare Japan Co., Tokyo, Japan) for column chromatography and eluted by methanol, and divided into six fractions (100-mL each, AF-1/2-1 to AF-1/2-6). AF-1/2-3 (83 mg) was purified by HPLC [column: Cosmosil πNAP (10 × 250 mm); mobile phase: gradient elution from 40% aq. methanol with 0.1% TFA to 95% aq. methanol with 0.1% TFA (0–60 min)] to obtain compound **3** (2.2 mg). AF-1/2-5 (97.1 mg) was purified by HPLC [column: Cosmosil πNAP (10 × 250 mm); mobile phase: gradient elution from 40% aq. methanol with 0.1% TFA to 95% aq. methanol with 0.1% TFA (0–60 min)] to obtain a peak. The peak was purified by HPLC [column: Cosmosil PBr (10 × 250 mm, Nakalai Tesque, Inc.); mobile phase: 70 aq. methanol with 0.1% TFA] to obtain compound **4** (3.3 mg).

Fraction AF-3 (325.3 mg) was purified by HPLC [column: Cosmosil πNAP (10 × 250 mm, Nakalai Tesque, Inc.); mobile phase: 65% aq. methanol with 0.1% TFA] to obtain two major peaks. Each peak was purified by HPLC [column: Cosmosil πNAP (10 × 250 mm); mobile phase: 40% aq. acetonitrile with 0.1% TFA] to obtain compounds **5** (3.0 mg) and **6** (4.8 mg).

Data of the isolated compounds

Silychristin A (**2**): ^{1}H-NMR (500 MHz, rt, methanol-d_4) 3.53 (1H, dt, J=6.2, 6.1 Hz), 3.83 (3H, s), 3.80-3.87 (2H, m),

4.54 (1H, d, *J*=11.5 Hz), 4.94 (1H, d, *J*=11.5 Hz), 5.56 (1H, d, *J*=6.2 Hz), 5.88 (1H, d, *J*=2.1 Hz), 5.92 (1H, d, *J*=2.1 Hz), 6.77 (1H, d, *J*=8.2 Hz), 6.86 (1H, dd, *J*=1.8, 8.2 Hz), 6.90 (1H, d, *J*=1.6 Hz), 6.95 (1H, br s), 6.99 (1H, d, *J*=1.8 Hz) ppm; ^{13}C-NMR (125 MHz, rt, methanol-d_4) 55.53, 56.41, 64.82, 73.76, 85.29, 89.11, 96.29, 97.35, 101.84, 110.60, 116.15, 116.63, 116.93, 119.76, 130.10, 131.59, 134.75, 142.17, 147.53, 149.12, 149.12, 164.49, 165.33, 168.74, 198.35 ppm; ESI-MS (positive) *m*/*z*=505 $[M+Na]^+$.

Data was compared with the reported values (Cheng et al. 2013).

Mariamide B (**3**): colorless solid, cis-isomer ^{1}H-NMR (500 MHz, rt, methanol-d_4) 1.55-1.60 (4H, m), 3.22-3.37 (4H, m), 3.81 (3H, s), 5.83 (1H, d, *J*=12.7 Hz), 6.61 (1H, d, *J*=12.7 Hz), 6.73 (1H, d, *J*=8.2 Hz), 6.82 (2H, d, *J*=8.4 Hz), 6.93 (1H, dd, *J*=1.3, 8.2 Hz), 7.35 (1H, d, *J*=1.3 Hz), 7.67-7.70 (2H, m) ppm; ^{13}C-NMR (125 MHz, rt, methanol-d_4) 27.70, 27.97, 40.10, 40.48, 56.39, 113.93, 115.82, 116.04, 116.04, 121.78, 124.78, 126.56, 128.61, 130.19, 130.19, 138.22, 148.51, 148.51, 161.95, 170.11, 170.46 ppm; trans-isomer ^{1}H-NMR (500 MHz, rt, methanol-d_4) 1.60-1.69 (4H, m), 3.30-3.42 (4H, m), 3.88 (3H, s), 6.42 (1H, d, *J*=15.8 Hz), 6.79 (1H, d, *J*=8.1 Hz), 6.80 (2H, d, *J*=8.4 Hz), 7.02 (1H, dd, *J*=1.0, 8.1 Hz), 7.11 (1H, d, *J*=1.0 Hz), 7.43 (1H, d, *J*=15.8 Hz), 7.67-7.70 (2H, m) ppm; ^{13}C-NMR (125 MHz, rt, methanol-d_4) 28.03, 28.05, 40.22, 40.50, 56.39, 111.55, 116.04, 116.04, 116.47, 118.77, 123.19, 126.56, 128.29, 130.19, 130.19, 142.00, 149.29, 149.82, 161.95, 169.23, 170.11 ppm; ESI-MS (positive) *m*/*z*=385 $[M+H]^+$.

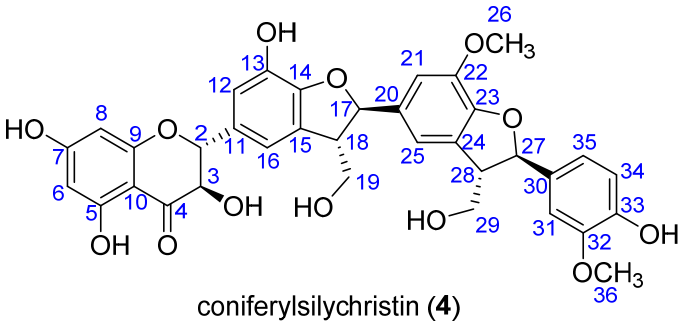
Data was compared with the reported values (Choudhary et al. 2008; Qin et al. 2017).

Coniferylsilychristin (**4**): brown solid, see Table S1 for NMR data. HR-ESI-MS (positive): found *m*/*z* 683.17583 $[M+Na]^+$, $C_{35}H_{32}O_{13}Na^+$ requires 683.17351; IR (neat) ν = 726, 783, 849, 1030, 1089, 1145, 1206, 1278, 1343, 1465, 1498, 1516, 1635, 1677, 2285, 2349, 2925, 3376 cm^{-1}; $[\alpha]_D^{23}$ +52.7° (c = 0.073, methanol).

8-dehydrodiconyferylsilychristin (**5**): brown solid, see Table S2 for NMR data. HR-ESI-MS (positive): found *m*/*z* 845.24252 $[M+Na]^+$, $C_{45}H_{42}O_{15}Na^+$ requires 845.24159; IR (neat) ν = 1158, 1212, 1278, 1339, 1361, 1436, 1456, 1498, 1517, 1541, 1558, 1636, 1684, 1698, 2882, 2938, 3239, 3319, 3363, 3376, 3567, 3588, 3609, 3629 cm^{-1}; $[\alpha]_D^{27}$ +62.0° (c = 0.132, acetone).

6-dehydrodiconyferylsilychristin (**6**): brown solid, see Table S3 for NMR data. HR-ESI-MS (positive): found *m*/*z* 845.24139 $[M+Na]^+$; $C_{45}H_{42}O_{15}Na^+$ requires 845.24159; IR (neat) ν = 780, 816, 861, 967, 1033, 1090, 1121, 1145, 1208, 1274, 1341, 1453, 1497, 1518, 1636, 3381 cm^{-1}; $[\alpha]_D^{24}$ +71.0° (c = 0.400, acetone).

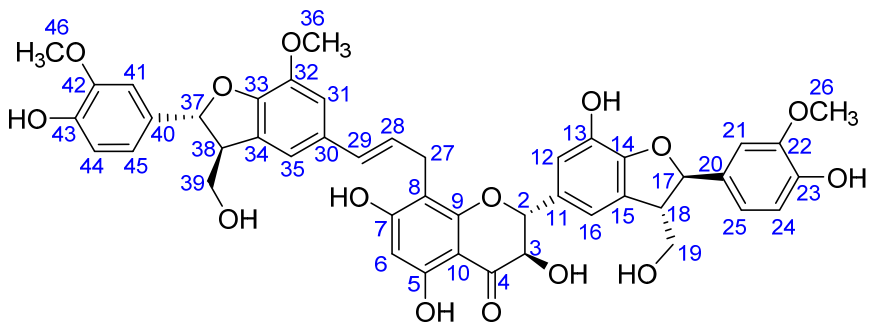
NMR data of compound 4-6



coniferylsilychristin (**4**)

Table S1. NMR data of compound **4** (500 MHz, rt, methanol-d_4)

No.	^{1}H	^{13}C	No.	^{1}H	^{13}C
2	4.94 (d, *J*=11.4)	85.27	20	-	136.84
3	4.54 (d, *J*=11.4)	73.78	21	6.97 (s)	112.04
4	-	198.31	22	-	145.50
5	-	165.33	23	-	149.35
6	5.92 (d, *J*=1.4)	97.39	24	-	130.41
7	-	168.75	25	6.97 (s)	115.82
8	5.88 (d, *J*=1.4)	96.32	26	3.85 (s)	56.91
9	-	164.50	27	5.53 (d, *J*=6.3)	89.29
10	-	101.86	28	3.49 (dt, *J*=6.3, 5.9)	55.30
11	-	131.64	29	3.74-3.83 (2H, m)	64.88
12	6.91 (br s)	117.00	30	-	134.62
13	-	142.19	31	6.94 (d, *J*=1.3)	110.71
14	-	149.11	32	-	149.14
15	-	130.10	33	-	147.63
16	6.95 (br s)	116.62	34	6.76 (d, *J*=8.2)	116.22
17	5.60 (d, *J*=6.3)	89.29	35	6.82 (dd, *J*=1.3, 8.2)	119.75
18	3.57 (dt, *J*=6.3, 5.8)	55.56	36	3.81 (s)	56.47

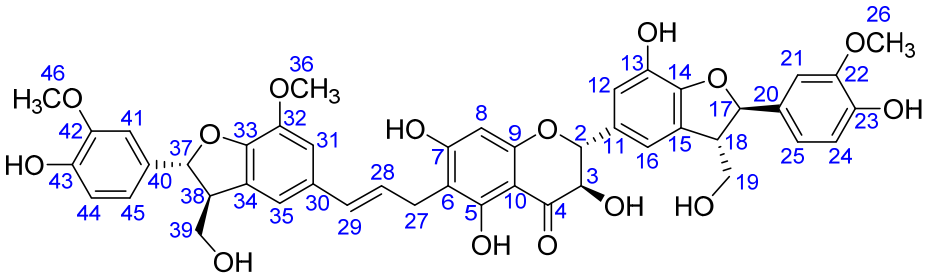


8-dehydrodiconiferylsilychristin (**5**)

Table S2. NMR data of compounds **5** (500 MHz, rt, acetone-d_6)

No.	^{1}H	^{13}C	No.	^{1}H	^{13}C
2	5.07 (d, *J*=11.4)	84.56	25	6.92 (dd, *J*=1.3, 8.2)	119.84
3	4.57 (d, *J*=11.4)	73.52	26	3.80 (s)[a]	56.33[b]
4	-	198.44	27	3.33 (dd, *J*=6.0, 14.3)	26.32
				3.39 (dd, *J*=6.0, 14.3)	
5	-	162.91	28	6.13 (td, *J*=6.0, 15.8)	126.16
6	6.12 (s)	96.77	29	6.30 (d, *J*=15.8)	131.10
7	-	165.64	30	-	132.55
8	-	107.31	31	6.84 (br s)	111.38
9	-	161.18	32	-	145.15
10	-	101.60	33	-	148.64
11	-	131.68	34	-	130.34
12	7.05 (br s)	116.77	35	6.90 (br s)	115.69
13	-	141.75	36	3.81 (s)	56.38
14	-	148.48	37	5.51 (d, *J*=6.3)	88.43
15	-	130.11	38	3.48 (dt, *J*=6.0, 6.0)	54.73
16	7.07 (br s)	116.22	39	3.78-3.90 (m)	64.51
17	5.57 (d, *J*=6.6)	88.73	40	-	134.41
18	3.59 (dt, *J*=6.0, 6.0)	55.08	41	7.00 (br s)	110.56
19	3.78-3.90 (m)	64.43	42	-	148.48
20	-	134.25	43	-	147.31
21	7.10 (br s)	110.66	44	6.79 (d, *J*=8.2)	115.75
22	-	148.45	45	6.85 (d, *J*=8.2)	119.65
23	-	147.33	46	3.82 (s)[a]	56.42[b]
24	6.82 (d, *J*=8.2)	115.75	5-OH	11.67 (s)	

Note: a–b: values with the same superscript may be interchanged.



6-dehydrodiconiferylsilychristin (**6**)

Table S3. NMR data of compounds 6 **(500 MHz, rt, acetone-*d*$_6$)**

No.	^{1}H	^{13}C	No.	^{1}H	^{13}C
2	5.01 (d, *J*=11.7)	84.85	25	6.85 (dd, *J*=1.3, 8.2)	119.63
3	4.63 (d, *J*=11.7)	73.39	26	3.79 (s)	56.32
4	-	198.42	27	3.42 (2H, d, *J*=6.5)	25.91
5	-	162.11^c	28	6.19 (dt, *J*=6.5, 15.7)	126.1
6	-	108.07	29	6.37 (d, *J*=15.7)	130.98
7	-	165.66	30	-	132.59
8	6.08 (s)	95.62	31	6.86 (br s)	111.35
9	-	162.15^c	32	-	145.16
10	-	101.4	33	-	148.60^d
11	-	131.35	34	-	130.13
12	6.98 (br s)	116.84	35	6.90 (br s)	115.67
13	-	141.8	36	3.82 (s)	56.38
14	-	148.62^d	37	5.57 (d, *J*=6.4)	88.64
15	-	130.35	38	3.58 (dt, *J*=6.4, 6.2)	55.08
16	7.00 (br s)	116.58	39	3.78-3.91 (m)	64.5
17	5.52 (d, *J*=6.4)	88.47	40	-	134.31
18	3.50 (dt, *J*=6.4, 6.2)	54.82	41	7.07 (d, *J*=1.3)	110.68
19	3.78-3.91 (m)	64.57	42	-	148.43^e
20	-	134.47	43	-	147.30^f
21	7.00 (br s)	110.56	44	6.81 (d, *J*=7.8)	115.74
22	-	148.47^e	45	6.91 (dd, *J*=1.3, 7.8)	119.78
23	-	147.35^f	46	3.82 (s)	56.38
24	6.78 (d, *J*=8.2)	115.74	5-OH	12.03 (s)	

Note: c–f: values with the same superscript may be interchanged.

NMR spectrums
Silychristin (2)

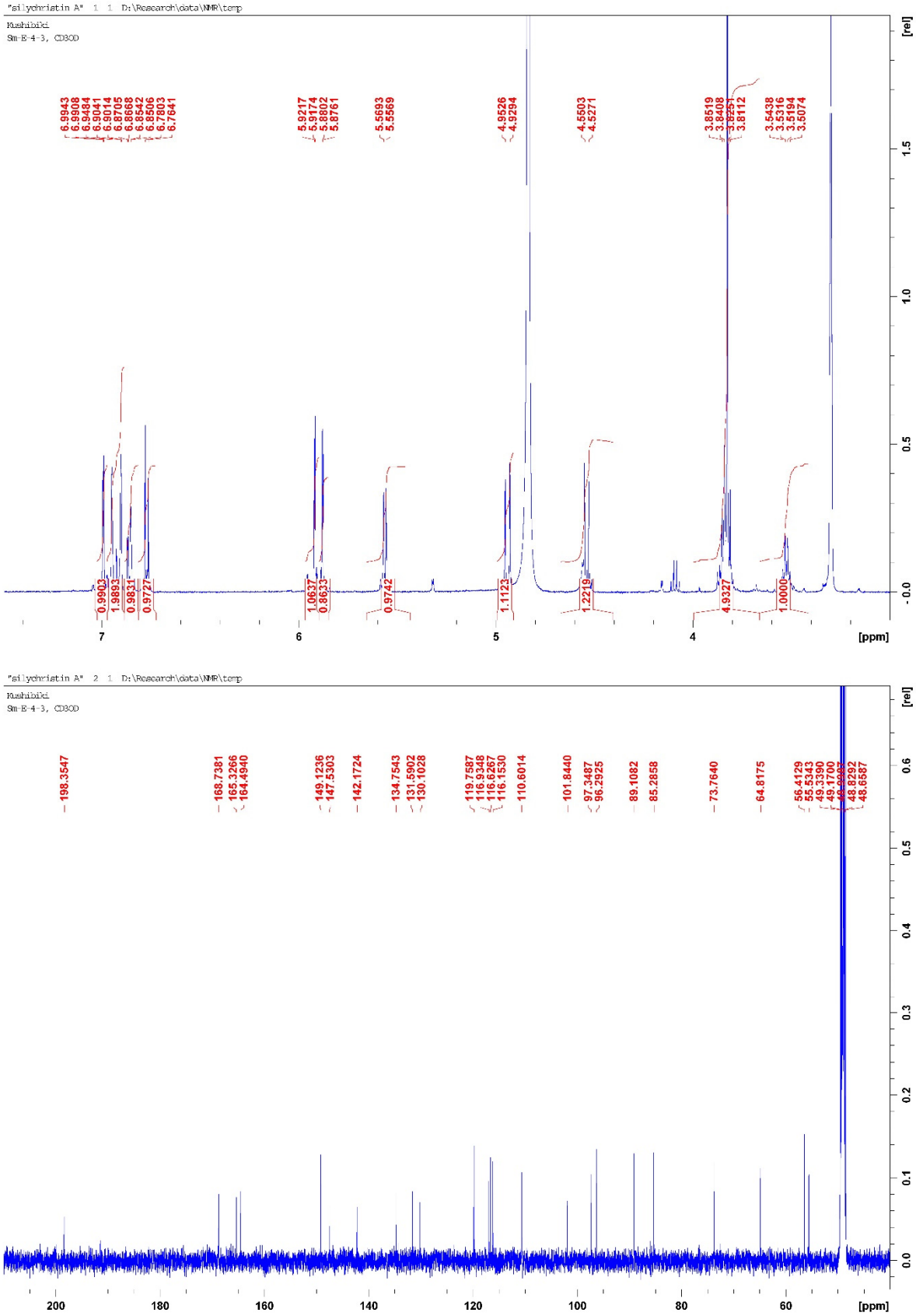
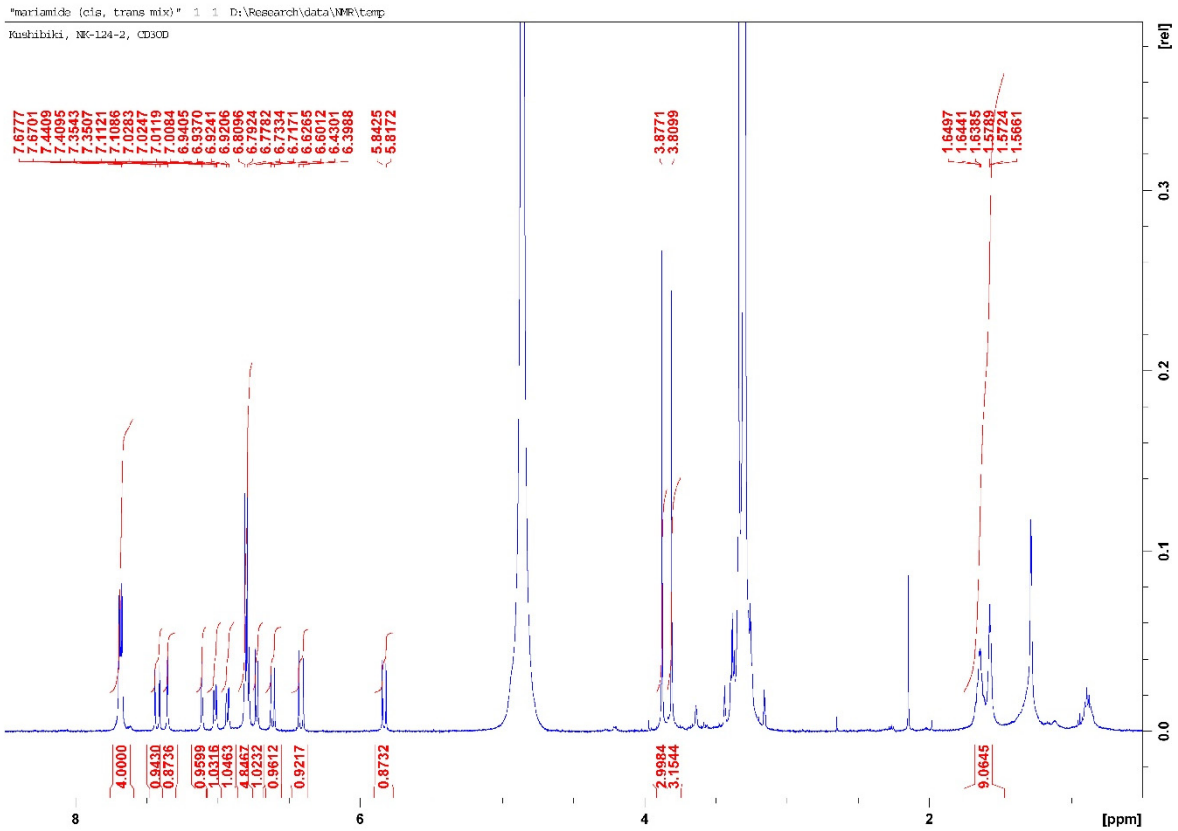


Figure S3. ^{1}H, ^{13}C-NMR spectrum of silychristin (**2**)

Mariamide B (3, cis/trans mixture)



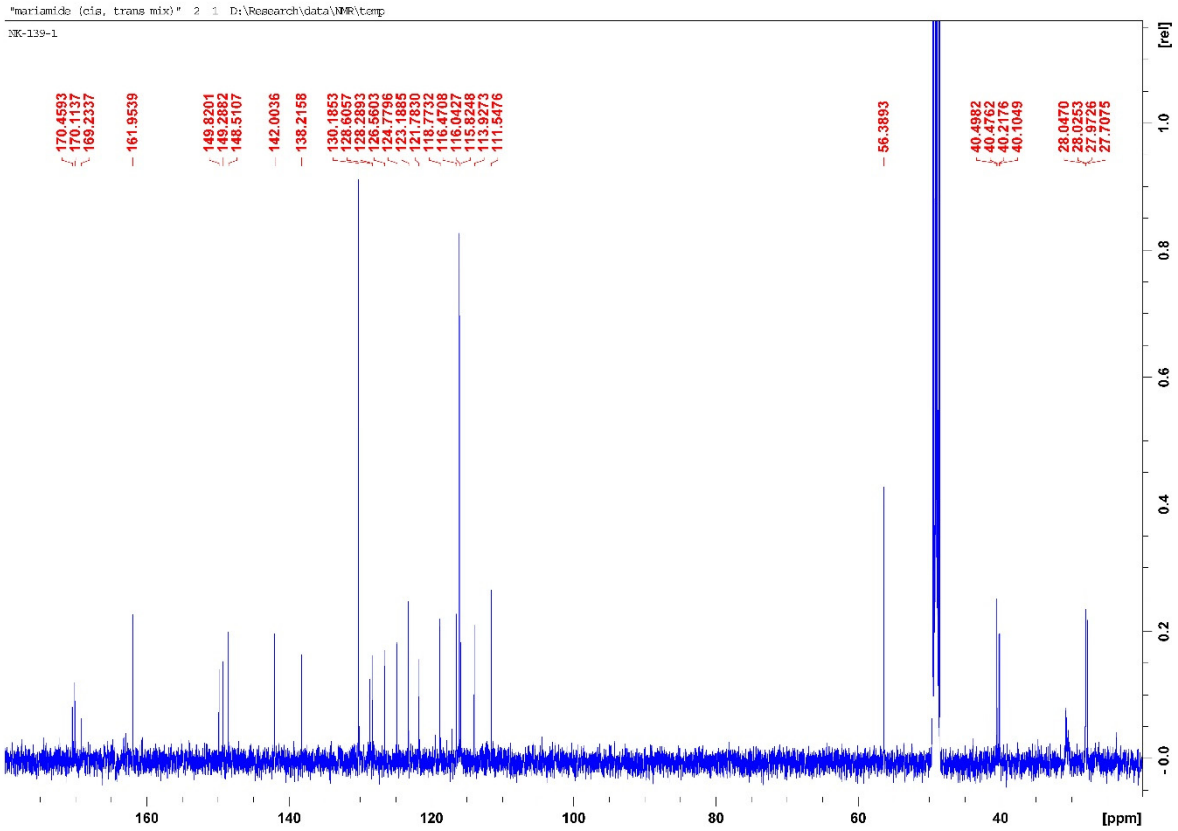
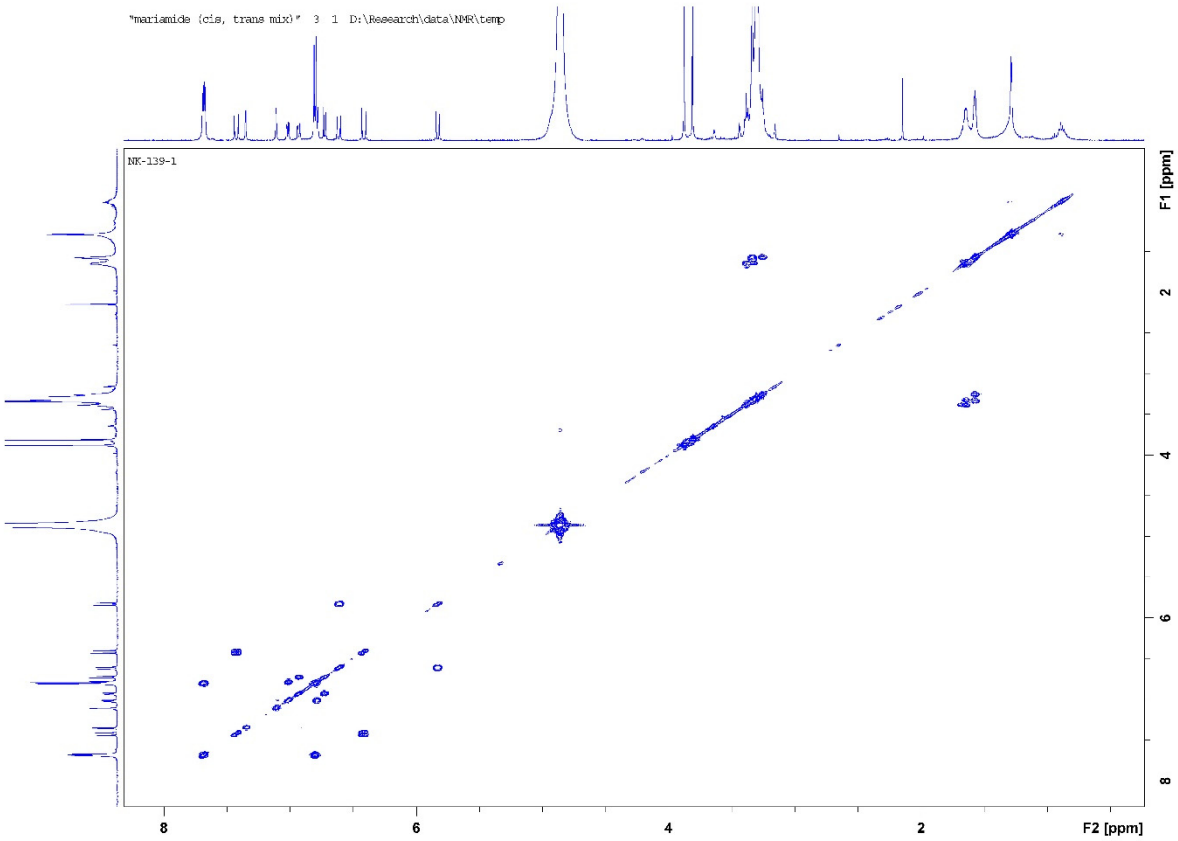


Figure S4. ^{1}H, ^{13}C-NMR spectrum of mariamide B (**3**, *cis*/*trans* mixture)



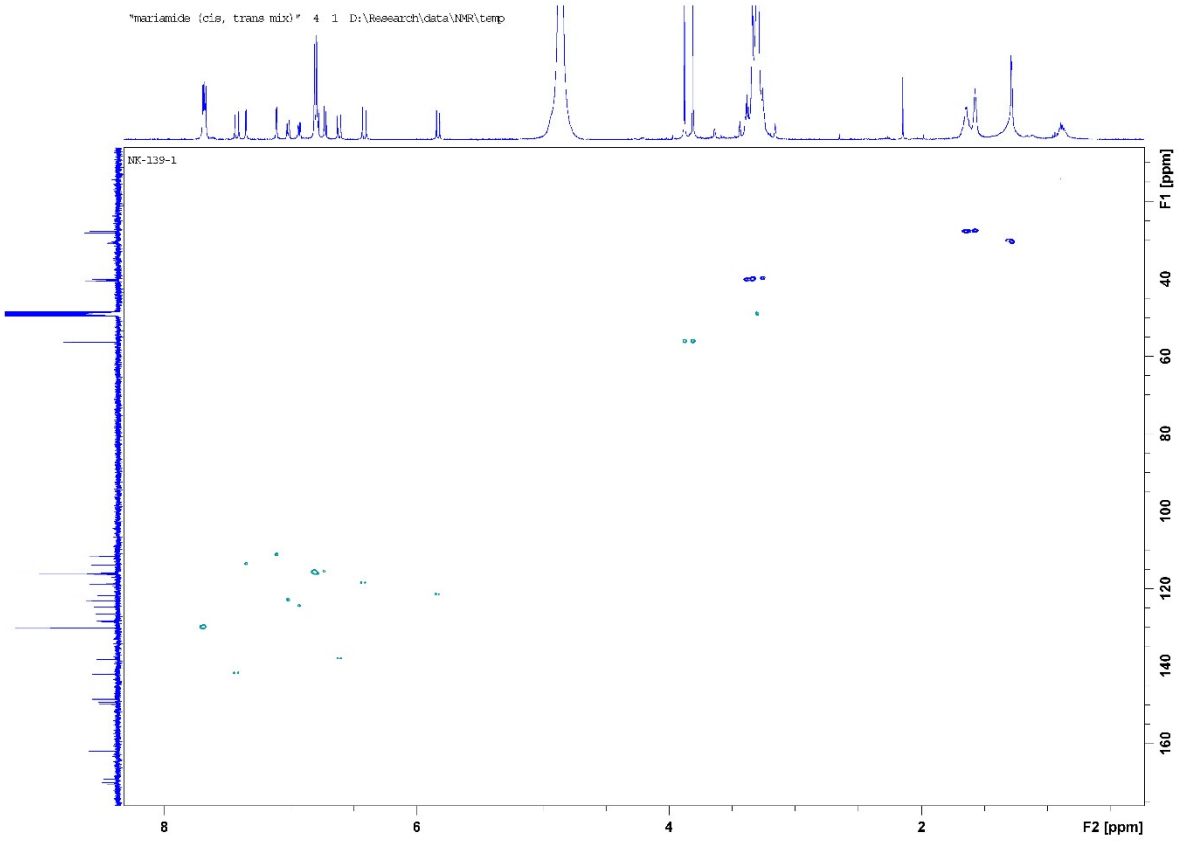


Figure S5. H-H COSY, HSQC spectrum of mariamide B (**3**, *cis*/*trans* mixture)

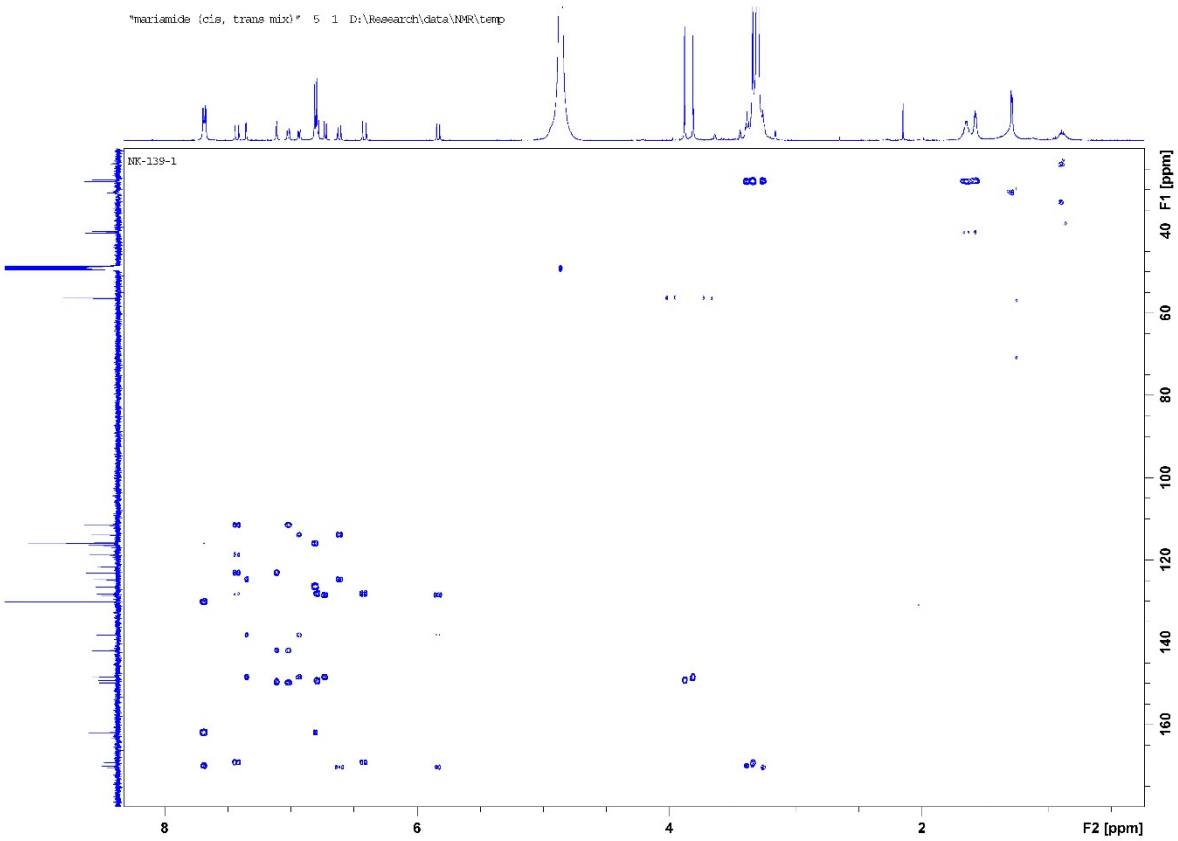


Figure S6. HMBC spectrum of mariamide B (**3**, *cis*/*trans* mixture)

Coniferylsilychristin (4)

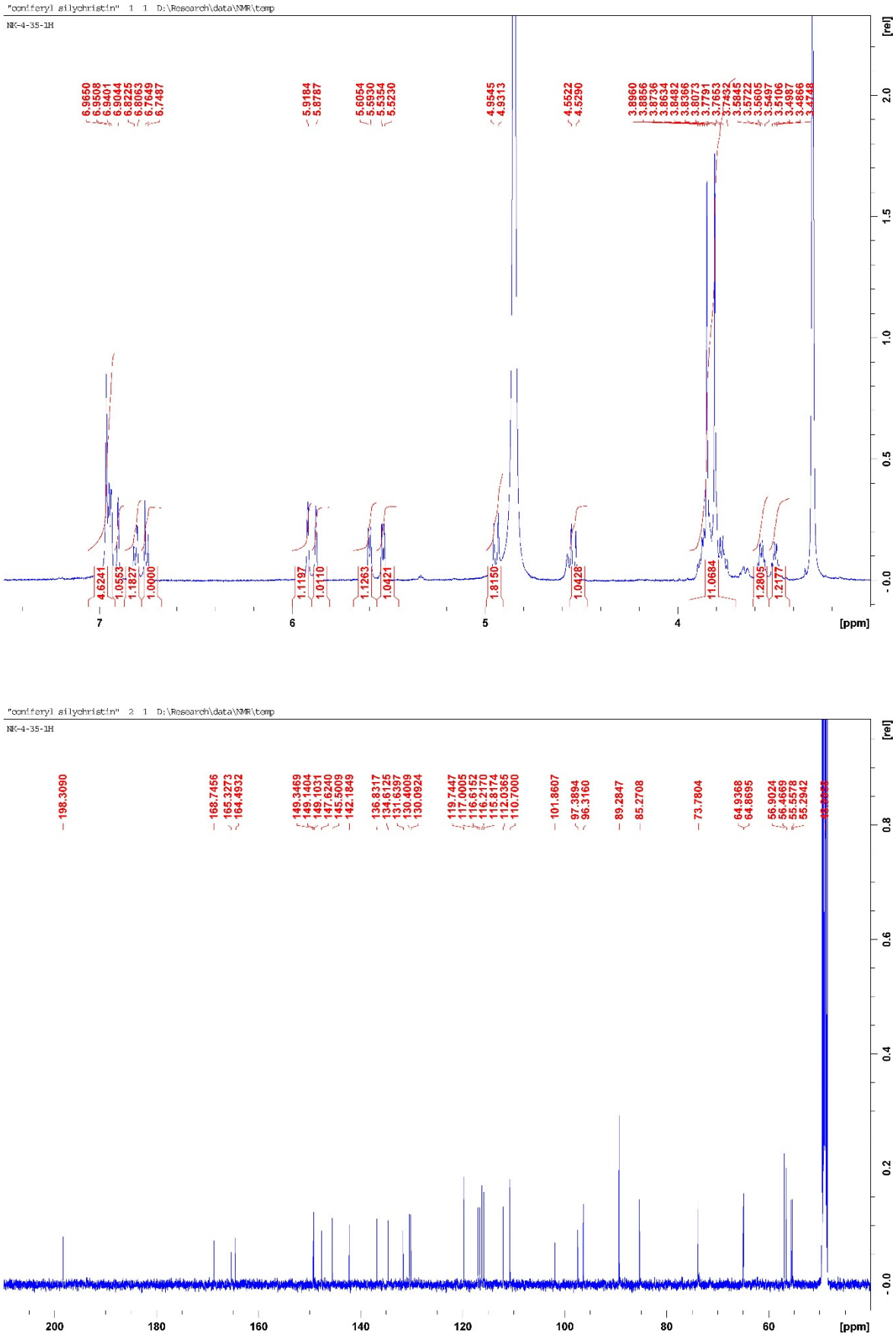
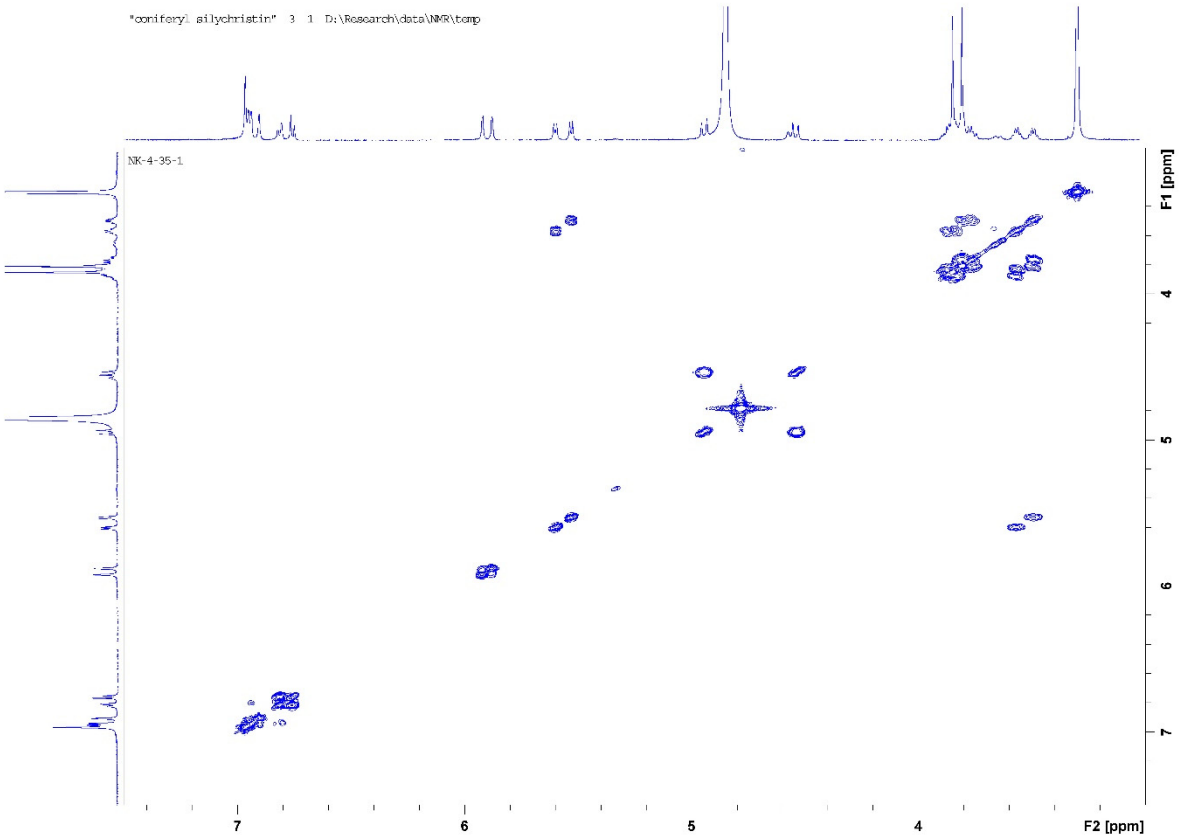


Figure S7. ^{1}H, ^{13}C-NMR spectrum of coniferylsilychristin (**4**)



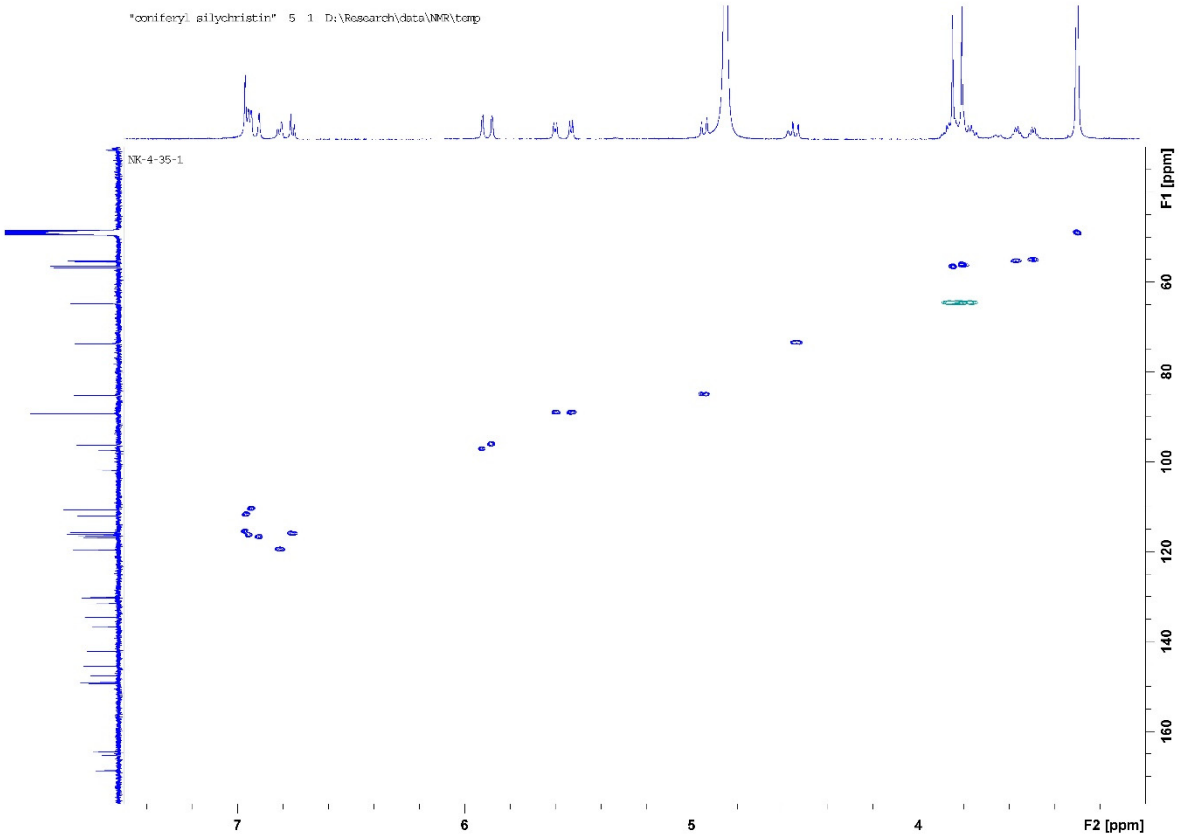


Figure S8. H-H COSY, HSQC spectrum of coniferylsilychristin (**4**)

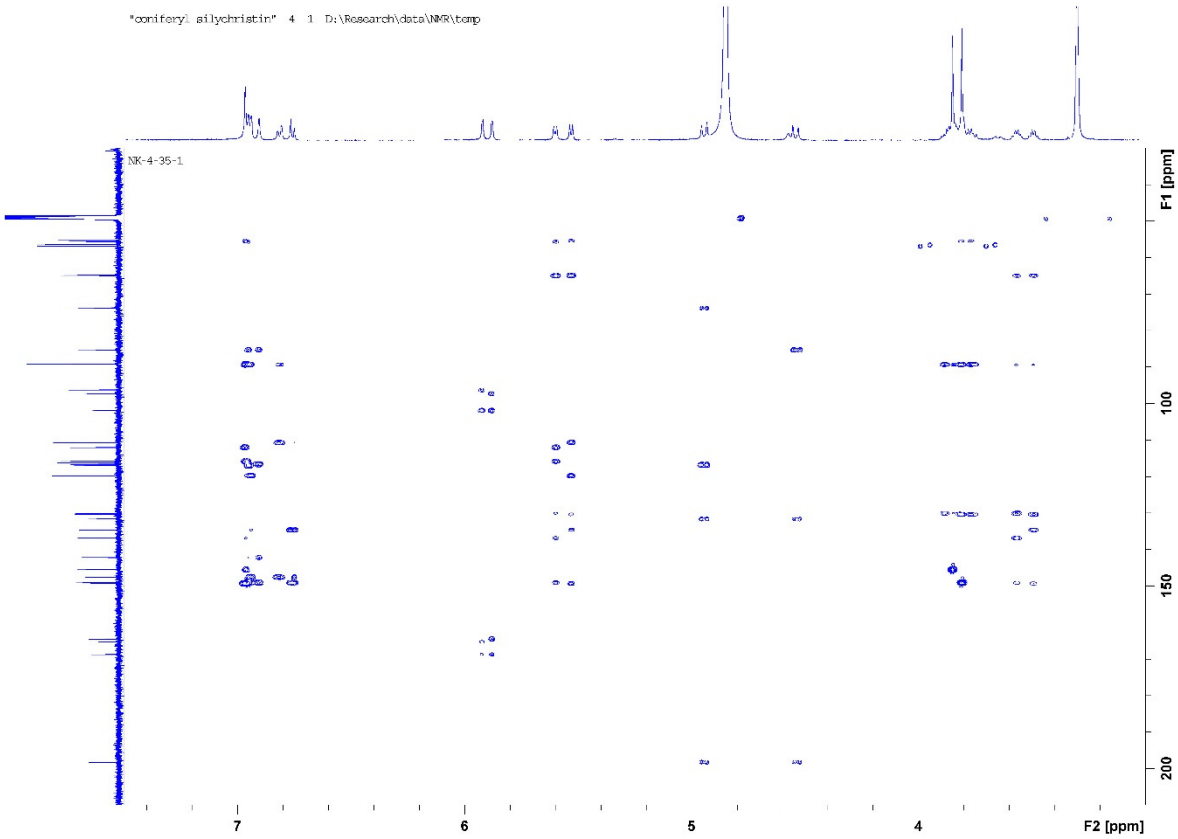


Figure S9. HMBC spectrum of coniferylsilychristin (**4**)

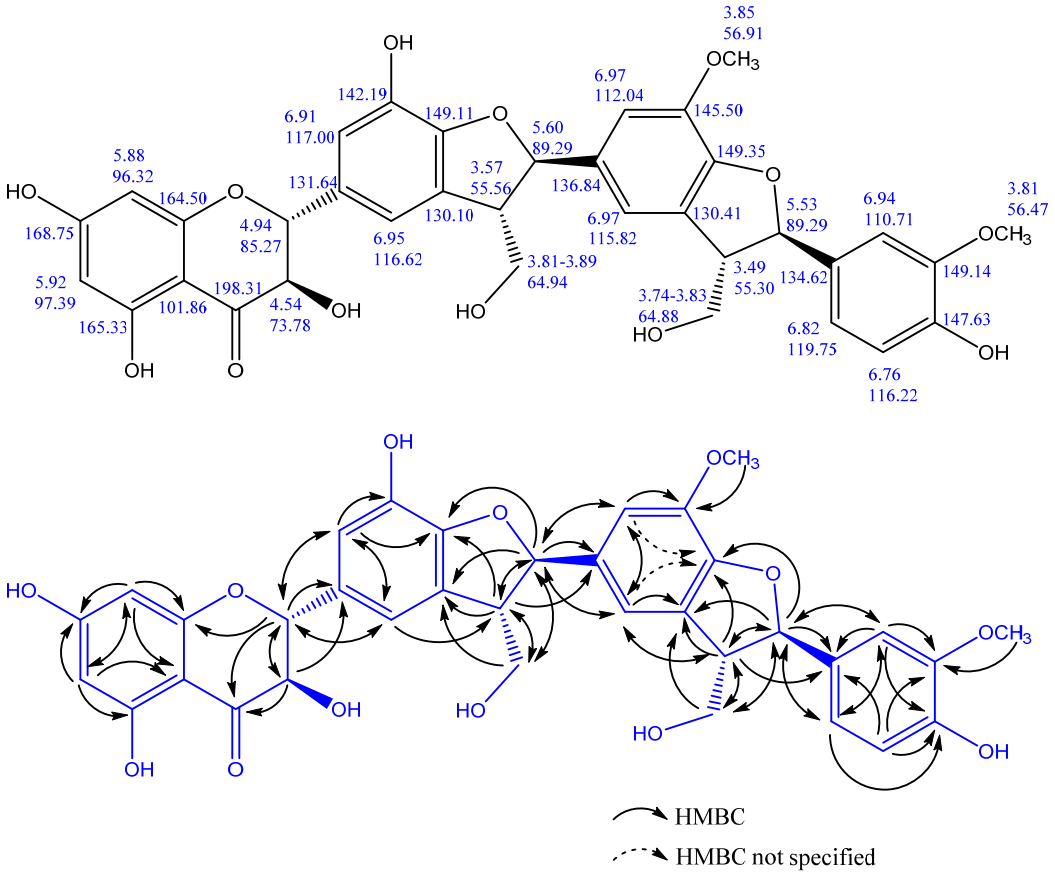
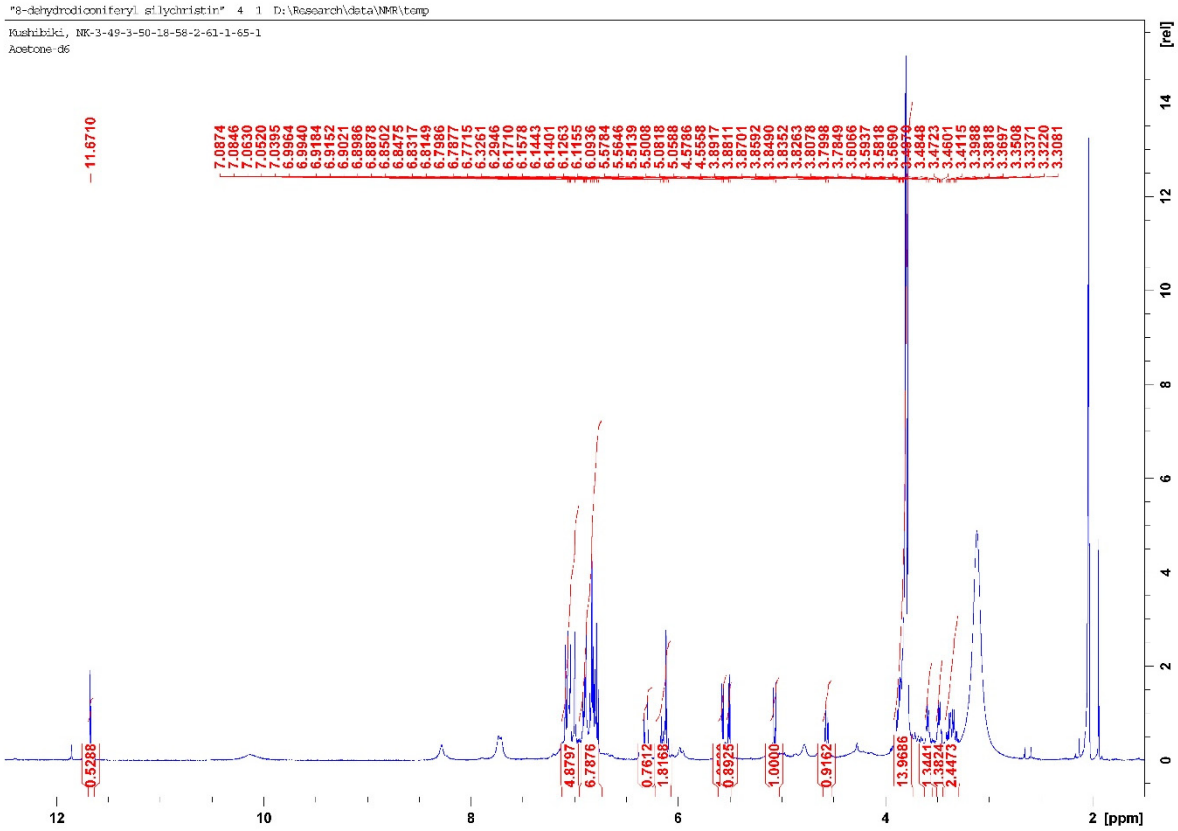


Figure S10. Shifts and HMBC correlations of coniferylsilychristin (**4**)

8-Dehydrodiconiferylsilychristin (5)



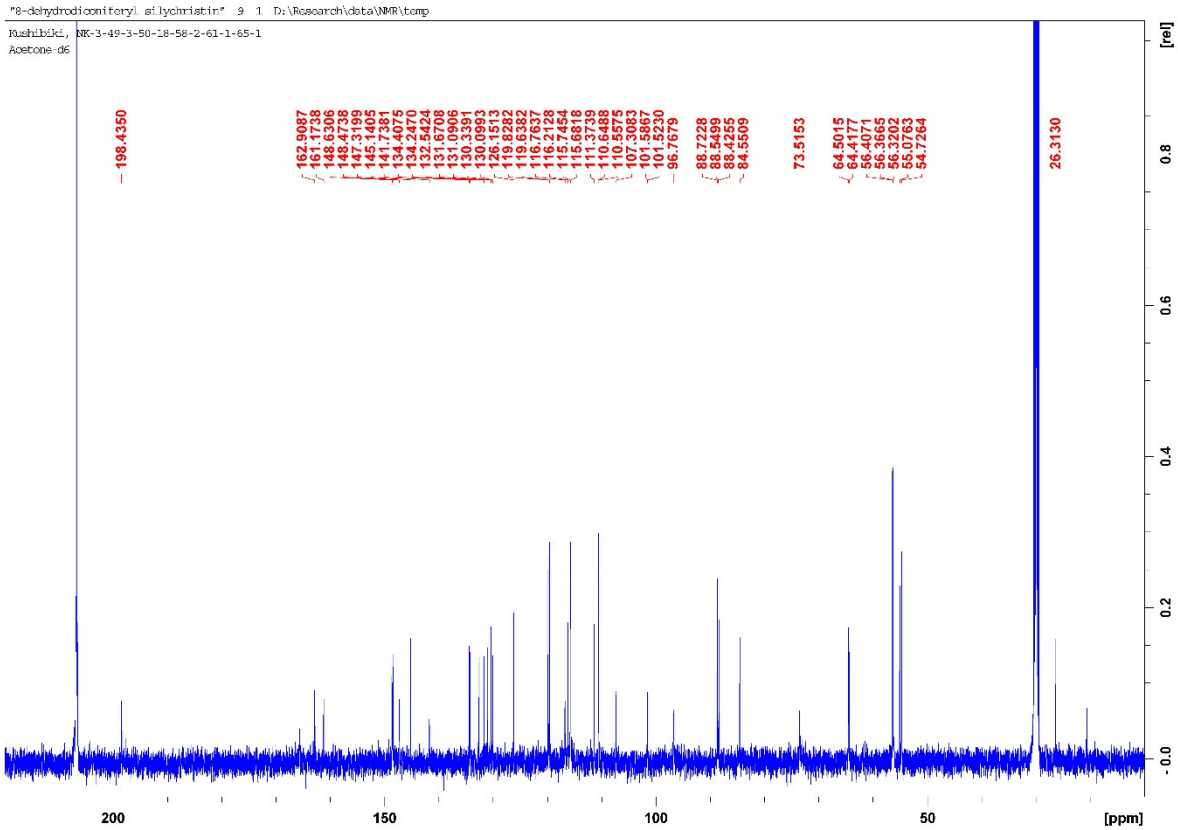
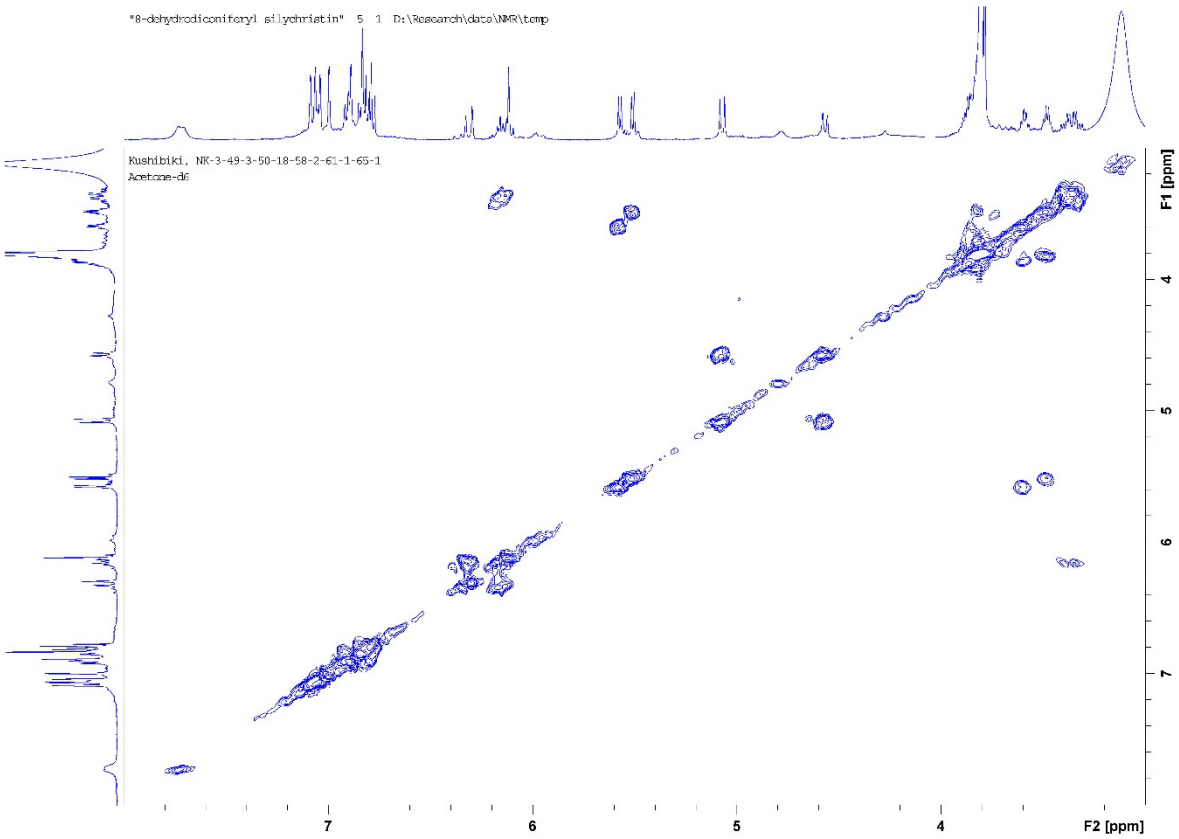


Figure S11. ^{1}H, ^{13}C-NMR spectrum of 8-dehydrodiconiferylsilychristin (**5**)



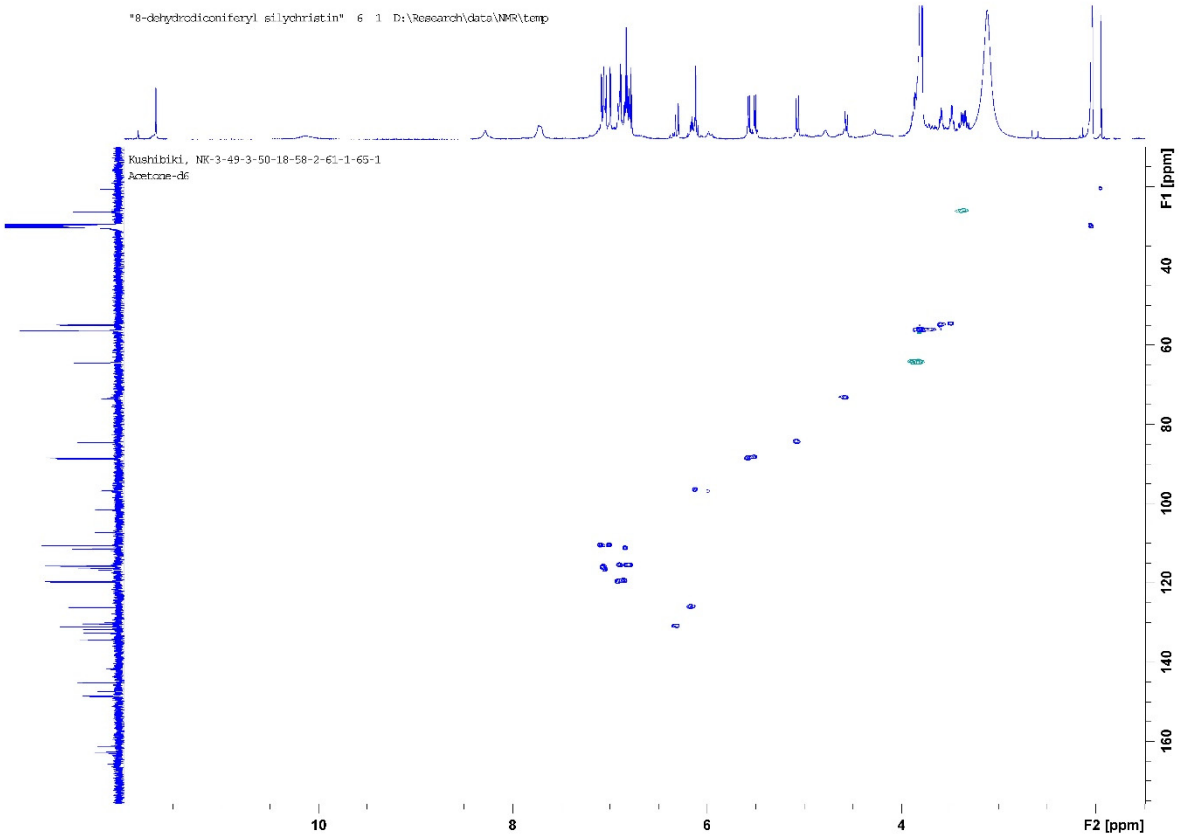


Figure S12. H-H COSY, HSQC spectrum of 8-dehydrodiconiferylsilychristin (**5**)

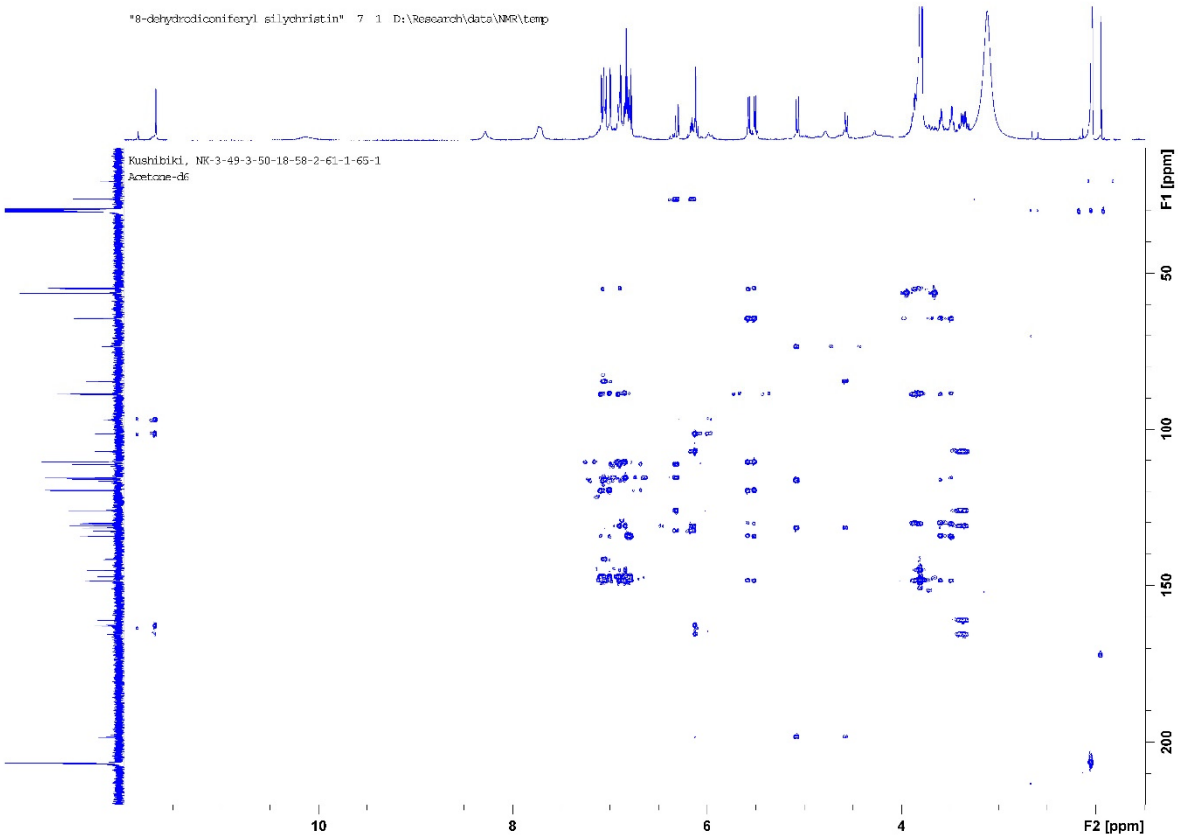


Figure S13. HMBC spectrum of 8-dehydrodiconiferylsilychristin (**5**)

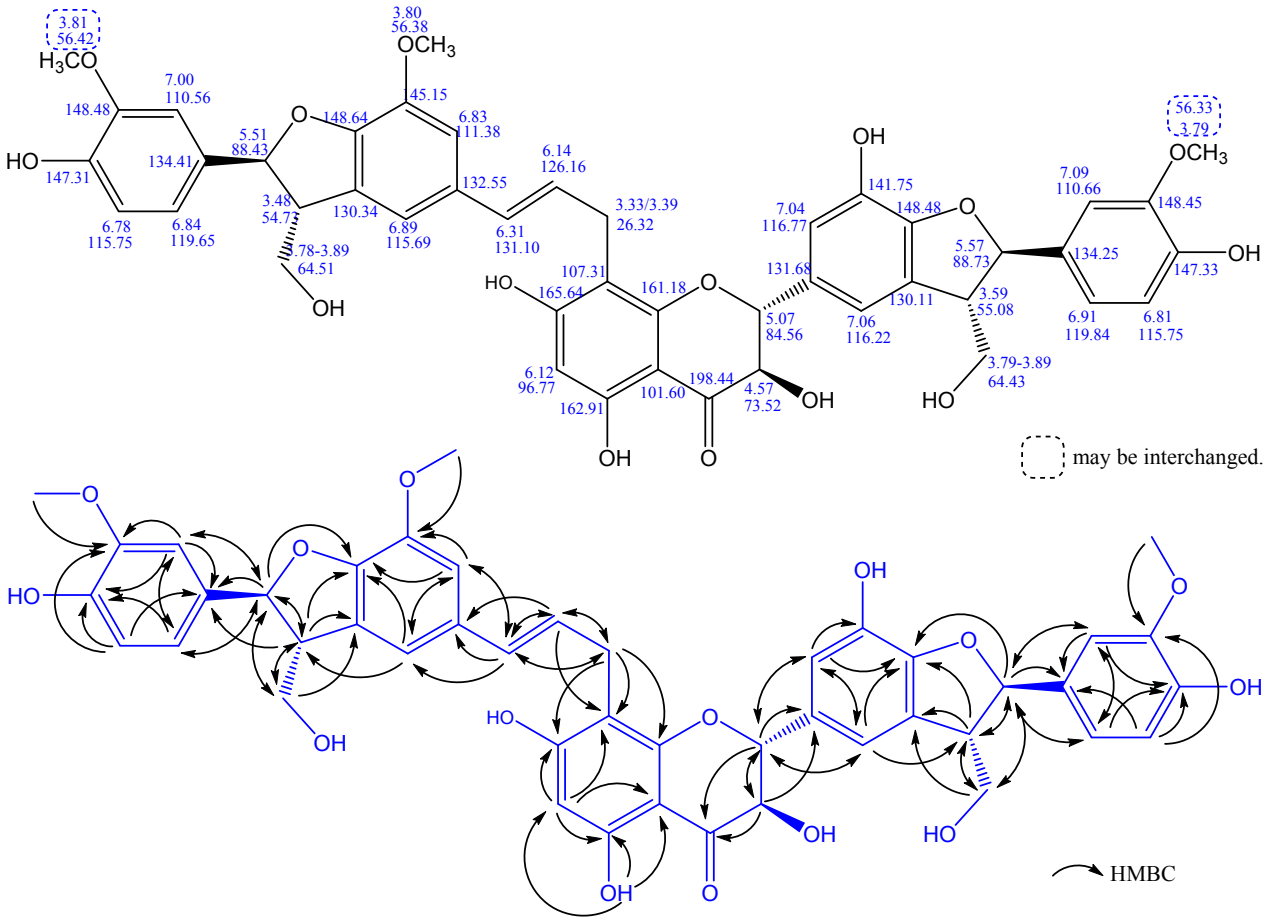
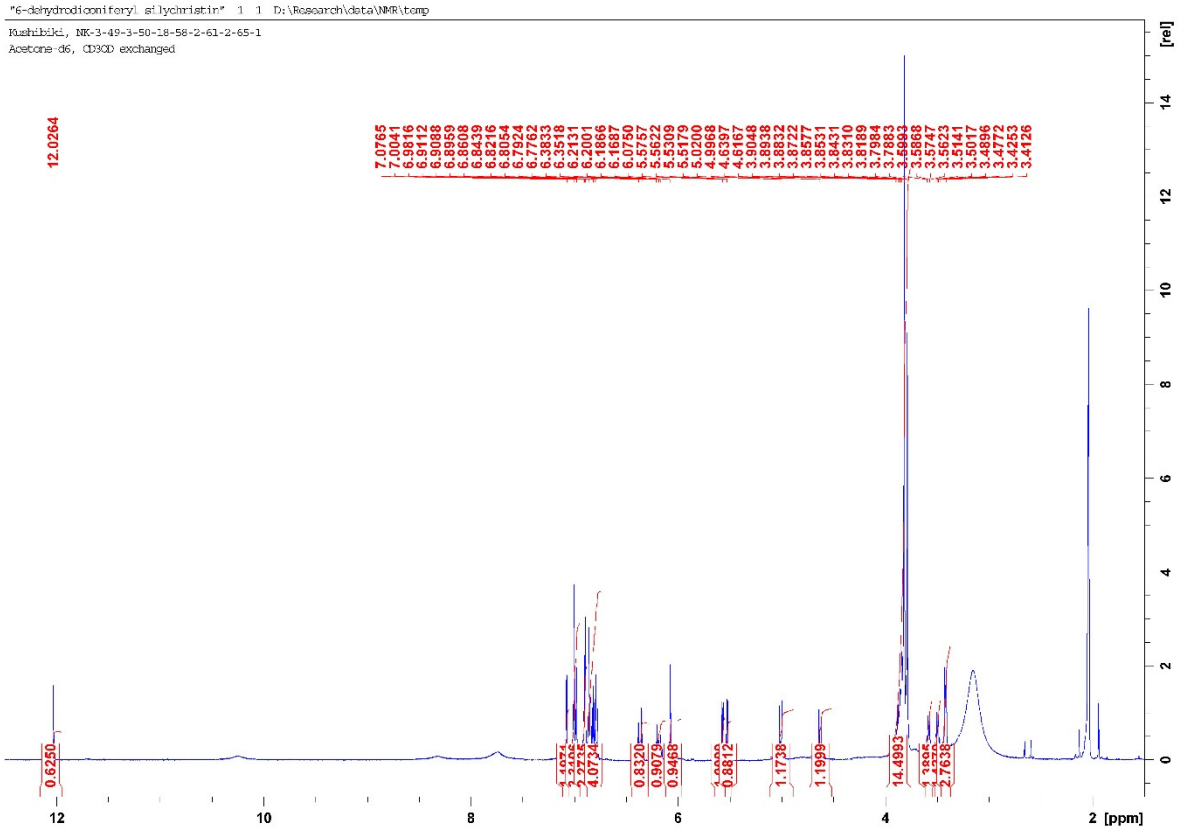


Figure S14. Shifts and HMBC correlations of 8-dehydrodiconiferylsilychristin (**5**)

6-Dehydrodiconiferylsilychristin (6)



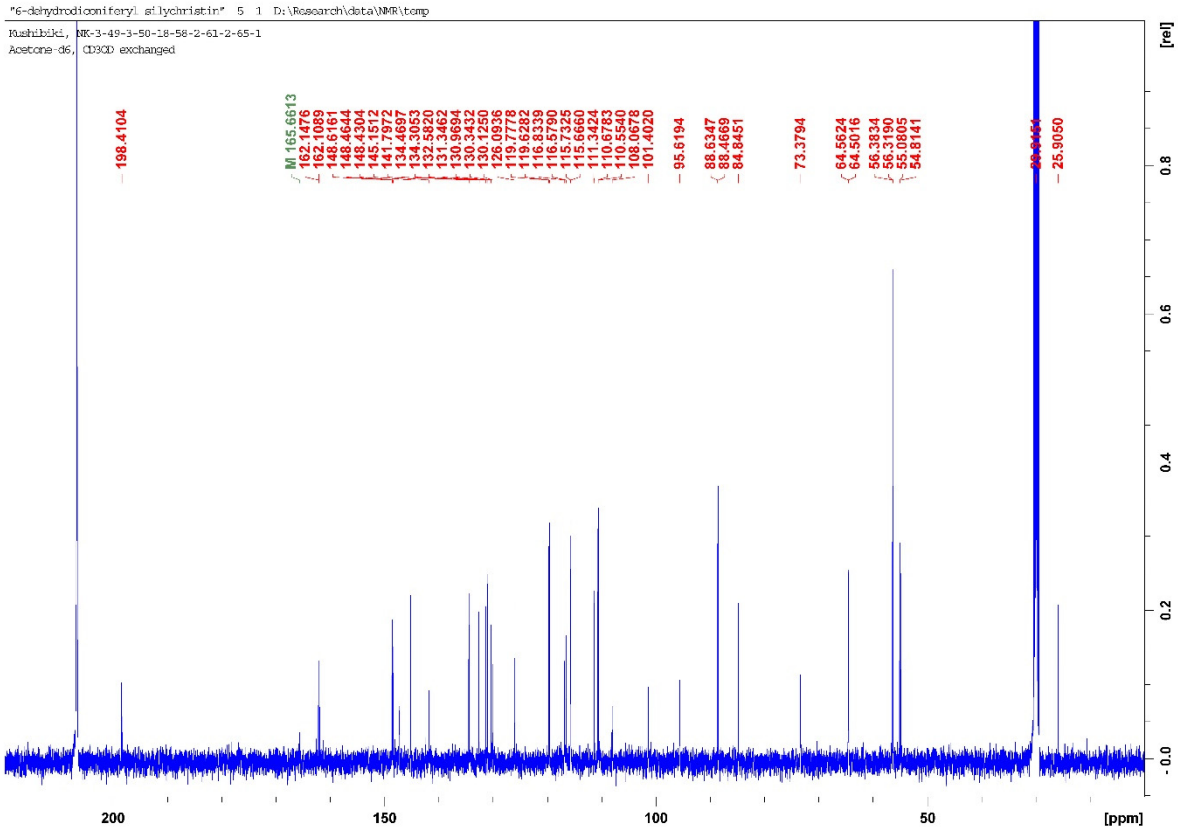


Figure S15. ^{1}H, ^{13}C-NMR spectrum of 6-dehydrodiconiferylsilychristin (**6**)

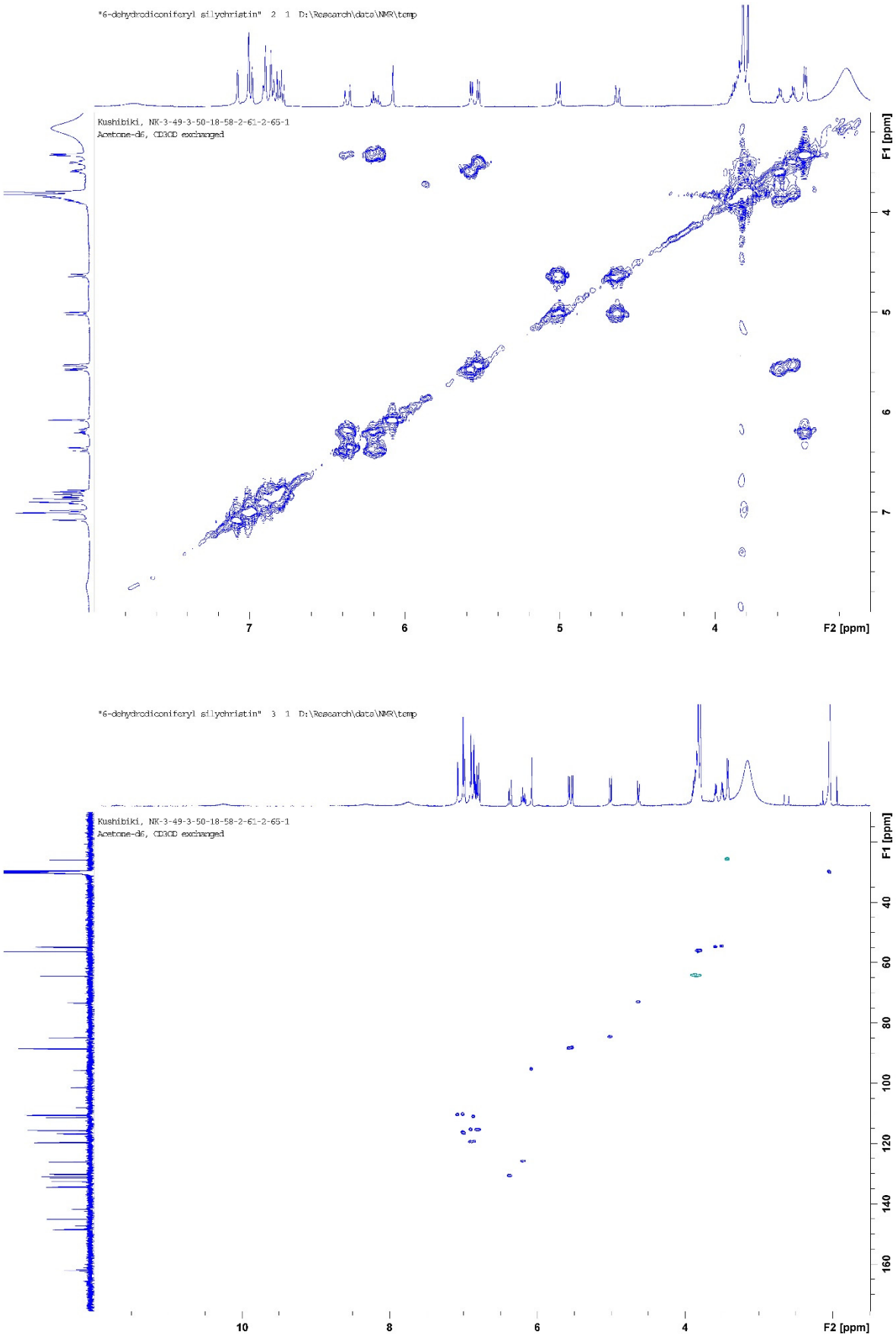


Figure S16. H-H COSY, HSQC spectrum of 6-dehydrodiconiferylsilychristin (**6**)

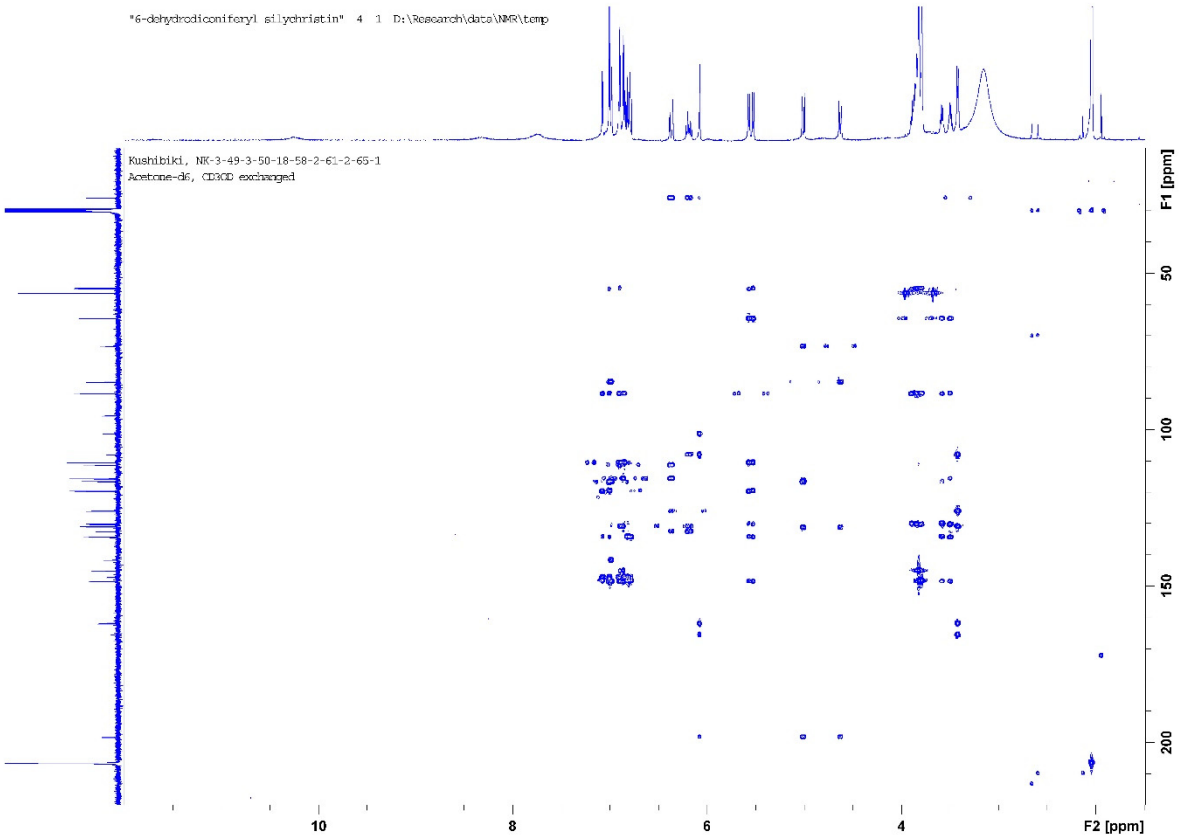


Figure S17. HMBC spectrum of 6-dehydrodiconiferylsilychristin (**6**)

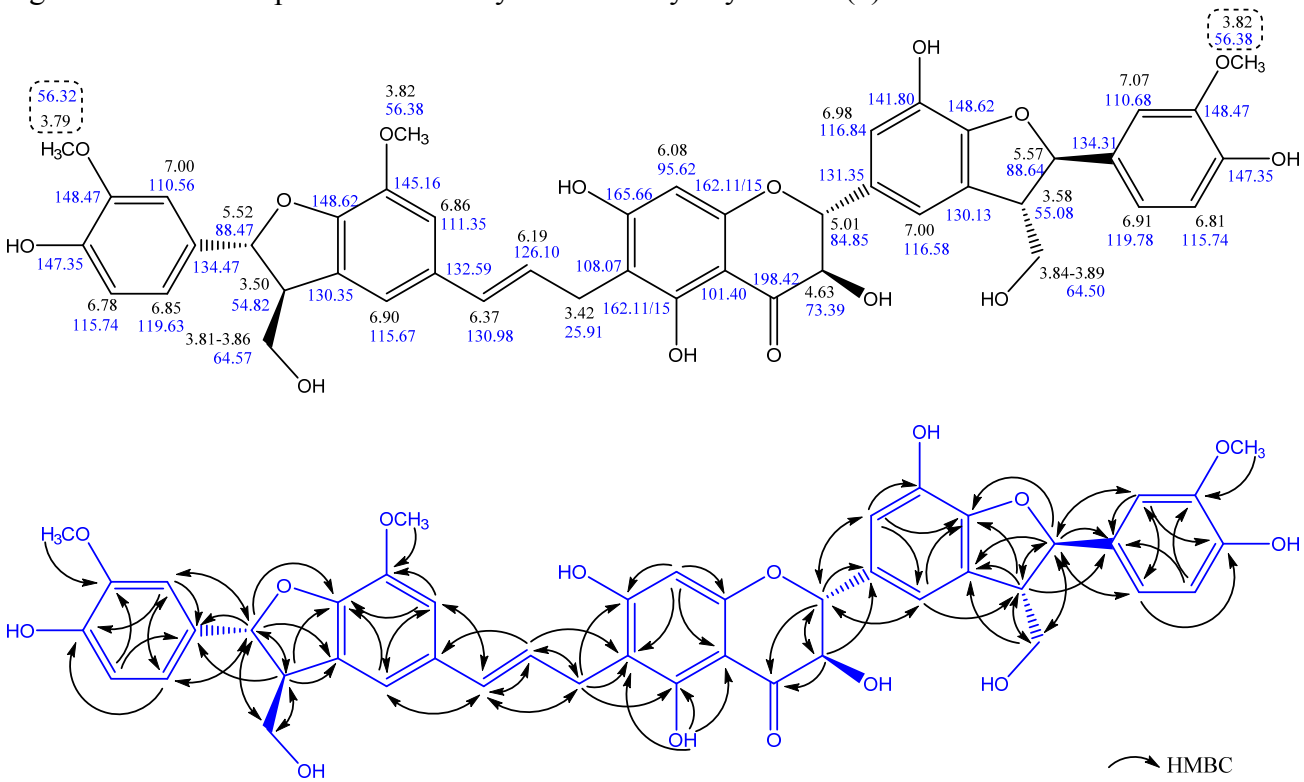


Figure S17. Shifts and HMBC correlations of 6-dehydrodiconiferylsilychristin (**6**)

References

Cheng X-R, Jie R, Wang C-H, Guan B, Jin H-Z, Zhang W-D. 2013. Chemical Constituents from Inula wissmanniana. Chem Nat Compd. 49:815–818.

Choudhary MI, Begum A, Abbaskhan A, Musharraf SG, Ejaz A, Atta-ur-Rahman. 2008. Two New Antioxidant Phenylpropanoids from Lindelofia stylosa. Chem Biodivers. 5:2676–2683.

Qin N bo, Jia C cui, Xu J, Li D hong, Xu F xing, Bai J, Li Z lin, Hua H ming. 2017. New amides from seeds of Silybum marianum with potential antioxidant and antidiabetic activities. Fitoterapia. 119:83–89.