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Article

Cardenolides in *Asclepias syriaca* Seeds: Exploring the Legacy of Tadeus Reichstein

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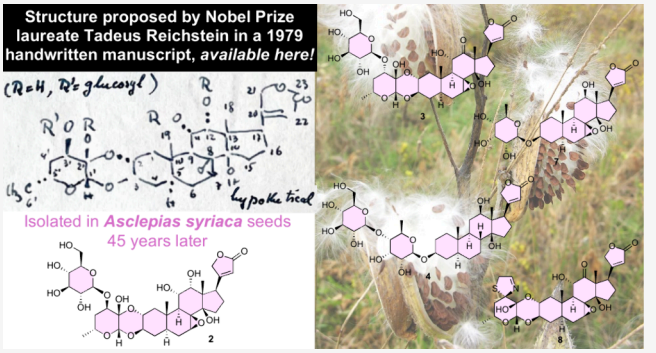
ABSTRACT: The common milkweed *Asclepias syriaca* is widespread in North America and produces cardenolide toxins that deter herbivores by targeting the transmembrane enzyme Na^+/K^+-ATPase. In 1979, Nobel Laureate Tadeus Reichstein elucidated the structure of novel cardenolides isolated from *A. syriaca* roots and proposed structures for several other cardenolides that could not be confirmed. In this study, we investigate the cardenolide composition of *A. syriaca* seeds, focusing on their abundance and *in vitro* inhibitory potency on the sensitive porcine Na^+/K^+-ATPase and that of the highly resistant large milkweed bug, *Oncopeltus fasciatus*. We identify five previously unreported cardenolides (**1**−**5**), three of which are predominantly found in seeds, in addition to the known syrioside (**6**), aspecioside (**7**), and the 2-thiazoline ring-containing cardenolide labriformin (**8**). Glucopyranosyl-allomethylosyl-12-deoxy aspecioside (**5**) is distinguished by lack of oxidation at C-12, and compounds **2**, **3**, **6**, and **8** contain a rare 1,4-dioxane motif. Inhibitory efficacy of the isolated cardenolides for sensitive and resistant enzymes appears to be correlated. Finally, we confirmed the structure of compound **2**, originally proposed by Tadeus Reichstein, and are pleased to share his original 1979 handwritten manuscript.



It is impossible to talk about the history of cardenolides without mentioning Reichstein, laureate of the 1950 Nobel Prize in Medicine and Physiology for his work on human steroid hormones, which culminated in the characterization of cortisone.[1] Reichstein was a pioneer in the chemistry of steroids, not only in humans but also in plants, which he had been studying since the 1930s, with a particular passion for the cardenolide glycosides produced by plants of the Apocynaceae family.[2] Cardenolides are notorious poisons that have been used by many human societies on the tips of arrows for hunting, as they are toxic to animals due to their inhibition of the essential enzyme Na^+/K^+-ATPase.[3,4] In 1964, Reichstein was contacted by the entomologist and ecologist Miriam Rothschild, who believed that certain aposematic insects sequester cardenolide from their host plants to defend themselves against predators.[5] Their collaboration resulted in two papers published in 1967 and 1968 that are among the foundational works of the discipline known as chemical ecology. The studies reported several cardiac glucosides sequestered in the emblematic North American monarch butterfly (*Danaus plexippus*) and in a North African grasshopper (*Poekilocerus bufonius*),[6,7] confirming Rothschild's hypothesis. Reichstein even corrected the hypothetical structure of one of the most complex cardenolides, voruscharin, isolated from *Asclepias curassavica*, which contains a thiazolidine heterocycle in position 3′ and a rare 1,4-dioxane motif.[8] Since then, cardenolide structures have been isolated from a number of plant families,[9,10] and their functions in plant−herbivore interaction continues to be revealed in the context of evolutionary ecology.[11−13]

Cardenolides from the genus *Asclepias* have been extensively studied chemically.[14−21] Previous X-ray analyses have determined the main stereogenic centers, indicating trans-fused rings A and B in their triterpene scaffold.[22−24] Although *A. syriaca* leaf phytochemistry has been reported,[25−28] it is surprising that important tissues of this plant have not yet been studied. This is particularly true for the seeds, which from an ecological perspective are expected to be chemically defended against seed predators such as the lygaeid bug *Oncopeltus fasciatus*.[29] In the present work we isolated five previously unreported compounds from the seeds of *A. syriaca* (**1**−**5**) in addition to the known **6**−**8**. The structures of **2** and **5** had been proposed already by Tadeus Reichstein in an article discussing the possible structures of syrioside (**6**) and

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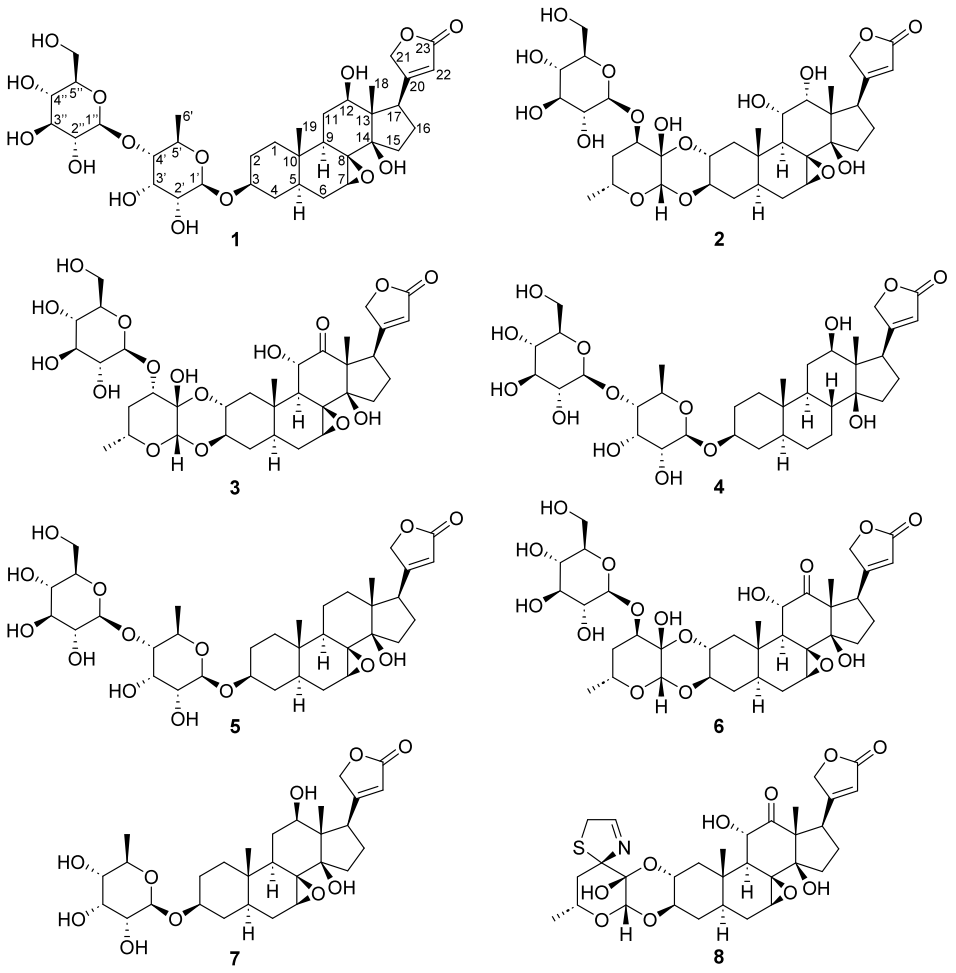

syriobioside.[30] This article, published in 1979 when Reichstein was 82 years old, was dedicated to Miriam Rothschild for her 70th birthday. In addition to our scientific findings in cardenolide chemistry, we present the original handwritten manuscript authored by Reichstein (Supporting Information), documenting the history of chemical ecology and natural products chemistry.

RESULTS AND DISCUSSION

In a previous report, we used UPLC-HRMS to quantify 21 cardenolide toxins in *A. syriaca* seeds across a latitudinal gradient in the USA, revealing a pattern of increasing cardenolide concentrations toward the center of the range of specialized lygaceous seed bugs.[29] Here we report the isolation and characterization of eight of these cardenolides from this plant material. A sample of the seeds of *A. syriaca* (100 g) collected in Ithaca, NY, was extracted with MeOH and then defatted of its wax content by liquid−liquid extraction with hexane. The dry methanolic extract was suspended in 16% MeCN, the solubilized fraction was subjected to successive preparative chromatography steps, and purity of isolated peaks was assessed by UPLC-HRMS. The process yields eight pure cardenolides, which were characterized by 1D and 2D NMR spectroscopy. We identified three known cardenolides: syrioside (**6**),[30] aspecioside (**7**),[25] and labriformin (**8**).[31] Compounds **6** and **8** were elucidated by NMR spectroscopic analysis (Table S1), while aspecioside was characterized based on UPLC-HRMS and MS/MS data. Furthermore, we isolated five new cardenolides, including glucopyranosyl aspecioside (**1**), glucopyranosyl syribioside (**2**), C-3′ epi-syrioside (**3**), glucopyranosyl allomethylosyl syriogenin (**4**), and glucopyranosyl-methyallosyl 12-deoxy aspecioside (**5**), all obtained as white solids.

Compound **1** had the molecular formula $C_{35}H_{52}O_{15}$, as determined by ^{1}H and ^{13}C NMR measurements with an ion peak at m/z 713.3355 $[M + H]^+$. 2D NMR experiments show a cardenolide featuring an epoxide at C-7 (δ_H 3.23, δ_C, 53.5) and C-8 (δ_C 64.2), with a signal at δ_H 3.61 (δ_C 76.1) corresponding to a hydroxylation at C-12. The NOESY signal between H-12 and H-17 agrees with a 12*S** configuration, all suggesting **1** as an aspecioside derivative.[25] However, **1** shows two signals characteristic of anomeric glycosyl positions in the ^{1}H and ^{13}C NMR spectra at δ_H 4.40 (δ_C, 105.9, H-1′) and δ_H 4.72 (δ_C, 99.8, H-1′), thus indicating a diglycosyl chain, with the first sugar unit being methylallose, judging by the spin coupling system that includes five carbinolic protons (H-1′−H-2′−H-3′−H-4′−H-5′), ending with a methyl doublet H_3-6′ ($^{3}J_{HH}$ > 6.3 Hz).

For the characterization of the second glycosyl group, we observed the triplet multiplicity of H-3″ with a large coupling constant ($^{3}J_{HH}$ > 8 Hz), plus the NOESY correlation between H-1″ and H-3″, both pointing to an antiperiplanar arrangement of H-3″ with H-2″ and H-4″, typical of a glucosyl moiety. The correlation found in HMBC data between H-1″ and C-4′ places the linkage of the second sugar at that position. The coupling constant above 7 Hz of both anomeric protons suggests *β* linkages. Therefore, compound **1** corresponds to 4′-*O*-*β*-glucopyranosyl aspecioside.

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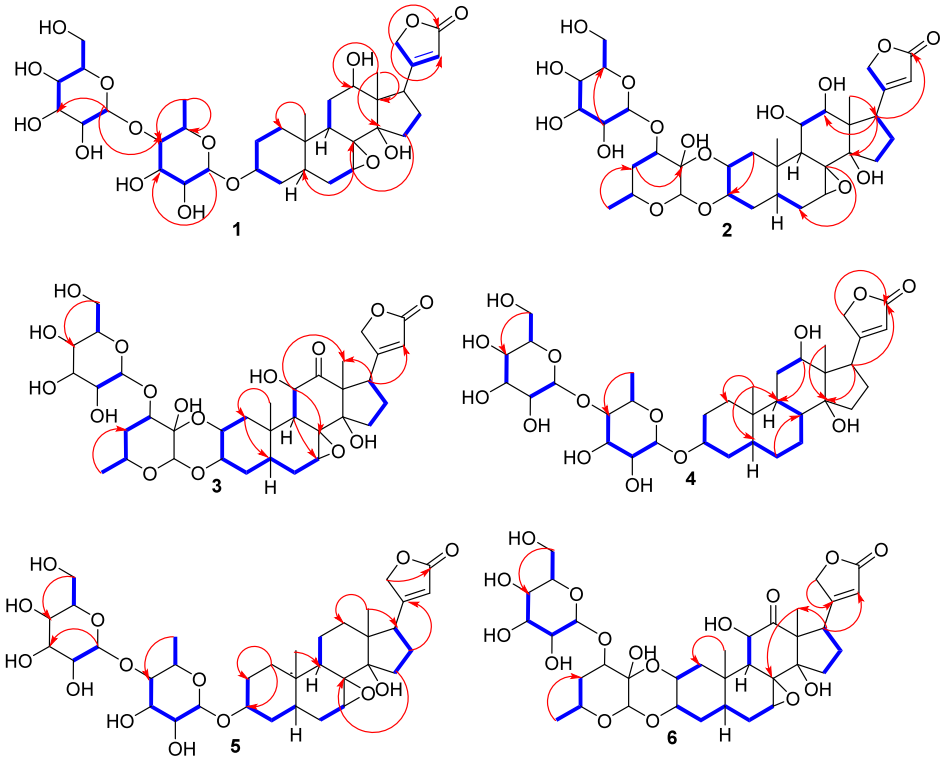

Figure 1. Key COSY (in bold blue lines) and HMBC correlations (in red arrows) of compounds **1**−**6**.

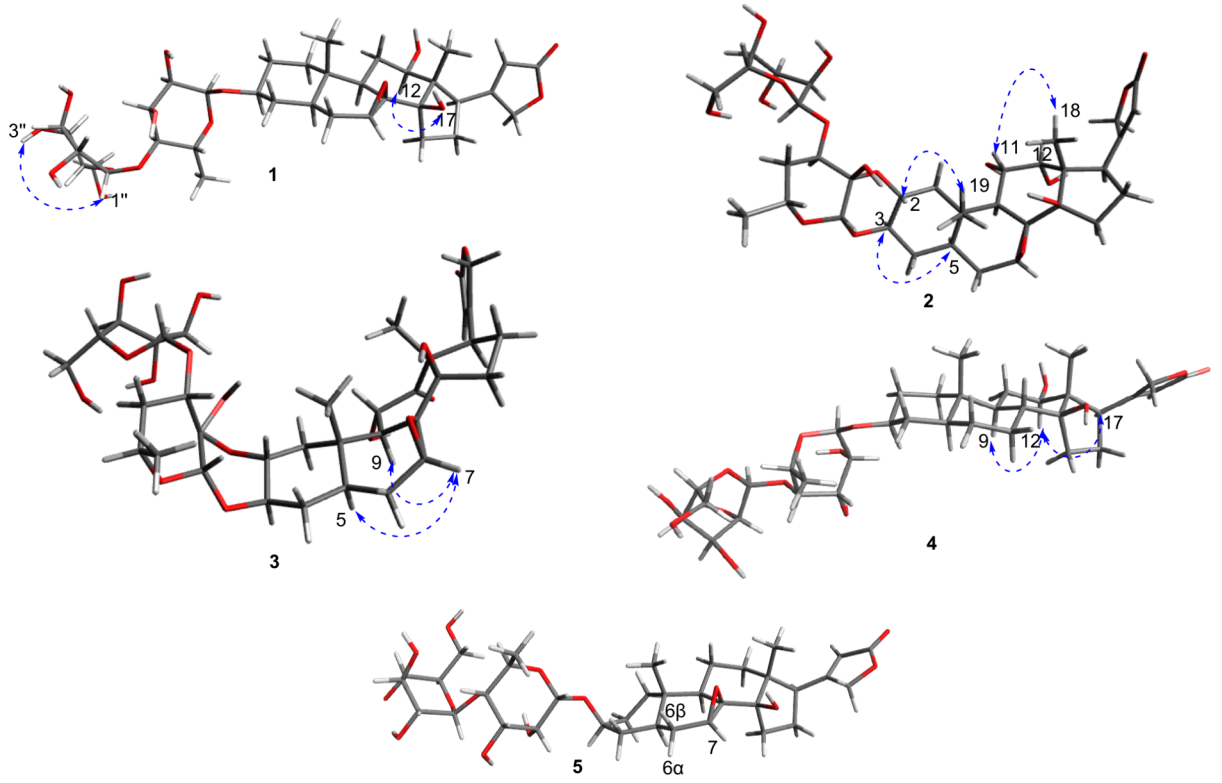

Figure 2. Key ROESY (blue ↔) correlations for compounds **1**−**5** generated with Avogadro 1.2.0 software.

Compound **2** has a molecular formula of $C_{35}H_{50}O_{16}$ and an ion peak at m/z 709.3069 $[M - H_2O + H]^+$. Signals at δ_H 3.28 (δ_C 53.7) and δ_C 64.6 suggest an epoxide between C-7 and C-8. The signals at δ_H 3.57 (δ_C 79.8) and δ_H 4.15 (δ_C71.4) describe two hydroxylations at C-11 and C-12 in a syn/cis arrangement according to a NOESY correlation between H_3-18 and H-11 and the small coupling constant of H-12 ($^3J_{HH}$ = 2 Hz). The presence of two anomeric protons indicates a diglycosylated cardenolide, with glucose as the second sugar moiety, going by similarities with **1**. Nonetheless, a key

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difference between **1** and **2** is a non-hydrogenated carbon at 90.8 ppm (C-2′) and a HMBC correlation between the anomeric proton H-1′ (δ_H 4.70) and the methylene (CH_2-4′), thus suggesting a dioxane ring fusion between the sterol and the first sugar moiety. NOESY correlations between H-3 and H-5, and H-2 and H_3-19, describe a trans/anti arrangement in this ring at C-2/C-3. These characteristics align with the structure identified by Reichstein as syribioside,[30] isolated here in its glucosylated form at position C-3′ with a β linkage; therefore we characterize **2** as 3′-*O*-β-glucopyranosyl syribioside.

Compound **3**, with a molecular formula of $C_{35}H_{48}O_{16}$ determined by ^{1}H and ^{13}C NMR with an ion peak at *m*/*z* 742.3286 $[M + NH_4]^+$, shares the same pattern of epoxide in C-7 and C-8, a hydroxylation on C-12, and the fusion at C-2 and C-3 with the first sugar moiety, to give compound **2**. However, instead of hydroxylation at C-11, a carbonyl group is present in that position (δ_C, 213.5), aligning with the cardenolide proposed by Reichstein as a syrioside, identified here as compound **6**. Further ROESY experiments revealed a correlation between H-1′ and H-3′, which suggests a syn-periplanar position of the two carbinolic protons. Therefore, this cardenolide is the epimer of the reported substance, making compound **3** a C-3′ epi-syrioside.

Compound **4**, with a molecular formula of $C_{35}H_{54}O_{14}$ and an ion peak at *m*/*z* 699.3586 $[M + H]^+$, displays signals at δ_H 3.34 (δ_C 75.6) indicative of a cardenolide with a hydroxylation at C-12, with an *R** stereocenter defined by the NOESY signals of H-12 with H-9 and H-17. All of the above is characteristic of a syriogenin derivative, first described in 1962[32] and later reported with a monoglycosylated unit.[33] The signals correlated to two sugar units, as in compound **1**. The spin coupling system that includes the carbinolic proton H-3 place the linkage of an allomethylose unit at that position. The second unit corresponds to a glucose moiety at position C-4′. The coupling constant above 7 Hz of anomeric protons H-1′ and H-1″ suggests a β linkage for both sugars. Therefore, we describe compound **4** as 4′-*O*-β-glucopyranosyl-3-*O*-β-allomethylosyl syriogenin. This structure was hypothesized by Reichstein et al. but never confirmed.

Compound **5**, with a molecular formula of $C_{35}H_{52}O_{14}$ and an ion peak at *m*/*z* 714.3695 $[M + NH_4]^+$, resembles the cardenolide described as aspecioside, originally reported from caterpillar tissues reared on *Asclepias fruticosa*.[25] The H-7 signal multiplicity (d, 6.0 Hz) confirms a syn-periplanar position of H-6α and H-7, with an angle close to 90° between H-6β and H-7. Here an additional glucose moiety is observed, making **5** similar to **1** linked at the same C-4′ and β linkage, although it differs from it by lacking the common hydroxylation at C-12. Compound **5** is described here as 4′-*O*-β-glucopyranosyl-3-*O*-β-allomethylosyl-12-deoxy aspecioside.

The eight compounds revealed five distinct oxidation patterns in the steroidal core, with compounds **1** and **7** differing only in the number of sugar units, and **3**, **6**, and **8** sharing similar structural features, including an epoxide ring, a carbinolic group at C-12, and hydroxylation at C-11. We then quantified the cardenolide **1**−**8** concentrations in seeds (Figure 3 and Table S2). Among them, 4′-*O*-β-glucopyranosyl aspecioside **1** emerges as the most abundant cardenolide in *A. syriaca* seeds, with a concentration of 720 ± 40 μg/g, approximately 2.5 times higher than that of compound **5** with 280 ± 40, followed by that of compound **4** (220 ± 40).

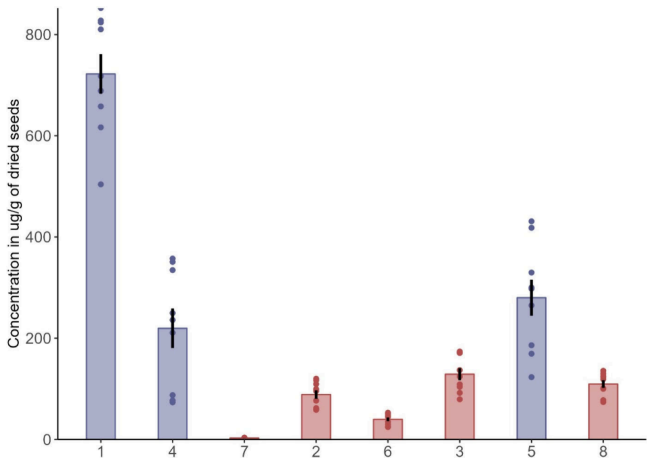

Figure 3. Quantification for compounds **1**−**8** in *A. syriaca* seeds sorted from left to right by retention time under the described chromatographic conditions. Blue bars correspond to compounds with glucopyranosyl-allomethylose moieties (**1**, **4**, and **5**), and compounds with other glycosylation patterns are shown in red. Shown are means ± standard errors.

Notably, the three most abundant compounds, each exceeding 200 μg per g, share the same glycosylation pattern composed of allomethylosyl and glucopyranosyl moieties, differing from the remaining cardenolides (Table S3).

A correlational heat map confirms the lack of correlation of **5** with the other seven compounds (Figure S1), suggesting a divergence for the biosynthesis of this compound. Cardenolide **5** is the only one is this study without the characteristic hydroxylation at C-12, commonly described in phytochemical reports from *A. syriaca*.[13,29,30,33] We hypothesize a key branching in the biosynthetic pathway of this milkweed, wherein a compound with the epoxide group, but without further oxidations, can accumulated at significant levels.

We assessed the *in vitro* inhibitory activity of cardenolides **1**−**8** on purified porcine (*Sus domesticus*) Na^+/K^+-ATPase, along with the enzyme from neural tissues of *Oncopeltus fasciatus*, a well-known adapted herbivore of milkweed seeds. The standard *in vitro* assay to determine the IC_{50} of each compound used ouabain as a reference (Table 3). The inhibitory capacity of the compounds was correlated across the two enzymes, despite the almost 1:500-fold difference in the values obtained for the porcine enzyme and that from the resistant enzyme from the cardenolide specialist insect. To further explore this, we analyzed the association between the IC_{50} values of the compounds in both enzymes (Figure 4), revealing a positive correlation of the toxic potential of isolated compounds against both the sensitive and resistant species (Estimate = 0.304 ± 0.065, t = 4.681, p-value = 0.003).

Labriformin **8**, as described by Agrawal et al.,[29] exhibits the highest inhibitory potency against both enzymes tested, closely followed by glucopyranosyl allomethylosyl syriogenin **4** (although no significant difference was observed) (Table S4). Consistent with previous studies on the inhibitory activity of monoglycosylated versus diglycosylated cardenolides,[20,34] the number of glycosylations does not necessarily have a strong impact on inhibitory activity: compounds **1** and **6**, differing by only one glucose moiety, display nonsignificantly different IC_{50}, regardless of the enzyme. C-3′ *epi*-syrioside **3** consistently exhibited the lowest inhibitory activity among the tested cardenolides. Despite epimers **3** and **6** differing by a single

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Table 1. ^{1}H NMR (800 MHz) for Compounds 1−6 in CD_3OD (J in Hz, Chemical Shifts in ppm.)

position	1	2	3	4	5
1	1.81, m	2.56, dd (12.9, 4.2)	2.69, dd (12.9, 4.1)	1.76, m	1.79, m
	1.07, td (13.6, 3.6)	1.14, dd (13.4, 11.8)	1.10, m	1.04, td (13.9, 4.0)	1.04, m
2	1.52, qd (13.1, 3.6)	4.12, m	4.08, m	1.71, m	1.81, m
	1.70, m			1.31, m	1.48, m
3	3.62, m	3.90, m	3.89, ddd (11.7, 10.1, 4.7)	3.63, tt (11.3, 4.7)	3.58, m
4	1.79, m	1.57, m	1.61, ddd (12.5, 4.7, 3.2)	1.88, m	1.77, m
	1.20, m	1.33, m	1.38, ddd (12.8, 5.1,11.7)	1.51, m	1.19, m
5	1.25, m	1.38, m	1.45, tdd (12.8, 5.1, 3.2)	1.10, m	1.66, m
6	1.82, m	1.84, m	1.90, m	1.37, m	1.79, m
	1.63, dd (15.4, 12.3)	1.62, m	1.69, m	1.28, m	1.61, m
7	3.23, m	3.28, m	3.47, d (6.2)	1.70, m	3.24, d (6.0)
8				1.31, m	
9	1.70, m	2.20, d (10.2)	1.91, m	0.98, td (12.3, 3.6)	1.21, m
11	1.84, m	4.15, m		2.01, m	1.70, m
	1.70, m			1.09, m	1.63, m
12	3.61, m	3.57, d (3.0)		3.34, m	1.70, m
13					1.65, m
15	2.24, m	2.44, ddd (12.8, 10.9, 9.4)	1.85, m	1.89, m	2.39, m
	1.70, m	1.59, m	1.75, ddd (13.5, 6.6, 3.2)	1.73, m	1.68, m
16	2.24, m	2.14, m	2.01, m	2.11, m	2.27, m
	2.04, m	1.95, m	2.01, m	1.92, m	1.96, m
17	3.35, m	3.59, m	4.10, m	3.33, m	2.89, dd (9.5, 5.5)
18	0.83, s	0.92, s	1.10, s	0.78, s	0.92, s
19	0.91, s	1.10, s	1.20, s	0.83, m	0.88, s
21	4.97, dd (18.4, 1.9)	5.03, dd (18.4, 1.8)	4.99, dd (18.4, 1.9)	4.96, dd (18.3, 1.8)	5.02, dd (18.3, 1.8)
	4.90, dd (18.4, 1.9)	4.95, dd (18.4, 1.8)	4.94, dd (18.4, 1.9)	4.89, dd (18.3, 1.8)	4.89, dd (18.3, 1.8)
22	5.92, s	5.91, s	6.00, s	5.89, s	5.89, s
1′	4.72, d (8.0)	4.70, s	4.48, s	4.72, d (8.1)	4.70, d (8.0)
2′	3.24, m			3.28, m	3.27, m
3′	4.32, t (2.9)	3.79, t (2.8)	3.73, dd (12.2, 4.7)	4.30, t (2.9)	4.30, t (3.0)
4′	3.3, m	1.84, m	2.04, m	3.28, m	3.28, m
		1.68, m	1.69, m		
5′	3.85, m	4.13, m	3.68, m	3.84, m	3.84, m
6′	1.29, d (6.3)	1.20, d (6.2)	1.23, d (6.2)	1.28, d (6.3)	1.27, d (6.2)
1″	4.4, d (7.7)	4.31, d (7.8)	4.47, m	4.38, d (7.8)	4.38, d (7.8)
2″	3.30, m	3.24, m	3.28, m	3.21, t (8.4)	3.21, t (8.4)
3″	3.36, t (8.4)	3.34, m	3.29, m	3.33, m	3.33, m
4″	3.34, m	3.27, m	3.28, m	3.32, m	3.32, m
5″	3.29, m	3.30, m	3.36, m	3.27, m	3.26, m
6″	3.86 m	3.89, m	3.86, m	3.85, m	3.82, m
	3.71 dd (11.9, 5.1)	3.64, dd (11.9, 6.3)	3.65, m	3.69, dd (11.9, 5.1)	3.69, dd (11.8, 5.1)

configurational difference at C-3′, they displayed significantly different inhibitory activities (p-value <0.001), with a 4.6- and 5.9-fold difference in their IC_{50} values for the adapted bug and sensitive porcine enzyme, respectively. This result resonates with another pair of C-3′ cardenolide epimers, known from *A. curassavica*, calotropin and calactin,[35] which display a 3-fold difference when tested against the adapted monarch butterfly enzyme.[12] A key unresolved question in pharmacology and chemical ecology is whether the most abundant toxins are also the most toxic. For both the highly specialized *O. fasciatus* and the porcine Na^+/K^+-ATPses, IC_{50} values varied approximately 10-fold, but as shown in Figure S48, no correlation exists between cardenolide concentration and toxicity for either enzyme.

Chemo-prospecting guided by the natural history of organisms leads to the discovery of unique structures. Conversely, molecules isolated from specific tissues can reveal their functional roles in biological interactions. As Reichstein noted in 1967, "The cardenolide and pregnane glycosides are often found in the seeds in high concentrations. This suggests that they have a biological role, because it is unlikely that the plant would accumulate unwanted byproducts in the seeds."[15] It is now accepted that these seed toxins protect them from herbivorous insects, and that some of these herbivores, such as *O. fasciatus*, are remarkably adapted to these toxins.[29]

Honoring the memory and significant contributions of chemists from the last century is important for our community because it is a poignant reminder of their ability to characterize complex structures with remarkable accuracy, despite limited resources in analytical chemistry. We were fortunate enough to acquire Reichstein's handwritten manuscript from 1979 (see Supporting Information and compare with the published manuscript)[19] in which he described magnificent cardenolide structures, underscoring the richness of his legacy. We have humbly described new cardenolide structures from *A. syriaca* seeds but are above all delighted to have characterized a

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Table 2. ^{13}C NMR (150 MHz) for Compounds 1−6 in CD_3OD (Chemical Shifts Are Given in ppm)

position	1	2	3	4	5
1	39.1, CH_2	45.7, CH_2	45.1, CH_2	38.3, CH_2	38.8, CH_2
2	29.8, CH_2	69.8, CH	69.3, CH	35.5, CH_2	29.6, CH_2
3	79.1, CH	73.2, CH	73.2, CH	79.4, CH	78.9, CH
4	34.9, CH_2	32.96, CH_2	32.9, CH_2	30.4, CH_2	34.7, CH_2
5	40.7, CH	41.6, CH	41.6, CH	45.8, CH	46.8, CH
6	29.4, CH_2	28.5, CH_2	28.1, CH_2	29.9, CH_2	29.1, CH_2
7	53.5, CH	53.7, CH	54.8, CH	30.8, CH_2	53.2, CH
8	64.2, C	64.6, C	63.8, C	42.1, CH	64.6, CH
9	44.1, CH	45.5, CH	49.1, CH	47.0, CH	40.2, CH
10	35.4, C	37.9, C	38.6, C	36.9, C	35.2, C
11	29.9, CH_2	71.4, CH	75.2, CH	28.9, CH_2	21.2, CH_2
12	76.1, CH	79.8, CH	213.5, C	75.6, CH	41.1,CH_2
13	59.1, C	55.9, C	65.2, C	57.2, C	52.8, C
14	82.4, C	81.9, C	82.4, C	86.6, C	82.1, C
15	35.8, CH_2	37.2, CH_2	37.0, CH_2	33.5, CH_2	35.6,CH_2
16	29.6, CH_2	31.1, CH_2	29.2, CH_2	28.3, CH_2	28.9,CH_2
17	47.4, CH	46.7, CH	43.6, CH	49.8, CH	51.6,CH
18	10.3, CH_3	14.5, CH_3	18.6, CH_3	9.8, CH_3	16.9,CH_3
19	13.3, CH_3	18.0, CH_3	13.9, CH_3	12.6, CH_3	13.1,CH_3
20	177.8, C	178.1, C	175.2, C	178.5, C	176.9, C
21	75.4, CH_2	75.7, CH_2	75.5, CH_2	75.5, CH_2	75,CH_2
22	117.8, CH	117.9, CH	118.8, CH	117.7, CH	117.7,CH
23	177.2, C	177.2, C	176.7, C	177.3, C	177.4, C
1′	99.8, CH	96.1, CH	97.1, CH	99.6, CH	99.5,CH
2′	75.1, CH	90.8, C	92.9, C	72, CH	71.7,CH
3′	72.3, CH	78.7, CH	83.8, CH	72.3, CH	71.9,CH
4′	83.8, CH	35.8, CH_2	38.8, CH_2	83.9, CH	83.6,CH
5′	69.4, CH	67.4, CH	69.4, CH	69.4, CH	69.1,CH
6′	18.2, CH_3	21.2, CH_3	21.3, CH_3	18.2, CH_3	17.9,CH_3
1″	105.9, CH	102.7, CH	106.6, CH	105.9, CH	105.6,CH
2″	72, CH	74.6, CH	75.5, CH	75.1, CH	74.8,CH
3″	77.9, CH	77.9, CH	78.1, CH	77.9, CH	77.6,CH
4″	71.2, CH	71.7, CH	71.5, CH	71.2, CH	70.9,CH
5″	77.8, CH	78.1, CH	77.9, CH	77.8, CH	77.5, CH
6″	62.3, CH_2	62.7, CH_2	62.7, CH_2	62.4, CH_2	62.2, CH_2

Table 3. Comparison of Na^+/K^+-ATPase Inhibition Activity (IC_{50} in μM ± Std. Error) of Compounds 1−8 Using Seed Bug and Porcine Proteins

compound	*Oncopeltus fasciatus*	*Sus domesticus*
8[a]	220 ± 40	0.9 ± 0.1
4[a]	220 ± 30	1.1 ± 0.1
6	640 ± 70	0.9 ± 0.1
5	830 ± 80	1.1 ± 0.1
1[a]	1040 ± 150	1.4 ± 0.1
7[a]	1080 ± 20	1.4 ± 0.1
2	1960 ± 80	2.2 ± 0.2
3	2950 ± 170	5.4 ± 0.2
ouabain	2100 ± 200	0.7 ± 0.1

[a]Data of compounds **1**, **4**, **7**, and **8** previously reported in Agrawal, et al. 2022.[29]

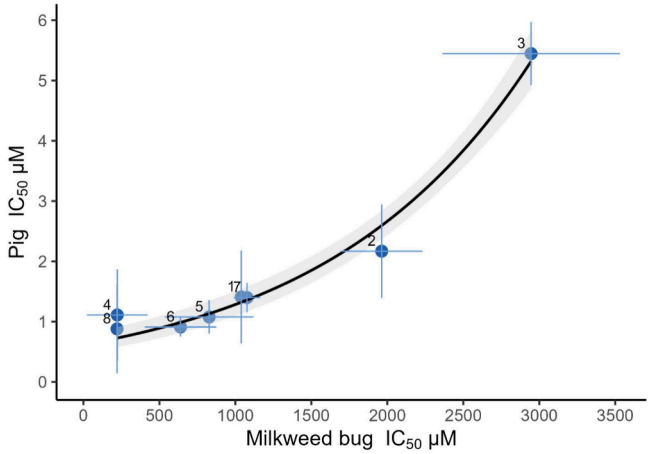

Figure 4. Correlation between inhibitory activity of the eight *A. syriaca* seed cardenolides on the Na^+/K^+-ATPase of a sensitive and resistant species. Blue lines represent the standard errors of the shown means. The black line shows the fitted exponential model, and the gray area shows the 95% confidence interval.

structure that Reichstein had proposed many years ago. We hope that his manuscript will inspire younger generations and shape vocations in the elucidation of natural products.

EXPERIMENTAL SECTION

General Experimental Procedures. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker 800 Avance III HD console and a TCI HCN cryoprobe. ^1H data were obtained using Bruker's standard pulse sequence zg. COSY 2D data were obtained with Bruker's double-quantum filtered pulse sequence cosydfphpp.

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HSQC used Bruker's edited adiabatic pulse sequence hsqcedetgpsisp2.3 with ^{1}H decoupling removed during acquisition.

HMBC used Bruker's constant time pulse sequence hmbcctetgpl2nd. NOESY used Bruker's gradient-enabled phase-sensitive pulse sequence noesygpph. The ^{13}C NMR data were obtained using a Bruker 600 Avance III HD console and BBFO^{+} probe using Bruker's standard proton decoupling power-gated pulse sequence zgpg30. Data processing was performed using MestReNova version 15.0.0-34764 Mestrelab Research S.L. Samples were measured in CD_3OD (99.5%). For compounds measured in CD_3OD, the residual solvent signals were δ_H 3.31/δ_C 49.15 as reference peaks.

For UPLC-HRMS and MS/MS analysis, we utilized a Dionex 3000 LC reversed-phase chromatography system coupled to an Orbitrap Q-Exactive mass spectrometer, controlled by Xcalibur software (Thermo Fisher Scientific). Methanolic extracts were chromatographically separated on an Agilent Zorbax Eclipse XDB-C18 column (150 × 2.1 mm, particle size 1.8 μm) maintained at 40 °C with a flow rate of 0.5 mL/min. Solvent A comprised 0.1% formic acid (FA) in H_2O, while solvent B contained 0.1% FA in MeCN. The gradient started at 5% B for 2 min postinjection, then increased linearly to 98% B at 11 min, held for 3 min, returned to 5% B over 0.1 min, and finally maintained at 5% B for 2.9 min to reequilibrate the column. Mass spectrometer parameters were set as follows: spray voltage (−3.0 kV, +3.5 kV), capillary temperature 380 °C, probe heater temperature 400 °C, with sheath, auxiliary, and sweep gas at 60, 20, and 2 AU, respectively. The S-Lens radio frequency level was 50, resolution 240,000 at m/z 200, and automatic gain control (AGC) target set at 3e6. Samples were analyzed in positive electrospray ionization mode with an m/z range of 70 to 1000. Data-dependent tandem mass spectrometry (MS/MS) (dd-MS2) parameters included MS1 resolution at 60,000 with an AGC target of 1e6, while MS2 resolution was set at 30,000 with an AGC target of 2e5. The maximum injection time was 50 ms, isolation window 1.0 m/z, stepped normalized collision energy (NCE) at 10 and 30, and a dynamic exclusion of 1.5 s, with the top five masses selected for MS/MS per scan.

Absolute quantification via HPLC-HRMS was performed on a reversed-phase liquid chromatography system consisting of an Agilent 1260 Infinity II coupled to an Agilent 6545 Q-TOF mass spectrometer. Methanolic extracts were chromatographically separated on an Agilent Infinity Lab Poroshell 120 ER-C18 column (150 × 2.1 mm, particle size 1.9 μm) maintained at 40 °C with a flow rate of 0.3 mL/min. Separation was achieved using an MeCN−H_2O gradient with 10 mM ammonium formate and 0.1% formic acid: 5% MeCN at 0 min, followed by 5−95% MeCN from 0 to 14 min, 100% MeCN from 14 to 17 min, and a 3 min postrun at 5% MeCN. Each sample was analyzed in positive electrospray ionization mode with an m/z range of 100−900. Optimal parameters for sensitive detection of solanidine included a gas temperature of 225 °C, drying gas at 10 L/min, nebulizer pressure set to 35 psi, sheath gas temperature of 325 °C, and sheath gas flow at 11 L/min. Quantification of cardenolides **1**−**8** was performed using the Agilent MassHunter Quantitative Analysis Software. Sample analysis involved injecting three replicates of the cardenolides **1**−**8** mixed together (standards solution) in MeOH at concentrations of 12.5 ng/mL, 125 ng/mL, 1.25 μg/mL, and 25 μg/mL, collectively used to construct a calibration curve for absolute quantification of each cardenolide in samples.

Plant Material. The seeds of *Asclepias syriaca* were collected at three locations in Tompkins, NY, USA: Ronz Pond (42.4731242, −76.3223601), Ellis Hollow Primrose site (42.430843, −76.386320), and Durland Bird Preserve (42.437641, −76.397634) in September 2018. Plants were identified by A. A. Agrawal in relation to many specimens collected and compared to *A. syriaca* preserved in the Herbarium of the L.H.B. Hortorium at Cornell University.

Extraction and Isolation. *Extraction.* Seeds (100 g) were collected and deflossed when brown (mature) but before dehiscence (opening and dispersal). Seeds were freeze-dried, ground, and then extracted with MeOH (1 L) for a week. The extract was washed with hexane (100 mL) a few times, followed by drying. The remaining residue was then suspended in 16% MeCN and H_2O (9 mL), sonicated for 30 s, vortexed, and centrifuged at 20800*g* for 12 min. After centrifugation, the clear supernatant was immediately injected for preparative HPLC fractionation.

All prepared samples were injected into an Agilent 1260 series preparative LC system with an Agilent 21.2 × 150 mm, C8, 5 μm column. Each first-pass injection was eluted at a constant flow rate of 14.87 mL/min with a gradient of MeCN and H_2O: 0 to 2 min at 16% MeCN, 2 to 25 min from 16% to 70%, 25 to 30 min from 70% to 95%, and 30 to 35 min at 95%. Target peaks were detected at 218 nm. In many cases, each first-pass target fraction required drying, resuspension in 16% MeCN, and reinjection for further cleanup. Fractions needing reinjection often required adjustments to the method gradients to increase column retention times for better isolation of the target peaks. The isolated fractions were pooled, dried, resuspended in 0.5 mL of 100% MeOH, and then analyzed on the Dionex 3000 LC reversed-phase chromatography system coupled to an Orbitrap Q-Exactive mass spectrometer UPLC-HRMS system in positive ionization mode for quality check.

Conformational Analyses. 3D models of the isolated compounds were obtained using Avogadro 1.2.0 software. Conformational analyses were performed using molecular mechanics (MMFF) to select among conformers with the lowest energy according to the Boltzmann distribution values.

The isolated cardenolides, together with their chemical data, are listed below. For ^{1}H NMR and ^{13}C NMR data, see Table 1 and Supporting Information, Table S1.

*4′-O-β-Glucopyranosyl aspecioside (**1**):* $[\alpha]_D^{25}$ −48 (*c* 0.005, MeOH); HRESIMS m/z 713.3355 $[M + H]^+$ (calcd for $C_{35}H_{53}O_{15}$, 713.3379, Δppm = 3 ppm); UV spectrum (Figure S49).

*3′-O-β-Glucopyranosyl syribioside (**2**):* $[\alpha]_D^{25}$ −24 (*c* 0.005, MeOH); HRESIMS m/z 709.3069 $[M - H_2O + H]^+$ (calcd for $C_{35}H_{50}O_{15}$, 709.3066, Δppm = 0.4 ppm).

*C-3′-epi-Syrioside (**3**):* $[\alpha]_D^{25}$ −16 (*c* 0.005, MeOH); HRESIMS m/z 742.3286 $[M + NH_4]^+$ (calcd for $C_{35}H_{52}O_{16}N$, 742.3281, Δppm = 0.6 ppm).

*4′-O-β-Glucopyranosyl allomethylosyl syriogenin (**4**):* $[\alpha]_D^{25}$ −24 (*c* 0.005, MeOH); HRESIMS m/z 699.3586 $[M + H]^+$ (calcd for $C_{35}H_{55}O_{14}$, 699.3586, Δppm = 0 ppm).

*4′-O-β-Glucopyranosyl-allomethylosyl 12-deoxy aspecioside (**5**):* $[\alpha]_D^{25}$ −48 (*c* 0.005, MeOH); HRESIMS m/z 714.3695 $[M + NH_4]^+$ (calcd for $C_{35}H_{57}O_{14}N$, 714.3695, Δ 0 ppm).

*Syrioside (**6**):* white powder; HRESIMS m/z 747.2828 $[M + Na]^+$ (calcd for $C_{35}H_{48}O_{16}Na$, 747.2835, Δppm = 1.6 ppm).

*Aspecioside (**7**):* white powder; HRESIMS m/z 551.2856 $[M + H]^+$ (calcd for $C_{29}H_{43}O_{10}$, 551.2851, Δppm = 0.9 ppm).

*Labriformin (**8**):* white powder; HRESIMS m/z 600.2264 $[M - H_2O + H]^+$ (calcd for $C_{31}H_{39}NO_9S$, 600.2262, Δppm = 0.3 ppm).

Sample Preparation for the Quantification of Cardenolides in *Asclepias syriaca* Seeds. Fifteen collections (one fruit pod per plant) were made from individual milkweed stems at least 5 m apart at a single field site (Durland Bird Preserve (42.437641, −76.397634)) in Ithaca, NY. Those were separated in three biological replicates, each composed of 20 seeds, four from each fruit pod, i.e., representing the chemical diversity of five collections. The seeds were exposed to liquid nitrogen, later pulverized, and divided into three technical replicates each of 20 mg of dried weight (d.w.).

The dried samples were placed into a fast prep matrix tube with zirconium/glass-pellets and 1 mL of MeOH. The sample was processed in a FastPrep-24 homogenizer (MP Biomedicals). Samples were centrifuged at 20,817*g* for 12 min to remove particulates, and the supernatant was taken to dryness in a rotary evaporator (Labconco CentriVap). Extracts were defatted twice by dissolving residues in 250 mL of MeOH, adding 750 mL of hexane, vortexing 3 times for 30 s, centrifuging for 10 min at 19,480*g*, and pipetting off the hexane layer. Defatted samples were dried and reconstituted in 100 μL of MeOH. These samples were filtered through a (0.2 μm) Millipore syringe filter during the sample preparation for HPLC-MS analysis. Three biological replicates and three technical replications from each resulted in nine data points per compound. The concentration per injection was later expressed in μg of cardenolide per gram of seed d.w. We employed Bonferroni-adjusted significance tests for pairwise

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comparisons between the concentration values of the isolated compounds.

Na^+/K^+ ATPase Inhibitory Activity Assay. We quantified the inhibitory potential of isolated cardenolides using Na^+/K^+-ATPase from porcine cerebral cortex (Millipore Sigma) and dissected *Oncopeltus* neural tissues following methods of Petschenka et al.[36] Briefly, each compound was resuspended in 20% dimethyl sulfoxide (DMSO)/H_2O to 5×10^{-3} M. We then prepared 1/10 serial dilutions to produce a six-point inhibition curve for each compound (5×10^{-3} M, 5×10^{-4} M, 5×10^{-5} M, 5×10^{-6} M, 5×10^{-7} M, 5×10^{-8} M). The compound solutions were diluted 1:5 with a buffered reaction mix containing Tris-buffered ATP, NaCl, KCl, $MgCl_2$, and Na^+/K^+-ATPase solution and incubated on a BioShake iQ microplate shaker (Quantifoil Instruments) at 200 rpm and 37 °C for 20 min. Each milkweed cardenolide was run in three technical replicates alongside equivalent molar solutions of ouabain. Reactions were terminated with 10% sodium dodecyl sulfate; then inorganic phosphate was stained with Taussky–Shorr reagent and absorbance measured spectrophotometrically at 700 nm. Absorbance values of reactions were corrected by their respective backgrounds (containing 10 mM ouabain, ATP, NaCl, $MgCl_2$, and appropriate enzyme but lacking KCl), and dose–response curves were fitted using a nonlinear mixed effects model with a four-parameter logistic function in the statistical software R studio.[36] We focus analyses on the cardenolide concentration at which the enzyme is inhibited by 50% (IC_{50}) compared to a control without toxins added.

We employed Bonferroni-adjusted significance tests for pairwise comparisons between the IC_{50} values for cardenolides **1**–**8** across both analyzed enzymes. For potential correlation between the inhibitory capacity of the compounds across both enzymes, IC_{50} values were analyzed using a GLM with a Gaussian distribution and identity link function.

■ ASSOCIATED CONTENT

Data Availability Statement

The NMR data for compounds **1**–**5** have been deposited in the Natural Products Magnetic Resonance Database (NP-MRD) and can be found at NP0341890 (compound **1**), NP0341891 (compound **2**), NP0341892 (compound **3**), NP0341893 (compound **4**), and NP0341894 (compound **5**).

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jnatprod.4c00960.

1D and 2D NMR and HR-ESI-MS spectra; Tadeus Reichstein's original handwritten manuscript (PDF)

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Notes

The authors declare no competing financial interest.

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Supporting information

Cardenolides in *Asclepias syriaca* seeds: exploring the legacy of Tadeus Reichstein in common milkweed

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Table of Contents

Table S1: NMR data of syrioside (6) and labriformin (8)[a]. The coupling constants (*J*) are in parentheses and reported in Hz; chemical shifts are given in ppm.

	Syrioside		**Labriformin**	
Position	^{13}C	^{1}H	^{13}C	^{1}H
1	45.5	1.08 (m)	44.1	1.07 td (12)
2	69.7	4.12 ()	68.9	4.14 ddd (11.8, 9.8, 4.0)
3	73.1	3.88 ()	71.4	4.01 ddd (11.3, 10.0, 4.5)
4	32.9	1.60 (m)	31.7	1.44 q (12.0)
5	41.7	1.45 tdd (12.8, 5.1, 3.3)	40.7	1.40 (m)
6	28.1	1.89 (m)	26.7[b]	1.75 td (13)
7	54.8	3.46 d(6.1)	54.1	3.45 d (6.2)
8	65.2		62.2	
9	48.9	1.88 (m)	48.3	1.71 d (12.8)
10	38.7		37.7	
11	75.2	4.79	73.5	4.79 dd (12.8, 4.6)
12	212.1		212.5	
13	63.8		63.1	
14	82.4		81.1	
15	37	1.85	36.0	1.72 qd (11)
16	29.2	2.01(2H)	28.4[b]	1.98–2.05 (2H, m)
17	43.6	4.10 t (8.2)	42.5	3.93 t (8.3)
18	18.5	1.09 s	18.3	1.07 s
19	13.9	1.20 s	13.6	1.20 s
20	173.8		170.5	
21	75.4	4.98 dd (18.5, 1.9 Hz)	73.7	4.78 dd (18.1, 1.9)
22	117.4	5.99 s	118.8	6.00 ddd (1.9, 1.9, 1.0)
23	175.3		173.8	
1’	96.1	4.69 s	95.0	5.08 s
2’	90.8		91.7	
3’	78.7	3.79 t (2.8)	99.5	
4’	35.7	1.82 (m)	47.2	2.23 dd (13.1, 11.2)
5’	67.4	4.12	68.2	4.26 ddd (11.2, 6.3, 2.0)
6’	21.2	1.19 d (6.3)	20.8	1.21 d (6.3)
1’’	102.7	4.31 d (7.8)		
2’’	74.6	3.24 dd (9.2, 7.8)		
3’’	77.9	3.34		
4’’	71.7	3.27		
5’’	78.1	3.3		
6’’	62.7	3.88		
		3.64 dd (11.9, 6.3)		
C=N			160.1	7.52 t (1.4)
C-S			42.7	3.85 dd (16.5, 1.4)
				3.89 dd (16.5, 1.4)

[a] James N. Seiber, et al. *Phytochemistry*, **1978**, (17), 967-970 doi: 10.1016/S0031-9422(00)88658-6.
[b] For labriformin the assignments for C-6, C-15 and C-16 were incorrectly identified as C-16, C-6 and C-15, respectively, in the original literature however the numerical values are in very close agreement.

Table S2. Spectrometric quantification of compounds 1-8 (concentration in μg/g of dried seeds)

Compound	Concentration ± std.error
5	280 ±40
8	110 ±8
1	720 ±40
4	220 ±40
7	2.6 ±0.2
2	89 ±8
6	40 ±4
3	129 ±12

Table S3. Multiple comparison test of the spectrometric quantification of compounds 1-8

group1	group2	df	statistic	p	p.adj	p.adj.signif
5	8	64	5.02524	4.30E-06	1.20E-04	***
5	1	64	-13.031	1.14E-19	3.20E-18	****
5	4	64	1.7786	8.01E-02	1.00E+00	ns
5	7	64	8.17288	1.60E-11	4.48E-10	****
5	2	64	5.64048	4.13E-07	1.16E-05	****
5	6	64	7.0814	1.34E-09	3.75E-08	****
5	3	64	4.45123	3.49E-05	9.77E-04	***
8	1	64	-18.056	8.26E-27	2.31E-25	****
8	4	64	-3.2466	1.86E-03	5.21E-02	ns
8	7	64	3.14764	2.50E-03	7.00E-02	ns
8	2	64	0.61524	5.41E-01	1.00E+00	ns
8	6	64	2.05616	4.39E-02	1.00E+00	ns
8	3	64	-0.574	5.68E-01	1.00E+00	ns
1	4	64	14.8099	2.37E-22	6.64E-21	****
1	7	64	21.2041	1.21E-30	3.39E-29	****
1	2	64	18.6717	1.36E-27	3.80E-26	****
1	6	64	20.1127	2.30E-29	6.45E-28	****
1	3	64	17.4825	4.63E-26	1.30E-24	****
4	7	64	6.39428	2.12E-08	5.94E-07	****
4	2	64	3.86189	2.65E-04	7.42E-03	**
4	6	64	5.3028	1.51E-06	4.23E-05	****
4	3	64	2.67263	9.54E-03	2.67E-01	ns
7	2	64	-2.5324	1.38E-02	3.86E-01	ns
7	6	64	-1.0915	2.79E-01	1.00E+00	ns
7	3	64	-3.7216	4.20E-04	1.18E-02	*
2	6	64	1.44092	1.54E-01	1.00E+00	ns
2	3	64	-1.1893	2.39E-01	1.00E+00	ns
6	3	64	-2.6302	1.07E-02	2.99E-01	ns

Table S3. Multiple comparison test of the inhibitory capacity of compounds 1-8

Sus domesticus (df: 28)				*Oncopeltus fasciatus* (df: 36)			
group1	**group2**	**p.adj**	**p.adj.signif**	**group1**	**group2**	**p.adj**	**p.adj.signif**
5	8	1.00E+00	ns	5	8	7.63E-03	**
5	1	1.00E+00	ns	5	1	1.00E+00	ns
5	4	1.00E+00	ns	5	4	1.04E-02	*
5	7	1.00E+00	ns	5	7	1.00E+00	ns
5	2	8.23E-04	***	5	2	1.53E-05	****
5	6	1.00E+00	ns	5	6	1.00E+00	ns
5	3	9.83E-17	****	5	3	4.80E-12	****
5	ouabain	1.00E+00	ns	5	ouabain	1.58E-05	****
8	1	2.91E-02	*	8	1	1.12E-07	****
8	4	1.00E+00	ns	8	4	1.00E+00	ns
8	7	7.41E-01	ns	8	7	4.41E-05	****
8	2	3.73E-06	****	8	2	2.04E-12	****
8	6	1.00E+00	ns	8	6	2.66E-01	ns
8	3	3.46E-19	****	8	3	1.96E-18	****
8	ouabain	1.00E+00	ns	8	ouabain	2.79E-11	****
1	4	1.00E+00	ns	1	4	3.19E-07	****
1	7	1.00E+00	ns	1	7	1.00E+00	ns
1	2	9.32E-03	**	1	2	2.18E-05	****
1	6	3.83E-01	ns	1	6	4.66E-01	ns
1	3	9.26E-18	****	1	3	4.44E-13	****
1	ouabain	1.18E-01	ns	1	ouabain	3.22E-05	****
4	7	1.00E+00	ns	4	7	6.85E-05	****
4	2	1.07E-04	***	4	2	4.06E-12	****
4	6	1.00E+00	ns	4	6	3.22E-01	ns
4	3	1.38E-18	****	4	3	4.25E-18	****
4	ouabain	1.00E+00	ns	4	ouabain	4.53E-11	****
7	2	1.22E-01	ns	7	2	9.76E-04	***
7	6	1.00E+00	ns	7	6	8.29E-01	ns
7	3	1.28E-14	****	7	3	1.54E-10	****
7	ouabain	6.10E-01	ns	7	ouabain	6.49E-04	***
2	6	9.98E-05	****	2	6	6.85E-07	****
2	3	1.60E-13	****	2	3	1.95E-04	***
2	ouabain	6.88E-05	****	2	ouabain	1.00E+00	ns
6	3	3.67E-17	****	6	3	4.07E-13	****
6	ouabain	1.00E+00	ns	6	ouabain	9.73E-07	****
3	ouabain	2.45E-16	****	3	ouabain	7.45E-03	**

Figure S1: Spearman correlation heat map of cardenolide abundance in Asclepias syriaca seeds. Only significant correlations are displayed with numbers, non-significant in white boxes.

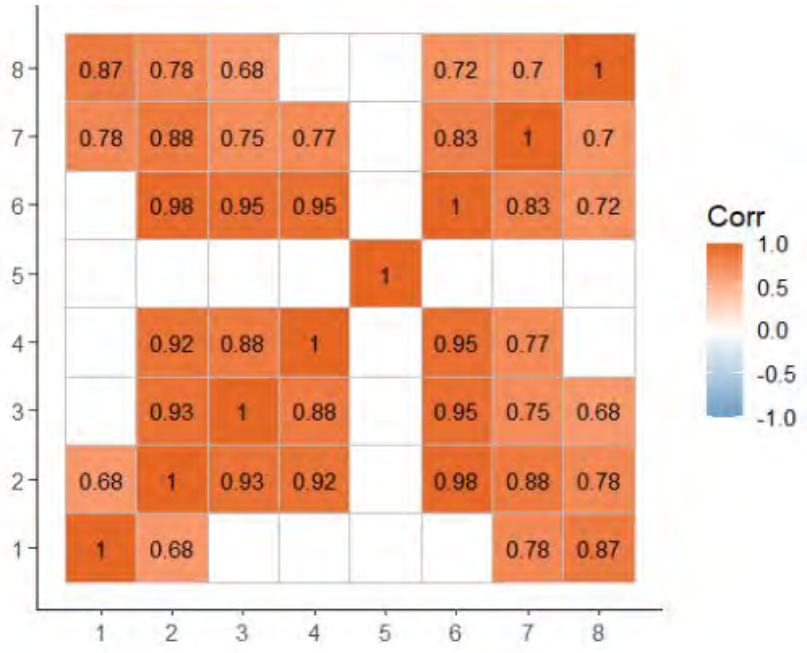
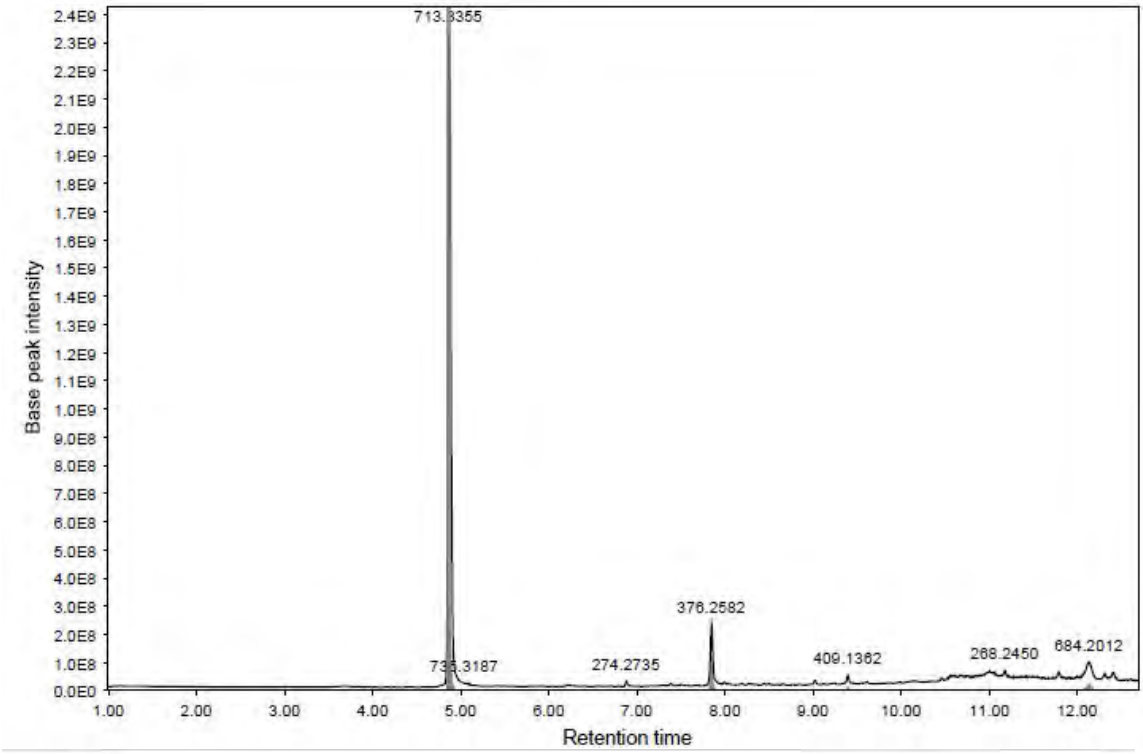


Figure S2: HR-ESI-MS spectrum of compound 1



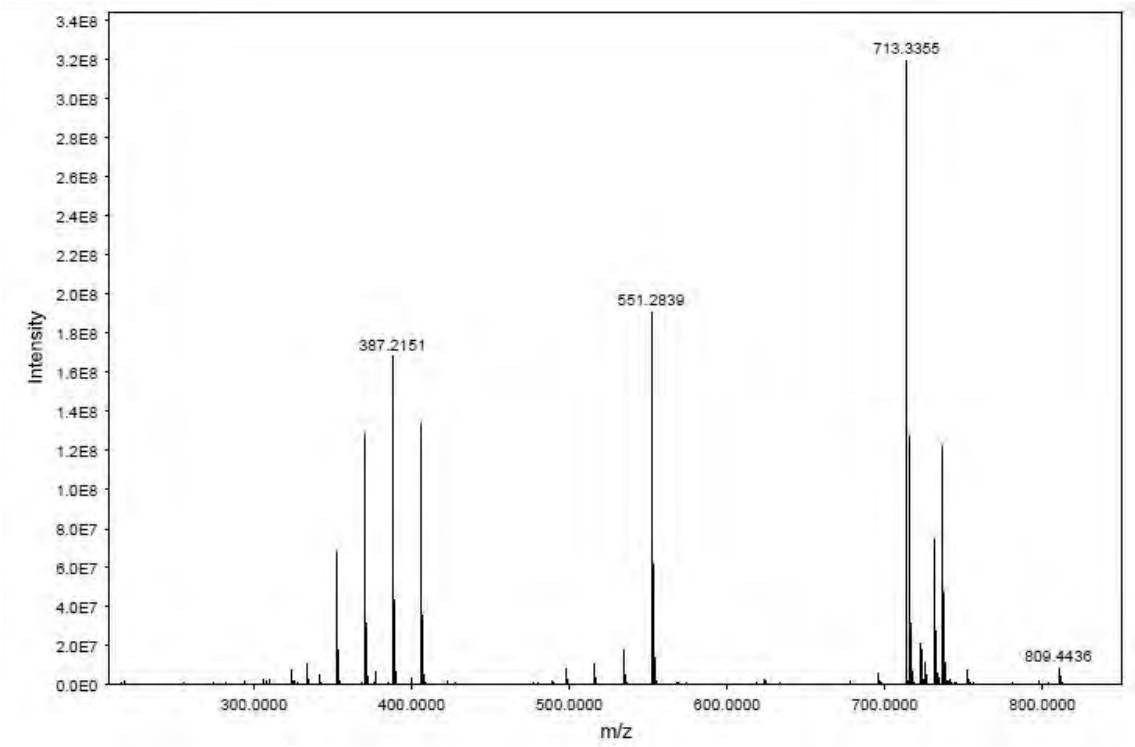


Figure S3: MS/MS spectrum of compound 1

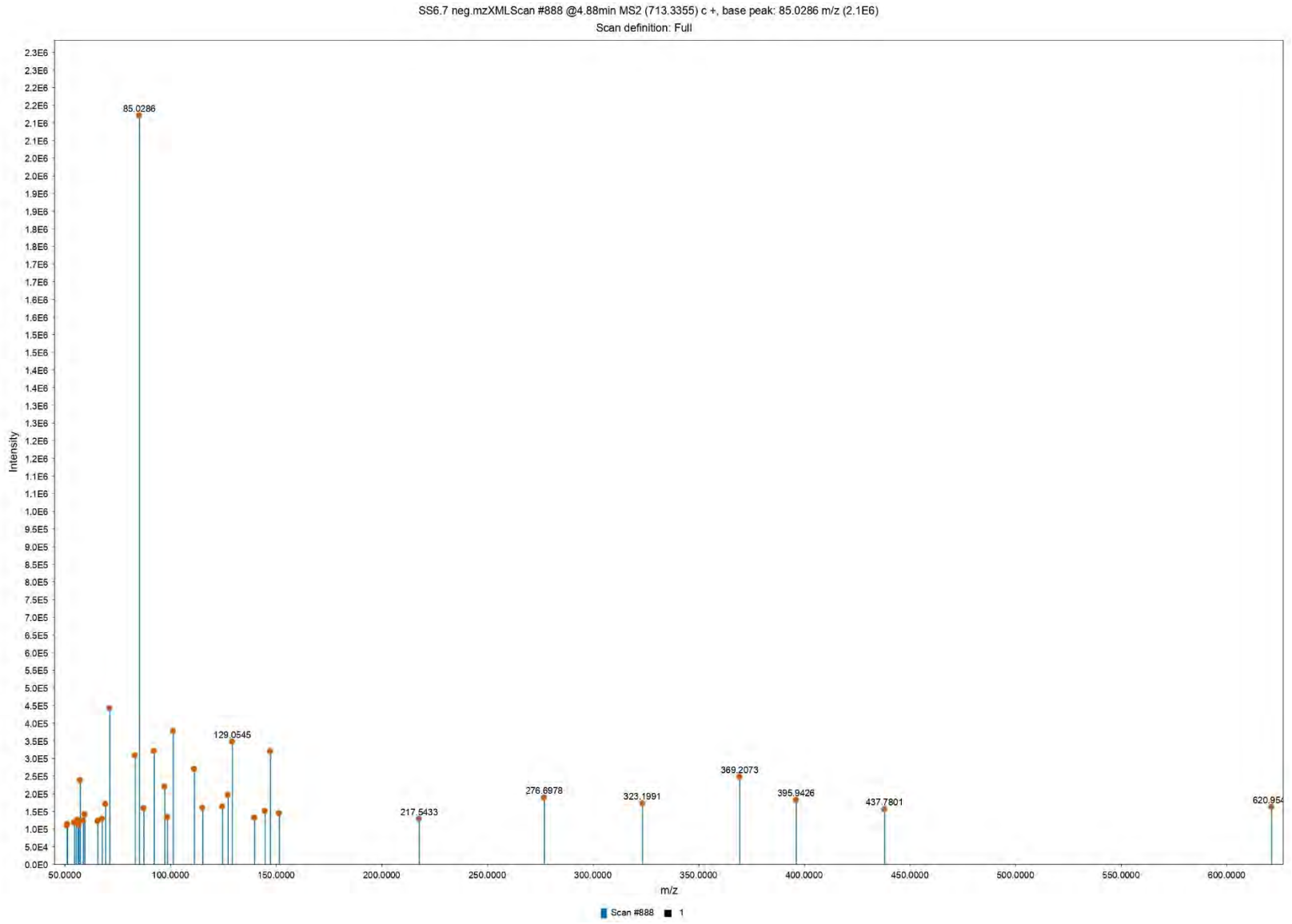


Figure S4: ^{1}H-NMR spectrum (800 MHz, CD_3OD) of compound 1.

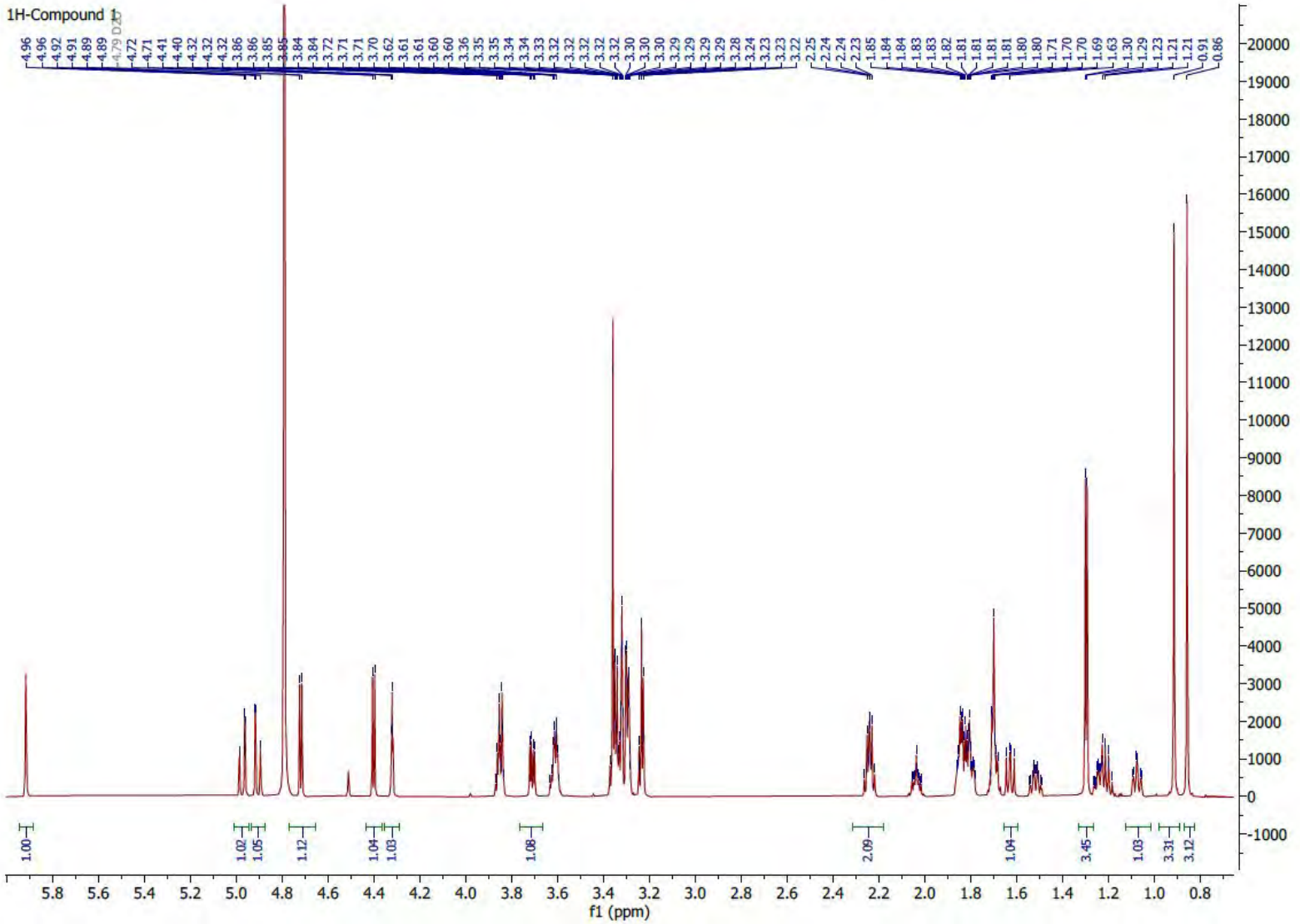


Figure S5: ^{13}C-NMR spectrum (600 MHz, CD_3OD) of compound 1.

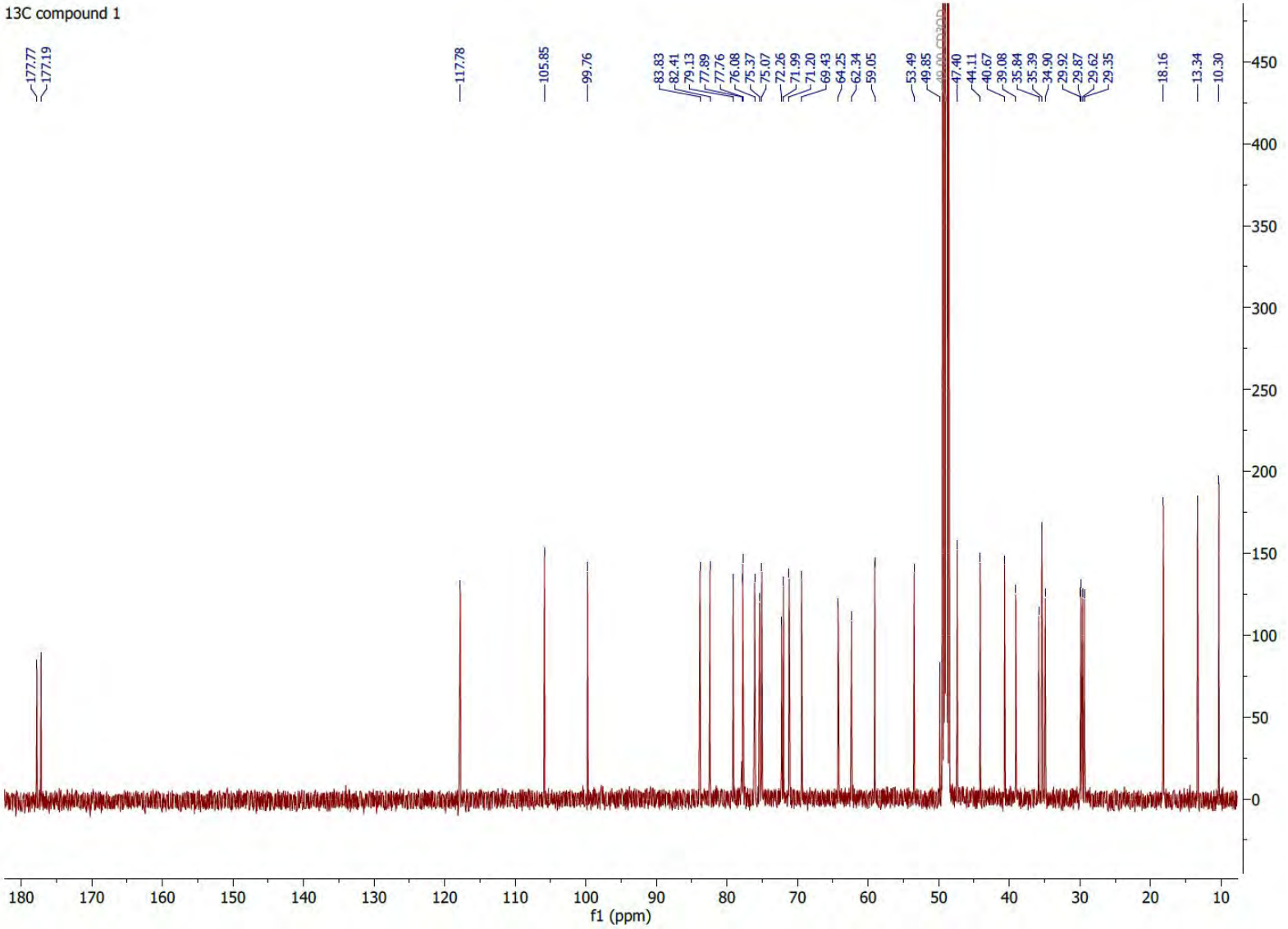


Figure S6: ^{1}H-^{13}C HSQC spectrum (800 MHz, CD_3OD) of compound 1.

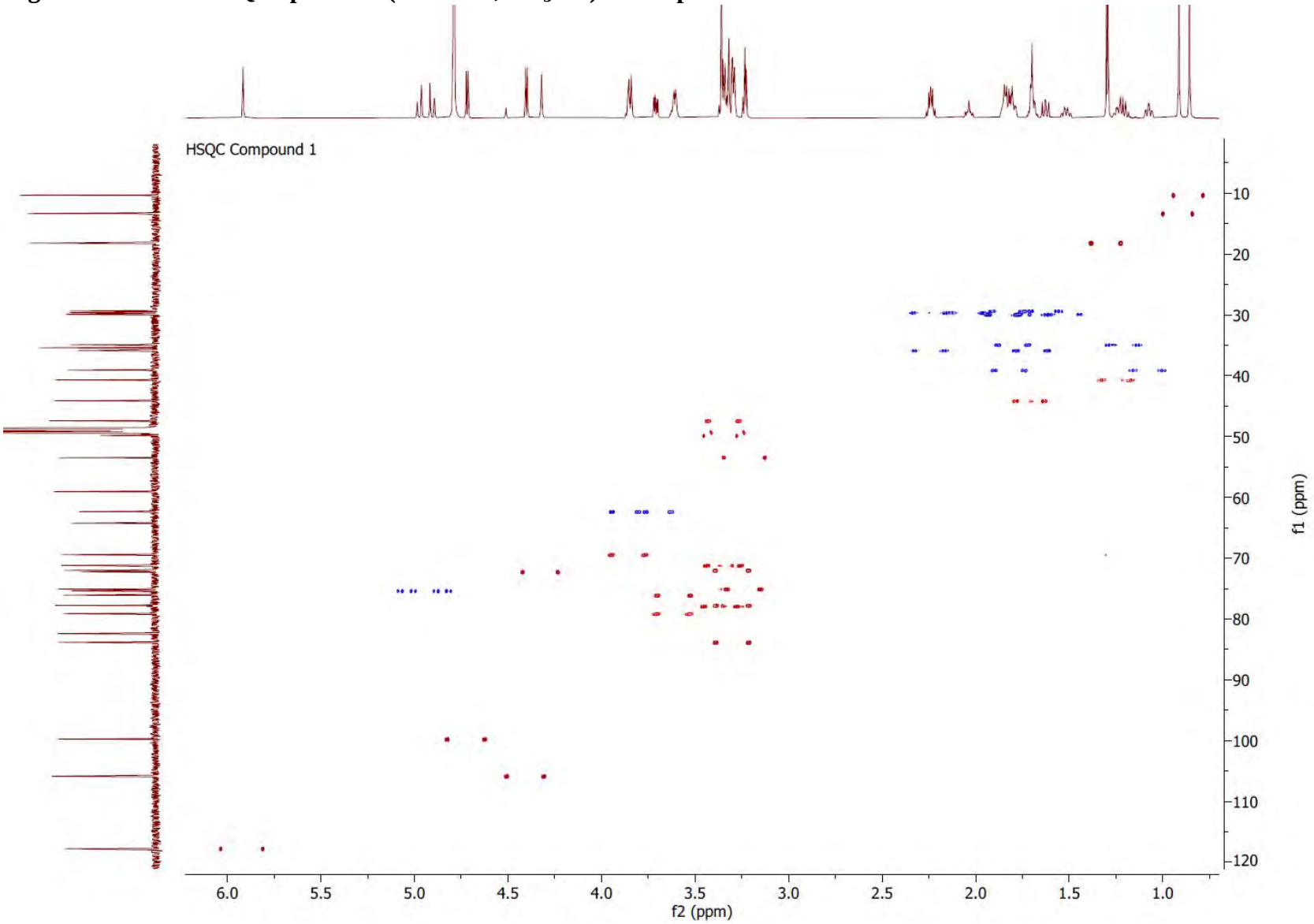


Figure S7: 1H-1H –COSY spectrum (800 MHz, CD3OD) of compound 1.

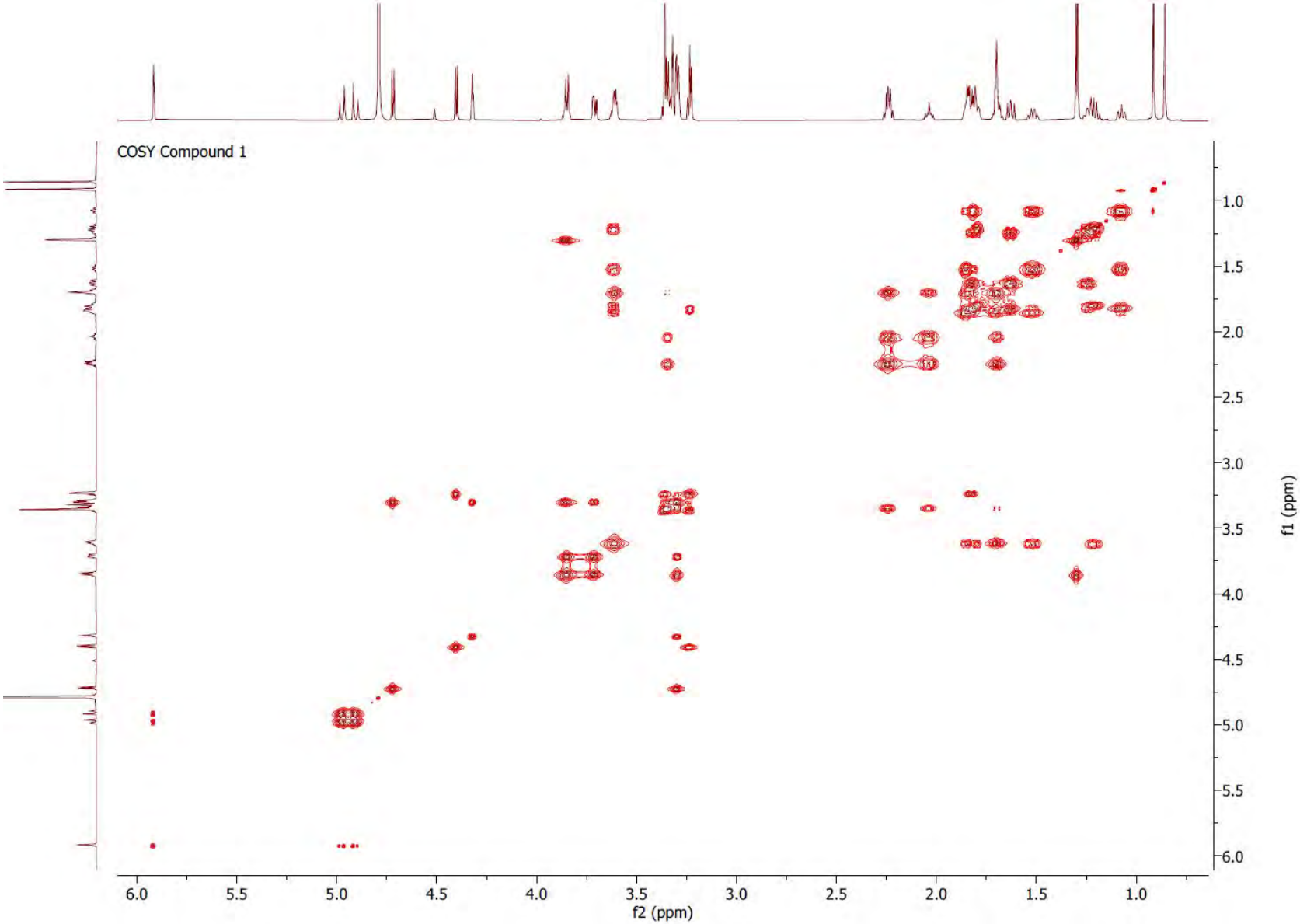


Figure S8: ^{1}H-^{13}C HMBC spectrum (800 MHz, CD_3OD) of compound 1.

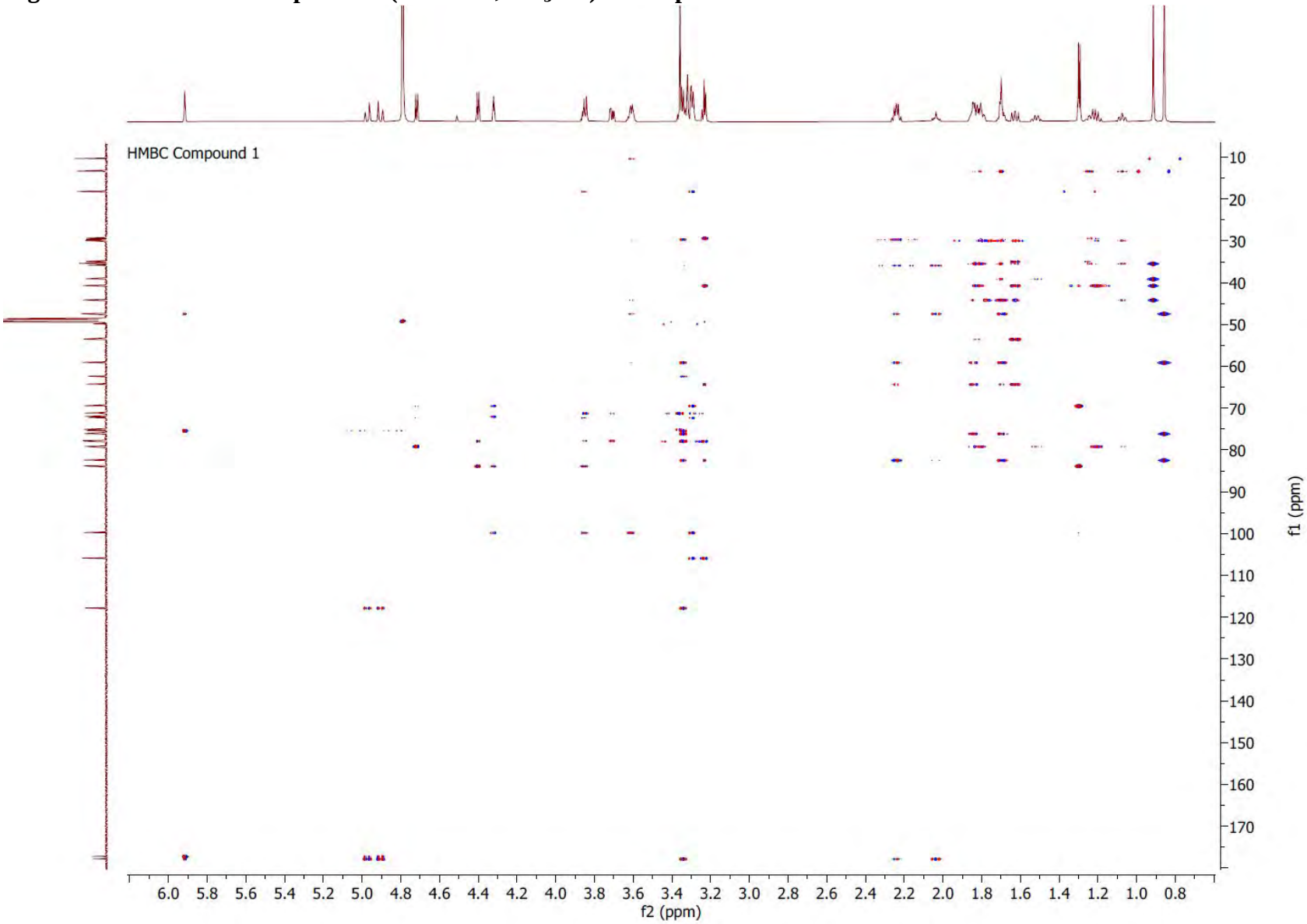


Figure S9: ^{1}H-^{1}H –NOESY spectrum (800 MHz, CD_3OD) of compound 1.

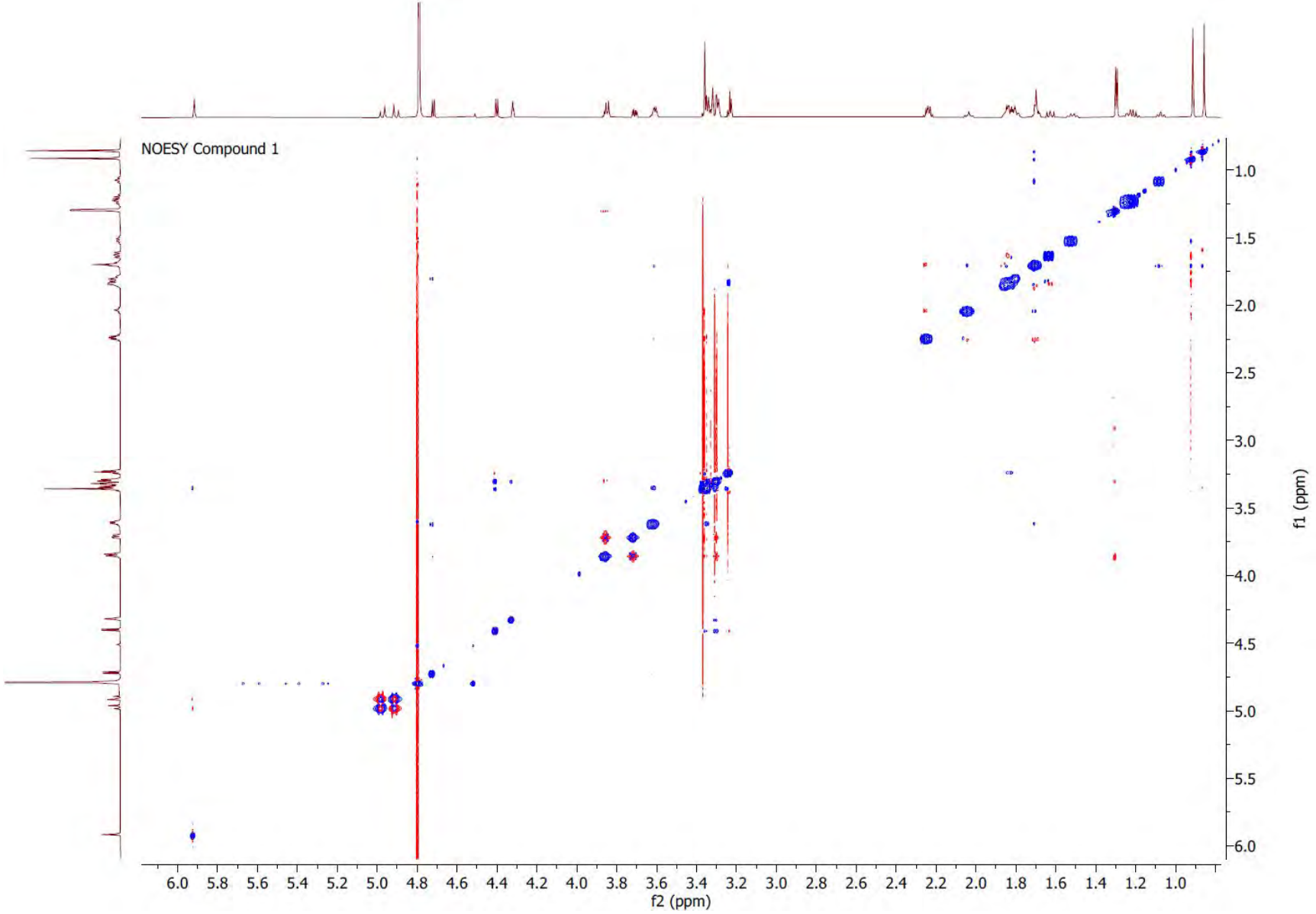


Figure S10: HR-ESI-MS spectrum of compound 2

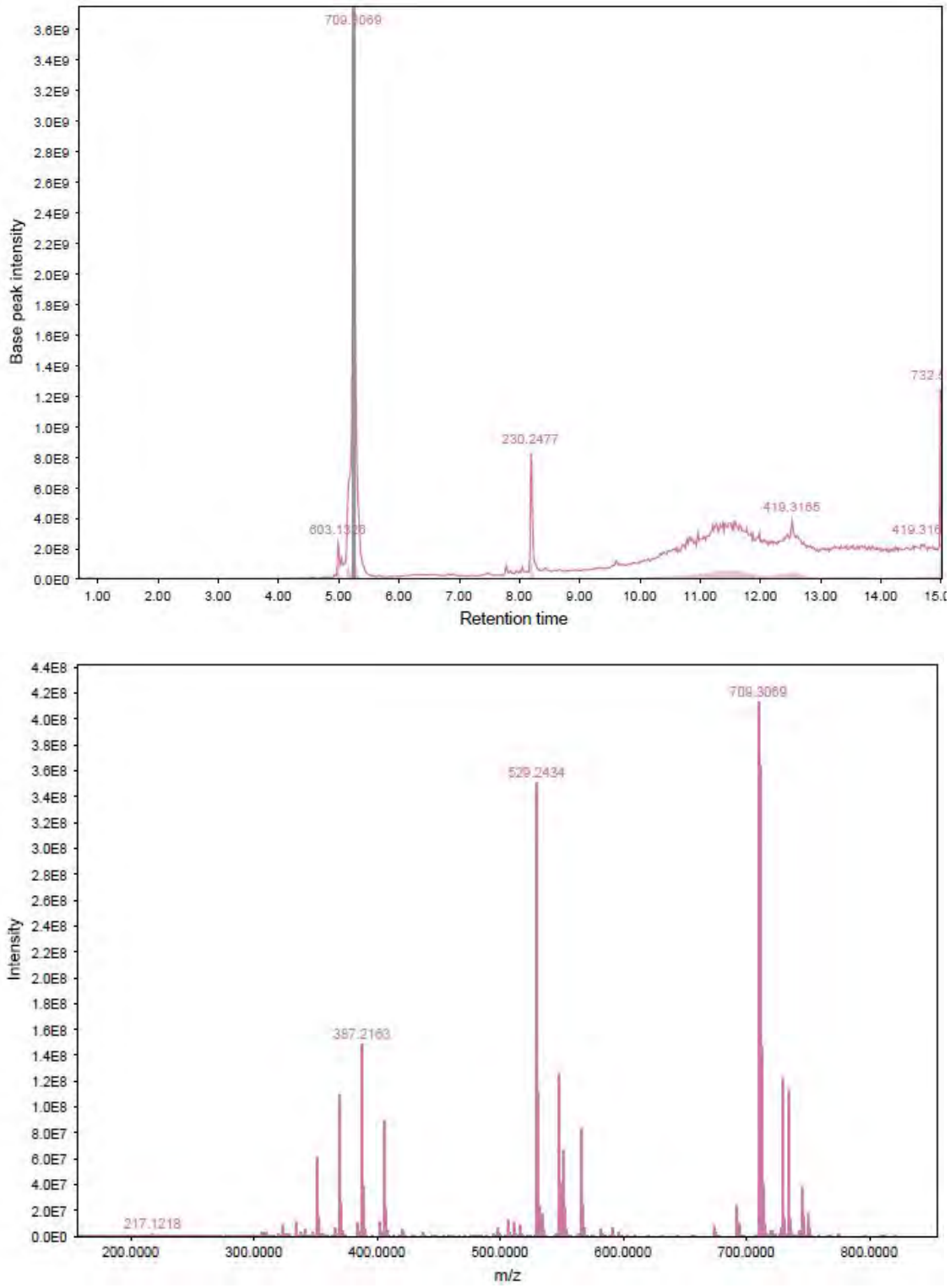


Figure S11: MS/MS spectrum of compound 2

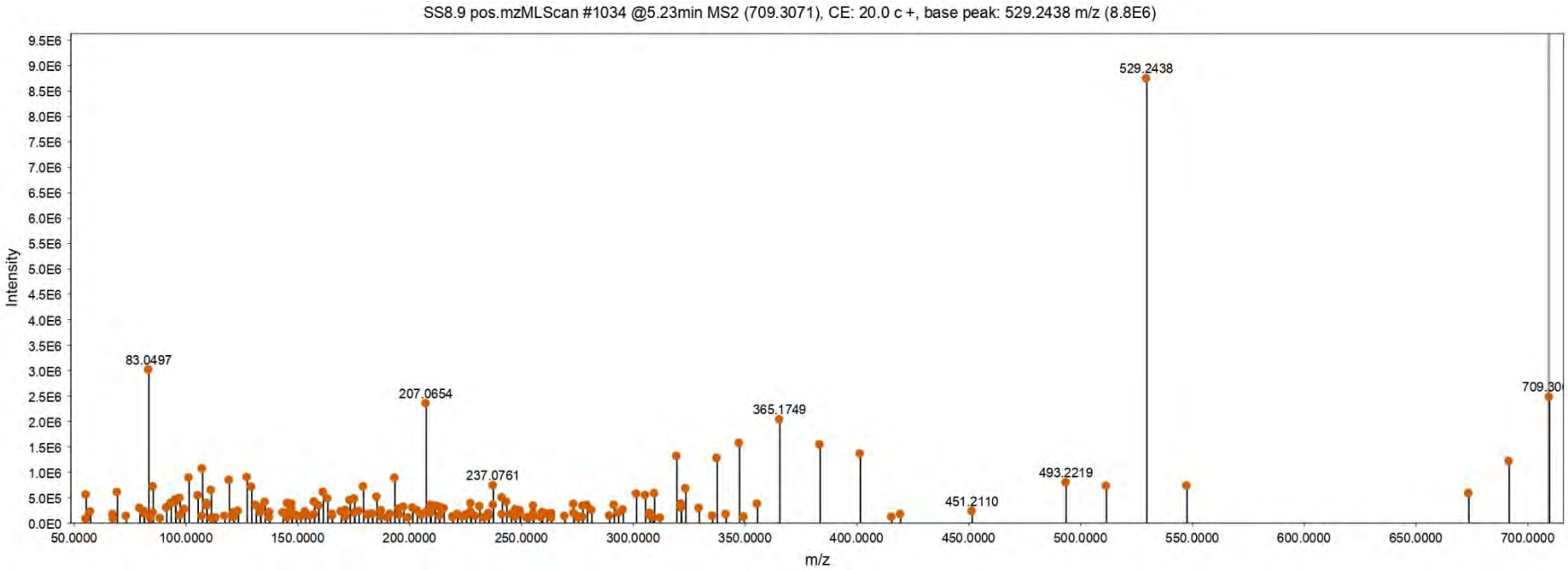


Figure S12: ^{1}H-NMR spectrum (800 MHz, CD_3OD) of compound 2.

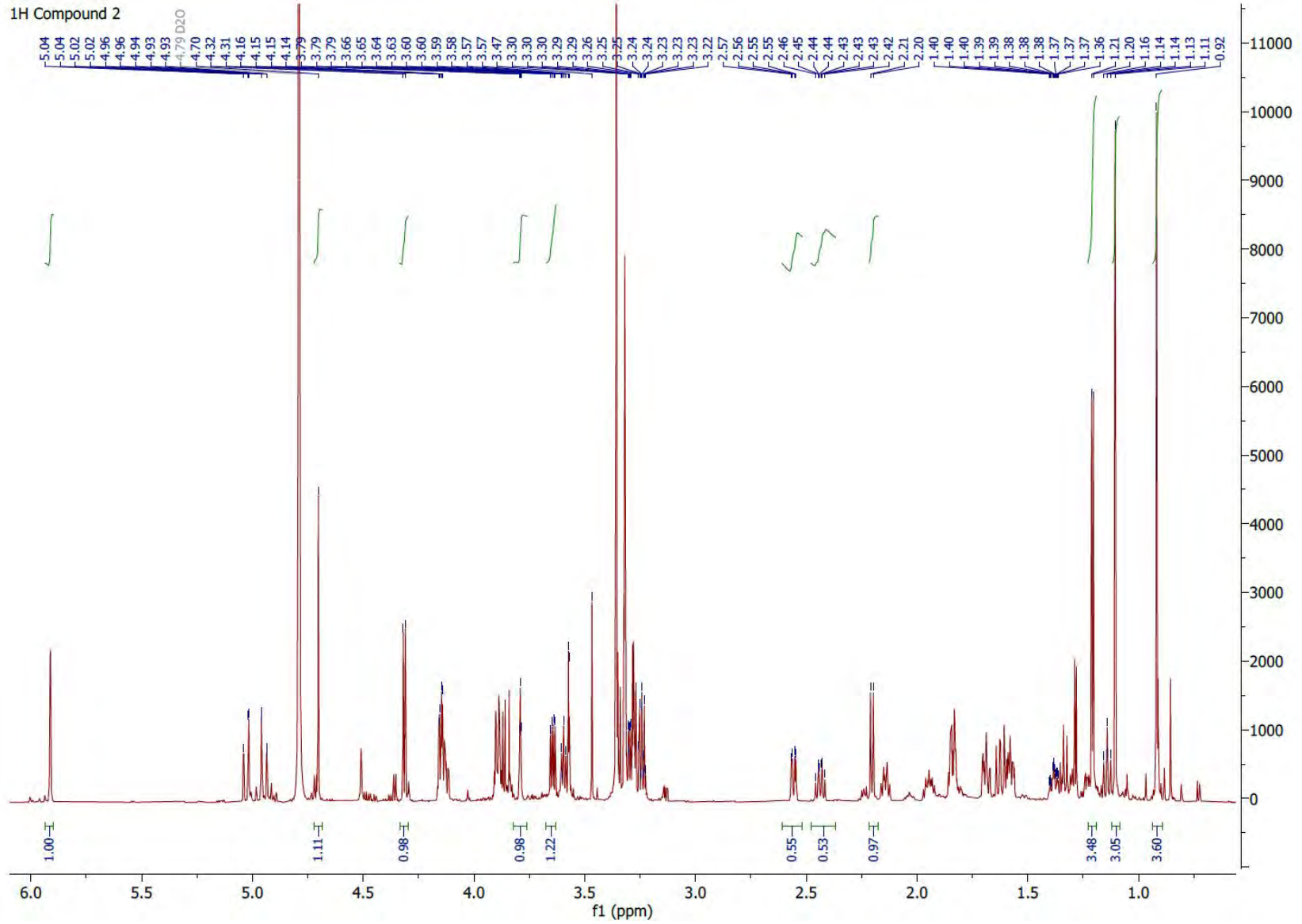


Figure S13: ^{13}C-NMR spectrum (600 MHz, CD_3OD) of compound 2.

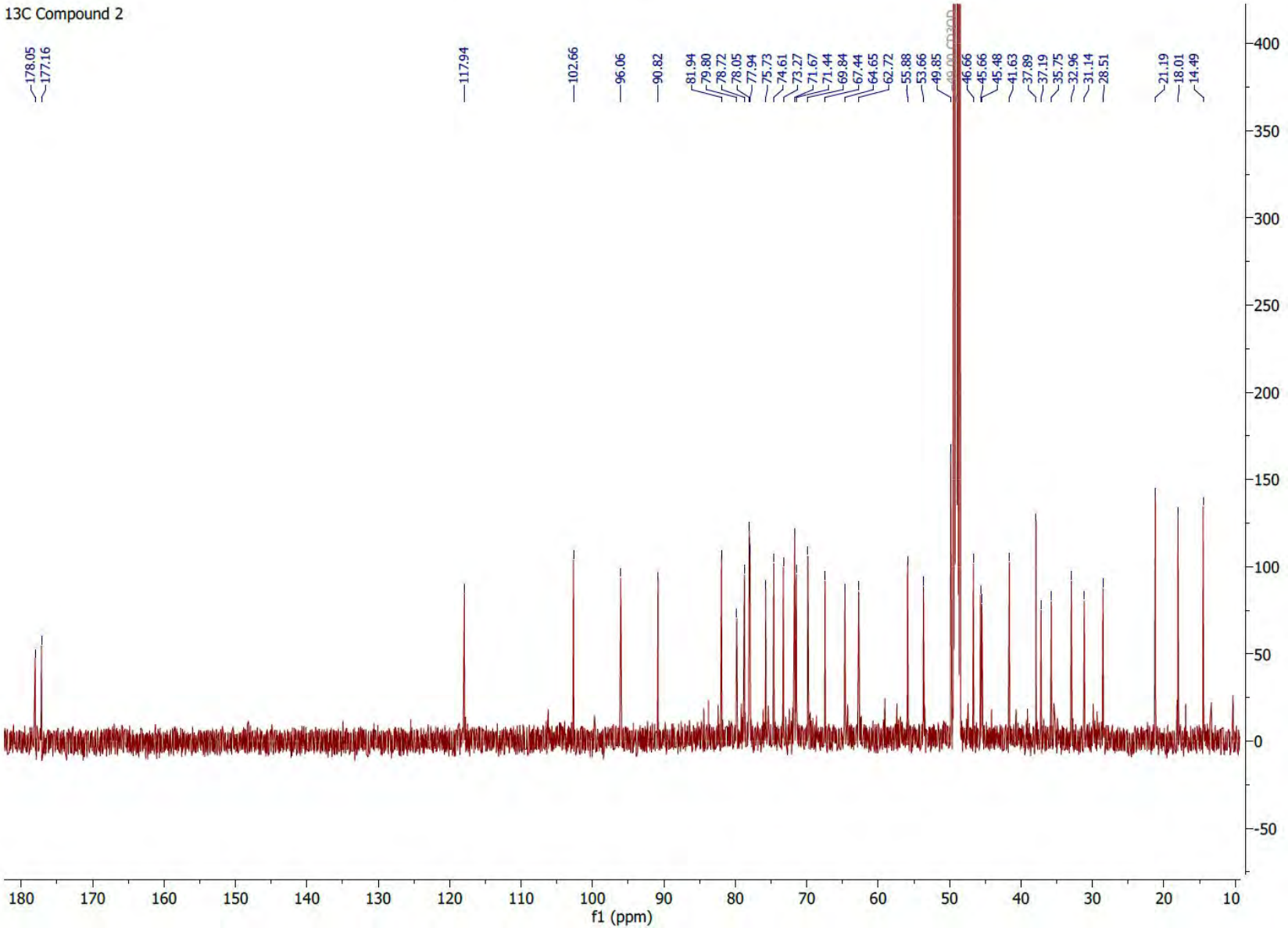


Figure S14: ^{1}H-^{13}C HSQC spectrum (800 MHz, CD_3OD) of compound 2.

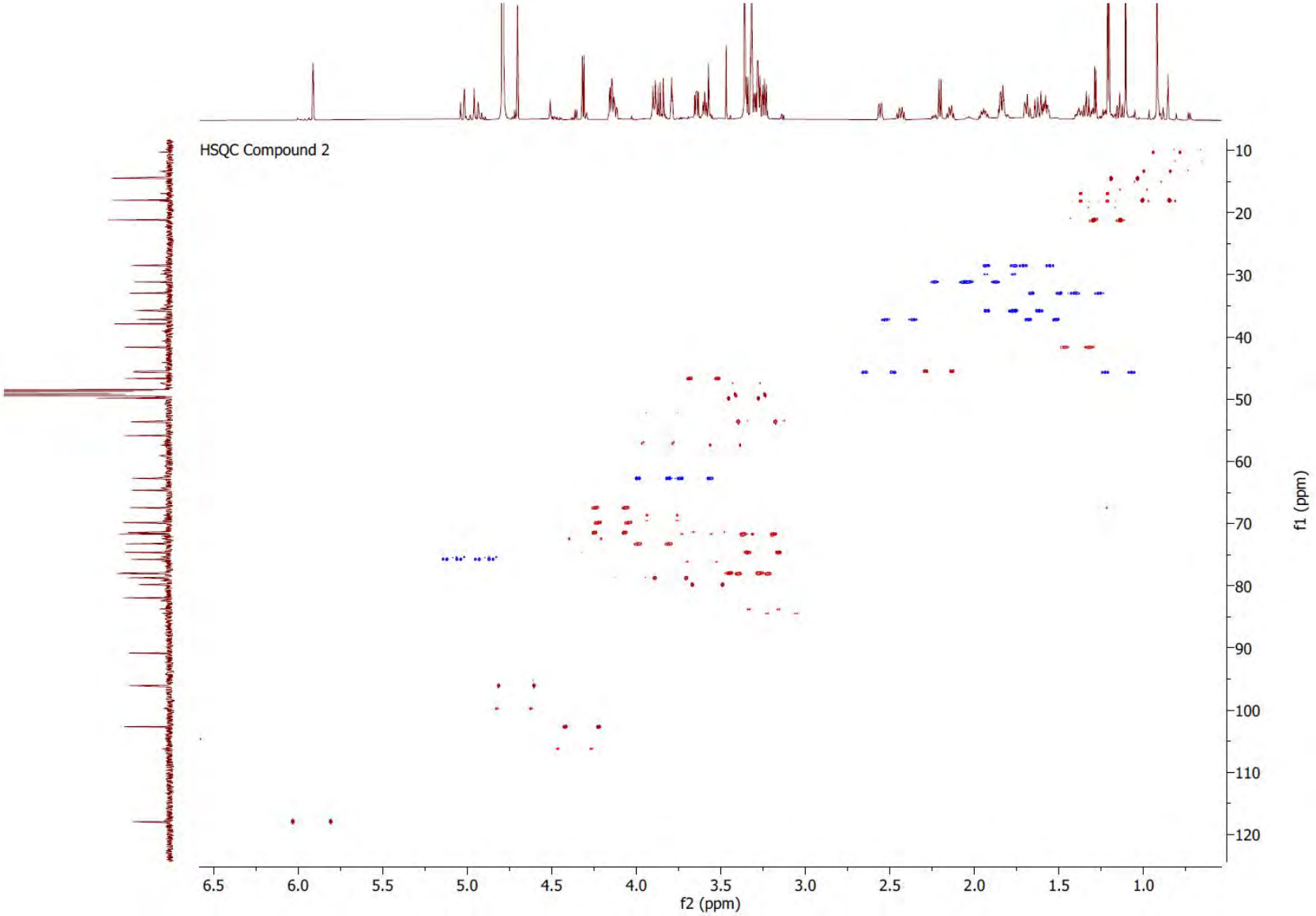


Figure S15: ^{1}H-^{1}H –COSY spectrum (800 MHz, CD_3OD) of compound 2.

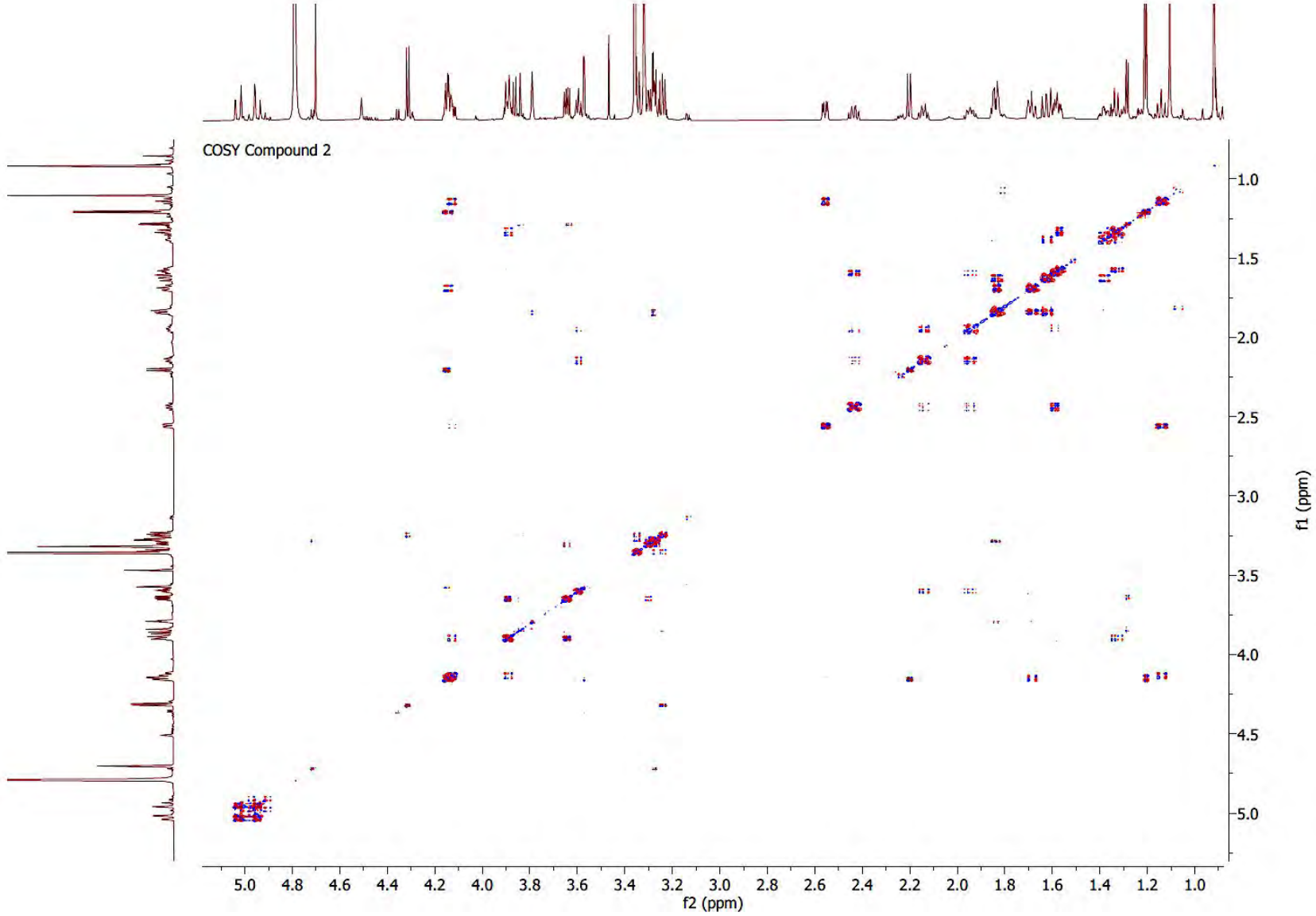


Figure S16: ^{1}H-^{13}C HMBC spectrum (800 MHz, CD_3OD) of compound 2.

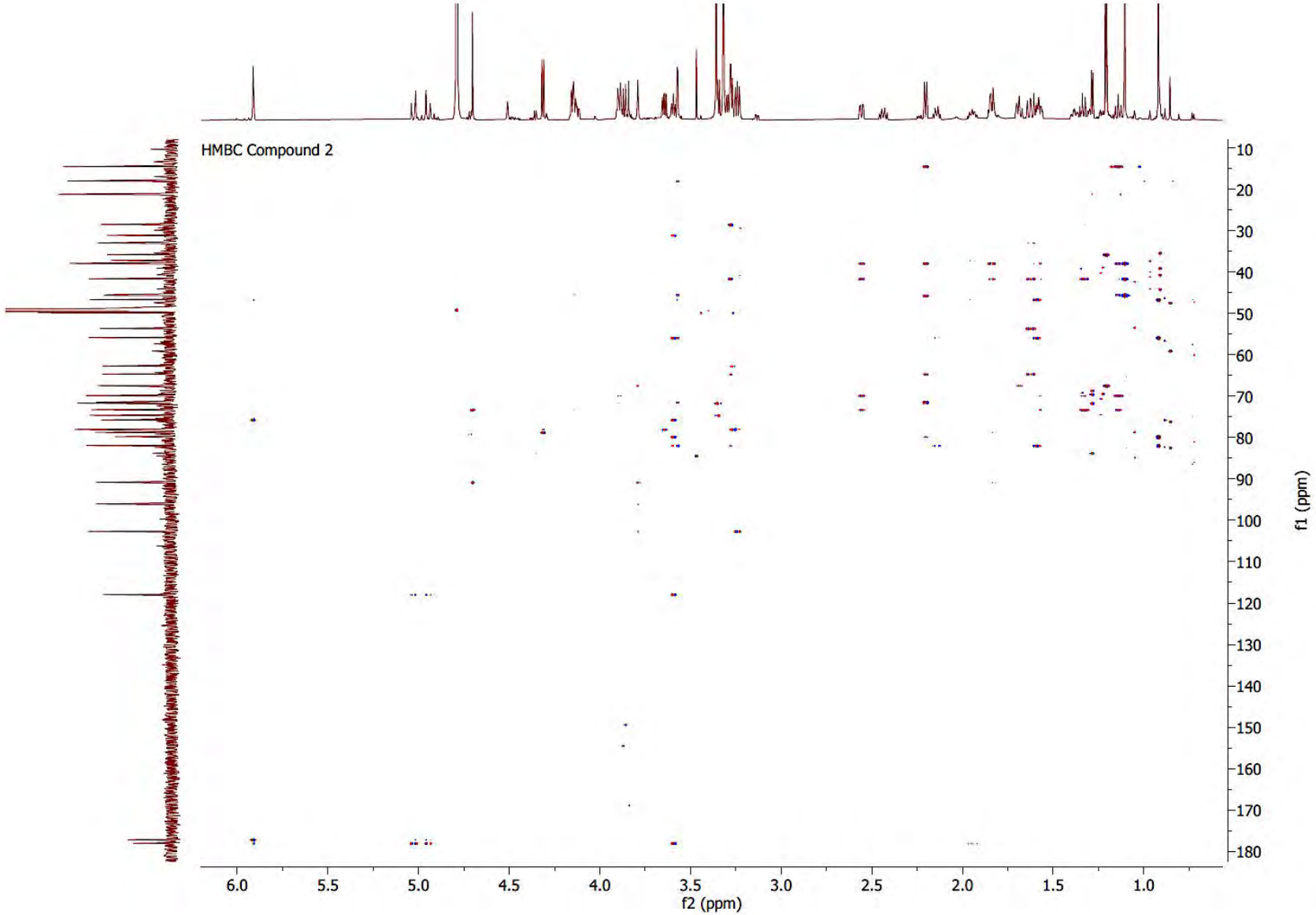


Figure S17: ^{1}H-^{1}H –NOESY spectrum (800 MHz, CD_3OD) of compound 2.

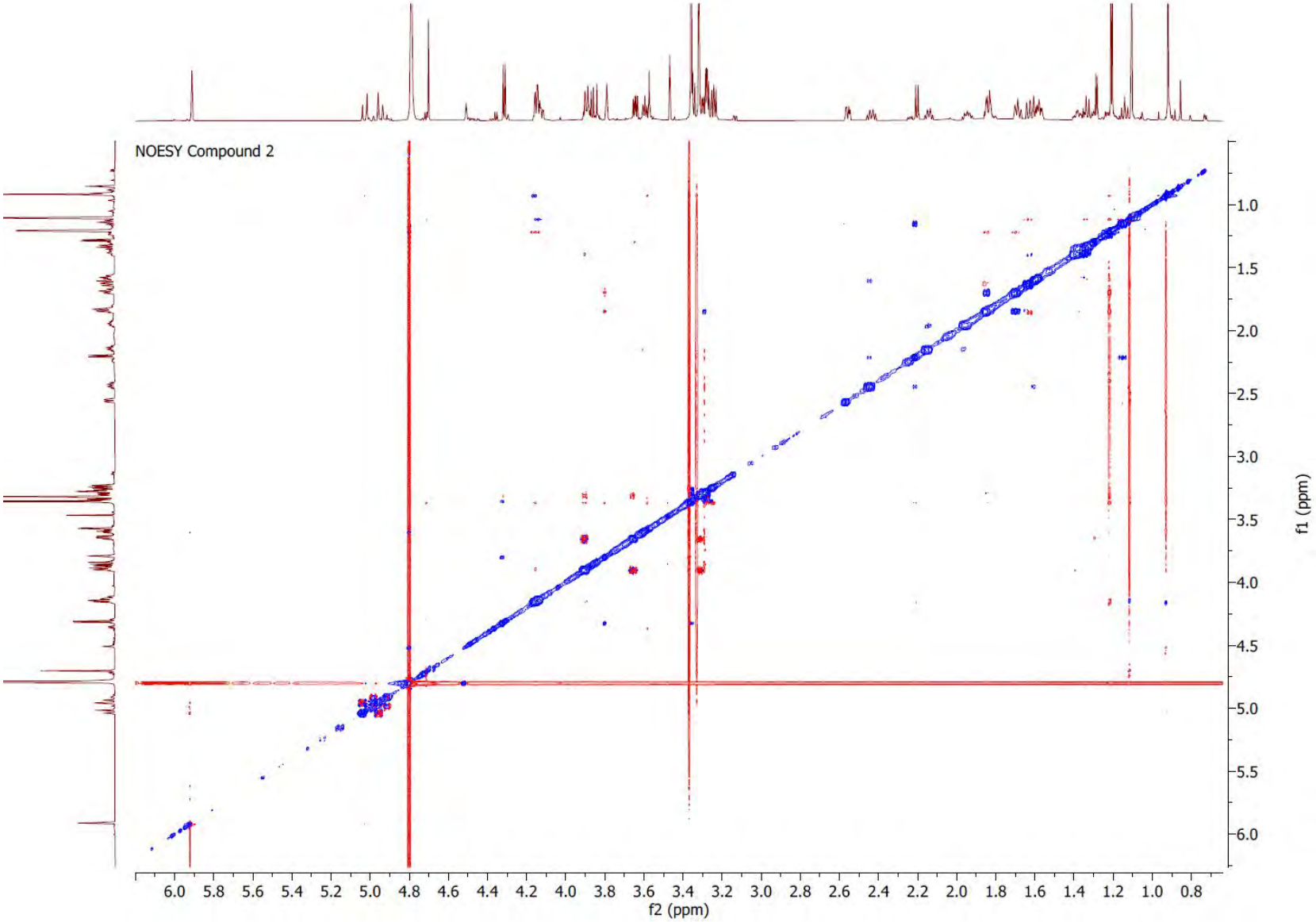


Figure S18: HR-ESI-MS spectrum of compound 3

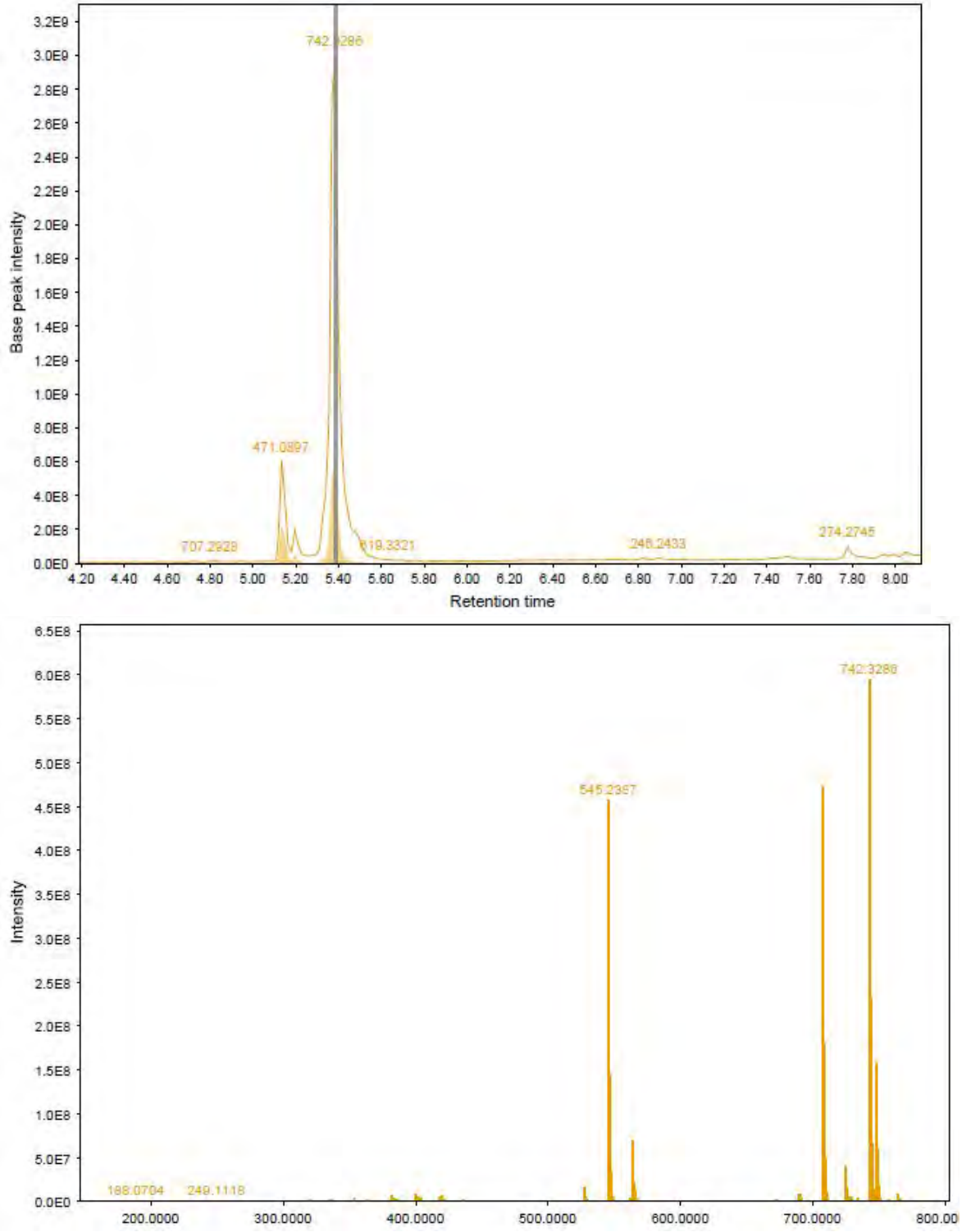


Figure S19: MS/MS of compound 3.

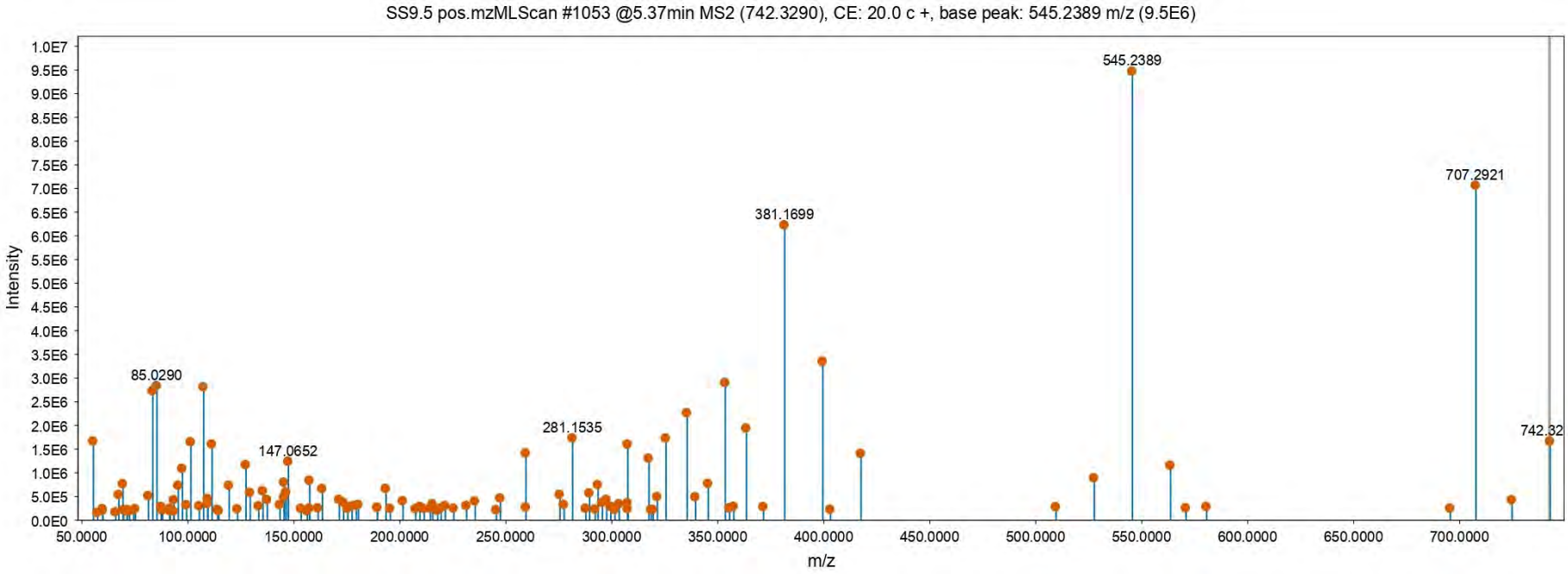


Figure S20: ^{1}H-NMR spectrum (800 MHz, CD_3OD) of compound 3.

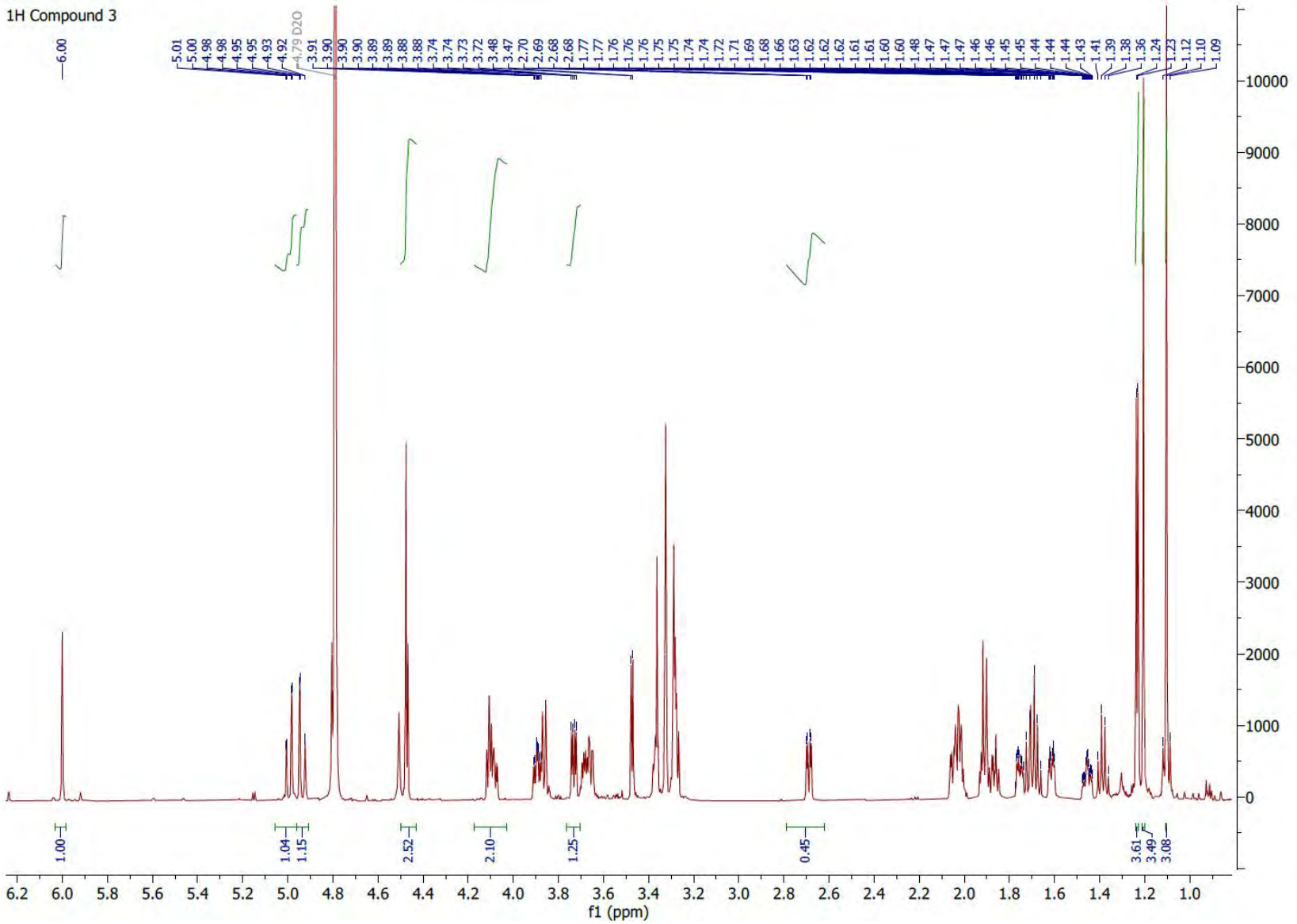


Figure S21: ^{13}C-NMR spectrum (600 MHz, CD_3OD) of compound 3.

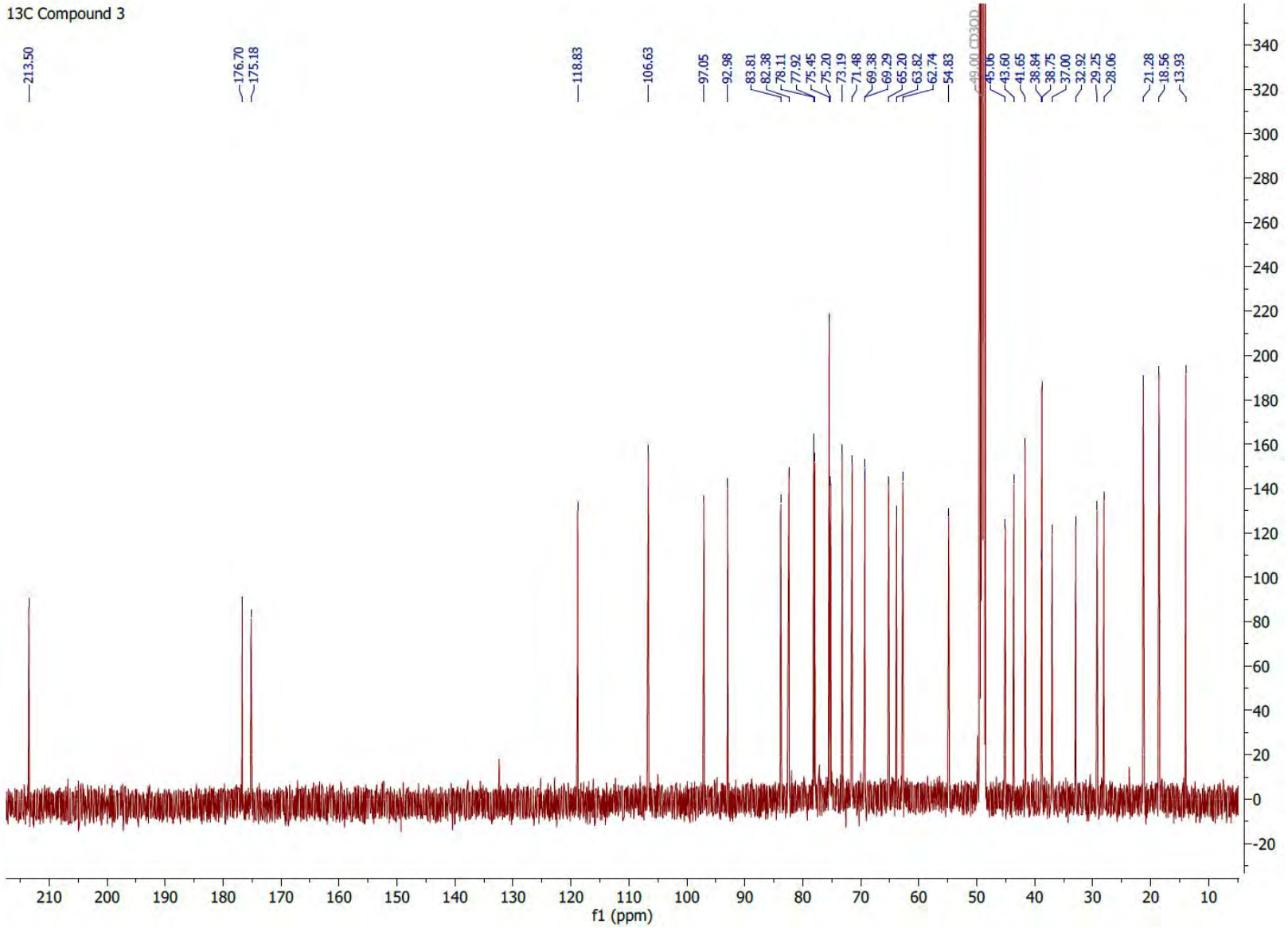


Figure S22: ^{1}H-^{13}C HSQC spectrum (800 MHz, CD_3OD) of compound 3.

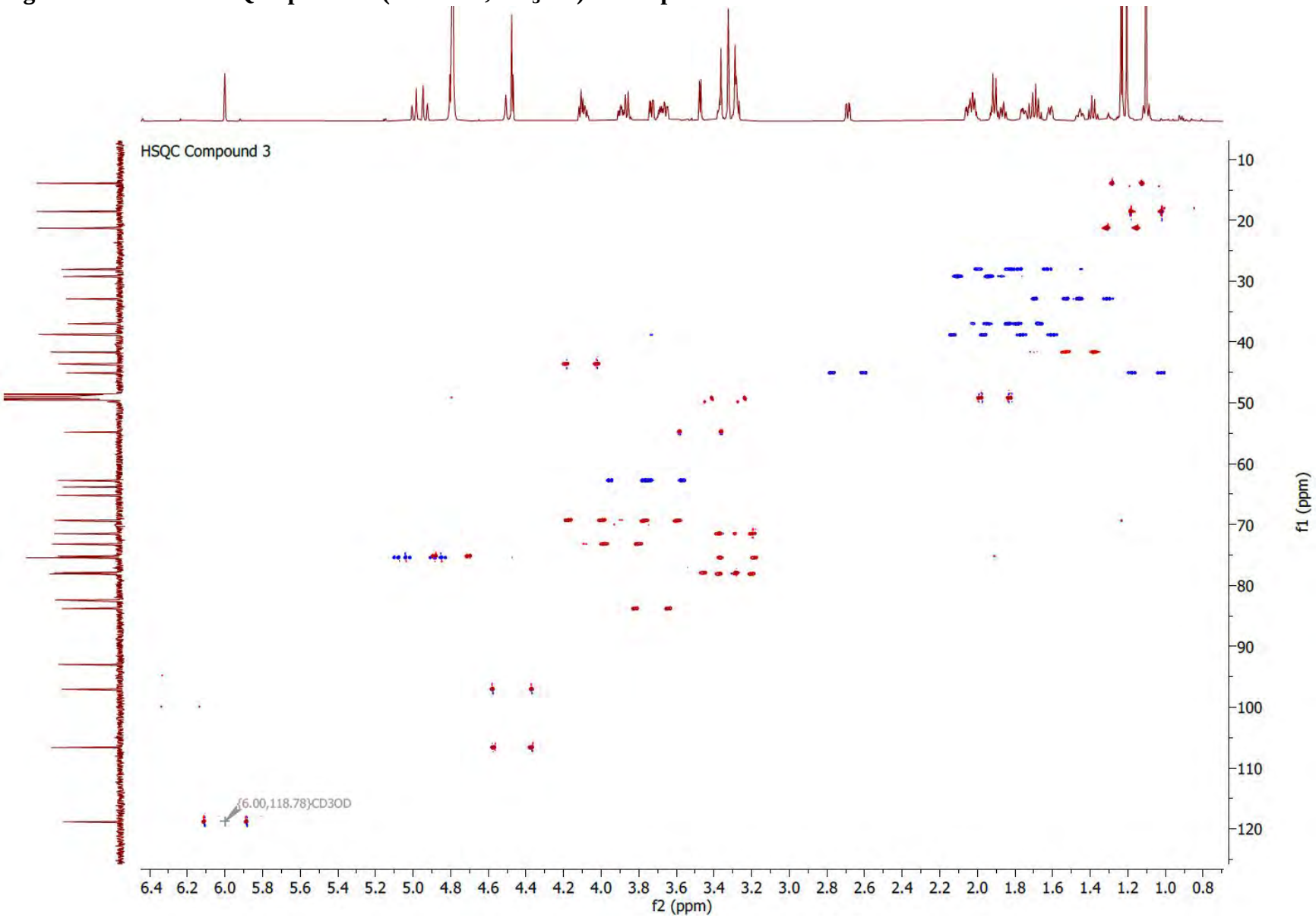


Figure S23: ^{1}H-^{1}H –COSY spectrum (800 MHz, CD_3OD) of compound 3.

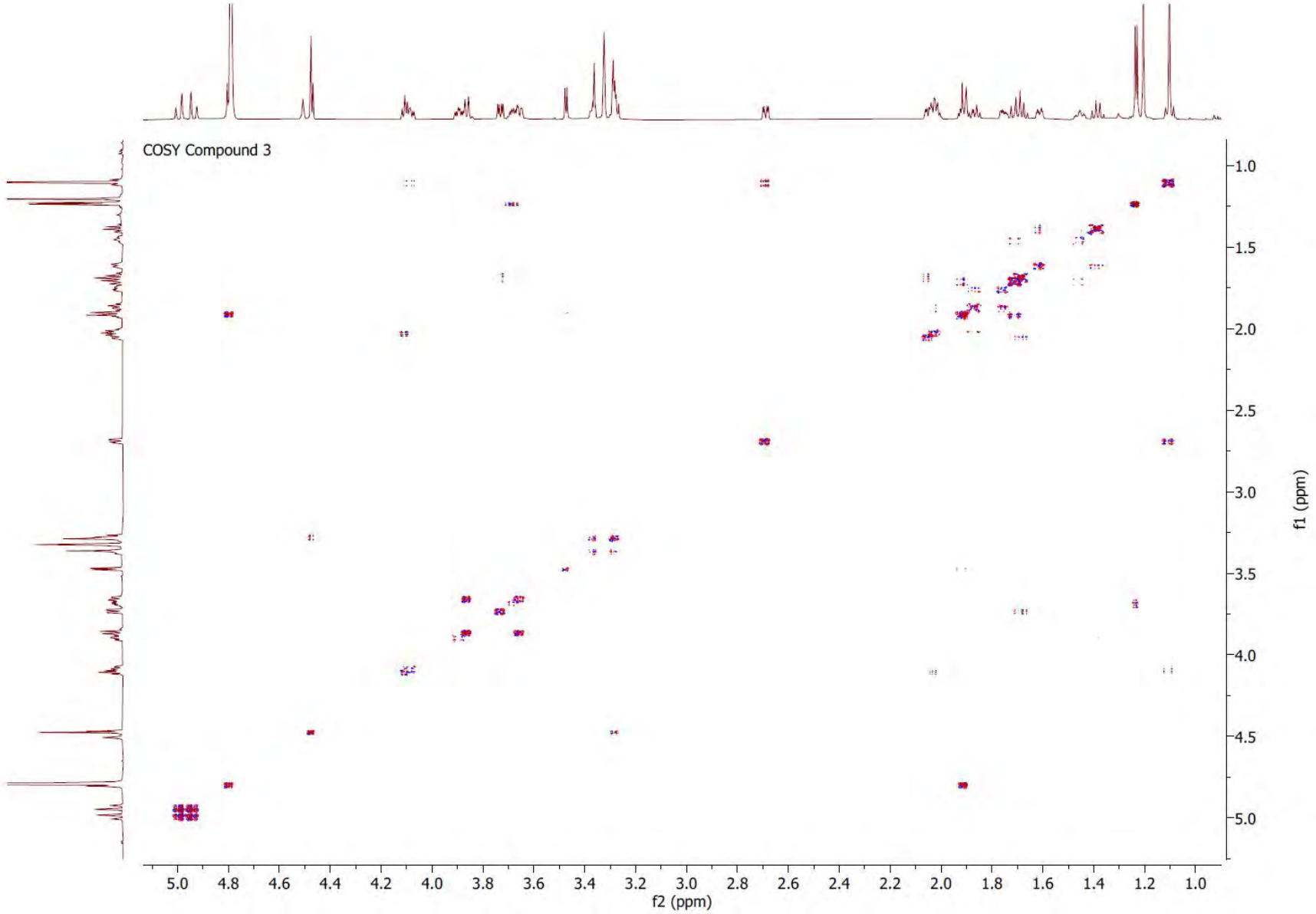


Figure S24: ^{1}H-^{13}C HMBC spectrum (800 MHz, CD_3OD) of compound 3.

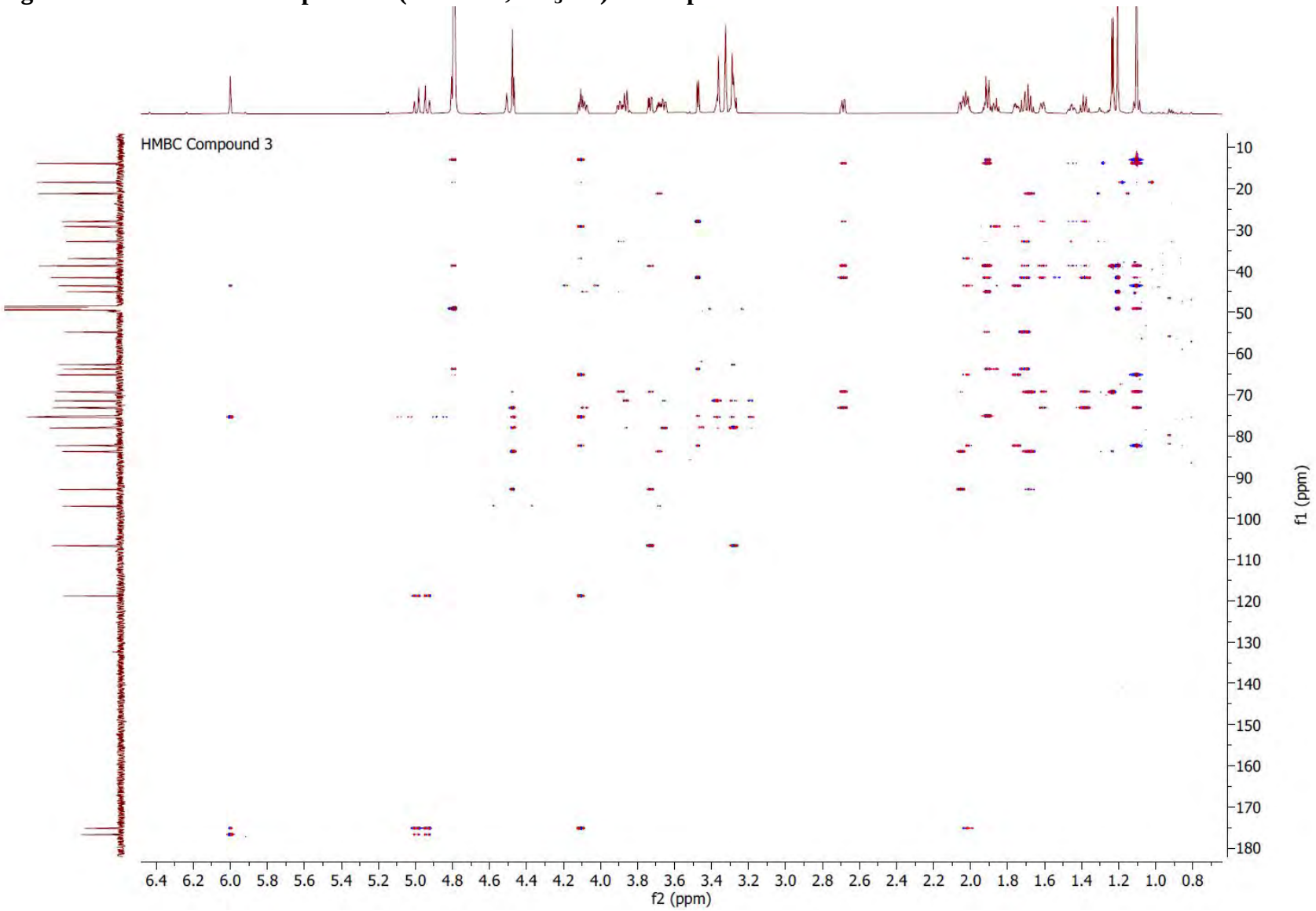


Figure S25: ^{1}H-^{1}H –NOESY spectrum (800 MHz, CD_3OD) of compound 3.

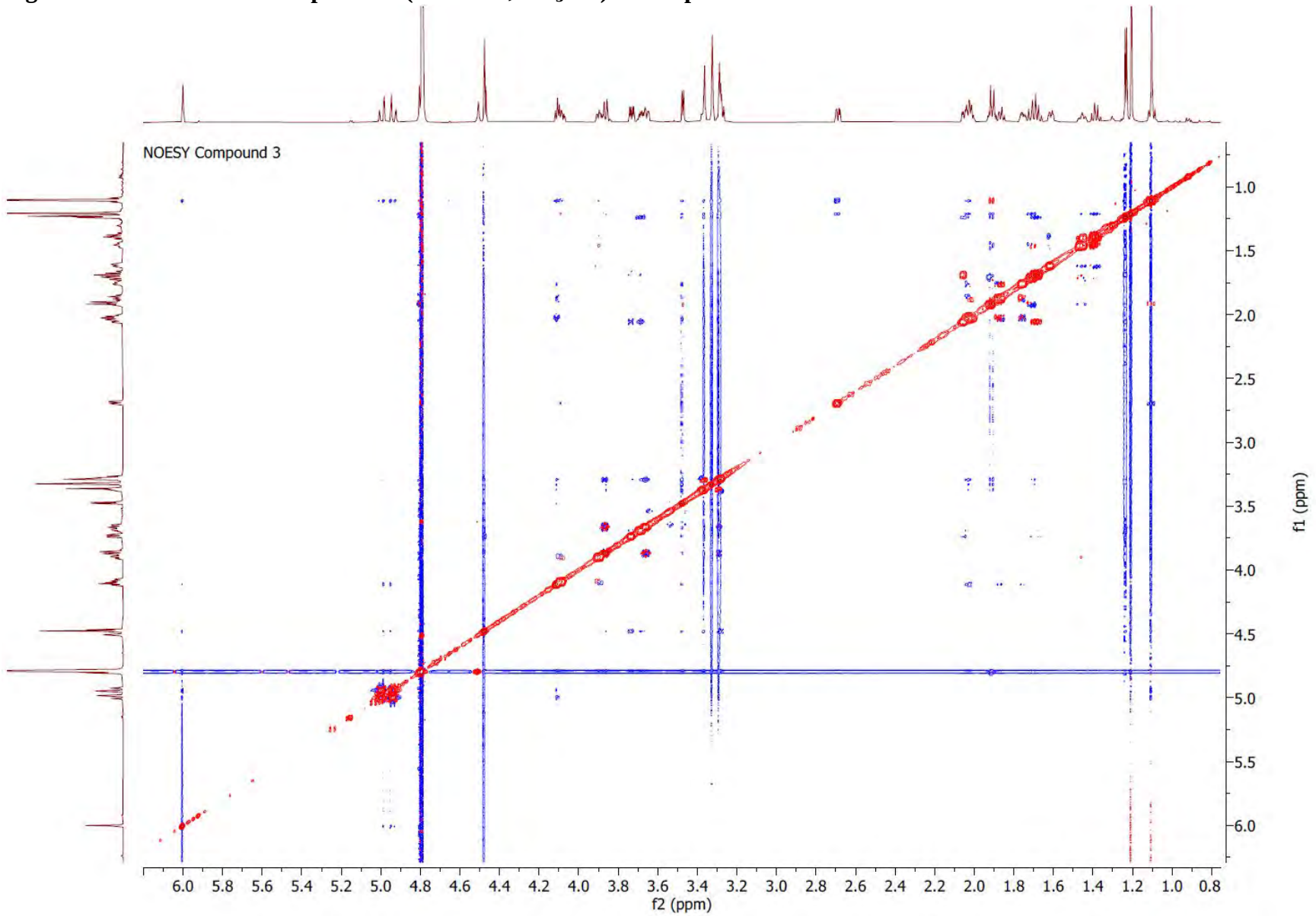


Figure S26: HR-ESI-MS spectrum of compound 4

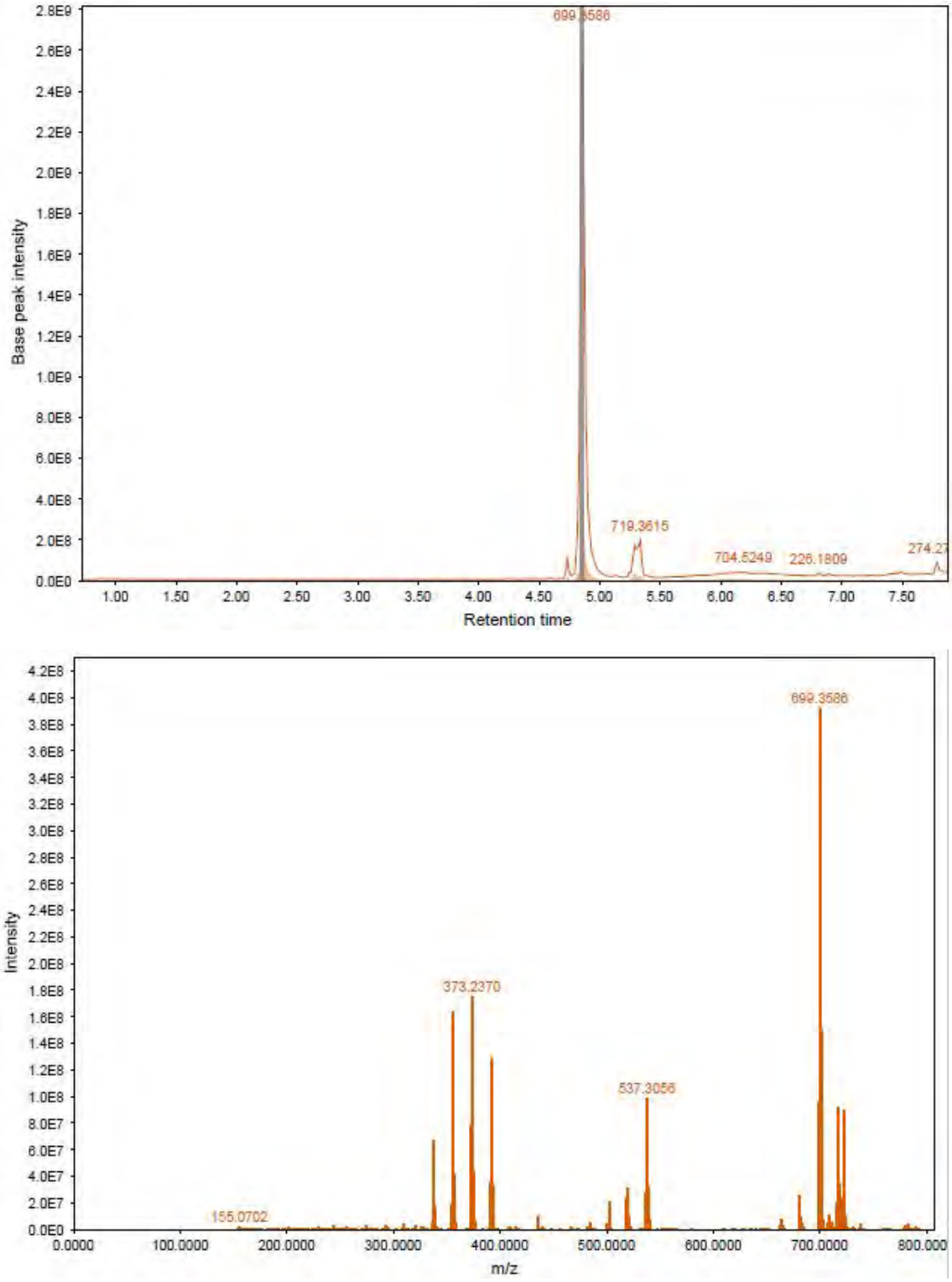


Figure S27: MS/MS spectrum of compound 4

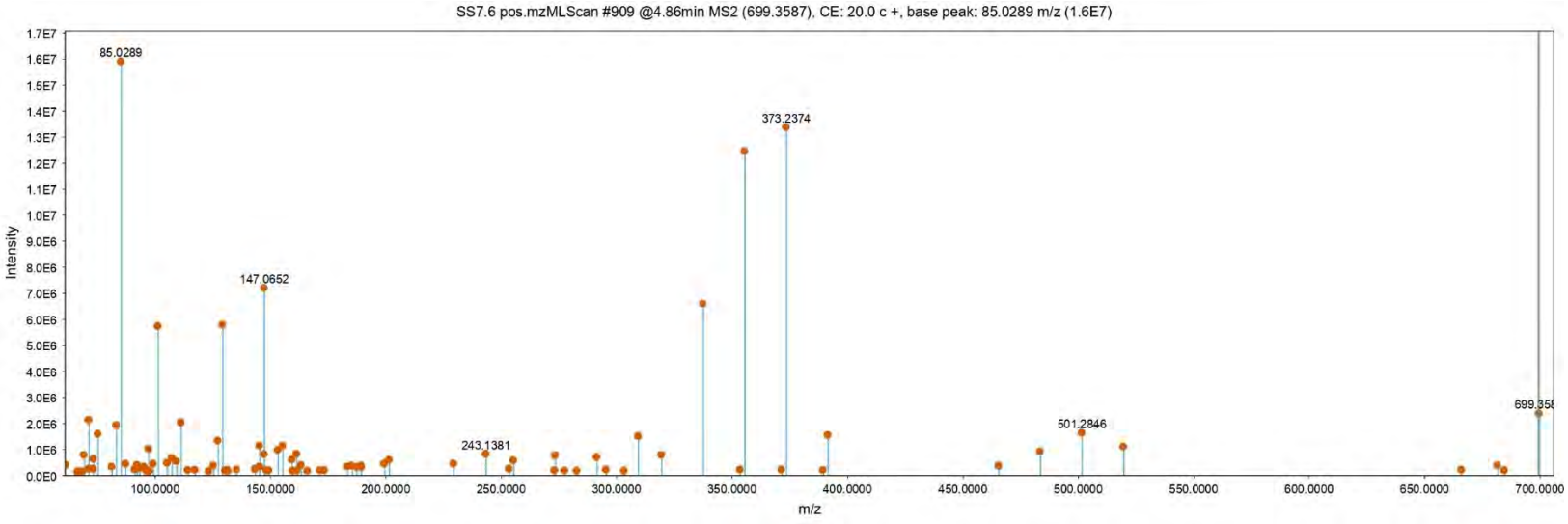


Figure S28: ^{1}H-NMR spectrum (800 MHz, CD_3OD) of compound 4.

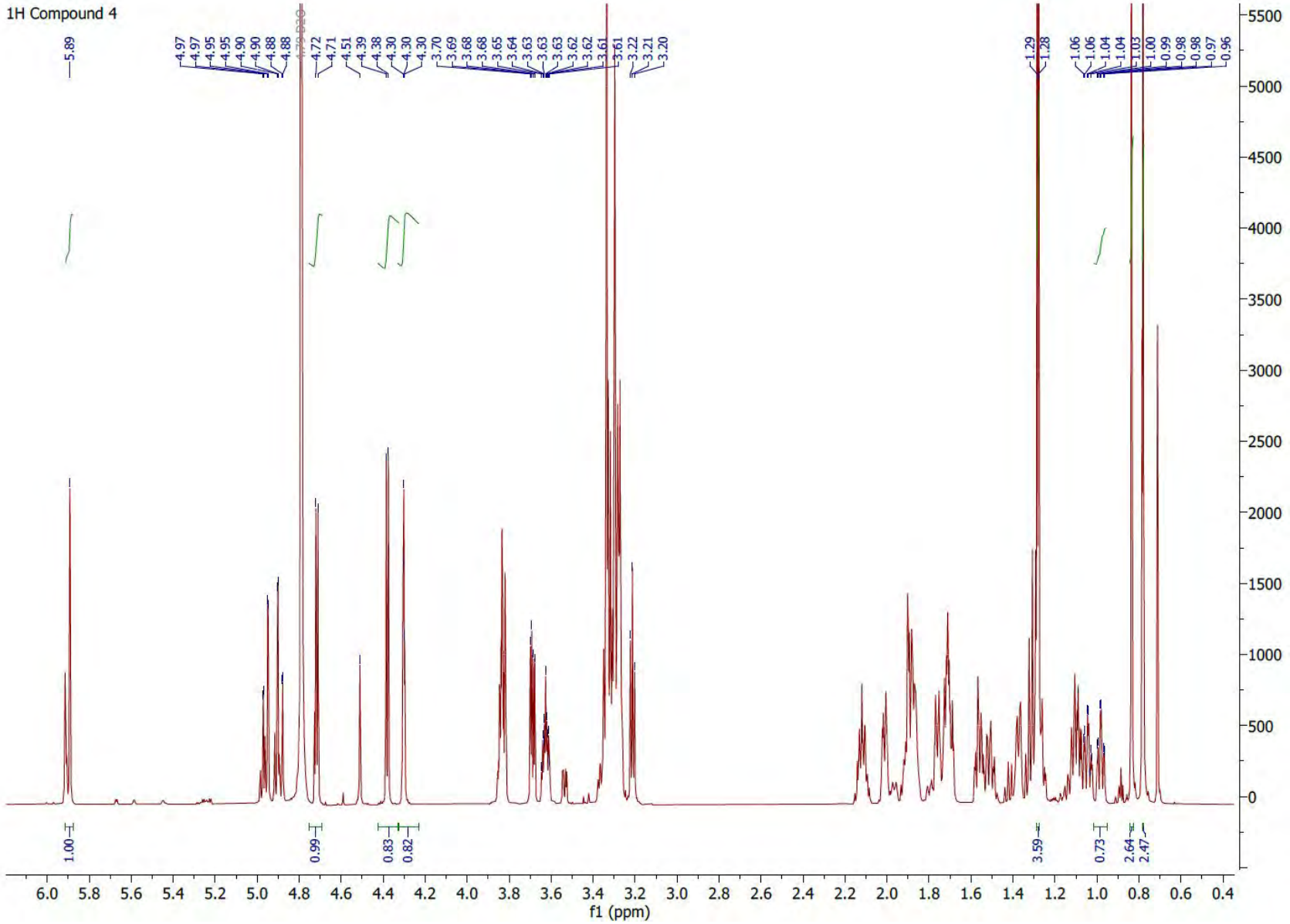


Figure S29: ^{13}C-NMR spectrum (600 MHz, CD_3OD) of compound 4.

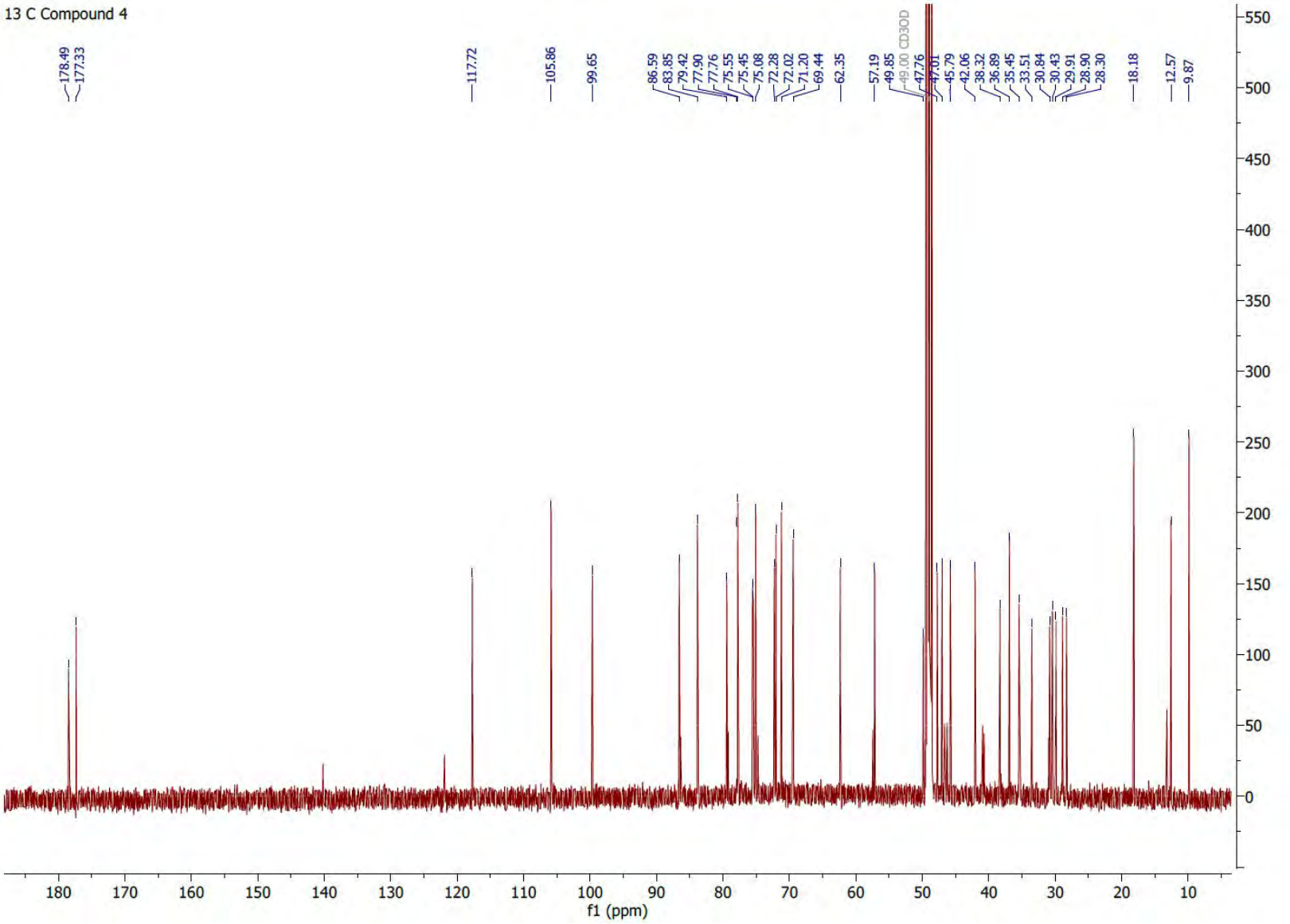


Figure S30: ^{1}H-^{13}C HSQC spectrum (800 MHz, CD_3OD) of compound 4.

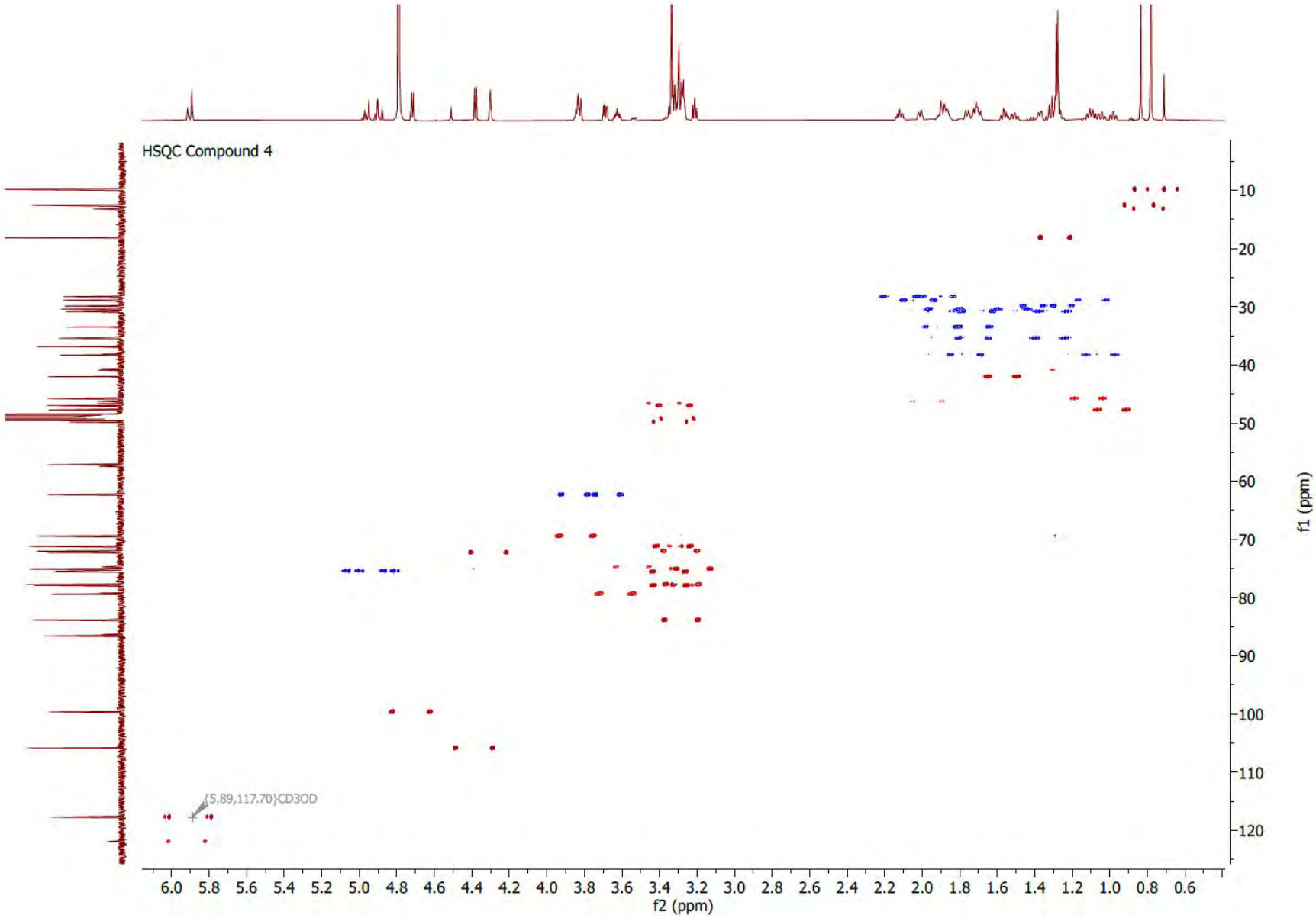


Figure S31: ^{1}H-^{1}H –COSY spectrum (800 MHz, CD_3OD) of compound 4.

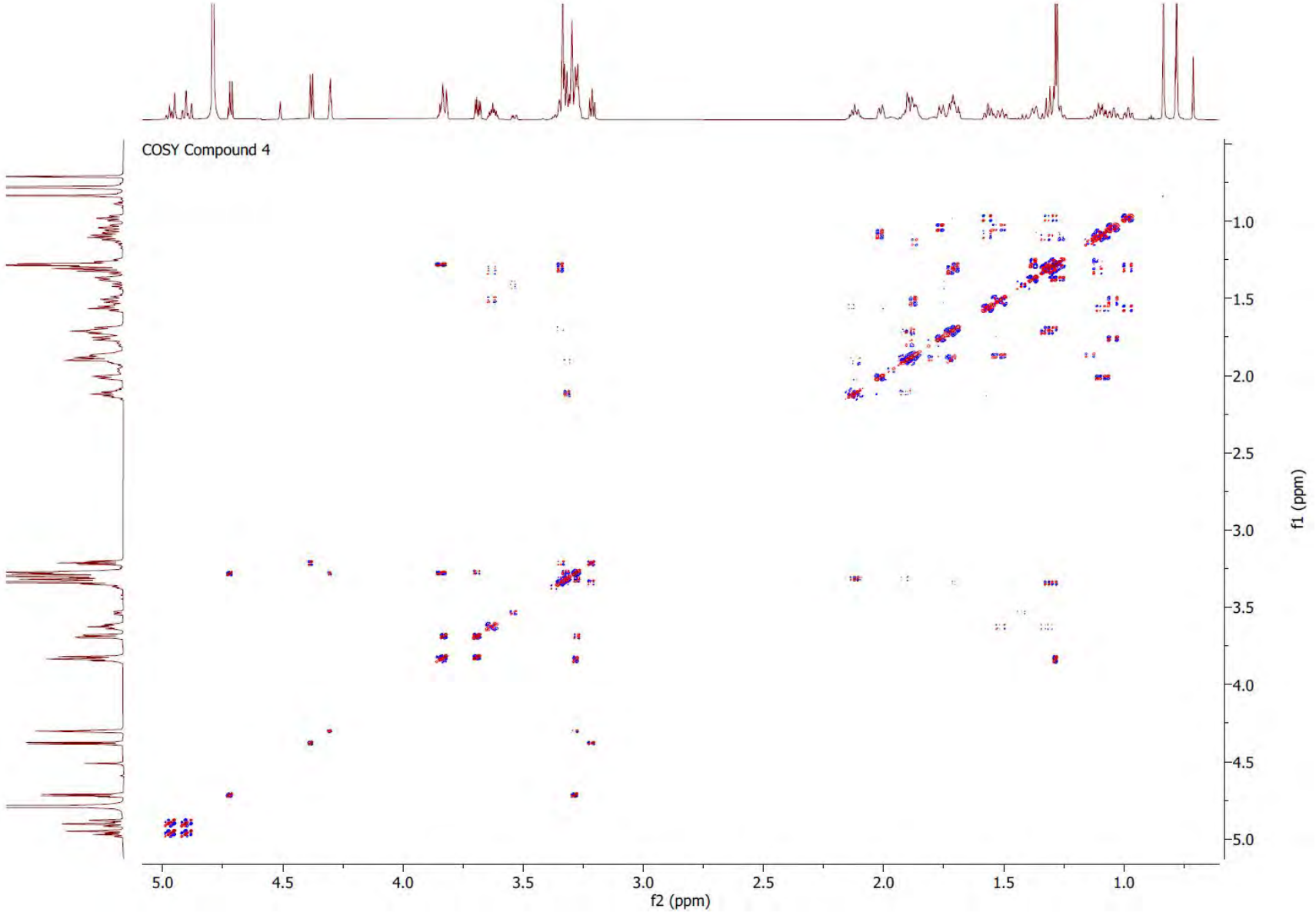


Figure S32: ^{1}H-^{13}C HMBC spectrum (800 MHz, CD_3OD) of compound 4.

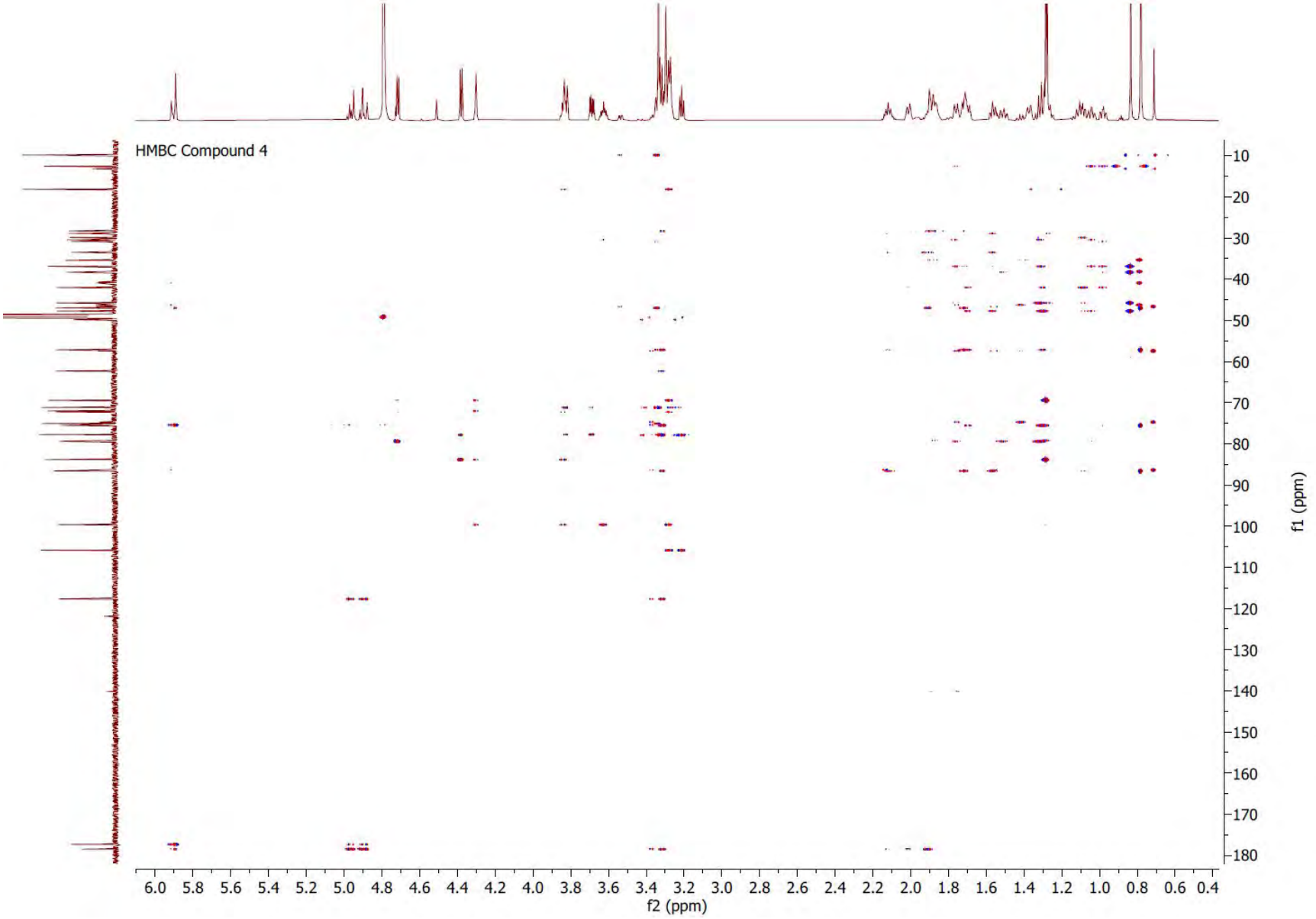


Figure S33: ^{1}H-^{1}H –NOESY spectrum (800 MHz, CD_3OD) of compound 4.

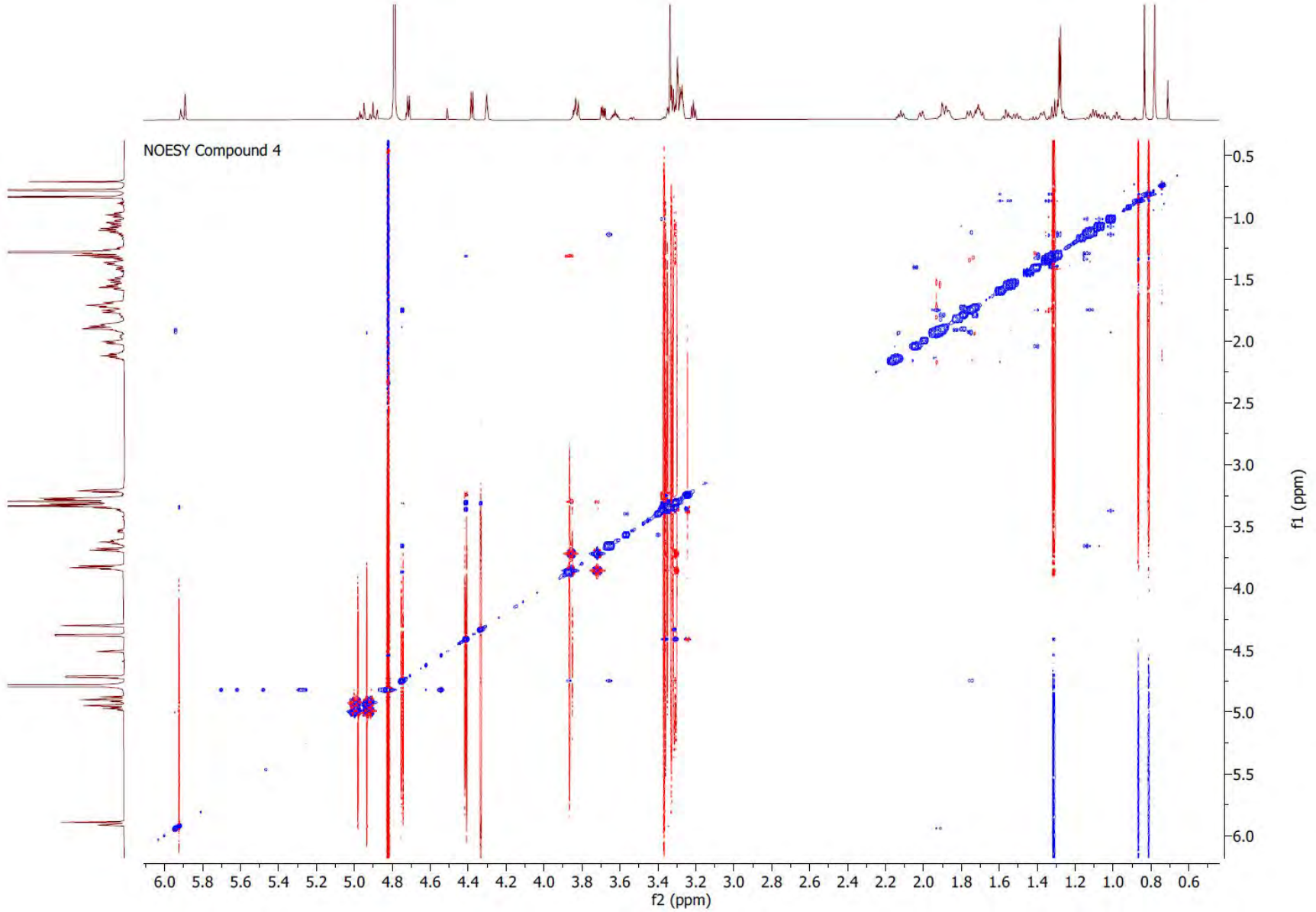


Figure S34: HR-ESI-MS spectrum of compound 5

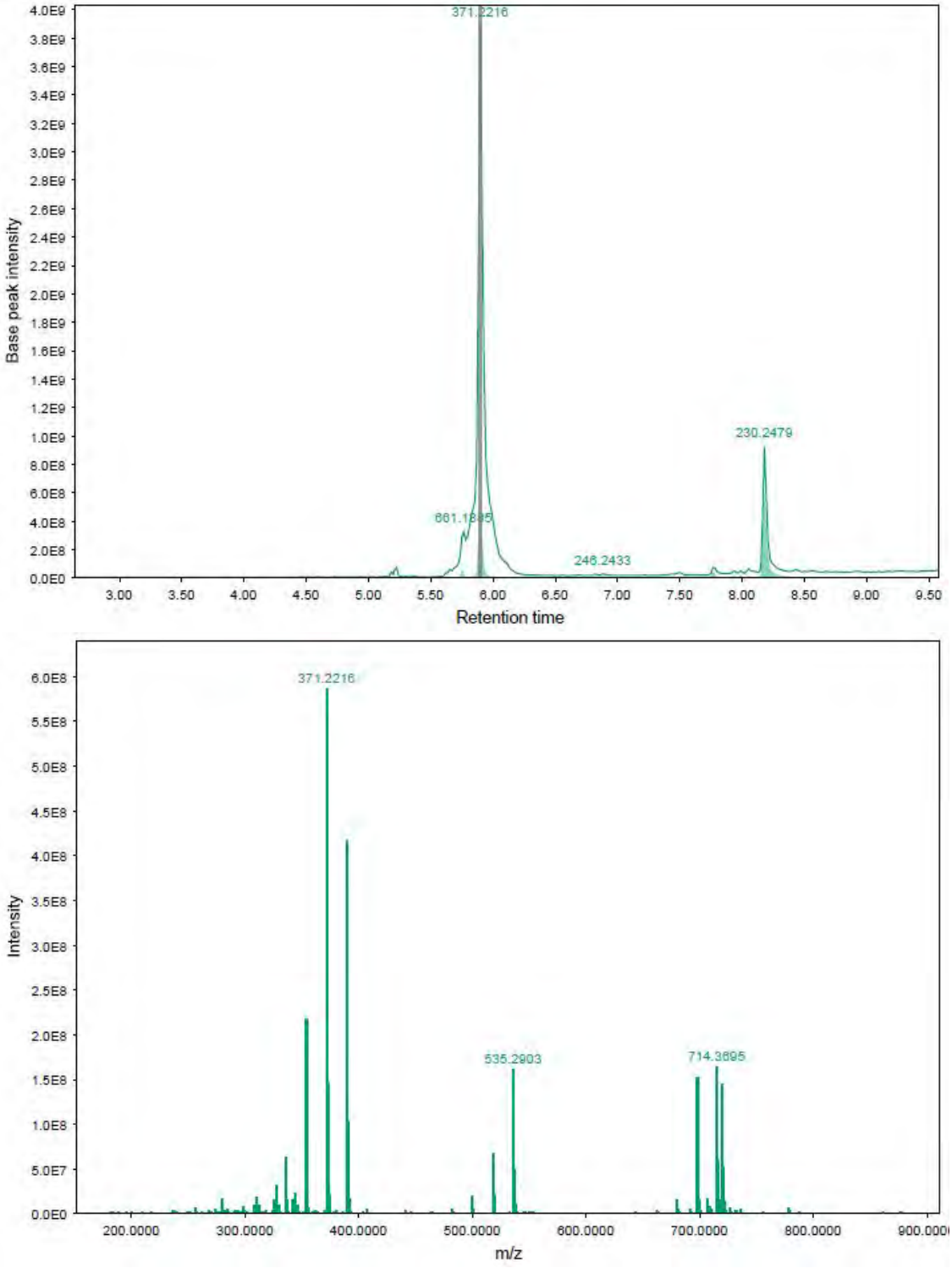


Figure S35: HR-ESI-MS spectrum of compound 5

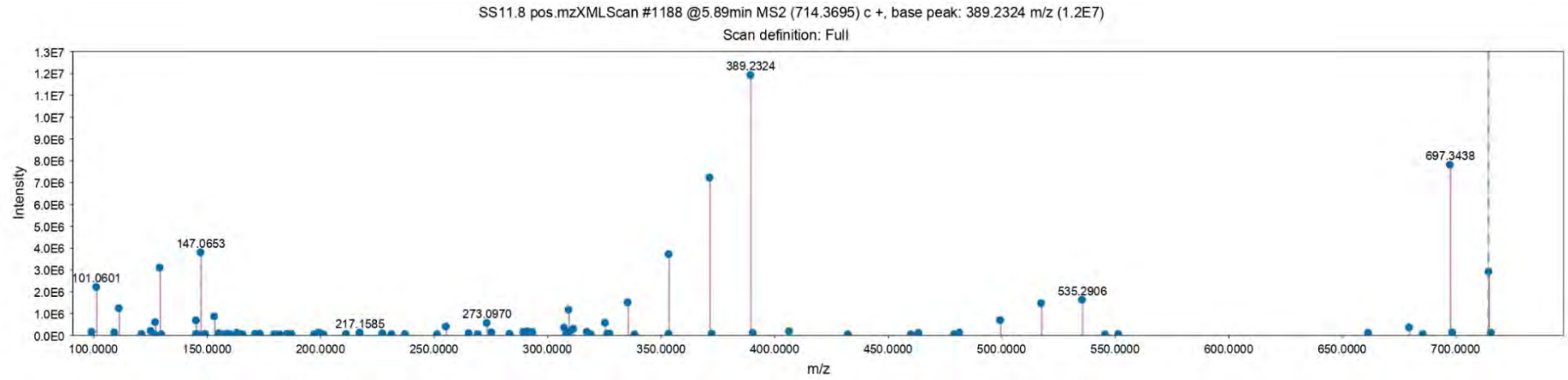


Figure S36: ^{1}H-NMR spectrum (800 MHz, CD_3OD) of compound 5.

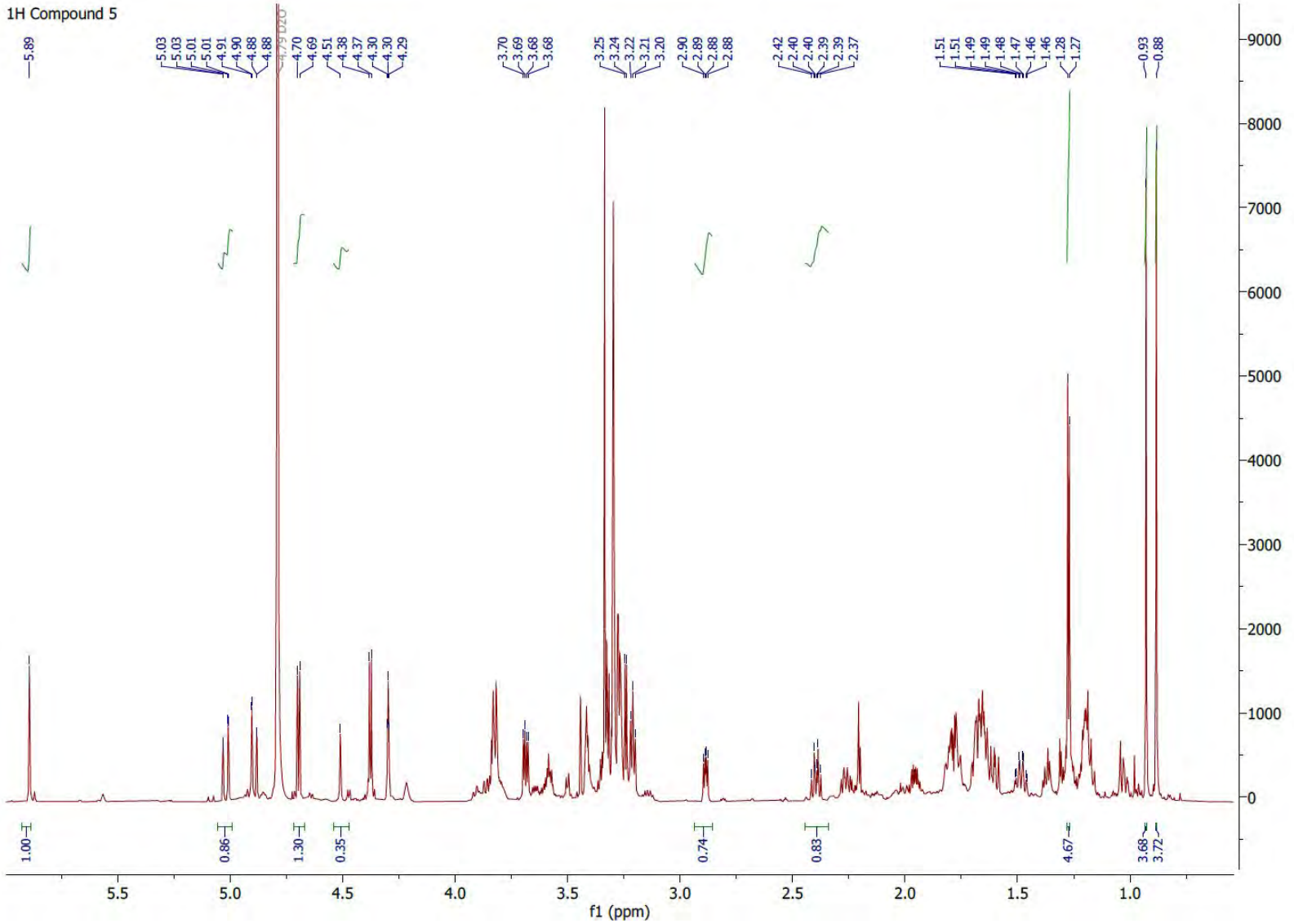


Figure S37: ^{1}H-^{1}H –COSY spectrum (800 MHz, CD_3OD) of compound 5.

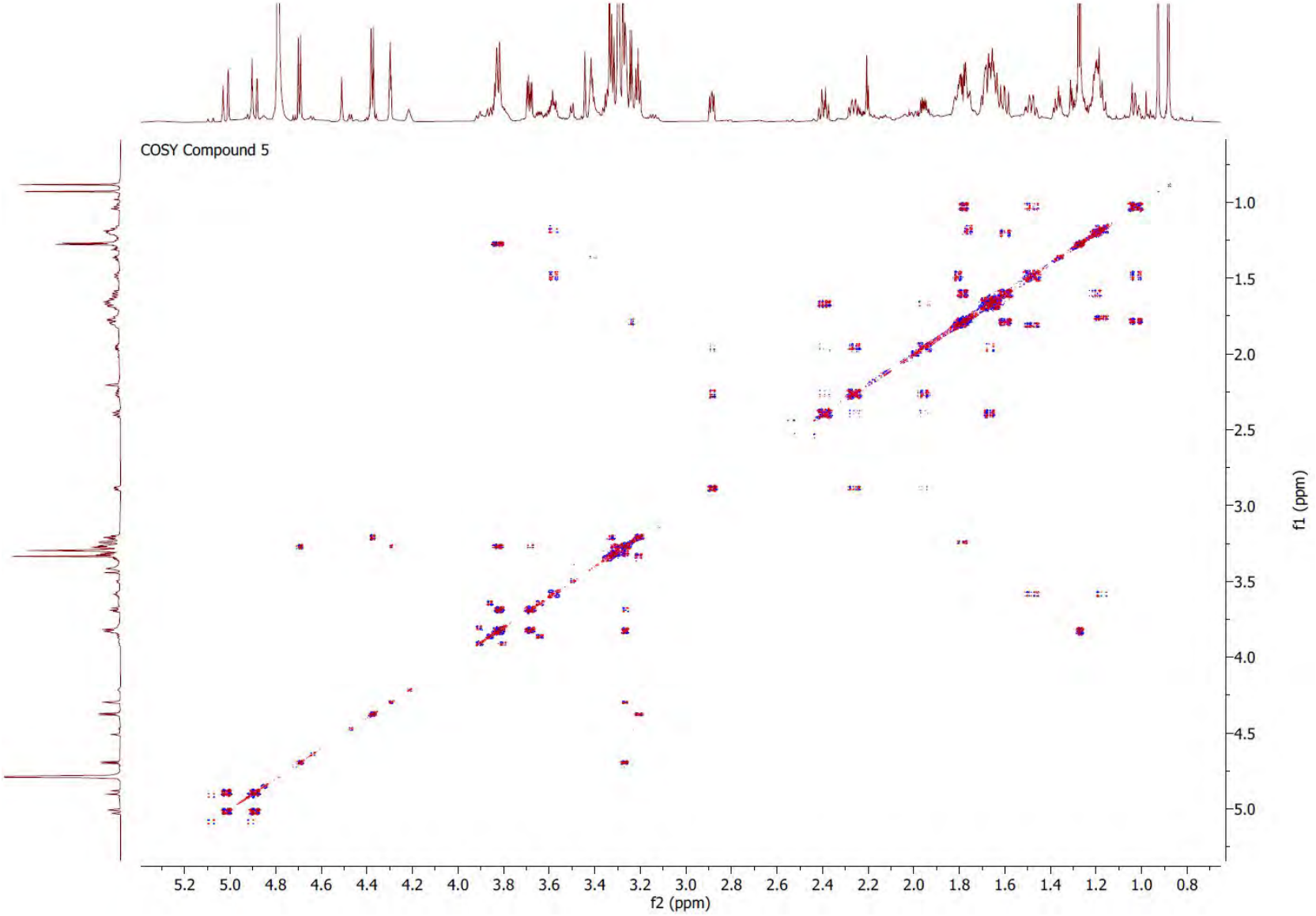


Figure S38: ^{1}H-^{13}C HMBC spectrum (800 MHz, CD_3OD) of compound 5.

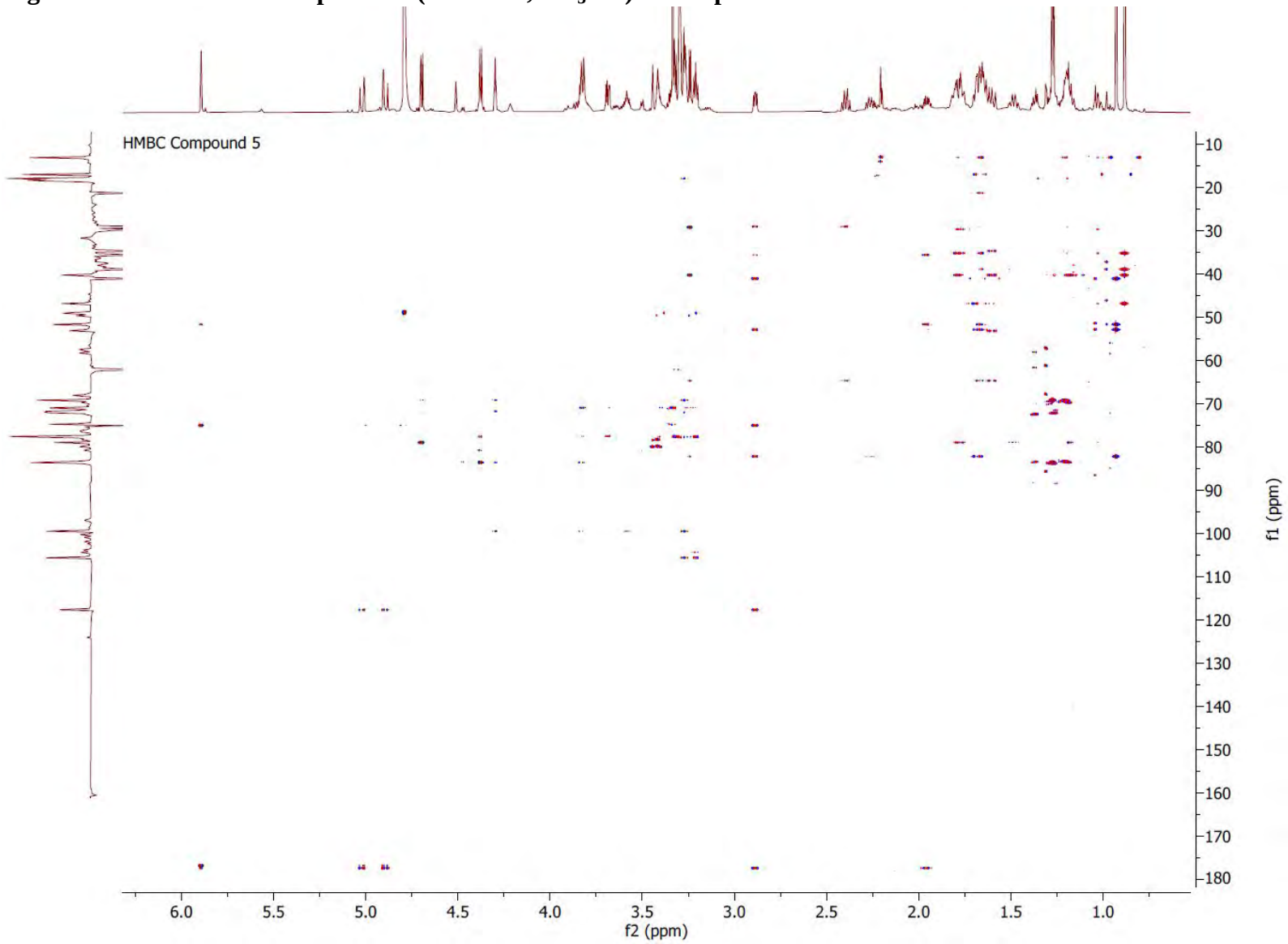


Figure S39: ^{1}H-^{1}H –NOESY spectrum (800 MHz, CD_3OD) of compound 5.

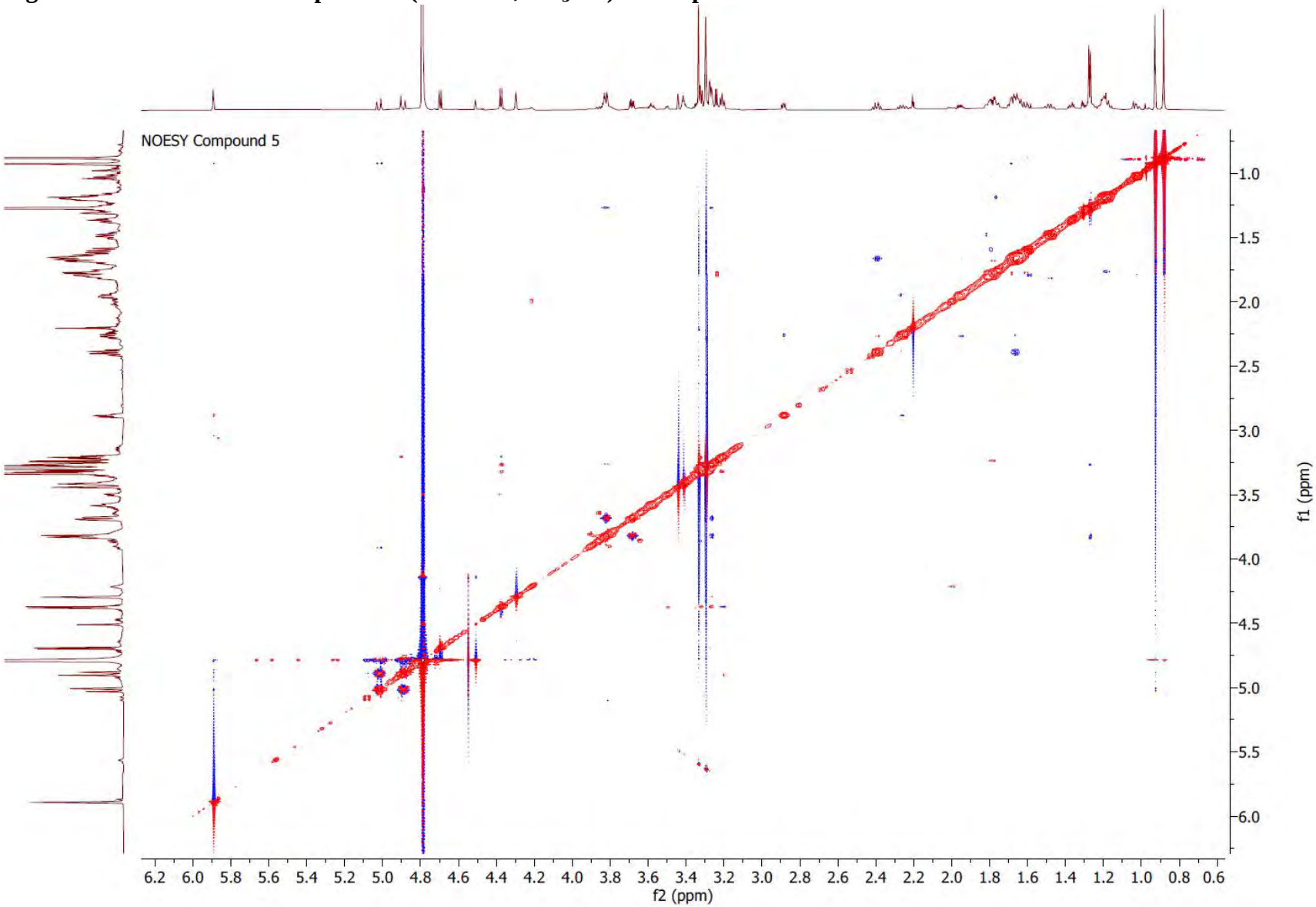


Figure S40: HR-ESI-MS spectrum of compound 6

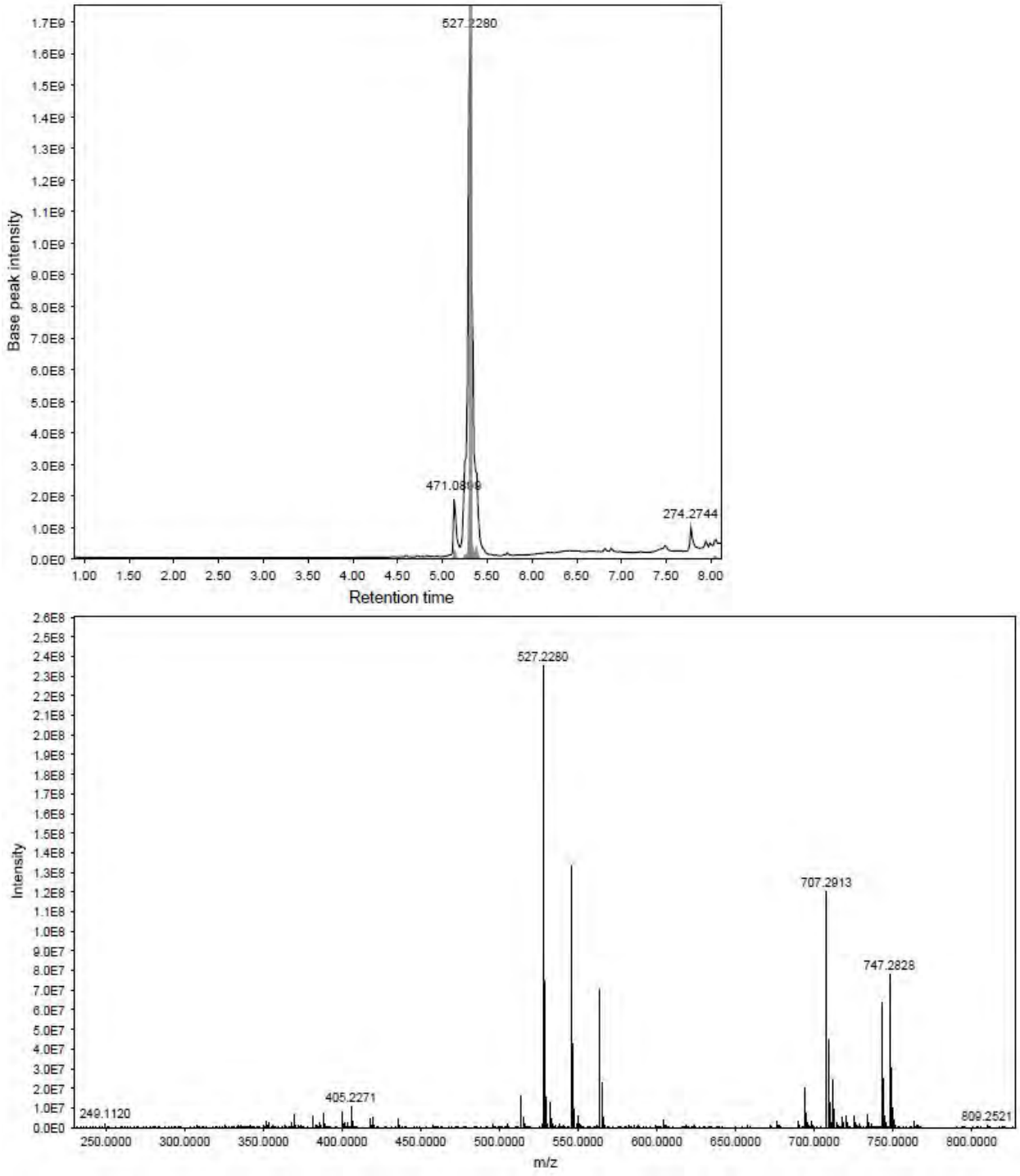


Figure S41: MS/MS spectrum of compound 6

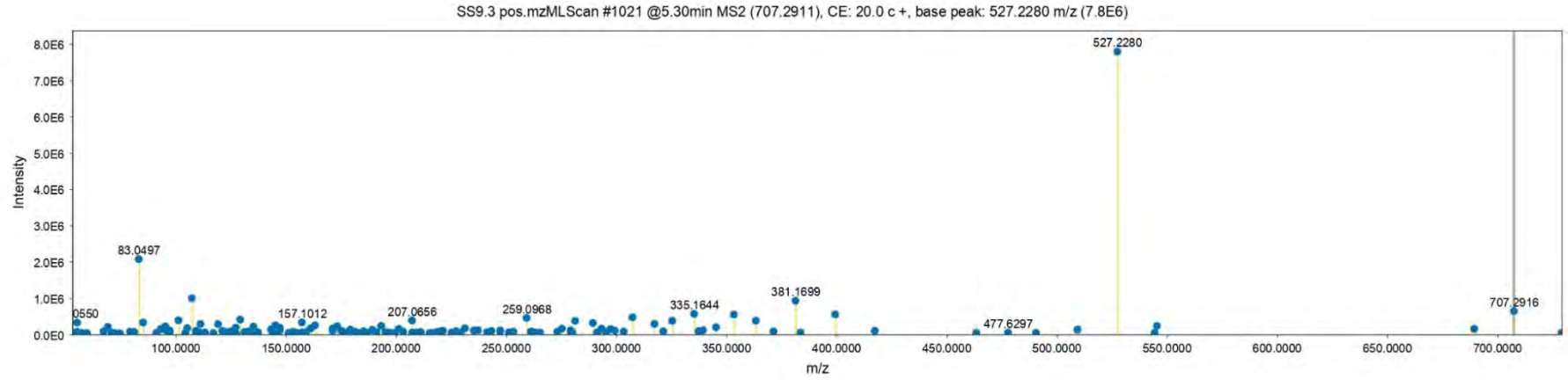
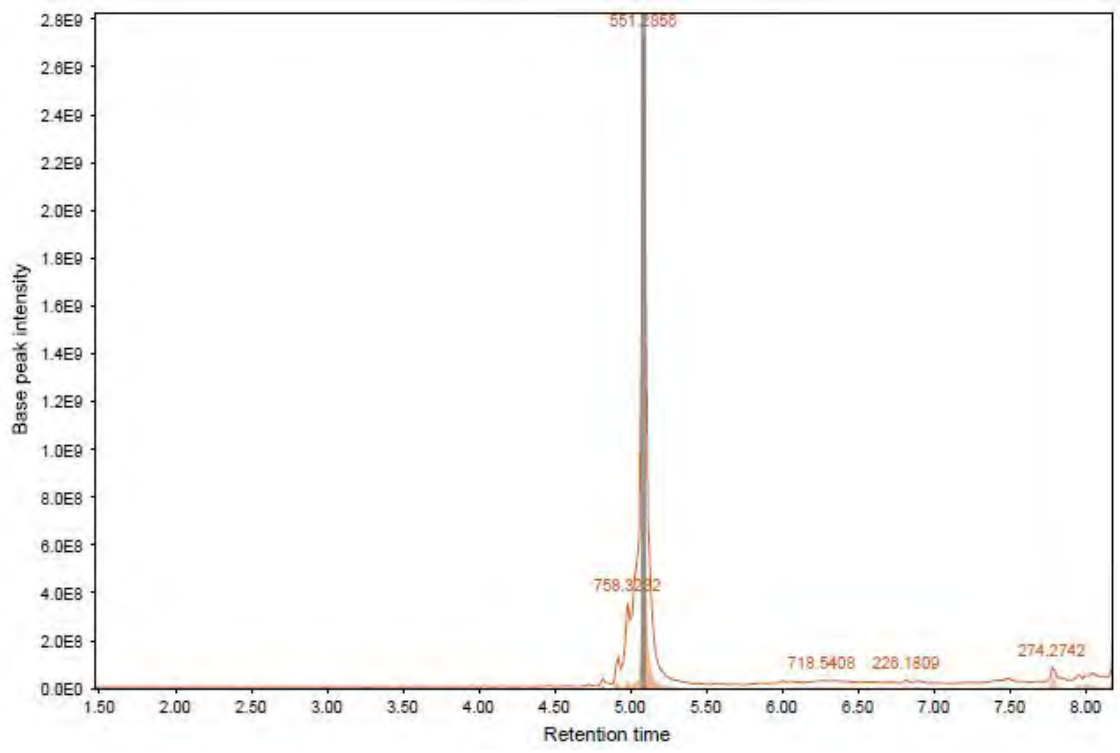


Figure S42: HR-ESI-MS spectrum of compound 7



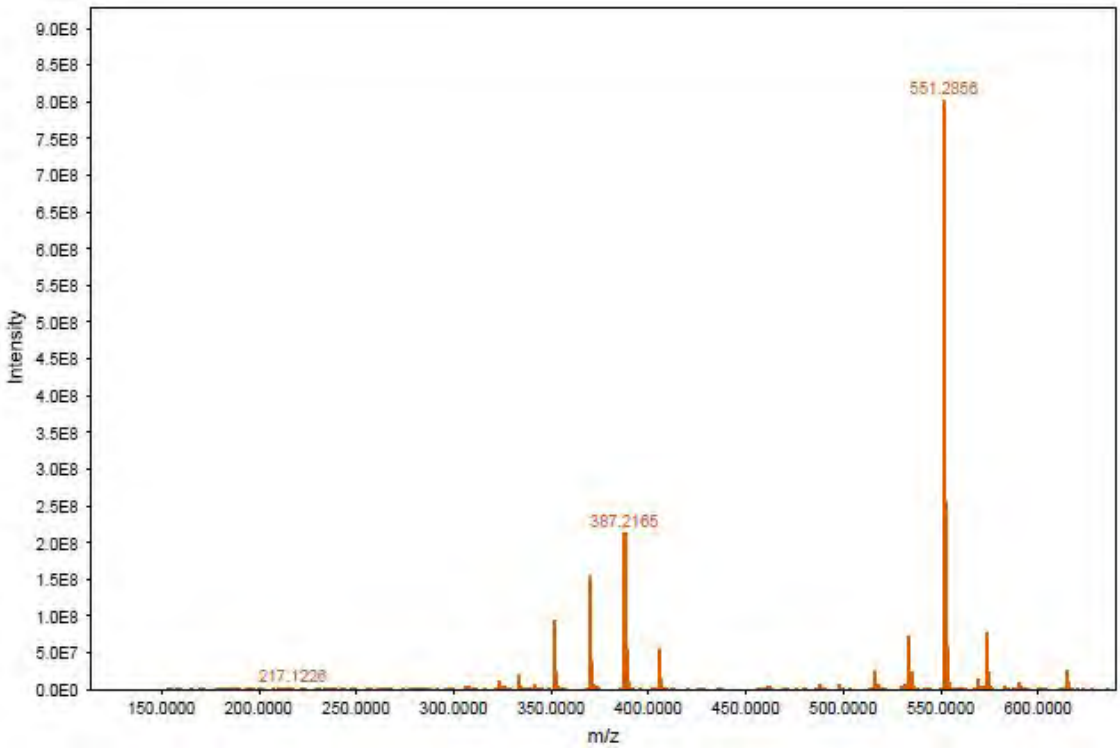


Figure S43: HR-ESI-MS spectrum of compound 7

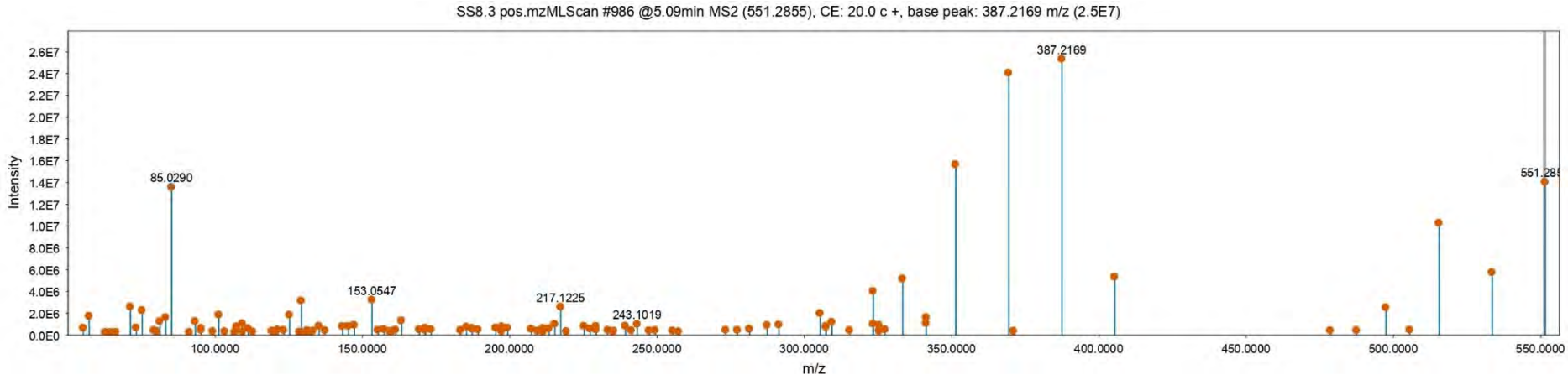


Figure S44: HR-ESI-MS spectrum of compound 8

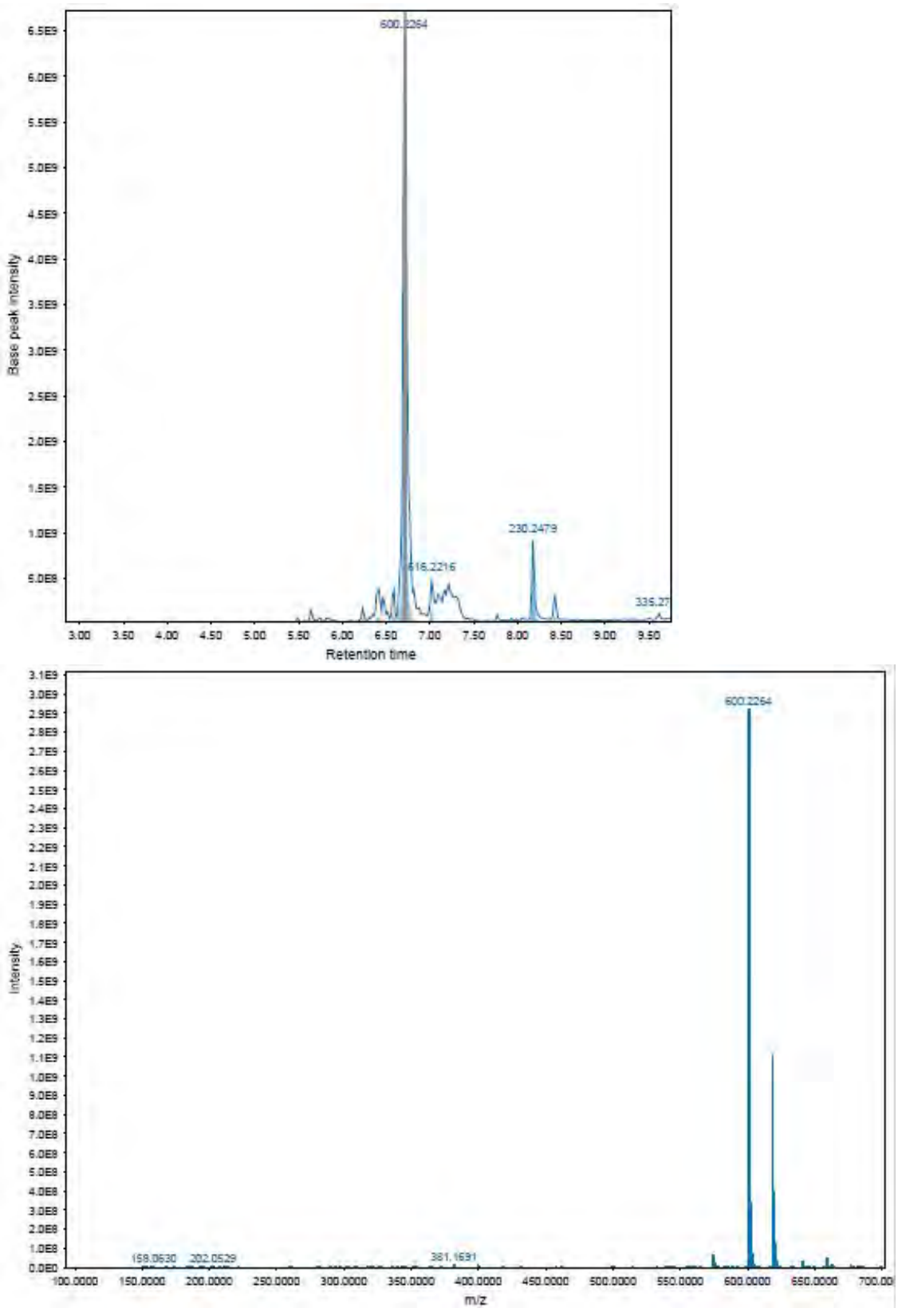


Figure S45: MS/MS spectrum of compound 8

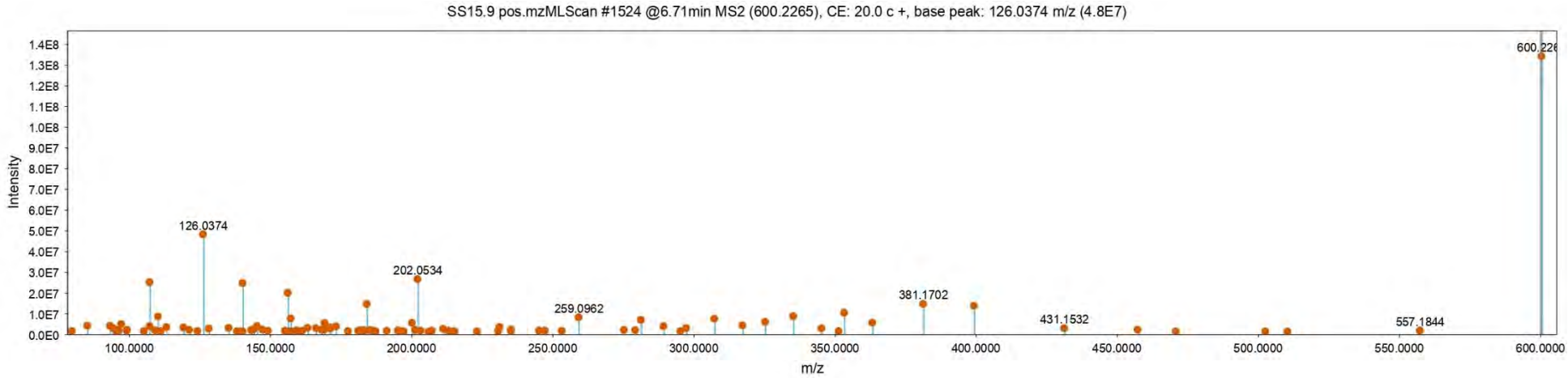


Figure S46: Inhibition curves of *Oncopeltus fasciatus* Na^+/K^+ ATPase by compounds from *Asclepias syriaca* seeds and ouabain

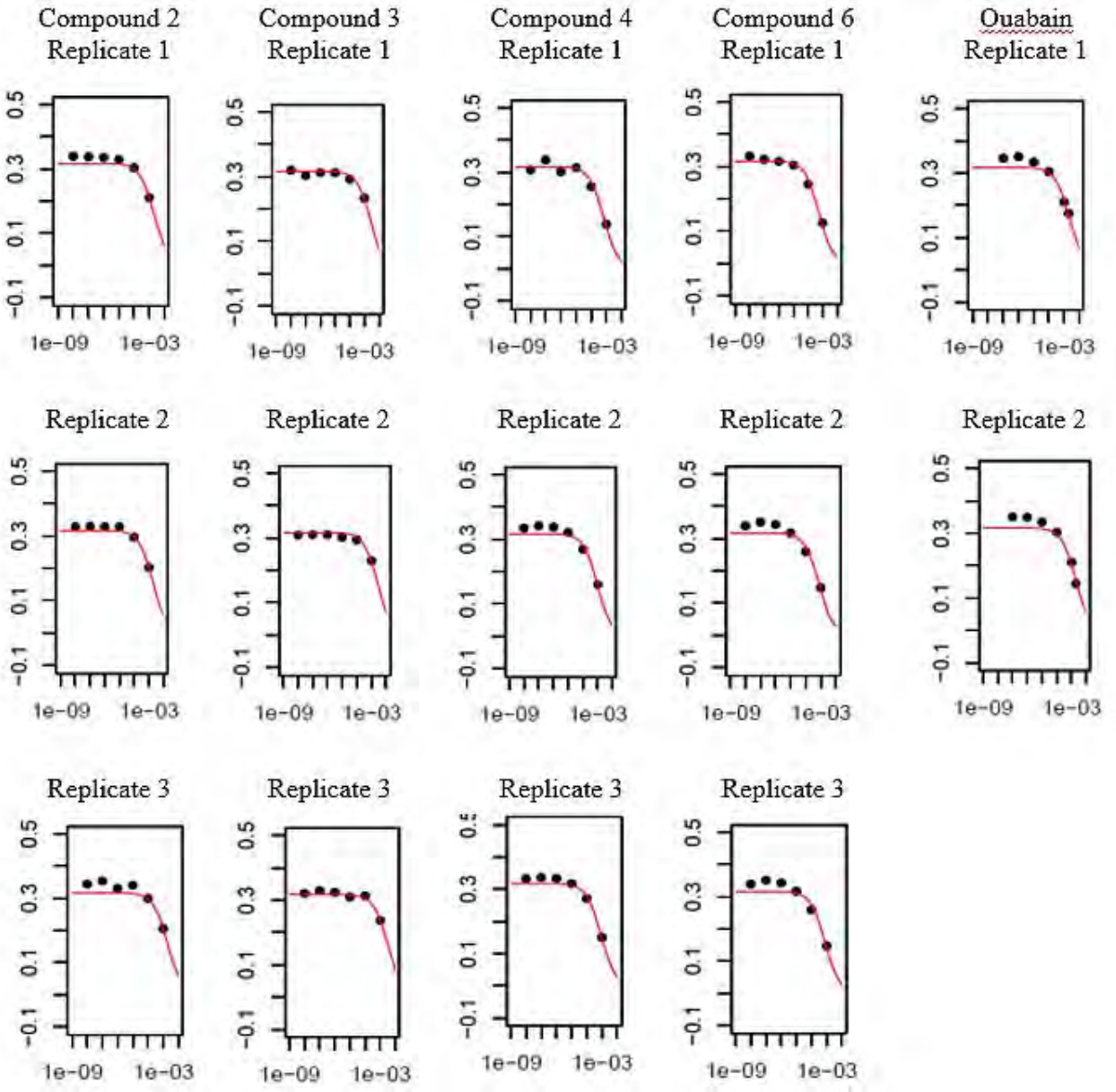


Figure S47: Inhibition curves of *Sus domesticus* Na^+/K^+ ATPase by compounds from *Asclepias syriaca* seeds and ouabain

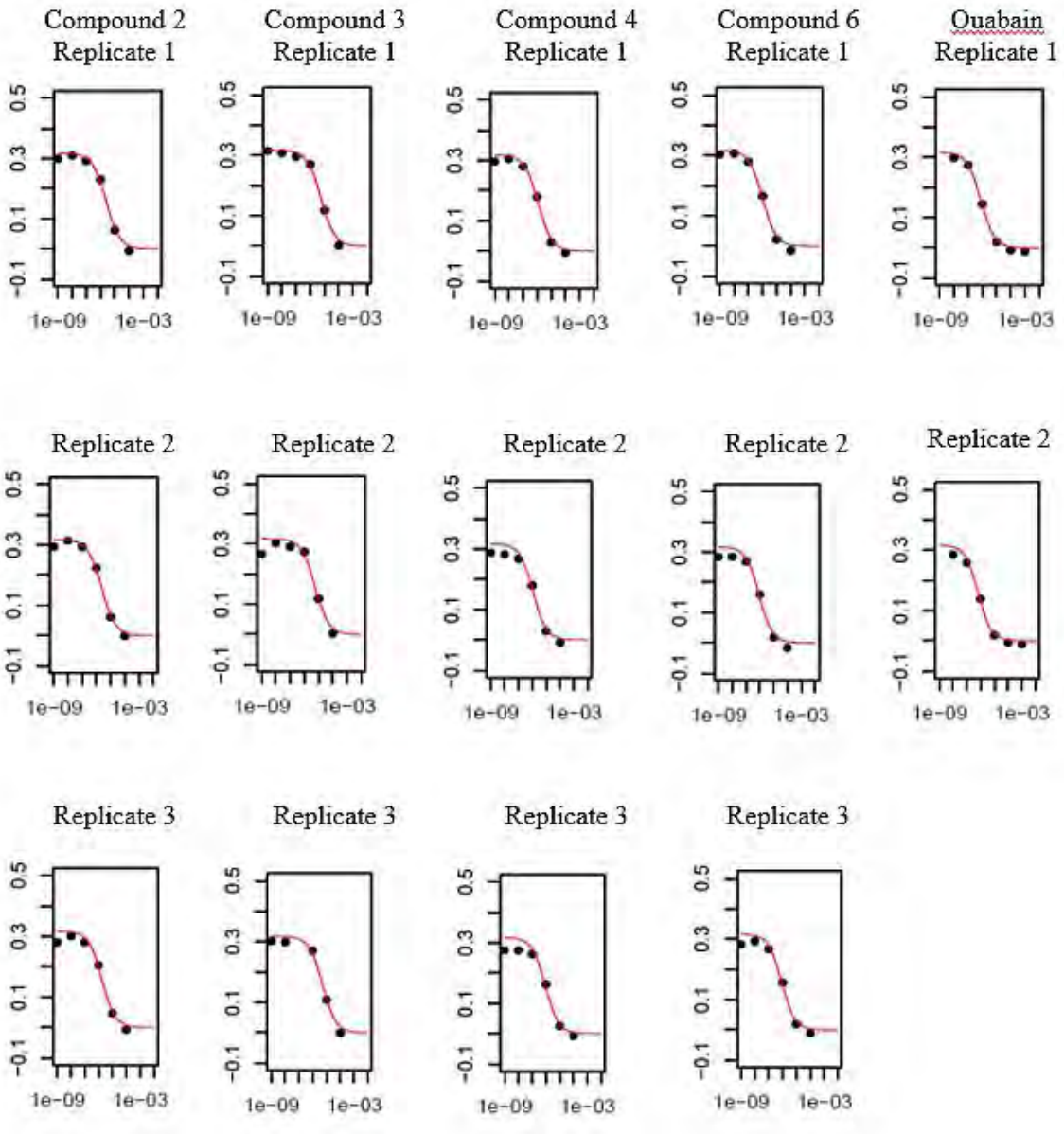


Figure S48 Pearson correlations between cardenolide concentration and inhibition potency against the porcine and *O. fasciatus* Na^+/K^+ ATPase

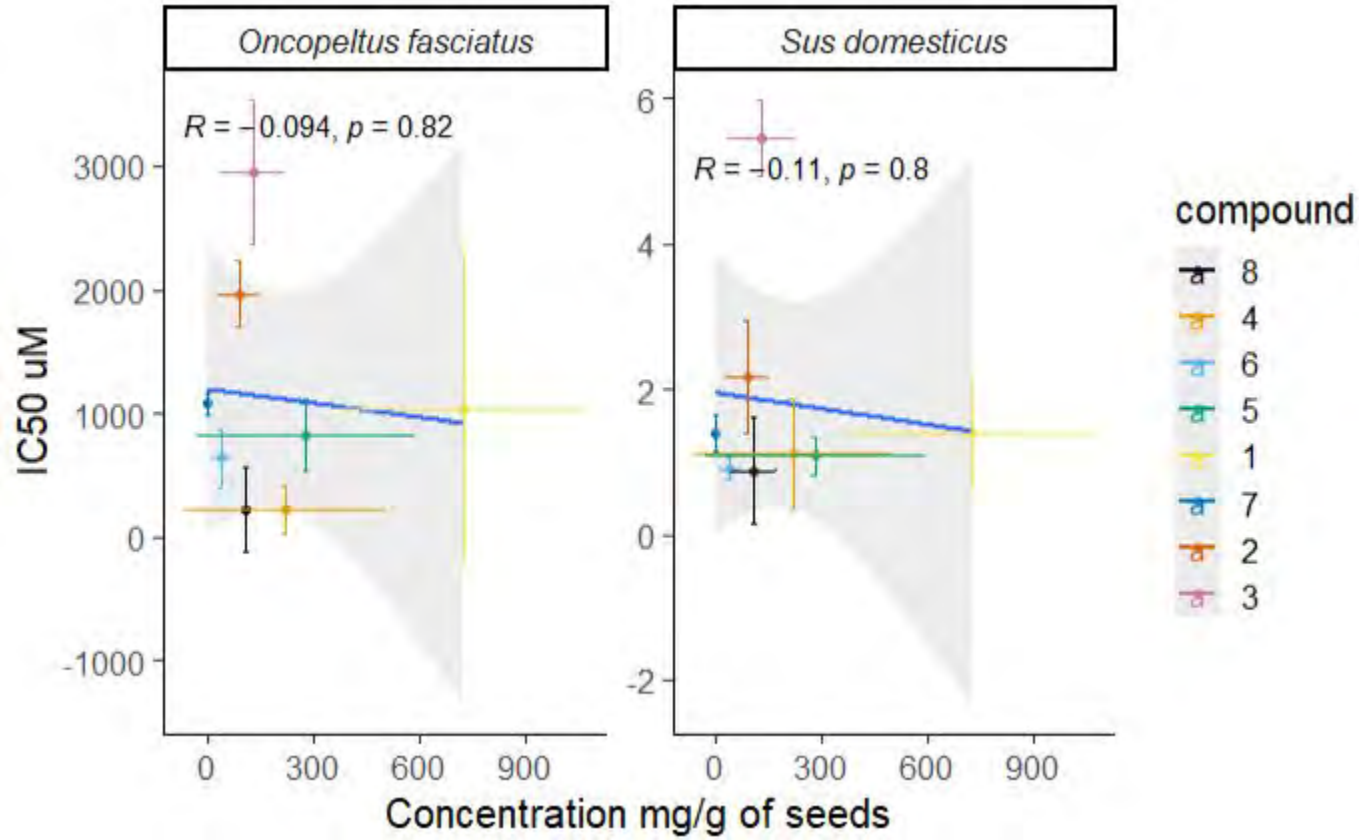
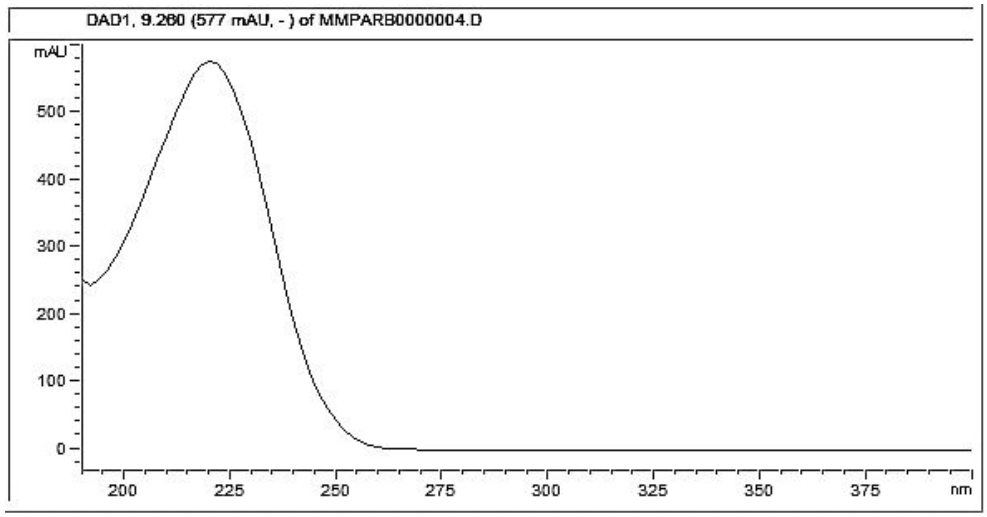


Figure S49: UV spectrum of compound 1. Reference for cardenolides.



Tadeus Reichstein handwritten 1979 manuscript.

Corresponding to the article Brown, P.; von Euw, J.; Reichstein, T.; Stöckel, K.; Watson, T. R. Cardenolides of Asclepias Syriaca L ., Probable Structure of Syrioside and Syriobioside. Glycosides and Aglycones, 334th Communication. *Helv. Chim. Acta* **1979**, *62* (2), 412–441. https://doi.org/10.1002/hlca.19790620207.

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270

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Isolierung von D-Glucose Derivat
entweder Penta-O-acyl-α-glucose
oder Tetra-O-acyl-α-propyl-D-glucosid!
fehlt noch sauberes Experiment!
auf p. 27 bei ^{13}N Tabelle und [40 b]
ändern Reichstein et al!

... der Vertilgung durch ... sind. Die Inhaltsstoffe ... Verfügung standen ... Spuren von Cardenoliden, ... solchen konnte leicht ... werden, die Hauptlinge ... Abbau mit β-Glucosid- ... zeigten, dass die

vorgeschlagenen Formeln 5 und 6 unrichtig sind. – Syriobiosid besitzt vermutlich Formel 7 und Syriosid Formel 10. Letzteres liefert bei fermentativem Abbau mit β-Glucosidasen nicht Syriobiosid, wie die Tschechischen Autoren glaubten sondern einen um 2 H-Atome ärmeren Stoff, den wir Desglucosyriosid (12) nennen. Die Formeln sind gut begründet aber nicht eindeutig bewiesen. Syriosid und Syriobiosid enthalten somit ~~denselben~~ als Zuckerbaustein eine (4,6-Didesoxy-hexosulose (32)) wie sie im Gomphosid (20) und den Calotropis-Cardenoliden (22, 24 etc) vorkommt, die ebenfalls von den Larven der genannten Schmetterlinge mit der Nahrung aufgenommen werden und als ~~Schutzstoffe~~ Abwehrstoffe wirksam sind.

~~Alle alten Formel nummern von 9 an aufwärts müssen um eins erhöht werden, nicht angezählt. Für den Fall dass diese vergessen wurde ist es zu korrigieren~~

Cardenolides of *Asclepias syriaca* L., probable structure of syrioside and syriobioside. (Glycosides & Aglycones 334 communication 1))

P. Brown 2), J. v. Euw 3), T. Reichstein 3), & K. Stöckel 3), T. R. Watson 5)

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4) 3) Present address: Pharmacy Dep.

5) 4) The University of Sydney, N.S.W. 2006, Australia.

Zusammenfassung: Aus den oberirdischen Teilen der Seidenpflanze *Asclepias syriaca* L. (Asclepiadaceae) isolierten Masler et al. [2.3] fünf krist. Cardenolide u.a. Syriobiosid (6) und Syriosid denen sie die Formeln 5 und 6 zuschrieben. *A. syriaca* ist eine der Futterpflanzen auf denen die Larven von Schmetterlingen leben, welche die Cardenolide der Nahrung zu speichern vermögen und dadurch vor der Vertilgung durch insektenfressende Tiere (bes. Vögel) geschützt sind. Die Inhaltsstoffe der Pflanze variieren stark. Bei dem uns zur Verfügung stehenden Material enthielten Blätter und Stengel nur Spuren von Cardenoliden, relativ viel enthielten die Wurzeln. Aus solchen konnte krist. Syriosid sowie eine Spur Syriobiosid direkt durch Chromatographie gewonnen werden, ~~wenig~~ die Hauptmenge des letzteren wurde ~~krist. Syriobiosid~~ erst nach fermentativem Abbau mit β-Glucosidasen erhalten. Chemische u. physikalische Methoden zeigten, dass die vorgeschlagenen Formeln 5 und 6 unrichtig sind. – Syriobiosid besitzt vermutlich Formel 7 und Syriosid Formel 10. Letzteres liefert bei fermentativem Abbau mit β-Glucosidasen nicht Syriobiosid, wie die Tschechischen Autoren glaubten sondern einen um 2 H-Atome ärmeren Stoff, den wir Desglucosyriosid (12) nennen. Die Formeln sind gut begründet aber nicht eindeutig bewiesen. Syriosid und Syriobiosid enthalten somit als ~~[illegible]~~ Zuckerbaustein eine (4,6-Didesoxy-hexosulose (32)) wie sie im Gomphosid (20) und den Calotropis-Cardenoliden (22, 24 etc) vorkommt, die ebenfalls von den Larven der genannten Schmetterlinge mit der Nahrung aufgenommen werden und als Abwehrstoffe ~~[illegible]~~ wirksam sind.

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Cardenolides of *Asclepias syriaca* L., probable structure of syrioside and syriobioside. (Glycoside & Aglucone 334 communication 1))

P. Brown 2), J. v. Euw 3), T. Reichstein 3), & K. Stöckel 4) & T. R. Watson 5)

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4) 3) Present address: ~~F. Hoffmann~~

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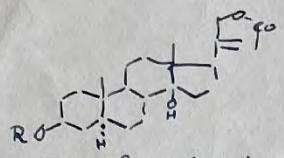
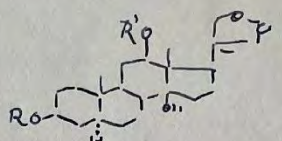
Zusammenfassung: Aus den oberirdischen Teilen der Seidenpflanze, *Asclepias syriaca* L. (Asclepiadaceae) isolierten Masler et al. [2.3] fünf krist. Cardenolide u.a. Syriobiosid und Syriosid denen sie die Formeln 5 und 6 zuschrieben. *A. syriaca* ist eine der Futterpflanzen auf denen die Larven von Schmetterlingen leben, welche die Cardenolide der Nahrung zu speichern vermögen und dadurch vor der Vertilgung durch insektenfressende Tiere (bes. Vögel) teilweise geschützt sind. Die Inhaltsstoffe der Pflanze variieren stark. Bei dem uns zur Verfügung stehenden Material enthielten Blätter und Stengel nur Spuren von Cardenoliden, relativ viel enthielten die Wurzeln. Aus solchen konnte krist. Syriosid sowie eine Spur Syriobiosid direkt durch Chromatographie gewonnen werden, die Hauptmenge des letzteren wurde ~~krist.~~ Syriobiosid erst nach fermentativem Abbau mit β-Glucosid-asen erhalten. Chemische u. physikalische Methoden zeigten, dass die vorgeschlagenen Formeln 5 und 6 unrichtig sind. - Syriobiosid besitzt vermutlich Formel 7 und Syriosid Formel 10. Letzteres liefert bei fermentativem Abbau mit β-Glucosidasen nicht Syriobiosid, wie die Tschechischen Autoren glaubten sondern einen um 2 H-Atome ärmeren Stoff, den wir Desglucosyriosid (12) nennen. Die Formeln sind gut begründet aber nicht eindeutig bewiesen. Syriosid und Syriobiosid enthalten somit ~~denselben~~ als Zuckerbaustein eine 4,6-Didesoxy-hexosulose (32) wie sie im Gomphosid (20) und den *Calotropis*-Cardenoliden (22, 24 etc) vorkommt, die ebenfalls von den Larven der genannten Schmetterlinge mit der Nahrung aufgenommen werden und als ~~Schutzstoffe~~ Abwehrstoffe wirksam sind.



1 (R=H) Uzarigenin
(= Odorigenin B) [9]
m.p. 243-250°, $[\alpha]_D$ = +14.9 (ethanol) [1] $C_{23}H_{34}O_7$ (374)

2 (R = β-D-glucosyl-)
= Desgluco-uzarin m.p. 260-272°
$[\alpha]_D$ = -44.1 (pyridine) $C_{29}H_{44}O_{12}$ (536) [1] [4]
Tetra-O-acyl derivative, m.p. 174-176;
$[\alpha]_D$ = -8.6° (chloroform) $C_{37}H_{52}O_{13}$

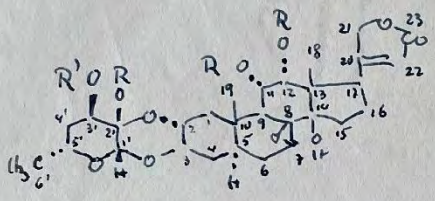


3 (R=H) Syriogenin m.p. 238-288° 3a
$[\alpha]_D$ = +9° (pyridine) $C_{23}H_{34}O_5$ (390) [2]

4 (R=Ac) Di-O-acyl syriogenin
m.p. 173-174, $[\alpha]_D$ = +26.2 (chloroform) [3]
$C_{27}H_{38}O_7$ (474) [3] 3b

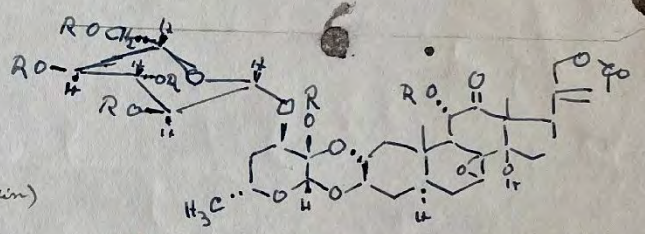
5 (R = D-glucosyl-L-rhamnosyl-; R'=H)
putative formula of syriobioside [2] 3a
m.p. 220-222°, $[\alpha]_D$ = +11.5 (pyridine)
assumed to be $C_{35}H_{54}O_{11}$ (698.8)

6 (R = D glucosyl-D-glucosyl-L-rhamnosyl-; R'=H)
putative formula of syrioside [2] 3a
m.p. 234-237°, $[\alpha]_D$ = -13.6 (pyridine)
assumed to be $C_{41}H_{64}O_{19}$ (896.9)
Acetate, m.p. 185-189°, $[\alpha]_D$ = -2.5° (chloroform)
assumed to be $C_{61}H_{84}O_{29}$ (1281.3)



7 (R=H) Syriobioside (TR-1525)
m.p. 221-223°, $[\alpha]_D$ = +26 → +0° (pyridine)
$C_{29}H_{40}O_{11}$ (564) with hypothetical genin $C_{23}H_{32}O_8$ (436)

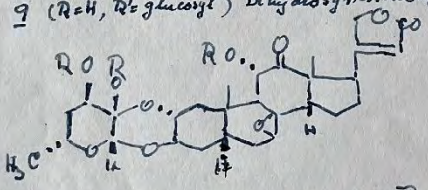
8 (R=Ac) Tetra-O-acyl-syriobioside (TR-1561) chloroform
m.p. 338-340°, $[\alpha]_D$ = +33.2 ($CHCl_3$)
$C_{37}H_{48}O_{15}$ (732) with hypothetical genin $C_{27}H_{36}O_{10}$ (520)



10
9 (R=H) Syrioside (TR-1524) m.p. 230-231°
$[\alpha]_D$ = -12.8 (pyridine) $C_{35}H_{48}O_{16}$ (724)
with hypothetical genin $C_{23}H_{30}O_8$ (434)

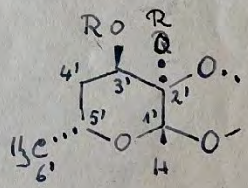
11
10 (R=Ac) Hexa-O-acyl-syrioside (TR-1527-B)
m.p. 192-193°, $[\alpha]_D$ = -1.2° (chloroform)
$C_{47}H_{60}O_{22}$ (976) with hypothetical genin $C_{25}H_{32}O_9$ (476)

9 (R=H, R'=glucosyl) Dihydrosyrioside (hypothetical)



12
11 (R=H) Desglucosyrioside (TR-1554)
m.p. 204-206°, $[\alpha]_D$ = +26 (chloroform)
$C_{29}H_{38}O_{11}$ (562) with hypothetical genin $C_{23}H_{30}O_8$ (434)

13
12 (R=Ac) Tri-O-acetyl-desgluco-syrioside (TR-1555-B) m.p. 302-303°,
$[\alpha]_D$ = +3.1° (chloroform) $C_{35}H_{44}O_{14}$ (688)
with hypothetical genin $C_{25}H_{32}O_9$ (476)



14
13. Other possibility with reversed ~~structure~~ configuration at C-2' of sugar moiety corresponding to 2

For isolation work we used a colony of *A. cyriaca* of garden origin cultivated since many years in Basel by the senior author (TR). Dried leaf and stems contained only little cardenolides. Roots although somehow erratic results gave roots. From 670 g dried roots dug up in the fall 1973 we could isolate 274 mgs of crystalline cyrioside after one single chromatography. No traces of free cyriobioside could be detected in this material. A little more was nevertheless found to be present as a D-gluco-derivative (3) after treatment of the highly polar amorphous material (dried mother liquors of cyrioside) with β-glucosidases (snail enzyme or commercial "cellulase") gave mixtures from which a total of 53 mgs of pure crystalline cyriobioside could be obtained after repeated chromatography. From 820 g dried root harvested in the fall 1974 only 35 mgs of crystalline cyrioside were obtained and tedious partition chromatography was necessary to obtain them. No pure cyriobioside was obtained in this experiment. This second batch obviously contained relatively more "polar" - negative material (perhaps polyhydroxy-pregnane glycosides (see Mitsuhashi et al [6a] and Papay et al [6b]) which impede isolation of cardenolides. Identity of our crystals was established by comparison with authentic material of cyriobioside, cyrioside and cyrioside-acetate kindly provided by Dr. S. Bauer. Melting points and rotations of our preparations were in good agreement with values given by the Czech authors [3a, b], mixed m.p. gave no depression and R_f-values in thin layer chromatography (TLC) and paper-chromatography (PC) in different systems were identical.

The material allowed us to show that the constitutive structures 5 and 6 suggested by Marles et al. [3a,b] for cyriobioside and cyrioside cannot be correct. Chemical degradation, combined with physical methods, particularly mass spectra and NMR-spectra allowed us to suggest formulae 7 and 8 (or most probably structures 13) for these compounds. X-ray work may be necessary for a final proof but we are confident that our formulae 7-12 in principle are correct, although some details (particularly chirality at C-2' of the sugar moiety) need further study and even a rigid proof that the steroid nucleus is present is still missing.

6) Marles et al [3a] give $[\alpha]_D^{22} = +11.6°$ in pyridine for cyriobioside. We found $[\alpha]_D^{25} = +26.4° \pm 8°$ in this solvent after 5 minutes and 0° after 30 and 45 minutes. The compound is obviously rapidly decomposed or rearranged in pyridine. All other compounds gave rotation values in good agreement with those reported by the Czech authors.

identified

to have identified syringin (3) rhamnose and glucose by PC.

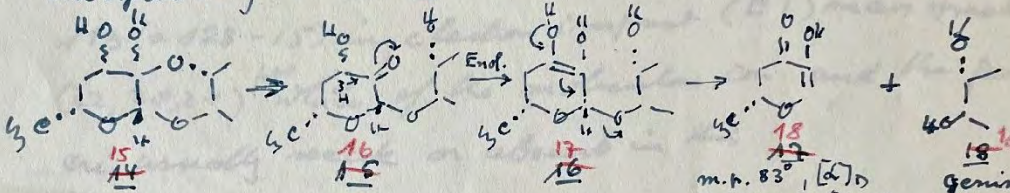
Masler et al. [3a,b] claim ~~that syringobioside~~ (after mild hydrolysis (method of Hamm & Siewert [15]) of syringoside and also after vigorous hydrolysis (with 5% H_2SO_4 in aqueous ethanol. They also claim to have obtained crystalline syringoside (m.p. 219-222°) after enzymatic cleavage of syringoside with β-glucosidases (exact enzyme or preparation from Aspergillus wentii [16]).

In our hands (rhamnose nor any other) no "normal" sugar could be traced in TLC or PC (methods see [17]) after vigorous hydrolysis of syringobioside (7) with Kiliani-mixture in micro scale [18]. Syringoside (10) under these conditions gave D-glucose as sole "normal" sugar, and this could be isolated as crystalline ~~2,3,4,6-tetra-O-acetyl-β-D-glucopyranoside~~ penta-O-acetyl-D-glucose by acetolysis of hexa-O-acetyl-syringoside (11) on preparative scale. Syringoside (10) after treatment with β-glucosidase (exact enzyme or commercial "cellulase" [7,1]) gave D-glucose (again ~~isolated as crystalline acetyl-β-D-glucopyranoside~~) and a compound which we call desgluco-syringoside (12). It has similar running properties and similar m.p. as syringobioside (7) but is slightly less polar and contains 2 hydrogen atoms less. Small amounts of free 12 are also present in the roots.

Exhaustive acetylation with acetanhydrid in pyridine at 35° for 6 days gives a tri-O-acetyl-derivative (13) while syringobioside (7) under these conditions yields a tetra-O-acetyl-derivative (8) with distinctly higher rotation. No "normal" sugar could be detected after vigorous hydrolysis of desglucosyringoside (12). ~~Small amounts of free 12 are also found in the roots~~

7,1) We thank the Ferment AG Basel for a gift of this very active material, prepared from ~~fungus~~ Aspergillus spec.

On the other hand syringoside (7) and desfuco-
syringoside (11) gave a very strong positive "osazone reaction
for methylreductinic acid" (Herse et al. [19] particularly p. 79 and
86). This can be performed on micro scale [20] and is
typical for the hexosulose moiety (14) in the Calotropis
glycosides e.s. calactin (21), calotropin (23), procerside [20]
as well as in gomphoside (19) and afzonde from Gomphocarpus
fruticosus [22,23,24], see Brüschweiler et al. [21, Tab. 1, p. 2776]. All
these compounds are decomposed on heating into "Herzgift-
methylreductinsäure" 17 and the genin (18), a reaction
for which Crout et al. [30, 31] suggested a mechanism
corresponding to 14 → 18. This thermal reaction can easily



be observed by prominent peaks for m/e 128 and
113 (= 128-15) in electron impact (EI) mass spectra
(provided registration is performed quickly after introducing
the probe) [31, 10, 23], while peaks of the molecular ion
or the genin (18) are usually weak or absent in this
procedure. As in other cardenolide glycosides [32] field
ionisation (FI) gave more informative results. Other
"soft" methods (see review [33]) may be as good (or better). Although
molecular ions are still weak or absent in FI-mass spectra of
this kind of compounds, they all showed strong peaks for 18
and the genin (19) (or genin -18). Molecule ions can often be
observed in the O-acetyl-derivatives (see below).

[32 p. 9-10, 20 p. 4, 21 p. 11]

Both syriobioside (7) and desglucosyriond (12/10) declared exactly in this manner (see fig. 2,3). We accept this as strong evidence that they ~~contain~~ may contain a similar sugar ~~residue~~ moiety. This was fully substantiated by the NMR-spectra (see below). As no other sugar could be detected in 7 and 11/12 we further conclude that both must contain highly oxygenated genins to explain their high oxygen content as found in combustion analyses [3]. This is fully confirmed from the mentioned mass spectra (fig. 2, 3) and results of acetylation (see below). The name syriobioside for compound 7 is therefore misleading as it contains only one sugar, nevertheless we continue to use it for historical reasons.

In the following we give our reasons for assigning structures 7, 11/12 and 9/10 to syriobioside, desglucosyriond and syriond

Biological activity of 7 and 9/10 has obviously never been checked. We could get at least some values for syrioside (9/10). In the atria of the isolated spontaneously beating guinea pig atria [34] it induced a concentration dependent digitalis-like increase in the amplitude of contraction but was somewhat less potent than digitoxin [35] while in the ATP-ase test [36] it was as active as this [5]. Digitalis-like activity (Sandor AG. PD. Dr. G. Scholtysik from the Biological and Medical Research Division/ Basle Dr. H. Heidmann of the Pharmac. Dep.) for the experiments and for allowing us to publish them (in litt. 7.1.1976, 20.12.1977).

5) We thank Dr. H.J. Portius and Prof. Dr. K.R.H. Repke, Forschungs Centrum für Molekularbiologie und Medizin, Akad. der Wiss. DDR Berlin-Buch very much for investigating our compound and sending us the results (in litt. 4.6.1976).

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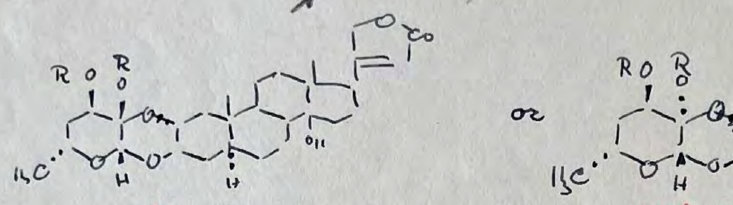
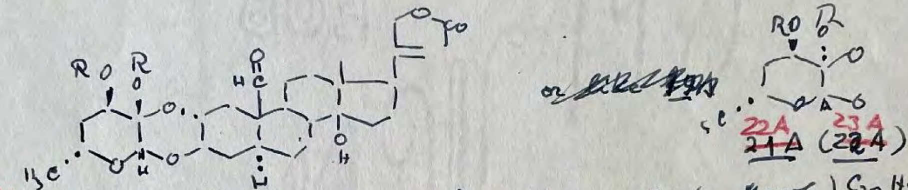


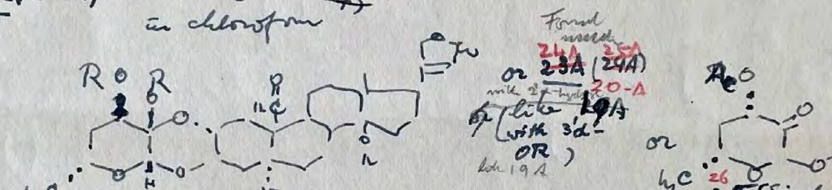
20 (or 20A) gomphoside (R = H): m.p. 232–243°, $[\alpha]_D^{18} = +15$ (methanol), $C_{29}H_{42}O_8$ (518) [22b, c, 23]
21 di-O-acetyl-gomphoside (R = Ac): m.p. 252–255°, $[\alpha]_D^{20} = +32$ (chloroform), $C_{33}H_{46}O_{10}$ (602) [22c]

Alternatives labelled 20A (19A) and 21A (20A). Marginal numbers 8) and 9).



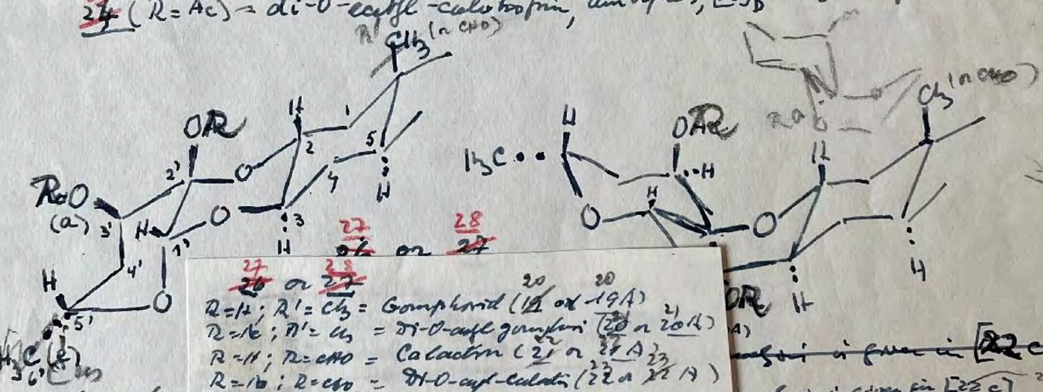
22 calactin (R = H): m.p. 262°–267°, $[\alpha]_D = +57.3$ (methanol) after 2 minutes and [+ 38.1° final minus] in methanol, $C_{29}H_{40}O_9$ (532) [21] 23
23 di-O-acetyl-calactin (R = Ac): m.p. 252–254°, $[\alpha]_D = +42.3$ in chloroform

Alternative labelled 22A, 21A (23A, 22A), "or like 19A". Marginal number 10).



24 calotropin (R = H): m.p. 221°, $[\alpha]_D = +65.3$ (methanol) $C_{29}H_{40}O_9$ (532) [21] 23
25 di-O-acetyl-calotropin (R = Ac): amorphous, $[\alpha]_D = 0°$ (chloroform)

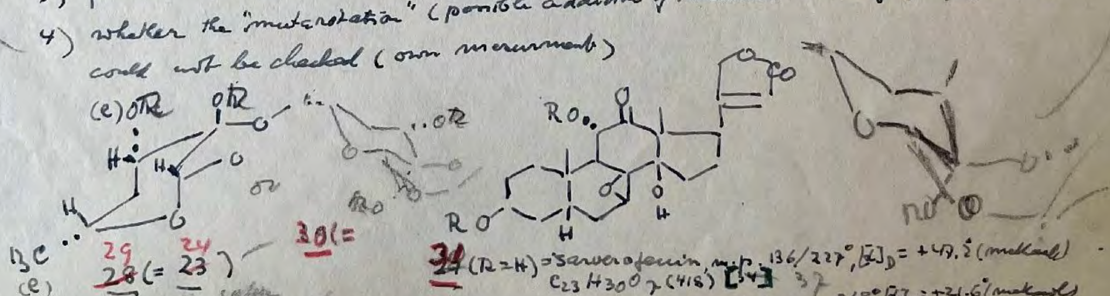
Alternatives: or 24A, 23A (25A, 24A) with 2'-hydroxy like 20-A, 19A, with 3'α-OR; 26 [Singh & Rastogi [33]] 23. Margin: [36]



27 (26) or 28 (27)

R = H; R' = CH_3 = Gomphosid (20 or 20A)
R = Ac; R' = CH_3 = Di-O-acetyl gomphosid (21 or 21A)
R = H; R = CHO = Calactin (22 or 22A)
R = Ac; R = CHO = Di-O-acetyl-calactin (23 or 23A)

2) a slightly ... alpha is given in [22c] 20
3) formula $C_{29}H_{48}O_8$ derived from combustion analysis is given in [22c] 20
4) whether the "mutarotation" (possible addition of methanol to aldehyde group) is real could not be checked (own measurement)



29 (28) (= 24 (23)) (e)
(R = A = ...)
30 (=
34 sarverogenin (R = H): m.p. 136/227°, $[\alpha]_D = +47.5$ (methanol) $C_{23}H_{30}O_7$ (418) [34] 37
32 di-O-acetyl-sarverogenin (R = Ac): m.p. 268°, $[\alpha]_D = +21.6$ (methanol) $C_{27}H_{34}O_9$ (502) [34] 37

Margin: [37]

has so far only been found in steroids containing a butenolide side chain and a 14β-hydroxy groups. Presence of the butenolide ~~side~~ ring chain is also well compatible with the UV.-[3a, 6] and IR.-spectra. Of the latter we give here only fig's for ~~cyrtobiside~~ syrioside (10) as example. All of the compounds checked in IR. gave absorption typical for the butenolide ring but bad resolution, perhaps due to water of crystallisation. This made it also difficult to check the empirical formulae by combustion analysis. To prevent termal decomposition we dried all samples at room temperature (20°) and 0,01 Torr over P_2O_5 for 24 hrs. Except compound ~~12~~ 13 which gave a correct result for $C_{35}H_{44}O_{14}$ all other compounds gave values corresponding to one molecul of H_2O in excess to those calculated. We assume that this is not covalently bound but rather firmly bound water of crystallisation, as otherwise it would be difficult to explain results of mass spectra. Free water was also visible in the ^1H-NMR spectra.

Acetylations. For structure determinations the O-acetyl derivatives have been used extensively. Acetylation of the sugar moiety proceeds stepwise, the secondary HO-groups at C-3' reacts quickly, the angular one at C-2' rather slowly as observed by Brüschweiler et al. [23, p. 2282] for compounds of this type and by Singh & Pastorey [36] for calotropin (24) in particular. In some cases the separation of fully and partially acetylated ~~mixed~~ compounds is difficult. In order to get pure material in high yield we sealed the samples with excess of acetic anhydride and abs. pyridine in vacuo and kept them at 35° for 8 days [23, p. 2282]. The

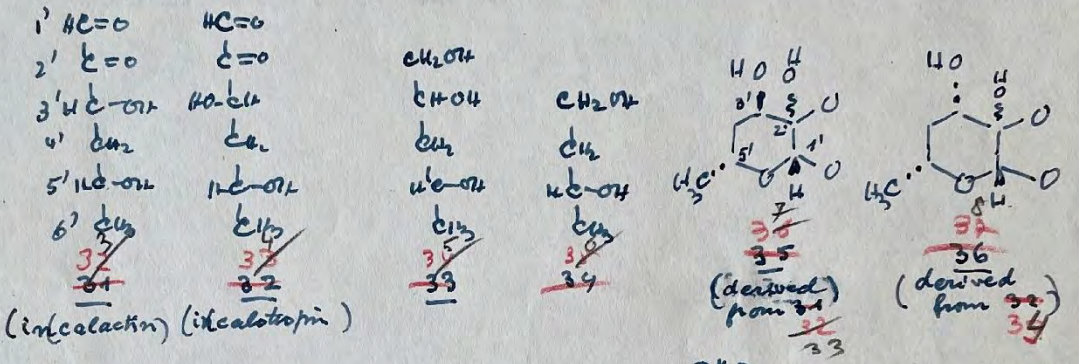
14-β hydroxy group is not attacked under these condition nor is the aldehyde group in 21 and 23 which would partially be destroyed by autoxydation if oxygen were not excluded.

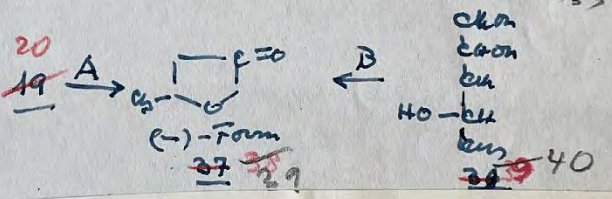
Model compounds. For interpretation of the results of mass spectra and NMR-spectra we used gomphoside (19) calactin (22), calotropin (24), sarverogenin (29) and their O-acetyl derivatives 20, 22, 24 or 25 and 32, as models with essentially known structures. In formulae 19-24 some details given in the original literature (reviewed in [23]) are altered to fit new result. Structures of the genins of 19, 21 and 23 (gomphogenin and calotropagenin) as well as of sarverogenin (30) are established (reviewed in [23]). Structure of the sugar is not completely proven, our present formulation in 19-23 is based on following facts:

21, 23, 25 or 26 and 32

Calactin (22) and calotropin (24) by pyrolysis produce identical products, compound 18 and calotropagenin [24-26]. They can therefore differ only in stereochemistry at C-1', C-2' or C-3' which loose their chirality when transformed into 18. As shown by Crout et al [29, 30, 31] and Crout et al [32] they are derived from 4,6-dideoxy-hexoses. For these only the two formulae 32 and 33 (corresponding to the D-series (written in Fischer projection)

Crout (5)





are possible, configuration at C-5' being established in two ways. Crout et al. [38] obtained (4R)-pentane-1,2,4-triol (35), $[\alpha]_D = -12°$ by degradation of calactin (21) and Combe & Watson [23] isolated D (–)-butane-1,3-diol (36) starting with gomphoside (20). – An earlier report by Curtis et al. [38a] seems at first sight to be in contradiction with these results. These authors [38a] obtained the same optically active (–)-tetrahydro-3-oxofuran (37) in several steps both from calactin (20) and from (4S)(+)-pentane-triol (40). We assume that in one of the two series of steps (A or B) inversion of the remaining center of chirality must have taken place.

Crout [38]

bonding

The two possible sugars (31 or 32) in the original glycosides are bound to the 2α, 3β-dihydroxy-steroid through bonding of glycoside and hemiacetal formation producing the dioxane derivatives 35 or 36. The NMR signals (see table 4) of the sugar moiety in gomphoside (19) and calactin (21) as well as in their di-O-acetyl derivatives 21 and 22 are virtually at the same field which we accept as evidence that these two compounds have identical structures in their sugar moieties and differ only at C-10 of the genin where gomphoside (19) carries a methyl group and calactin (21) an aldehyd group. – From the NMR it is also evident that the HO-group at C-3' is axial in these two compounds while it is equatorial in calotropin (23) see below. So far we do not see a possibility to assigne reliable configuration at C-1' and C-2' with the available spectra but according to Klyne's rule [39] all natural cardenolide glycosides have at C-1' the same absolute configuration i.e. β-D or α-L. As we are in the D-series we prefer to designate β-D which is the stereo type of writing gives 35 and 36 with β-H orientation at C-1'. Assuming chair conformation of the sugar moiety this brings also the 6'-methyl group in the preferred equatorial position (26-27 or 28-29 respectively).

39

gives

acetonide

Still less evidence is available for chirality at C-2'. Combe & Watson [23 p.95] state that the diol system in gomphoside (20) does not form an acetonide, suggesting a trans-position, as formulated by Brüschweiler et al. [21]. We now prefer not to attribute too much weight to the nonreactivity with acetone, it could also be caused by steric hindrance, and leave decision open. Gomphoside would then be either 20 or 20A. For 20 the all chair conformation 27 would probably be the preferred arrangement while for 20A a boat conformation of the dioxane ring (of the acetate) corresponding to 28 may also be favorable. Some ^{13}C-NMR data (see on 60) indicate that 28 (=22) is more likely to be correct than 27 (=21).

acetoxy

As mentioned above, NMR-spectra (table 4) show that the acetoxy group at C-3' in di-O-acyl gomphoside [21] and in di-O-acyl calactin (23) is axial, while in di-O-acyl calotropin (25 or 26) it is equatorial. Singh & Rastogi [36] suggested that the dioxane ring of calotropin (24) is opened during acylation and di-O-acyl-calotropin has structure 26. Their argument is the pronounced downfield shift of the signal for the 1' proton of calotropin (24) in the NMR-spectra after acylation. This explanation may be correct but the shift could also be reconciled with structure 25. We had not (sufficient material of di-O-acyl-calotropin) to check by an ^{13}C-NMR spectrum and leave decision open. All the other

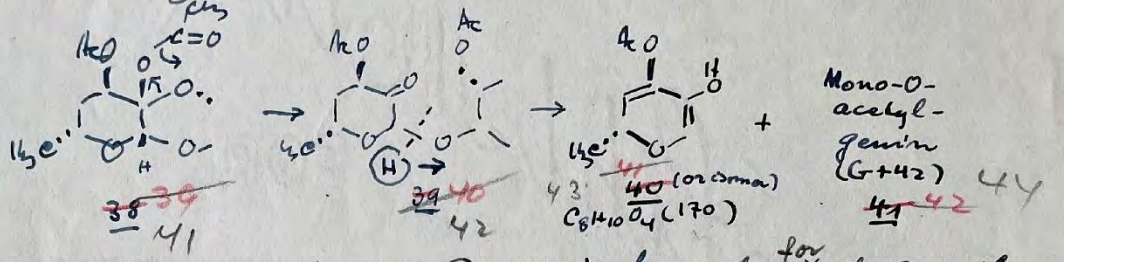
14

compounds ~~19~~ 20, ~~21~~ 22 as well as 7, ~~9~~ 10 and ~~11~~ 12 did not show this shift (table ~~II~~ 4) and there is no reason to assume they should rearrange in the suggested way during acylation. For di-O-acyl-gomphoside (~~20~~ 21) and di-O-acyl-calactin (~~22~~ 23) we could also get ^{13}C-NMR spectra which (see table 3) (are in agreement with the given structures and show no signals which could be assigned to a keto group at C-3'. ~~From ^{1}H-NMR data (see table 4)~~ We are confident that the same is true for 8 and ~~12~~ 13 as the signals for their sugar moieties in the ^{1}H-NMR spectra (see table 4) are virtually at the same positions.

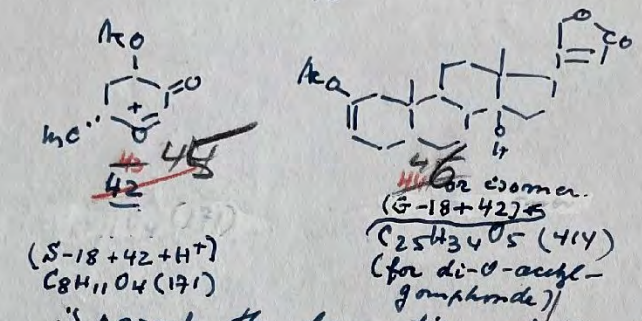
Mass spectra of free glycosides. In the FI spectrum of syriobioside (7) (fig ~~2~~ 4) no molecular peak is visible ~~both~~ but a weak one for M-18. Beside a prominent peak at m/e 128 (~~17~~ 18) a very ~~prominent~~ strong one for m/e 436 (= $C_{23}H_{32}O_8$ table 2) indicative of a genin. Desglucosyrioside (~~11~~ 12) (fig ~~3~~ 5) gave a small peak for the molecular ion and strong ones for m/e 128 (~~17~~ 18) (= $C_6H_8O_3$ table 2), 416 ($C_{23}H_{28}O_7$ table 2.) and a median one at m/e 434 indicative of the genin. In the FI spectrum of syrioside (~~9~~ 10) no molecular ~~no molecular~~ ion is visible but again prominent peaks at m/e 128 (~~17~~ 18) and 416 ($C_{23}H_{28}O_7$ table 2) corresponding to genin-18.

Mass spectra of acyl derivatives. In the acylated compounds (8, ~~10~~ 11, ~~12~~ 13, ~~20~~ 21, ~~22~~ 23, ~~24~~ 25) a thermic reaction corresponding to scheme ~~14~~ 15 → ~~17~~ 18 is only possible after elimination of ketene ~~or~~ the glucosyl residue in ~~10~~ 11. This may indeed take place in the FI spectra to a small extend as ~~minor~~ fairly strong peaks at m/e 170 and ~~41~~ 43 ~~(40 or 41 more)~~ ~~genin~~ minor ones for ~~probably thermic~~ the genin (G) ~~were observed in all cases~~. Another reaction only observed in ~~perhaps confined to~~ ~~due to peculiarities of~~ FI spectra gives rise to peaks at m/e 170 and genin+42, this we tentatively formulate as ~~assume is due to~~

an acetyl shift 38 → 39 producing an intermediate 39



Corresponding to Ringi's formula for di-O-acetyl-calohopin (26) leading to fragments 40 and 41. This may be a thermic process as production of the ions 40 and 41 can be minimised by lowering the temperature, but it could be due to particularities of the Fl. spectra as it was not observed in EI and H_2-CI (chemical ionisation using hydrogen as ionising gas) mass spectra (see fig. 6 and 10). In the H_2-CI spectrum of di-O-acetyl-gomphoside (21) as a model (fig 6) a distinct peak of $M+H^+$ is visible, the most prominent further peaks are attributable to loss of H_2O and one or two mol CH_3COOH. Peaks for $G+H^+$ (391) or $G+42+H^+$ (433) are absent but a distinct peak at m/e 171 ($40+H^+$) is again visible, perhaps the last formulated as 43 is again visible. From metastable peaks of ions obtained in defocussed spectra (see Table 1.) it can be deduced that the four most probable processes for forming the ion m/e 171 (as depicted in Table 1 ...) are the following two can be observed 585 ($M+H^+-18$) $\xrightarrow{414}$ 171 and 525 (585-60) $\xrightarrow{-354}$ 171. No peak for the last particle 414 corresponding to 44 is visible but a distinkt peak at m/e 355 corresponding to a di-anhydro-genin (390-36, or 43-60) perhaps 44 + H^+. If this interpretation

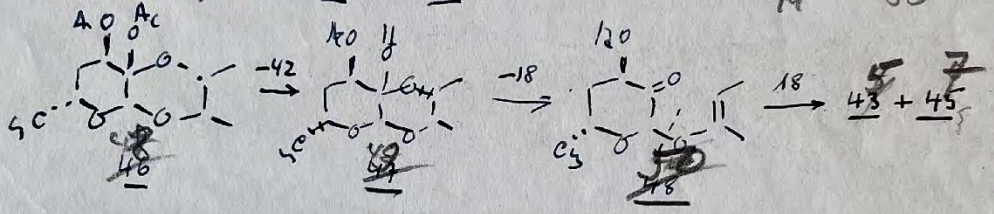


(S-18+42+H⁺)
$C_8H_{11}O_4$ (171)

46 or isomer.
(G-18+42)
$C_{25}H_{34}O_5$ (414)
(for di-O-acyl-gomphonde)

46 or isomer
G-36
$C_{23}H_{30}O_3$ (354) (for di-O-acyl gomphotriole)

is correct the formation of the last particle m/e 414 (perhaps 47) even if not directly visible in the spectrum is indicative of some acetyl migration after or concomitant with loss of water in 20 also in the CI mass spectrum. But the directly visible fragments in the H_2-CI spectrum are 43 and 45 + H^+ (355) which are both probably formed without acetyl-migration perhaps via the intermediates 47 and 48 or in a concerted process 46 → 48.



With these results in our model compounds we can interpret the structures of syringiloside (7), desglucosyringioside (11) and syringoside (9) with some confidence.

Syringiloside (7): The empirical formula $C_{29}H_{40}O_{11}$ (564) is evident from addition of the fragments 17 ($C_6H_8O_3$) and genin ($C_{23}H_{32}O_8$) seen in its FI mass spectrum (fig 2 and table 2). Probable structure of the sugar moiety is discussed above. The genin has two hydrogen less than $C_{23}H_{34}O_8$ calculated for a hexahydroxy-cardenolide. If our assumption is correct that it really is a normal cardenolide then a double bond, a carbonyl group or an epoxy ring must be present. Although evidence for the absence of a double bond (negative $C(NO_2)_4$ reaction in the O-acetyl derivative) and a carbonyl group (IR- and UV-spectra) are meagre due to insufficient

amount of material (no ^{13}C-NMR spectrum possible) we postulate the presence of an 7β, 8β-oxiran ring on the ^1H-NMR spectrum of the O-acyl-derivative. Acetylation of syriobiorside (7) gave a tetra-O-acyl-derivative (8) which in the FI mass spectrum (fig 7) showed a small but distinct molecular ion and aside other peaks ~~for~~ distinct peaks for the genin (520), genin+42 (562) probably formed through acyl migration corresponding to 41 and for ~~40~~ 42 (170). The ^1H-NMR-spectrum (fig 12) shows four signals for acyl groups of which two must be in the sugar moiety. The other two are best compatible with positions 11 and 12 in the sterol nucleus. As a 11β-hydroxy group is not acetylated under our conditions it must be in 11α orientation. From the coupling constants it is also evident that these two groups are in cis position i.e. 11α, 12α, a rare arrangement in natural steroids.

Constants

An important signal in the spectrum (fig 12) is the doublet at δ = 3.38 ppm (J = 6) which we take as strong evidence for the presence of the 7β, 8β-oxiran ring, as it is in excellent agreement with the similar signal in ~~the~~ the spectrum of di-O-acyl-sarmentogenin [~~37~~ 34] see table 4.

(32)

Perfluoro syriobiorside (~~11~~ 12). This compound gave even a peak of the molecular ion ($C_{29}H_{38}O_5$ = 562) in the FI mass spectrum (fig 3) and strong peaks for the fragments ~~17~~ 18 and ~~18~~ 19 (genin $C_{23}H_{30}O_8$ = 434). The latter therefore contains two hydroxyl ~~groups~~ atoms less than syriobiorside (7). Acetylation gave a tri-O-acyl-derivative (~~12~~ 13) showing only three signals for acyl groups in the ^1H-NMR-spectrum (fig ~~14~~ 16 and table 4.) and in the FI mass spectrum (fig 8) peaks for a molecular ion (688), genin (476), mono-O-acyl-genin (518) and ~~40~~ 41 (170). In the ^1H-CI mass spectrum (fig 9) corresponding peaks were only visible for M+1 (689, very weak)

and m/e 171 (~~42~~ 43) while no peaks for genin +1 (477) or mono-O-acyl-genin +1 (519) were detected. - The presence of a keto group (assigned to 12-position) is clearly visible from the ^{13}C-NMR-spectrum (table 3) (in the IR.- and as a doublet ($J = 11,5$) in the ^1H-NMR-spectrum (fig 14, table 4)). Positions are in good agreement with the spectrum of di-O-acyl sarsapogenin (~~31~~ 32) [37] (see table 4), small differences in ~~position~~ are probably ~~in part~~ mainly due to different conformation at C-5. The ^{13}C-NMR spectrum (table 3) is very well compatible with the ~~structure 12 and a little~~ all of structure 13 and probably better than with structure ~~13~~ 14 (with the dioxan ring as a boat (~~27~~ 28)) without excluding the latter. conformation 27

Syrioside (~~9~~ 10). This compound contains one mol D-glucose bound glycosidically to ~~11~~ 12. From relative stability to hydrolysis (excludes a) furanoside), rotation differences and ^1H-NMR of its hexa-O-acyl-derivative (table 4) it is bound as β-D-glucopyranoside. As place of attachment the HO-group at 11α can be excluded because in the ^1H-NMR spectrum (see table 4) of the hexa-O-acyl derivative (~~10~~ 11) the signal of the 11β-H is virtually at the same position as in ~~12~~ 13. We tentatively have therefore placed the glucose (suggested to be attached) in 3 position as the most likely place but no final proof is available. - The FI mass spectrum of free syriosid (~~9~~ 10) (fig. 4) shows no peak of a molecular ion (as expected) and even no peak for the genin (434) but strong peaks for G-18 (416) ek and ~~17~~ 18 (128) and also a small one for the glucosyl cation (163). In the FI mass spectrum of the hexa-O-acyl-derivative (~~10~~ 11) (fig 10) again no molecular peak (976) is visible

furanoside

only small peaks for M-60 and M-120 but distinct peaks for other fragments including mono-O-acetyl-genin (518), genin (476) etc., anhydro-tetra-O-acetyl-glucose (S2 = 330) and ~~M-60~~ 41 (170). The ^{1}H-NMR-spectrum (see table 4) of hexa-O-acetyl-sysiorid (~~M-60~~ 11) is in good agreement with the suggested structure and confirms the presence of six acetyl groups.

20a

bei Fig 1 ansehen wir in Manuscript: cm^{-1} $\frac{1}{cm}$

1783, 1746 and 1630 cm^{-1} correspond to the butenolide ring and 1707 to the ketogroup at C-12.

Fig 2–3 ~~Figure~~. Partial IR.-absorption ~~spectrum of~~ spectra, 2 = ~~desglucosyriodide~~ (12 = TR-1554), m.p. 204–206°, 0,65 mg cristals pured in ca 250 ~~300~~ mg KBr.[6]) showing bands at ca. 1782, 1747 and 1632 cm^{-1} corr. to the butenolide ring and 1709. to the keto group at C-12. Fig 3 ~~of~~ syriobiosid (7; TR-1525) m.p. 221–222°, 0,45 mg cristals pured in ca 200 mg KBr[6], showing only the butenolide-bands at ca 1780, 1740 and 1624 cm^{-1}. No band ~~~~ ~~1707~~ of a ketogroup ~~is visible~~ at ca 1708 cm^{-1} is visible.

Alle weiteren Figuren Nummern müssen und zwei erhöht werden

X3) ~~6.. Perkin Elmer~~ IR. spectrophotometer model 125.

besser nur je 2 Figuren auf ein Blatt

Fig. 1. IR.-absorption spectrum of syriobioside 10 (Prep. TR-1525) m.p. 230-237 ~~221-223~~
72 ~~0,5~~ mg cristals pressed in ca 300 mg KBr [6]) Resolution is bad probably owing
to water of cristallisation, the peaks at ca 1783, 1746 ~~1626, 1738~~ and 1630 cm⁻¹ ~~1782~~ and correspond to the
1782 1744 and 1626 cm⁻¹
butenolid ring), 1707 to the ketogroup at C-12.

Fortsetzung: (siehe S. 20a)

Fig. 2-3 (siehe S. 20a)

Fig. 4 ~~2~~ FI mass spectrum [7]) of syriobioside ~~7~~ (TR-1525) m.p. 221-223
$C_{29}H_{40}O_{11}$ (564), probe temp. 250°. ~~M⁺ not observed,~~ m/e ~~546 = M−H₂O,~~
~~436 (G),~~ 418 (G−18) and ~~128 (S) are prominent~~. Assignments: M⁺ not
observed; 546 (M−18); 436 (G); 418 (G−18); 400 (418−18); 382 (400−18)
128 (S),

Fig. 5 ~~3~~. FI mass spectrum [7]) of desglucosyrioside (12 ~~11~~ = TR-1534 A), m.p. 204-206°,
$C_{29}H_{38}O_{11}$ (562), probe temp. 235°. Assignments: 562 (M); 434 (G); 416 (G−18)
128 (S)

13 6) Recorded by K. Aegerter on a Perkin-Elmer IR. grating spectrophotometer,
Modell 125

14 7) Secured by Mr. Richard B. Scott details see exper. Part. Composition
of ions by high resolution mass spectroscopy see table 1.

15 21

6

Fig. 4 FI mass spectrum[7] of syringide (9 = TR-1524), m.p. 230-231°, $C_{35}H_{48}O_{16}$ (724), probe temp. 235°. Assignments: 526 (M-18-180=glucose); 416 (G-18); 398 (416-18); 370 (398-28); 163 ($C_6H_{11}O_5$=glycosylation); 128 (17).

7

Fig. 5 FI mass spectrum[7] of di-O-acyl gomphoside (20, prep. TLC purified -1563 by chromatography on SiO_2), m.p. 285-287°, $C_{33}H_{46}O_{10}$ (602), probe temp. ....
Assignments: 602 (M); 584 (M-18); 560 (M-42); 542 (M-60); 524 (542-18); 500 (542-42); 482 (M-120); 464 (482-18); 456 ; 432 (G+42); 390 (G); 372 (G-18); 356 , 170 (40).

(?)

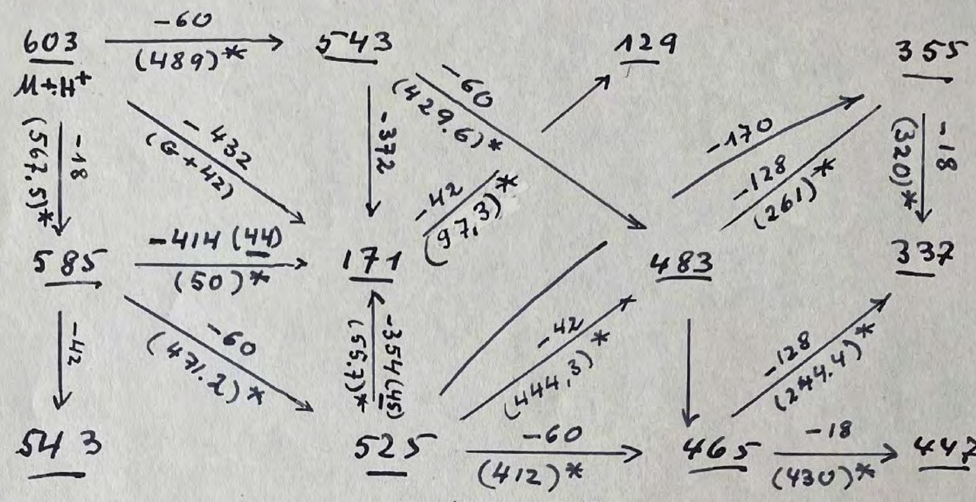
H_2^-

8

Fig. 6. (H_2)-CI mass spectrum[8] of di-O-acyl-gomphoside (20), source temp. 220°, probe temp. 180°. Assignments: 603 ($M+H^+$); 585 (603-18); 543 (603-60 and 585-42); 525 (585-60); 501 (543-42); 483 (543-60); 465 (525-60 and 483-18); 447 (465-18); 429 (447-18); 397 (525-128); 355 (483-128 and 525-170, corresp. 45 (=G-36)+H^+ to G-36+H^+ = 447 H^+); 337 (465-128 and 355-18 and 525-170); 171 (43); 129 (171-42); 111 (129-18). The processes summarized in scheme 1 could be

15 8) Secured by ..... on a AEI-MS 902 instrument, H_2^- pressure 0.3 Torr.

confirmed by metastable peaks in defocussing spectra



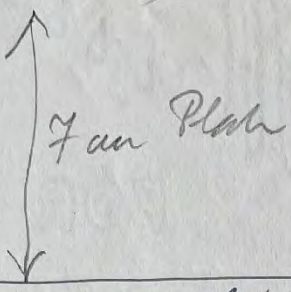
~~Scheme~~ Table 1: ~~for important peaks and~~ mestable ions in the H_2-CI mass spectrum of di-O-acetyl-gomphoside (21).

found: 567.5 ; 489 ; 471.2 ; 444.3 ; 430-429.6 ; 412 ; 320
calc.: 567.54 ; 488.97 ; 471.15 ; 444.36 ; 429.70 and 429.62 ; 411.85 ; 319.91

found: 261 ; 244.4 ; 97.3 ; 55.7 ; 50
calc.: 260.92 ; 244.23 ; 97.32 ; 55.70 ; 49.98

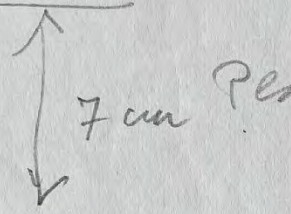
hier kein Platz lassen
weiter fahren

~~16~~ 23



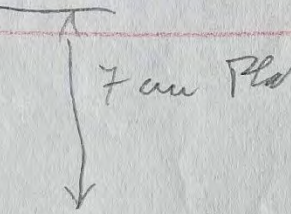
a

~~Fig 7~~ FI mass spectrum of tetra-O-acetyl syringitoxide (8 = TR-1561)
m.p. 338-340°, $C_{32}H_{48}O_{15}$ (732), probe temp. 260°. ~~Assignments~~:
732 (M); 690 (M-42); 673 (732-59); 672 (732-60); 630 (672-42); 612 (672-60)
594 (612-18); 586 (?); ~~562 (G+42)~~; 548 (?); ~~520 (G)~~; 502 (G-18);
170 (~~40~~ 41).



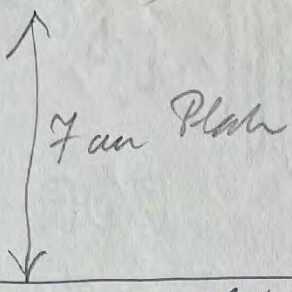
10

~~Fig 8~~ FI mass spectrum of tri-O-acetyl-desfuroyl-syringitoxide (~~12~~ 13, TR-1555)
with hypothetical genin $C_{25}H_{32}O_9$ (476)
m.p. 302-303°, $C_{35}H_{44}O_{14}$ (688) probe temp. 270°. Assignments: 688 (M);
652 (688-18-18); 628 (~~M~~ 688-60); 613 (628-15); 610 (628-18); 592 (610-18)
586 (628-42); 568 (628-60); 550 (568-18); 526 (568-42); 518 (G+42);
508 (568-60); 490 (508-18); 476 (G); 464; 432 (?); ; 416 (G-60);
280 (?); ; 170 (~~40~~ 41); 152 (170-18); 128 (~~40~~ 41-42).

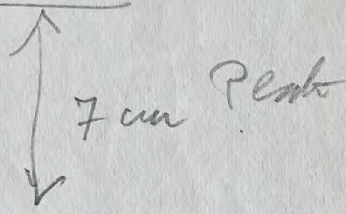


~~Fig 9~~ H_2-CI mass spectrum [6]) of ~~tri~~-O-acetyl-~~gomphoside (20)~~ desfuroyl-syringitoxide (~~12~~ 13), probe temp. ... Assignments: 689 (M+H⁺); 617 ≙ 689-18); 645 ≙ 689-44 (or M-1-42); 629 ≙ 689-60); 611 ≙ 671-60); 569 ≙ 629-60 [m* 515, calc 514.7); 551 ≙ 569-18); 527 ≙ 569-42); 509 ≙ 527-18 or (569-60); 399 ≙ 599-170 or (527-128); 381 ≙ 399-18 or (551-17 or 509-128); 363 ≙ 381-18); 171 (40+H⁺); 143 (171-28); 83 (143-60);
399 = G-18-60 (calc 519-120?); 381 = 399-18; 363 = 381-18
peaks for fragment (477) and mono-O-acyl-genin +1 (519) absent

~~16~~ 23



~~Fig 7~~ FI mass spectrum of tetra-O-acetyl synvinboside (8 = TR-1561)
m.p. 338-340°, $C_{32}H_{48}O_{15}$ (732), probe temp. 260°. ~~Assignments~~:
732 (M); 690 (M-42); 673 (732-59); 672 (732-60); 630 (672-42); 612 (672-60)
594 (612-18); 586 (?); 562 (G+42); 548 (?); 520 (G); 502 (G-18);
170 (~~40~~ 41).



~~Fig 8~~ FI mass spectrum of tri-O-acetyl-desfuco-syringoside (~~12~~ 13, TR-1555)
with hypothetical genin $C_{25}H_{32}O_9$ (476)
m.p. 302-303°, $C_{35}H_{44}O_{14}$ (688) ~~probe~~ temp. 270°. Assignments: 688 (M);
652 (688-18-18); 628 (~~M~~ 688-60); 613 (628-15); 610 (628-18); 592 (610-18)
586 (628-42); 568 (628-60); 550 (568-18); 526 (568-42); 518 (G+42);
508 (568-60); 490 (508-18); 476 (G); 464; 432 (?); ; 416 (G-60);
280 (?); 170 (~~40~~ 41); 152 (170-18); 128 (~~40~~ 41 - 42).

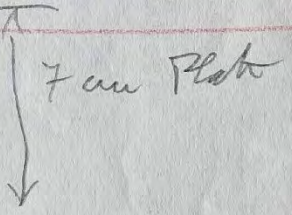


Fig 9 H_2-CI mass spectrum [6]) of ~~tri~~-O-acetyl-desfuco-~~gomphoside~~ syringoside (~~20~~ 13), probe temp. ... Assignments: 689 (M+H$^+$); 617 (689-18); 645 (689-44 for M-1-42); 629 (689-60); 611 (671-60); 569 (629-60 [m* 515, calc 514.7); 551 (569-18); 527 (569-42); 509 (527-18 or (569-60); 399 (599-170 or (527-128); 381 (399-18 or (551-17 or 509-128); 363 (381-18); 171 (40+H$^+$); 143 (171-28); 83 (143-60):
399 = G - ~~18~~ - 60 (or 519-120 ?); 381 = 399-18; 363 = 381-18
peaks for fragment (477) and mono-O-acetyl-furan (519) absent

Fig. 11. 270 MHz NMR-Spectrum of Di-O-acyl-calaction (23) Prep. 1555, m.p. 252°-254°, in $CDCl_3$ 9)16)
15&4
Assignment tentativ.

Fig 11. 100 MHz NMR-Spectrum of tetra-O-acyl-cyrtolivonde (8) Prep. TR-156, m.p. 338-340° in $CDCl_3$ 10)17)
Contained one mol. water of crystallization. Assignments tentativ

Fig 12 270 MHz NMR-spectrum of tetra-O-acyl cyrtolivonol (8) same as fig 12 but only showing lower field signals in higher resolution 9)16)

16 9) We express our thanks to Dr. W. Arnold, Zentrale Forschungsspektroskopie Roche Ltd. Basle for providing this spectrum and his help in interpretation. Performed on a Bruker HX 270 instrument with BNC 1180 computer

17 10) We express our thanks to Dr. H. Fehlhaber and Mr. A. Borer, Laboratory Physics Laboratory at CIBA Ltd Basel and their help in interpretation. Performed on Varian-Spectrograph, Modell HA-100. The signals labeled as HO- disappeared after shaking with D_2O.

Fig. 14 100 MHz NMR-spectrum of Tri-O-acetyl-desgluco-sympsonid (~~12~~ 13) Prep. TR-1555-A, m.p. 300-301° (decomp.) in $CDCl_3$ (~~10~~ 17) containing some water of crystallisation. Assignments tentative.

Table 3. Signals in ^{13}C NMR spectra in $CDCl_3$, assignments (attached proton test) interrelated with ^1H signals by Birdsall plots [40] [40a] (see also Prestch et al. [40b])

Carbon nr.	Type	Di-O-acetyl gomphonitol (20) 21	Di-O-acetyl-galactitol(i) (22) 23	Di-O-acetyl desgluco-sigmoside (12) 13	Di-O-acetyl sarmento-genin (31) 32
1	CH_2	41.7	42.6	43.9	36.2
2	CH-O- or CH_2	71.1 (a)(b)	70.7 (a)	66.7	25.5
3	CH-O-	66.4 (a)	66.6 (a)	69.8	69.0
4	CH_2	32.0 (b)	33.1 (b)	34.9	32.0
5	CH	44.8	43.6	40.5	32.9
6	CH_2	27.7 (c)	27.7 (c)	35.6	35.5
7	CH_2 or CH-O-	27.2 (c)	27.4 (c)	54.3	52.7
8	CH or C-O-	40.8	35.7 (e)	62.6	63.0
9	CH	49.6	48.6	45.2	32.9
10	>C<	38.0	70.8 (a)	37.7	34.4
11	CH_2 or CH-OAc	21.3	21.9	75.3	75.5
12	CH_2 or >C=O	39.6	39.5 (e)	204.6	204.6
13	>C<	49.6	49.4	64.1	64.3
14	-C(-OH)-	85.1	85.0	80.9	81.1
15	CH_2	32.9 (b)	32.4 (b)	28.4	28.5
16	CH_2	26.9 (c)	26.9 (c)	26.6	26.6
17	CH	50.8	50.7	41.8	41.8
18	CH_3	15.7	15.6	17.3	17.5
19	CH_3 or -C(=O)H	13.7	206.4	13.7	23.0
20	C=	174.9	174.2	170.9	171.1
21	CH_2-O-	73.5	73.4	73.7	73.7
22	CH=	117.5	118	118.9	118.7
23	C=O	174.6 (3)	173.9	173.8	173.9
1'	HC(-O-)(-O-)	93.2	93.2	93.1	—
2'	>C(-O-)(-O-)	95.6	95.6	95.6	—
3'	>CHOAc	70.3 (h)	70.5	70.9 (6)	—
4'	>CH_2	34.8	35.0	31.5	—
5'	>CHO-	71.8 (h)	71.2	70.3 (6)	—
6'	CH_3	20.9 (d)	20.8	20.9	—
	Acetyl CH_3-CO	20.7 (d)	20.8	20.7	20.8
	" "	21.7	21.6	21.8	21.4
	" "	—	—	21.2	—
	Acetyl CH_3-C=O	168.8	165.5	169.7	169.5
	" "	168.7	168.8	168.8	170.4
	" "	—	—	168.3	—

Assignment (number of H)	Sp. Nr. (MHz)	C-18 (3)	C-19 (3) (1)	C-2 (1)	C-3 (1)	C-7 (1)	C-9 (1)	C-11 (1)	C-12 (1)	C-17 (1)	C-21 (2)	C-22 (1)
Di-O-acetyl-gomphoside (TRW and TR-1564) (21)	98159 (270)	0,844 (s) 0,878 (s)		4,05 (m) J = ~13 J = ~10 J = 4.5	3.84 (m) J = 13 J = ~10 J = 4.5					2.78 (q) J = 10 J = 5	4.89 (AB) J = 18 J = 2 J = 1.5	5,88 (s)
Di-O-acetyl-sarverogenin (TR-1387) (32) [34]	22753 R-348 (100)	1,129 (s)	1,074 (s)		4.98	3.40 (d) J = 6	2.54 (d) J = 13	5,58 (d) J = 13		3.88 (t) J = 8 J = 8	4,75 (AB)	5,92 (s)
Di-O-acetyl-calotropin (25 or 26) (TR-1533)	72970 R-440 (100)	0,82 (s)	10,01 (s) (-CHO)	3.60-4.20 (m)							4.74 (d) 4.92 (d) J (AB) = 18	5,86 (s)
Di-O-acetyl-calactin (23) (TR-1534) fig 11	99004 (270)	0,81 (s)	10.03 (s) (-CHO)	4.04 (m) J ~ 12 J = 11 J = 4	3.7 (m) J = 13 J ~ 10 J = 4					2.76 (q) J = 10 J = 5	4.78 (q) 4.96 (q) J (AB = 18 J = 2 J = 1.5	5.86 (s)
Tetra-O-acetyl-syriobioside (8) (TR-1561) fig 12, 13	95187 (270)	1.048 (s)	0,921 (s)	ca 4.0 (m)	3.74 (m) J = 12 J = 10 J = 4.5	3.38 (d) J = 6	2.40 (d) J = 11.5	5.42 (q) J = 11.5 J = 2.5	5.23 (d) J = 2.5		4.78 4.83 J (AB) = 16	5,94 s
Tri-O-acetyl-desglucosyrioside (13) (TR-1555-A) fig 14	72644 R-434 (100)	1.12 (s)	1.01 (s)	3.60-4.20 (m)		3.50 (d) J ~ 5		5.66 (d) J = 11.5			4.69 4.89 J (AB) = 18	5,97 (s)
Hexa-O-acetyl-syrioside (11) (TR-1527)	94630 (270)	1.126 (s)	1.006 (s)	3.57-3.75 (m)		3.54 (d) J = 6.0		5,68 (d) J = 11,5			4.71 4.84 J (AB) = 18 J = 2 J = 1.5	5,88 (s)

Table 4. Signals in ^1H NMR spectra in $CDCl_3$, assignments tentative.

1.29

O-Ac gemin	O-Ac sugar	O-Ac glucose	C-1' (1)	C-3' (1)	C-4' (2)	C-5' (1)	C-6' (3)	C-1'' (1)	C-2'' (1)	C-3'' (1)	C-4'' (1)	C-5'' (1)	C-6'' 2	further signals
	2,08 (s) 2.10 (s)		4.83 (s)	5.74 (t) $J = 3$		ca 3,97 (m)	1.24 (d) $J = 6$							
2.0 (s) 2,15 (s)														
	2,03 (s) 2.09 (s)		5.54 (s)	5,80 (q) $J(a,a) = 10$ $J(a,e) = 6$	3.60 - 4.20 (m)		1,26 (d) $J = 6$							
	2.06 (s) 2.08 (s)		4,80 (s)	5.74 (q) $J(e,a) = 3$ $J(e,e) = 2,5$	ca 3.95 (m)		1.22 (d) $J = 6$							2,47 (q) 1-H equat.? $J = 12$ $J = 5$
2.07 (s) 2,20 (s)	2.05 (s) 2.07 (s)		4.83 (s)	5,75 (t) $J = 3$	ca 3.97 (m)		1,25 (d) $J = 6$							
2.23 (s)	2.06 (s) 2.06 (s)		4.82 (s)	5.74 (t) covered	3.60 - 4.20 (m)		1,22 (d) $J = 6$							
2.22 (s)	2.06 (s)	2.07 (s) 2,01 (s) 2,02 (s) 2.10 (s)	4.71 (s)		3.80 - 4,00 (m)		1.21 (d) $J = 6.5$	4,44 (d) $J = 8$	5,01 (t) $J = 8$ $J = 9$	5,08 (t) $J = 9$	5.13 (t) $J = 9$	3.57 - 3.75 (m)	4,03 (q) 4,35 (q) $J(AB) = 12$ $J = 4.0$ $J = 1.5$	2,34 (q) $J = 13, J = 4$ 2.59 (d) $J = 1.5$ (- OH)

table 3. Signals in ^{13}C NMR-spectra in $CHCl_3$, assignments tentative, interrelated with ^1H signals by Birdsall plots [40].

carbon nr.	type	di-O-acetyl-gomphoside (21)	di-O-acetyl-calactin(i) (23)	tri-O-acetyl-desgluco-syrioside(13)	di-O-acetyl-sarvero-genin(32)
1	CH_2	41.7	42.6	43.9	36.2
2	CH-O- or CH_2	71.1(a)(f)	70.7(a)	66.7	25.5
3	CH-O-	66.4(a)	66.6(a)	69.8	69.0
4	CH_2	32.0(b)	33.1(b)	34.9	32.0
5	CH	44.8	43.6	40.5	32.9
6	CH_2	27.7(c)	27.2(c)	35.6	35.5
7	CH_2 or CH-O-	27.2(c)	27.4(c)	54.3	52.7
8	CH or C-O-	40.8	35.7(e)	62.6	63.0
9	CH	49.6	48.6	45.2	32.9
10	$>C<$	38.0	70.8	37.7	34.4
11	CH_2 or CH-OAc	21.3	21.9	79.3	75.5
12	CH_2 or C=O	39.6	39.5(e)	204.6	204.6
13	$>C<$	49.6	49.4	64.1	64.3
14	C-OH	85.1	85.0	80.9	81.1
15	CH_2	32.9(b)	32.4(b)	28.4	28.5
16	CH_2	26.9(c)	26.9(c)	26.6	26.6
17	CH	50.8	50.7	41.8	41.8
18	CH_3	15.7	15.6	17.3	17.5
19	CH_3 or $C<^{O}_{H}$	13.7	206.4	13.7	23.0
20	C=	174.9	174.2	170.9	171.1
21	CH_2-O-	73.5	73.4	73.7	73.7
22	CH=	117.5	118.0	118.9	118.7
23	C=O	174.6(g)	173.9	173.8	173.9
1'	$HC<^{O-}_{O-}$	93.2	93.2	93.1	-
2'	$>C<^{O-}_{O-}$	95.6	95.6	95.6	-
3'	CHOAc	70.3(h)	70.5	70.9(b)	-
4'	CH_2	34.8	35.0	31.5	-
5'	CHO-	71.8(h)	71.2	70.3(b)	-
6'	CH_3	20.9(d)	20.8	20.9	-
acetyl	CH_3-CO	20.7(d)	20.8	20.7	20.8
"	"	21.7	21.6	21.8	21.4
"	"	-	-	21.2	-
acetyl	CH_3-CO	168.8	165.5	169.7	169.5
"	"	168.7	168.8	168.8	170.4
"	"	-	-	168.3	-

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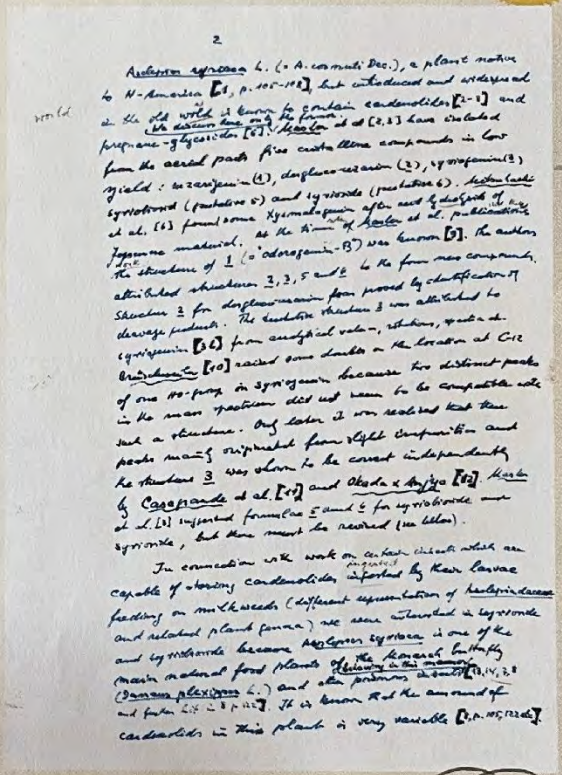

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