Supplementary Information

Deciphering sphaeropsidin A's chemical instability under physiological conditions – degradation studies and elucidation of the major metabolite

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List of Tables and Figures

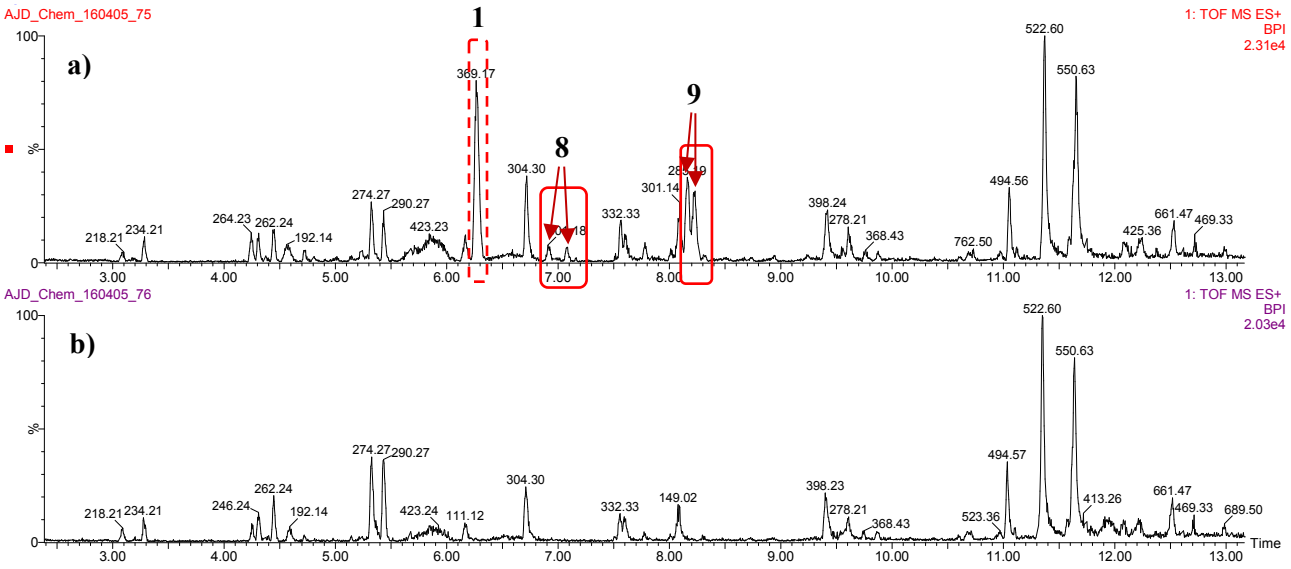

Figure S1: Base peak ion chromatogram (ESI positive mode) of SphA **1** incubated in water/DMSO (9:1) for 55 h. a) Sample containing SphA **1** along with minor metabolites **8** and **9** (arrows indicate separated peaks having the same MS i.e. potential isomers) compared to b) blank sample.

Table S1: LC-MS data of SphA **1** and minor metabolites **8** and **9**.

Product	**1**	**8**	**9**
Accurate mass (*m/z*), Elemental composition	$[M+H]^+$: 347.1861, ($C_{20}H_{27}O_5$)	$[M+H]^+$: 301.1773, ($C_{19}H_{25}O_3$)	$[M+H]^+$: 285.1852, ($C_{19}H_{25}O_2$)
	$[M+H-H_2O]^+$: 329.1750, ($C_{20}H_{25}O_4$)	$[M+H-H_2O]^+$: 283.1626, ($C_{19}H_{23}O_2$)	$[M+H-H_2O]^+$: 267.1736 ($C_{19}H_{23}O$)
	$[M+Na]^+$: 369.1673, ($C_{20}H_{26}O_5Na$)	$[M+Na]^+$: 323.1586, ($C_{19}H_{24}O_3Na$)	-
Retention time (min)	6.26	6.93, 7.08	8.16, 8.23

Note: Each of the two chromatographic peaks of products **8** and **9** appearing at the indicated retention times exhibit the same accurate mass, indicative of respective isomer formation.

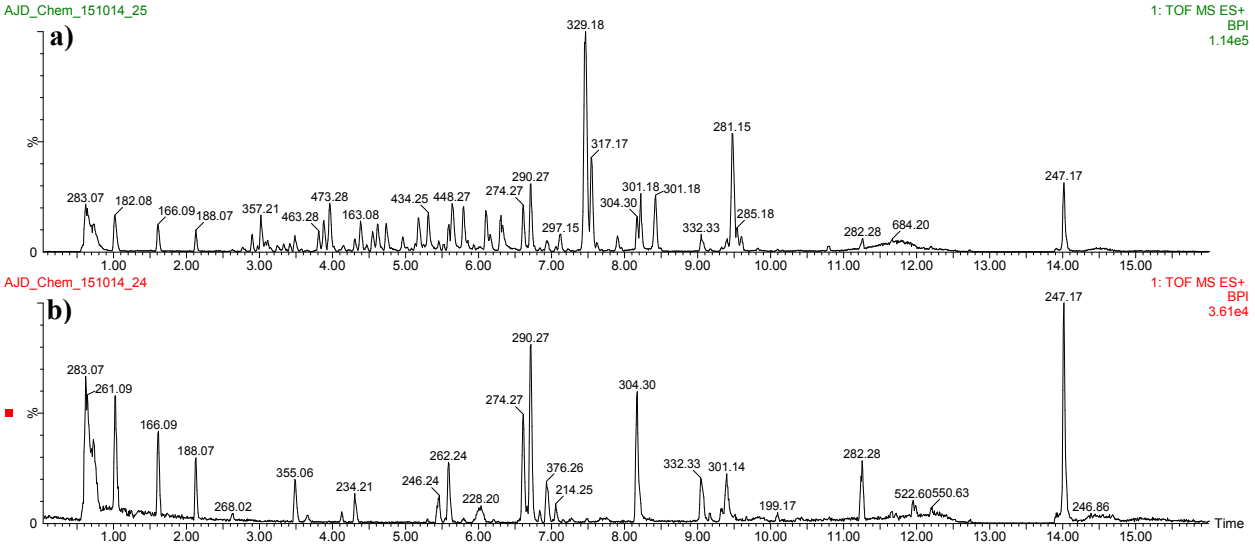

Figure S2: Comparison of base peak ion chromatogram of: a) SphA **1** incubated for 31 h in MEM and b) blank sample comprising of MEM/DMSO (9:1), incubated for 31 h under the same conditions. Note: the y-axis (intensity) scale for the blank sample in b) is ~3× higher to highlight the presence of minor peaks.

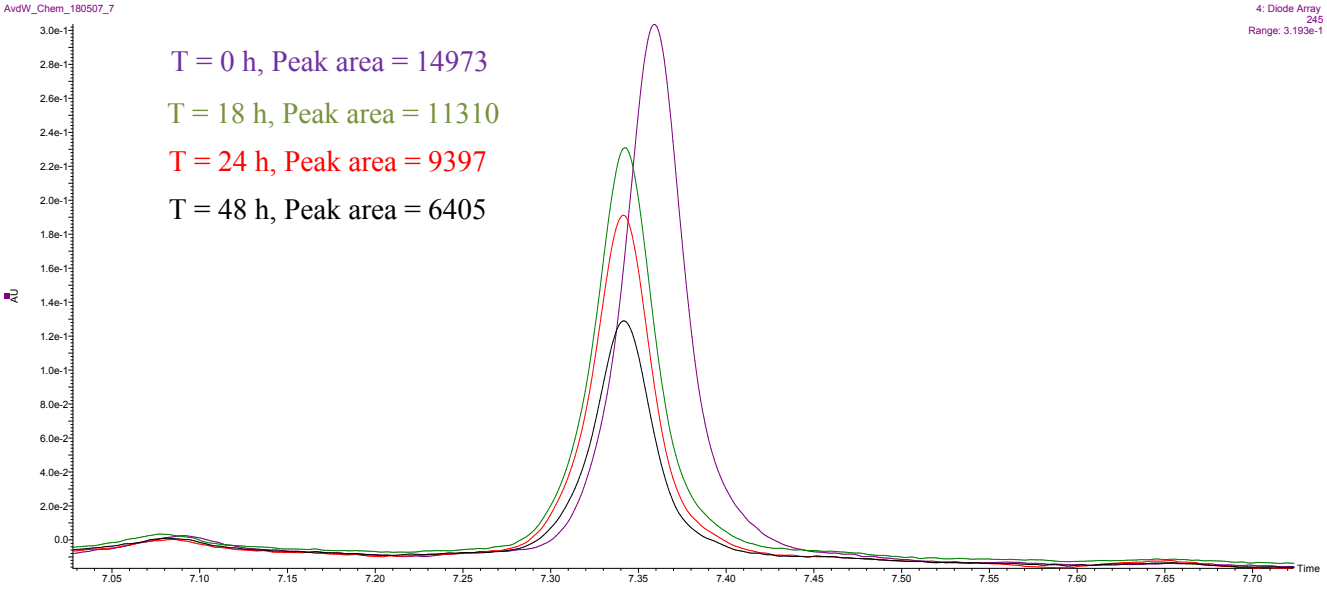

Figure S3: Overlaid UV chromatograms of the SphA **1** peak (extracted at 245 nm) acquired over period of 48 h when incubated in MEM/DMSO (9:1). Apart from SphA being clearly apparent at the retention time of 7.45 min, the complexity of the data over the extended incubation period complicated analysis of the degradation metabolites (see S2). However, the data did confirm the instability of SphA in cell culture medium. This behaviour was further corroborated by analysis of the UV (λ_{max} at 245 nm) data, displaying a clear decrease in concentration of SphA over 48 h of incubation (Figure S3 above). Peak area integration revealed that within 24 h of incubation, approximately 40% of SphA had degraded – further validating the results reported by Lallemand *et al.*, as well as our findings with conventional HPLC methodology.[1]

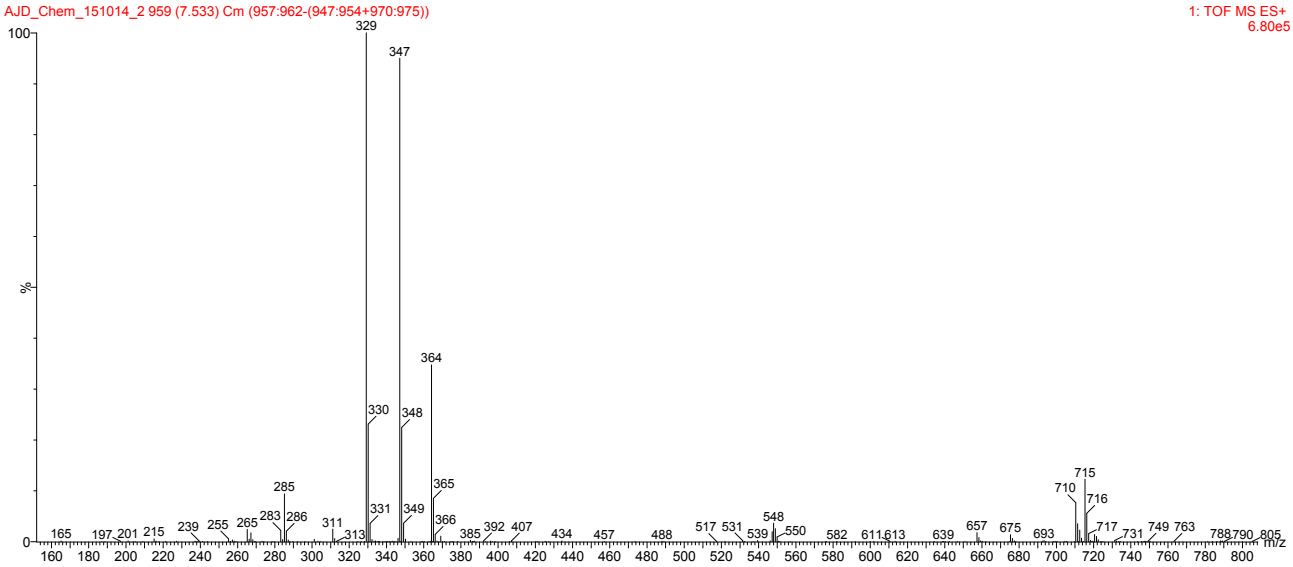

Figure S4: Mass spectrum (ESI positive mode) of SphA **1** (eluted at 7.45 min) incubated in MEM cell medium (t = 0).

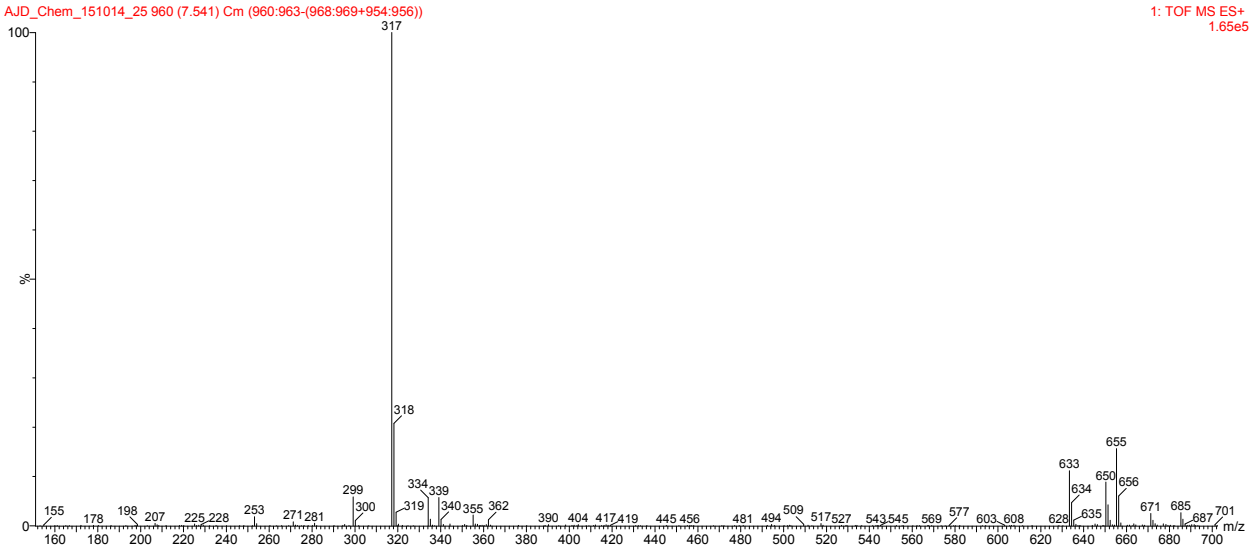

Figure S5: Mass spectrum (ESI positive mode) of major SphA degradation metabolite **10** (eluted at 7.54 min) incubated in MEM cell medium (t = 24).

Table S2: LC-MS data of SphA **1** and major metabolite **10**.

Product	**1**	**10**
Accurate mass (*m/z*)	$[M+H]^+$: 347.1862	$[M+H]^+$: 317.1743
	$[M+H-H_2O]^+$: 329.1756	$[M+H-H_2O]^+$: 299.1626
	$[M+NH_4]^+$: 364.2112	$[M+NH_4]^+$: 334.2017
	$[M+Na]^+$: 369.1682	$[M+Na]^+$: 339.1566
	$[2(M)+Na]^+$: 715.3419	$[2(M)+Na]^+$: 655.3236

	[M+H−CO_2−H_2O]$^+$:285.1839	[M+K]$^+$: 355.1382
Retention time (min)	7.45	7.54

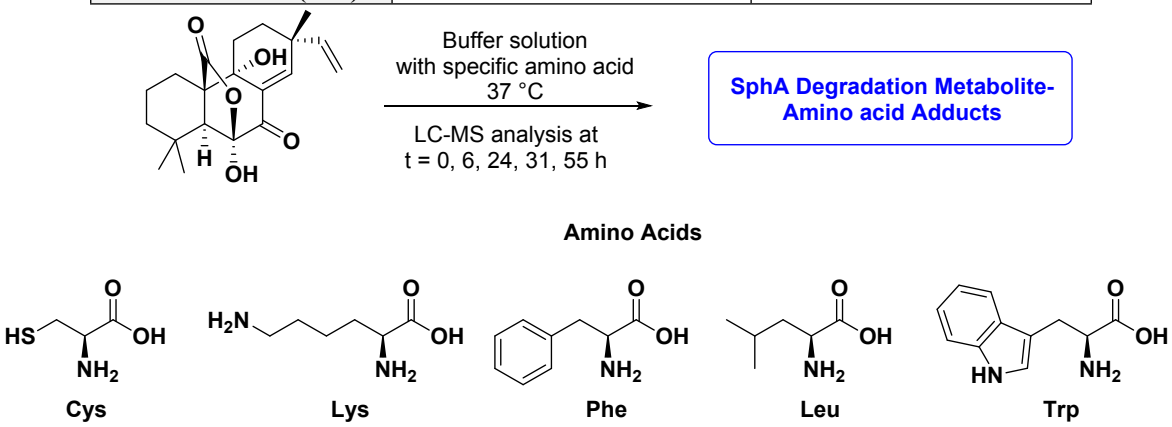

Figure S6: Investigating SphA degradation products-amino acid adducts. Initially studied SphA **1** in buffer-only media (buffer/DMSO, 9:1) to determine molecular ions [M+H]$^+$ of SphA **1** degradation products (DP). Experiments whereby a specific amino acid is introduced and analysed in the SphA **1**/buffer system propose the formation of several SphA degradation products-amino acid adducts. Analysis of adduct formation *via* LC-MS.

The exploration of the LC-MS data of SphA incubated in this buffer-only solution revealed a similar, yet less complex, degradation behaviour compared to experiments implementing MEM cell medium. The chromatographic and HR-MS data of SphA, incubated in the buffer-only solution, was scrutinised to reveal the generation of several possible SphA degradation products (Table S3).

Table S3: SphA degradation compounds as proposed from SphA **1**/buffer incubation experiment.

SphA & its Degradation Products	MW (g/mol)	Retention Time (min)	Experimental [MW+H]$^+$ *m/z*	Theoretical [MW+H]$^+$ *m/z*	Molecular Formula*	PPM
1	346	6.27	347.1855	347.1858	$C_{20}H_{26}O_5$	-0.9
10	316	6.36	317.1755	317.1753	$C_{19}H_{24}O_4$	0.6
	314	5.01	315.1949	315.1960	$C_{20}H_{26}O_3$	-3.5
8	300	6.91, 7.07	301. 1796	301.1804	$C_{19}H_{24}O_3$	-2.7
	298	4.19, 4.97	299.1652	299.1647	$C_{19}H_{22}O_3$	1.7
	296	4.01, 4,57, 4.73	297.1486	297.1491	$C_{19}H_{20}O_3$	-1.7
9	284	8.16, 8.23	285.1863	285.1855	$C_{19}H_{24}O_2$	2.8
	282	6.62, 7.08	283.1690	283.1698	$C_{19}H_{22}O_2$	-2.8
	182	3.52	183.1018	183.1021	$C_{10}H_{14}O_3$	-1.6
	162	3.26	163.0751	163.0759	$C_{10}H_{10}O_2$	-4.9

* Represents molecular formula of the neutral species.

Table S4: Proposed SphA degradation products (DP)-amino acid adducts as determined from the identified molecular ions.

SphA Degradation Product (DP) MW (g/mol)	Adduct MW (g/mol)	Experimental $[MW+H]^+$ *m/z*	Theoretical $[MW+H]^+$ *m/z*	Molecular Formula*	Description
SphA DP-Cys Adduct					
316	437.5496	438.1931	438.1950	$C_{22}H_{31}NO_6S$	SphA DP+Cys
298	419.5344	420.1845	420.1845	$C_{22}H_{29}NO_5S$	SphA DP+Cys
297	417.5185	418.1690	418.1688	$C_{22}H_{27}NO_5S$	SphA DP+Cys
297	538.6767	538.1883	539.1886	$C_{25}H_{34}N_2O_7S_2$	SphA DP+(2×Cys)
282	403.5350	404.1904	404.1896	$C_{22}H_{29}NO_4S$	SphA DP+Cys
SphA DP-Lys Adduct					
316	462.5790	463.2808	463.2808	$C_{25}H_{38}N_2O_6$	SphA DP+Lys
298	444.5637	445.2698	445.2702	$C_{25}H_{36}N_2O_5$	SphA DP+Lys
182	328.4039	329.2073	329.2076	$C_{16}H_{28}N_2O_5$	SphA DP+Lys
SphA DP-Phe Adduct					
316	481.5806	482.2537	482.2543	$C_{28}H_{35}NO_6$	SphA DP+Phe
298	463.5653	464.2429	464.2437	$C_{28}H_{33}NO_5$	SphA DP+Phe
182	347.4055	348.1803	348.1811	$C_{19}H_{25}NO_5$	SphA DP+Phe
SphA DP-Leu Adduct					
316	447.5644	448.2698	448.2699	$C_{25}H_{37}NO_6$	SphA DP+Leu
182	313.3893	314.1966	314.1967	$C_{16}H_{27}NO_5$	SphA DP+Leu
SphA DP-Trp Adducts					
316	520.6166	521.2642	521.2652	$C_{30}H_{36}N_2O_6$	SphA DP+Trp
298	502.6014	503.2534	503.2546	$C_{30}H_{34}N_2O_5$	SphA DP+Trp
182	386.4415	387.1917	387.1920	$C_{21}H_{26}N_2O_5$	SphA DP+Trp

* Represents molecular formula of the neutral species.

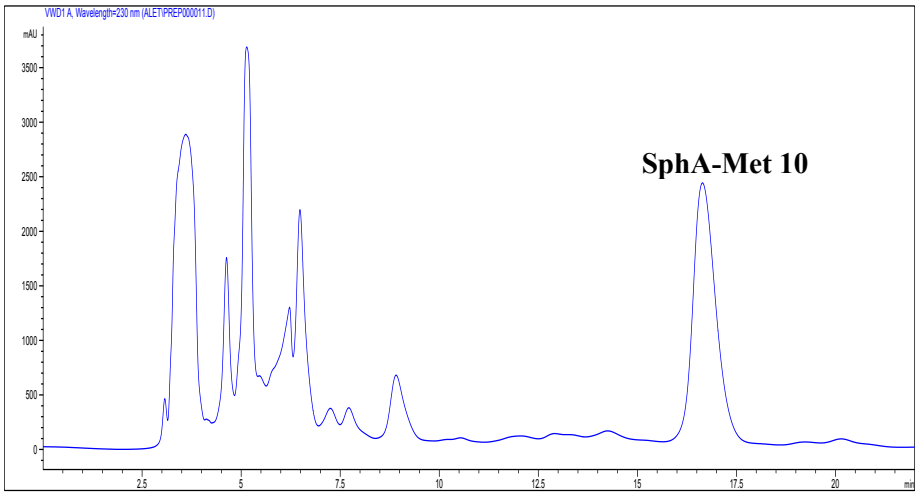

Figure S7: Sample of crude SphA **1** degradation mixture used for the isolation of SphA-Met **10**. Representation of UV chromatogram acquired and utilised during preparative HPLC isolation of SphA-Met **10** (15.8–17.7 min).

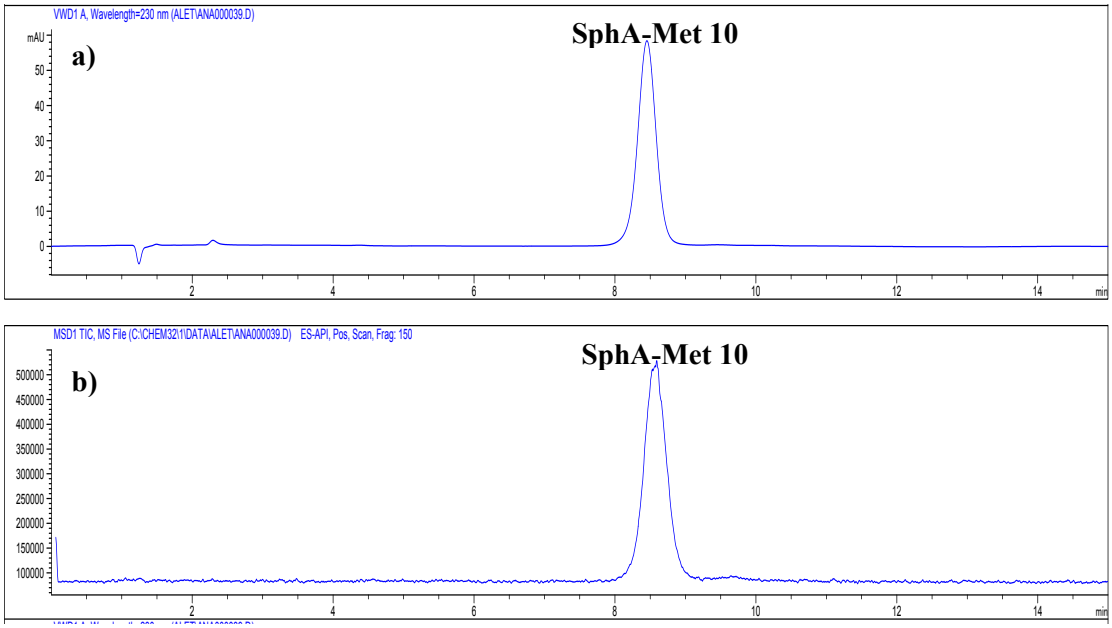

Figure S8: Purity analysis of isolated fraction of SphA-Met **10**. a) UV and b) total ion chromatogram of the analytical separation of SphA-Met **10**.

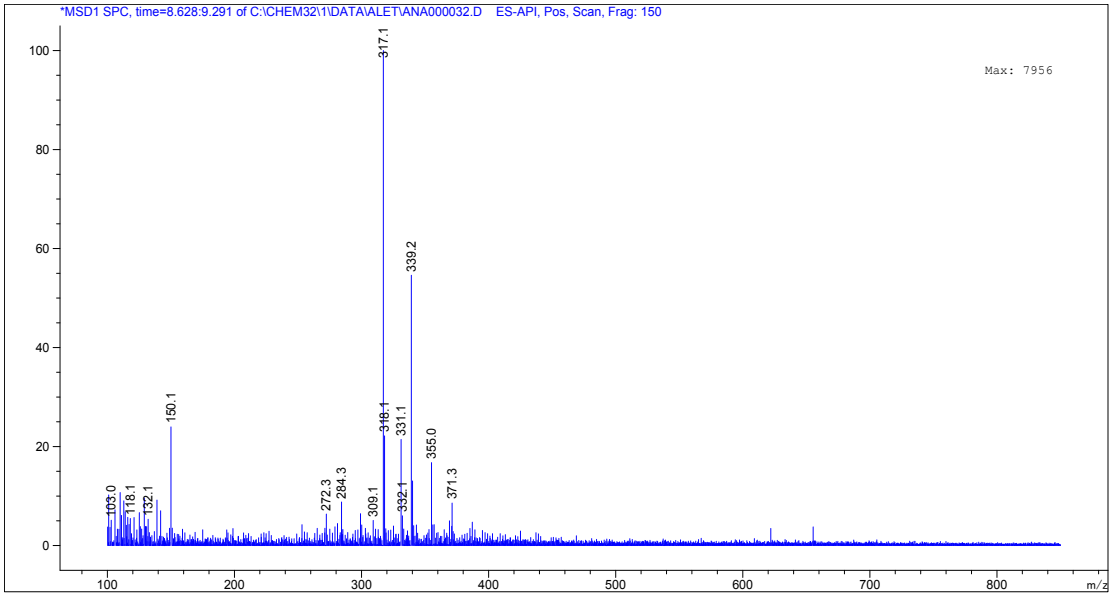

Figure S9: Mass spectrum of SphA-Met **10** peak eluted at 8.65 min (from chromatogram (b) in Figure S8) obtained on a single quadrupole instrument.

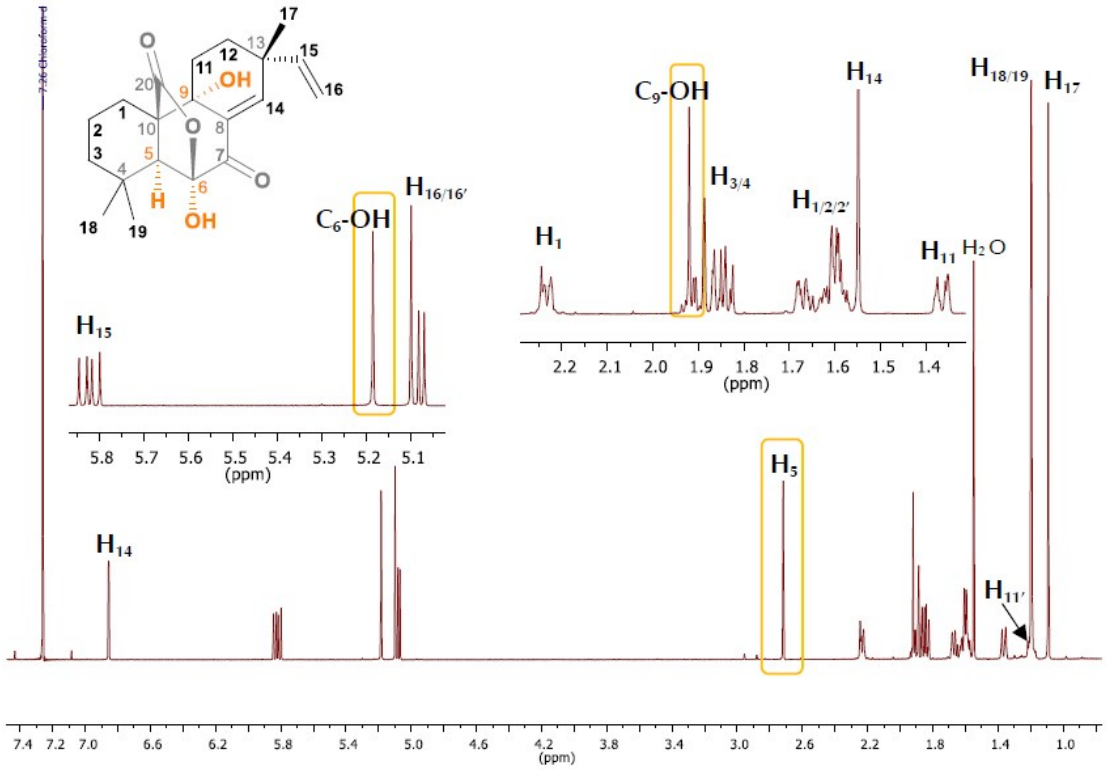

Figure S10: ^{1}H NMR spectrum ($CDCl_3$) of SphA **1**. Assignments made according to Evidente *et al*.[2] Highlighted signals C_6-OH, H_5 and C_9-OH are missing from the ^{1}H NMR spectrum of SphA-Met **10** (shown below).

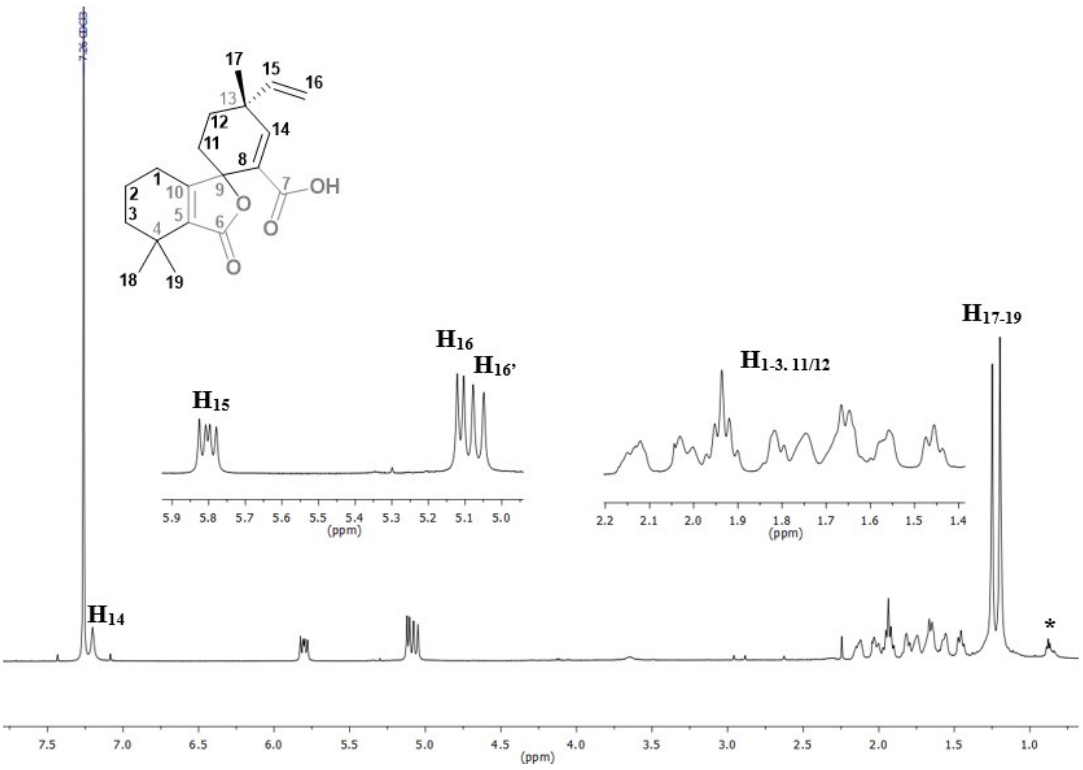

Figure S11: ^{1}H NMR spectrum ($CDCl_3$) of SphA-Met **10**. (* = grease)

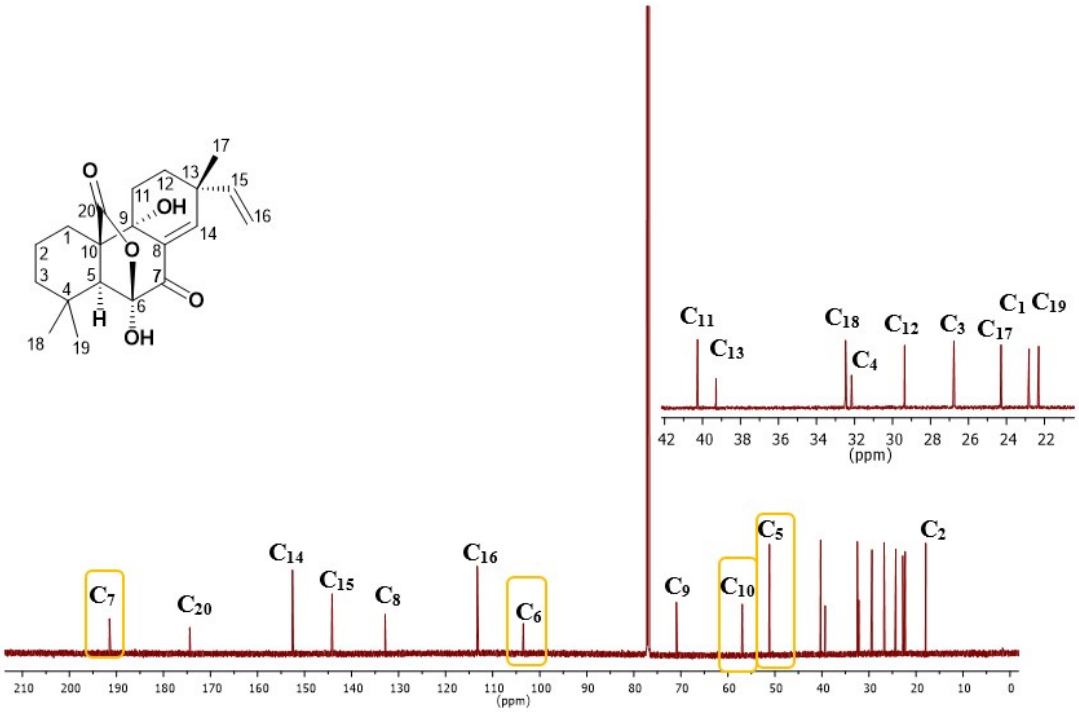

Figure S12: ^{13}C NMR spectrum ($CDCl_3$) of SphA **1**. Assignments made according to Evidente *et al*. Highlighted signals C_7, C_6, C_{10} and C_5 are missing from the ^{13}C NMR spectrum of SphA-Met **10** (shown below). C_{18}/C_{19} interchangeable.

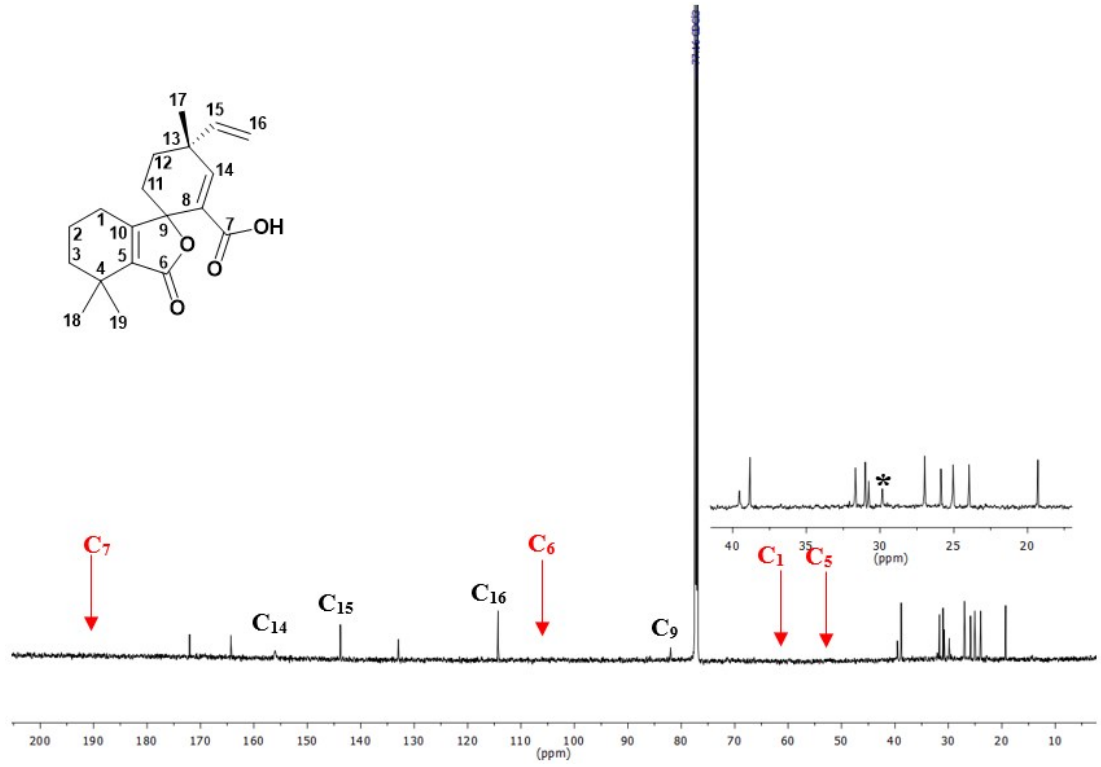

Figure S13: ^{13}C NMR spectrum ($CDCl_3$) of SphA-Met **10**. (* = grease)

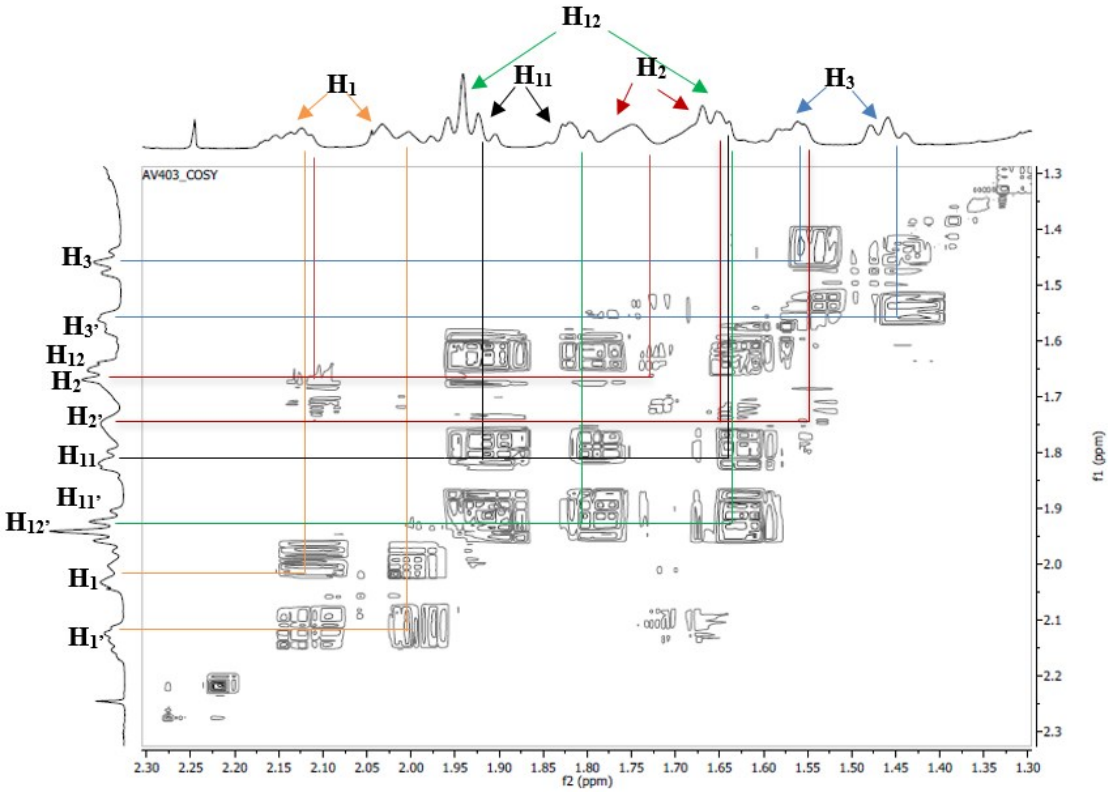

Figure S14: Expansion of COSY NMR spectrum of SphA-Met **10**.

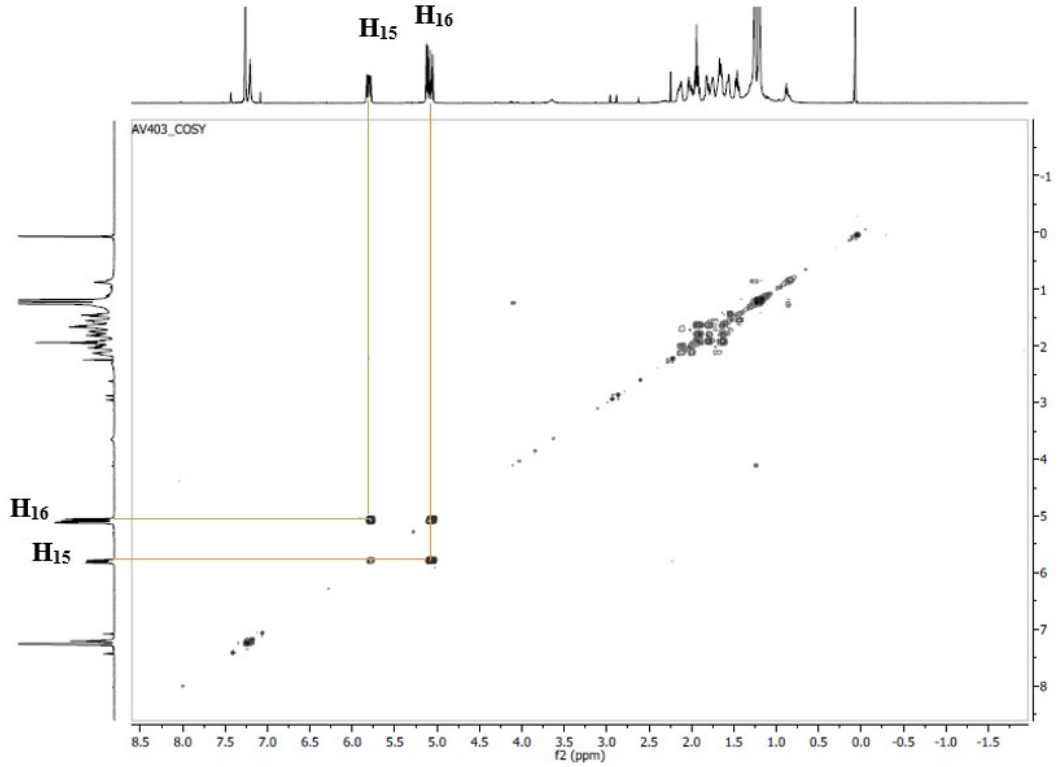

Figure S15: COSY NMR spectrum of SphA-Met **10**.

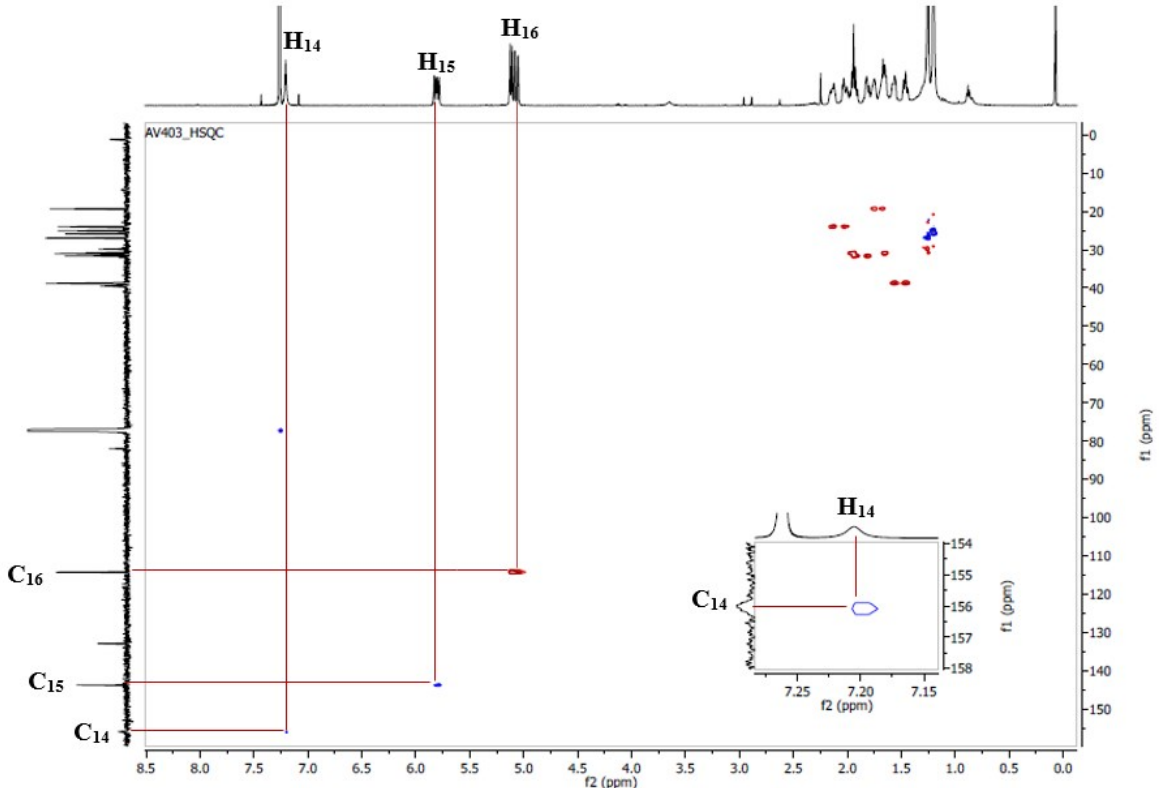

Figure S16: HSQC NMR spectrum of SphA-Met **10**.

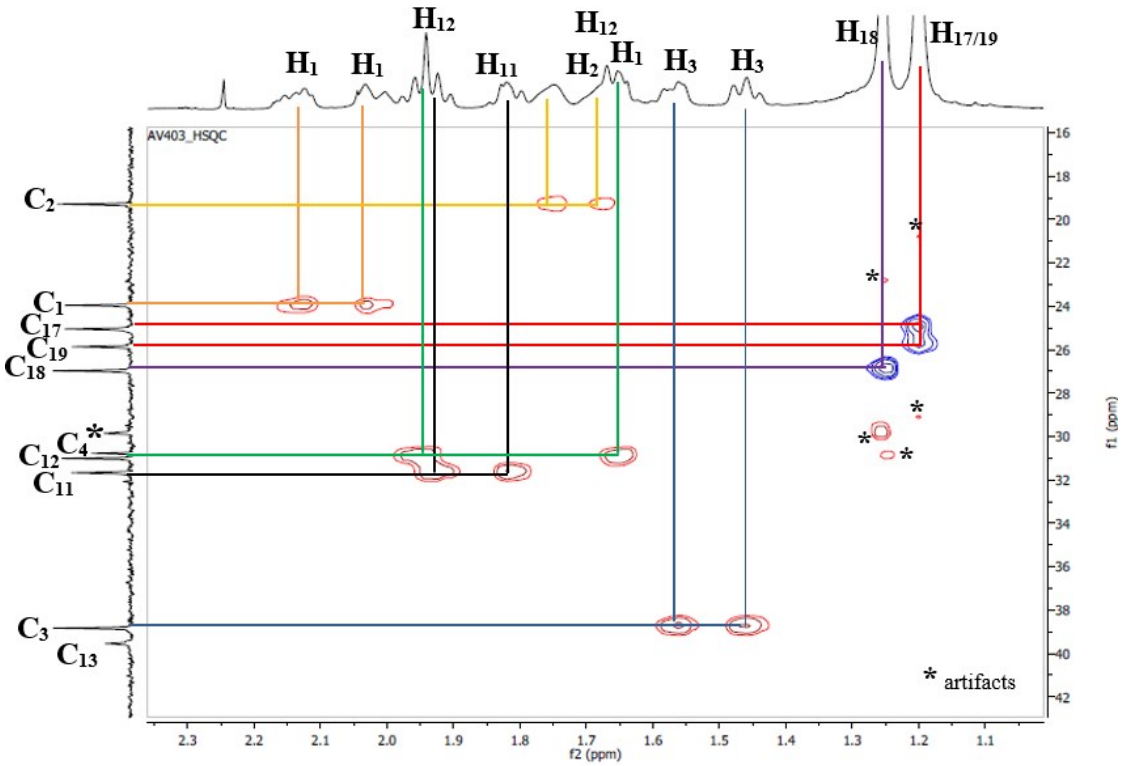

Figure S17: Expansion of aliphatic region of HSQC NMR spectrum of SphA-Met **10**. ^{13}C and ^{1}H NMR signals of 18 and 19 are interchangeable.

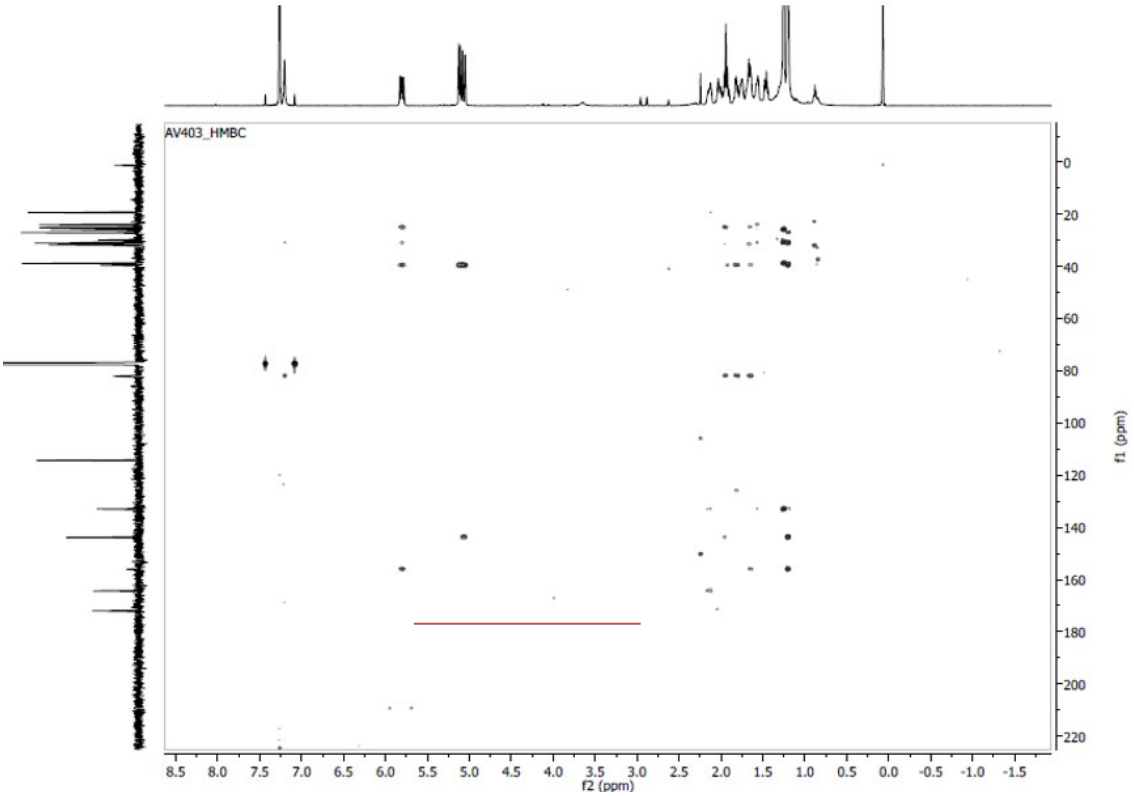

Figure S18: HMBC ($^{n}J_{CH}$ = 8.0 Hz) NMR spectrum of SphA-Met **10**.

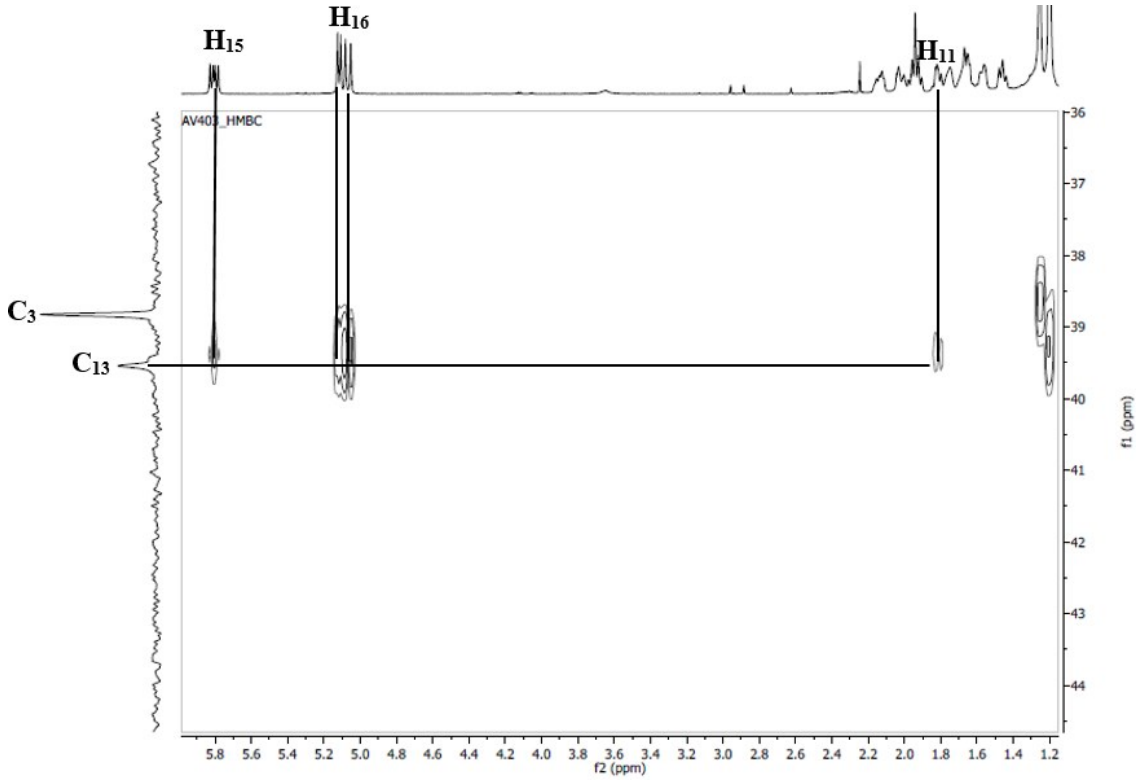

Figure S19: Expansion of HMBC NMR spectrum of SphA-Met **10**, showing coupling of C_{13}/H_{15}, H_{16} and H_{11}

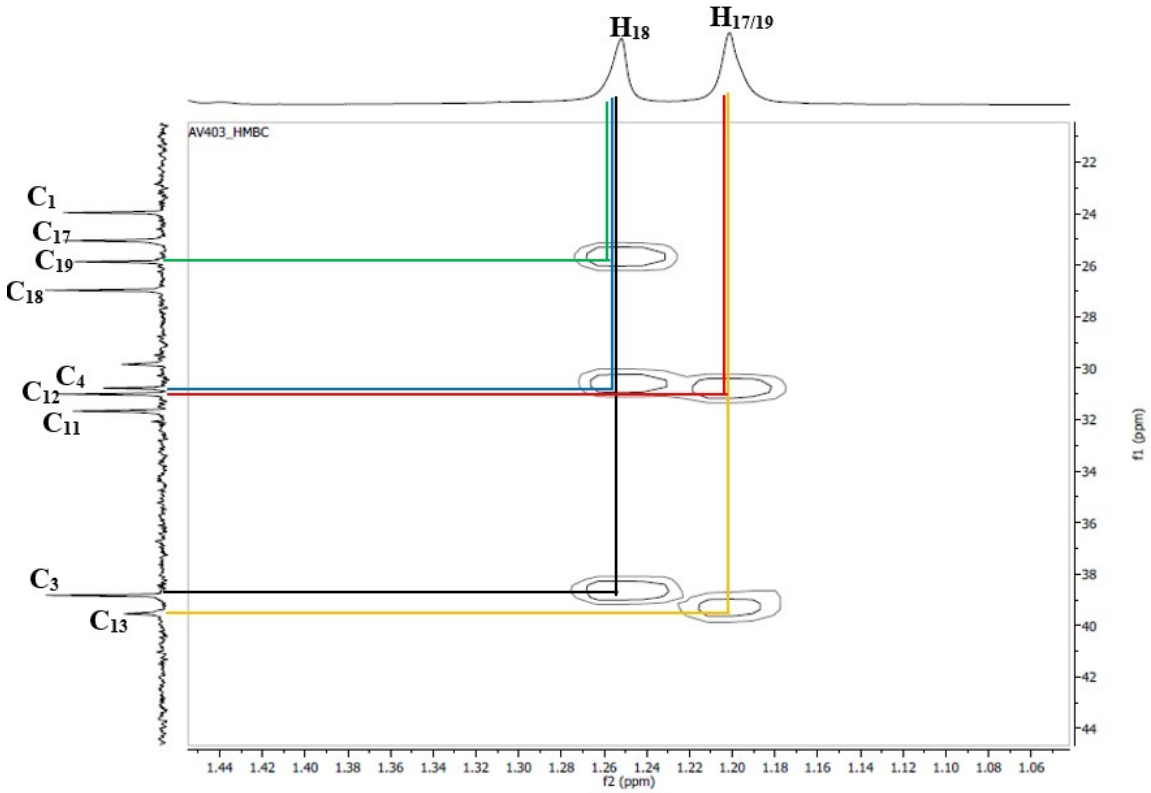

Figure S20: Expansion of HMBC NMR spectrum of SphA-Met **10**, showing coupling of C_{13}/H_{17}; C_3/H_{18}; C_{12}/H_{17}; C_4/H_{18}; C_{19}/H_{18}. ^{13}C and ^{1}H NMR signals of 18 and 19 are interchangeable.

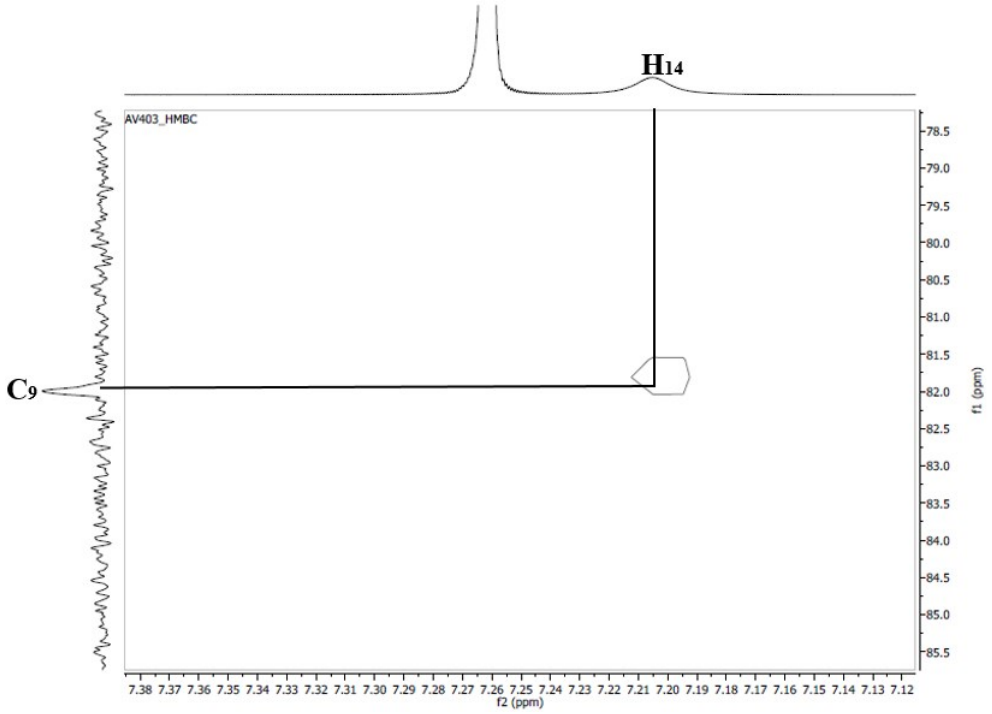

Figure S21: Expansion of HMBC NMR spectrum of SphA-Met **10**, showing coupling of C_9/H_{14}.

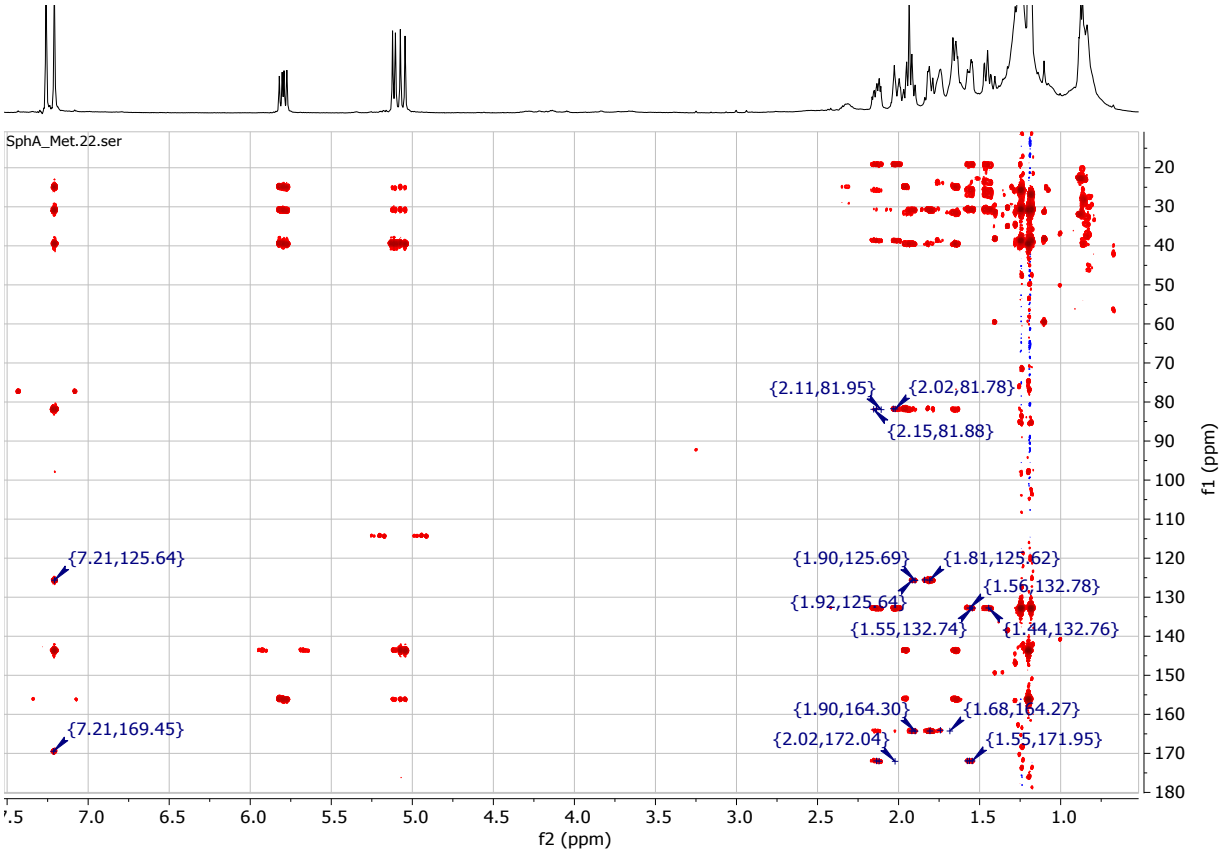

Figure S22: Additional HMBC ($^{n}J_{CH}$ = 3.5 Hz) NMR spectrum of SphA-Met **10**, showing weaker ^{1}H–^{13}C correlations and cross-peaks identifying C_7 (169.5 ppm) and C_8 (125.6 ppm).

References:

1. B. Lallemand, M. Masi, L. Maddau, M. De Lorenzi, R. Dam, A. Cimmino, L. Moreno y Banuls, A. Andolfi, R. Kiss, V. Mathieu and A. Evidente, *Phytochem. Lett.*, 2012, 5, 770-775.
2. A. Evidente, L. Sparapano, A. Motta, F. Giordano, O. Fierro and S. Frisullo, *Phytochemistry*, 1996, 42, 1541-1546.