Combining OSMAC, metabolomic and genomic methods for the production and annotation of halogenated azaphilones and ilicicolins in termite symbiotic fungi

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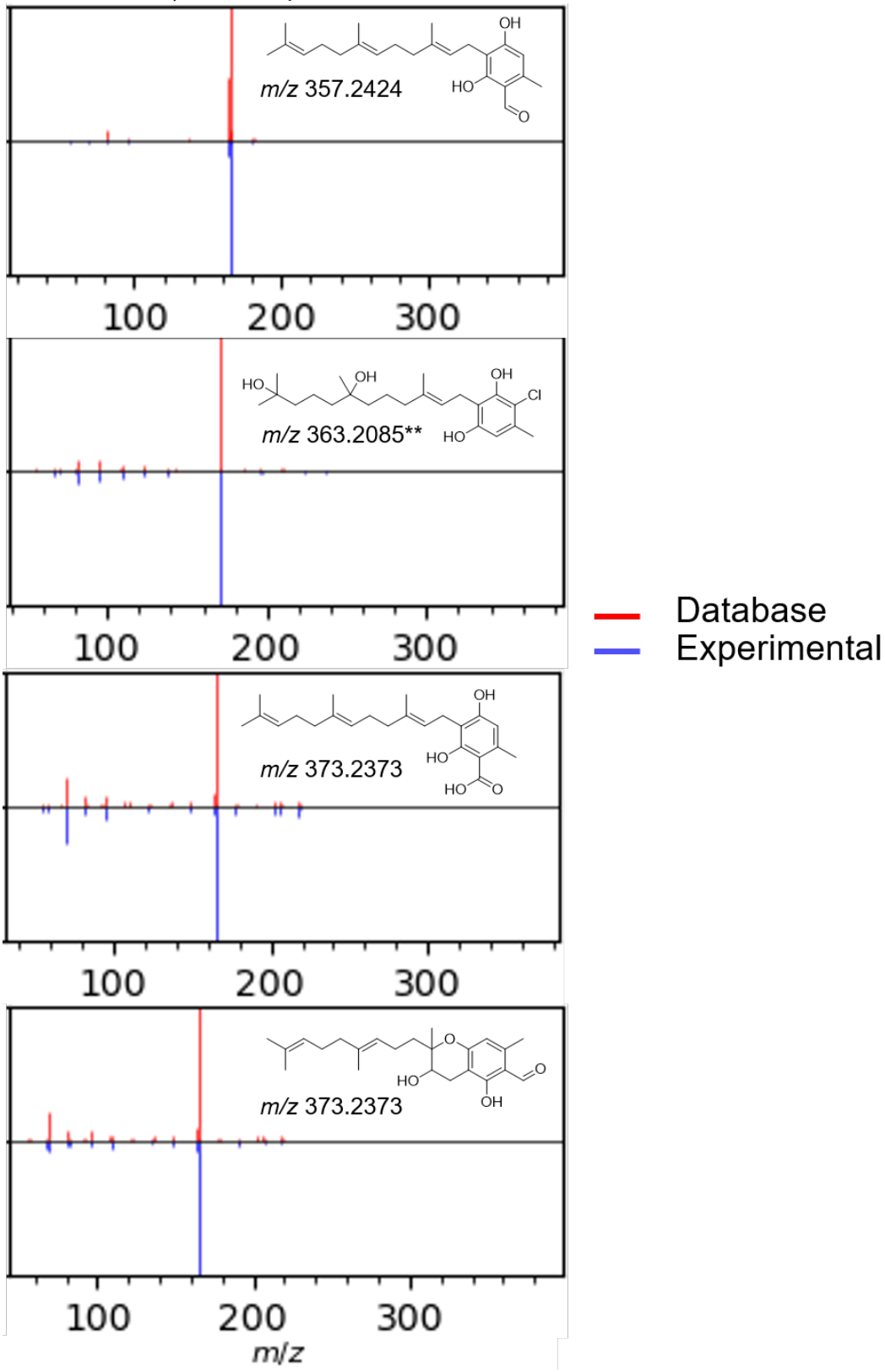
Supplementary data

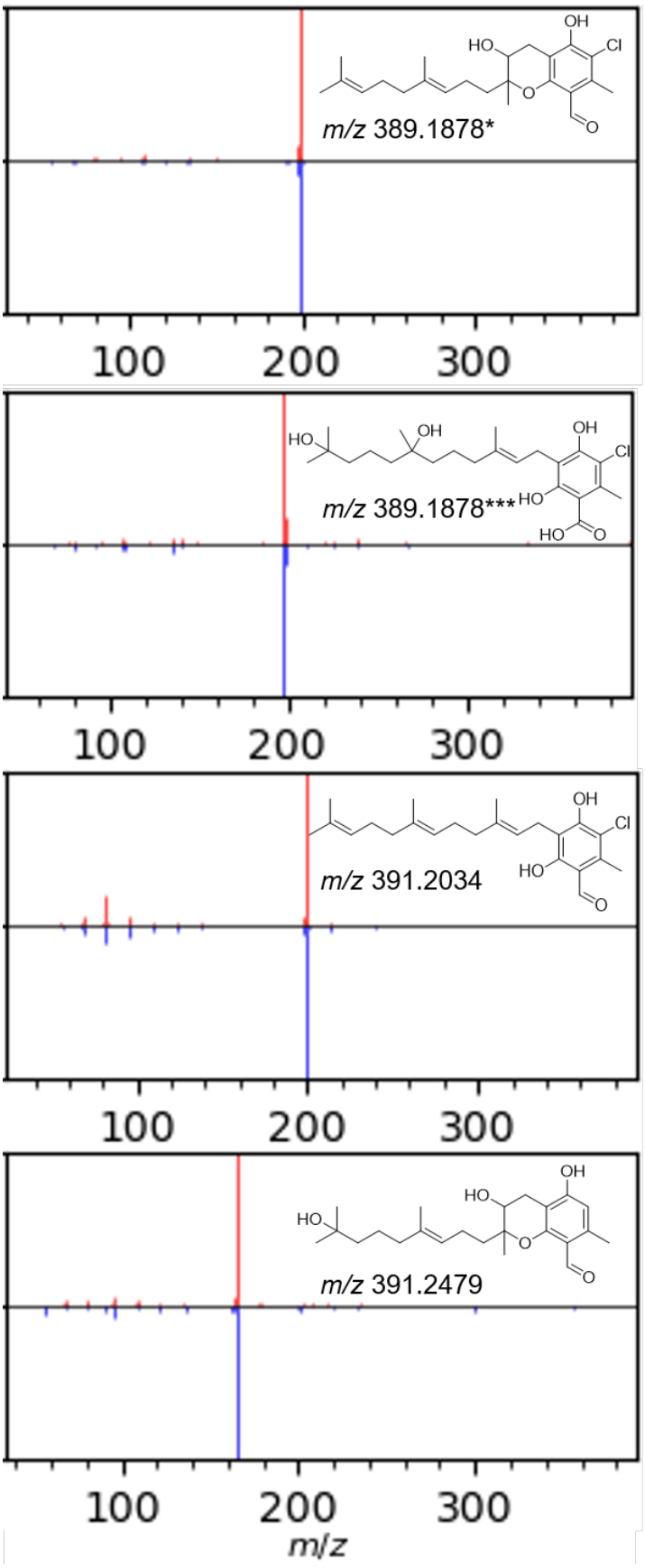
Table S1: Dereplicated ilicicolins from *N. discophora* crude extract.

CN63								
$[M+H]^+$	Error (ppm)	RT	Formula	Name	Database	Cosine score	Annotation*	Ref
357.2422	0.6	14.9	$C_{23}H_{32}O_3$	LL-Z 1272β	GNPS	0.91	Level 2	[6]
363.2080	1.5	8.6	$C_{22}H_{35}ClO_4$	Ilicicolinol-$2H_2O$	In-House	0.92	Level 1	[7]
373.2364	2.5	16.5	$C_{23}H_{32}O_4$	Ilicicolinic acid B	In-House	0.93	Level 1	[8]
373.2373	0.0	13.2	$C_{23}H_{32}O_4$	Ilicicolinal G	In-House	0.85	Level 1	[7]
389.1867	2.8	14.7	$C_{23}H_{31}ClO_4$	Ilicicolinal H-H_2O	In-House	0.92	Level 1	[7]
389.1873	1.3	8.5	$C_{23}H_{35}ClO_6$	Ilicicolinic acid E-$3H_2O$	In-House	0.93	Level 1	[7]
391.2028	1.7	15.9	$C_{23}H_{31}ClO_3$	LL-Z1272α	MS-DIAL	0.89	Level 2	[9]
391.2456	5.9	11.7	$C_{23}H_{34}O_5$	Ilicicolinal I	In-House	0.82	Level 1	[7]
407.1951	8.0	17.0	$C_{23}H_{31}ClO_4$	LL-Z 1272δ	MS-DIAL	0.69	Level 2	[10]
407.1985	-0.4	14.2	$C_{23}H_{31}ClO_4$	Ilicicolinic acid A	In-House	0.93	Level 1	[8]
421.1773	0.8	12.2	$C_{23}H_{29}ClO_5$	Ascofuranone	MS-DIAL	0.74	Level 2	[11]
425.2082	1.7	8.5	$C_{23}H_{33}ClO_5$	Ilicicolinic acid C	In-House	0.82	Level 1	[8]

*Level 0 corresponds annotations from isolated pure compounds; level 1 to annotations by comparison with standards; level 2 to putative annotations (e.g., MS/MS library comparison or tentative structure); level 3 to a chemical class assignment. RT = Retention Time

Figure S1: Dereplicated ilicicolins structures with their theoretical exact masses and experimental fragmentation spectrum comparison with databases (in red *m/z* of database, in blue experimental *m/z* obtained). * represents the amount of H_2O losses during ionization.

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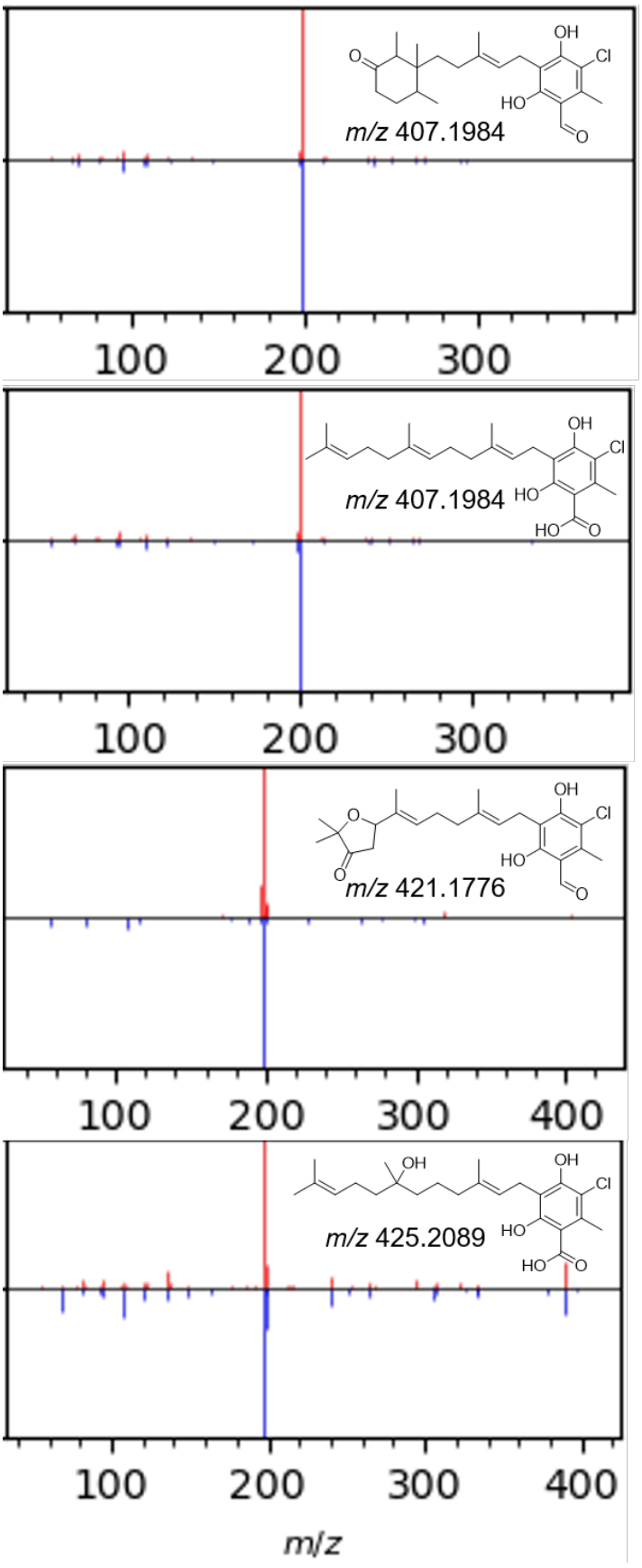
m/z
Database
Experimental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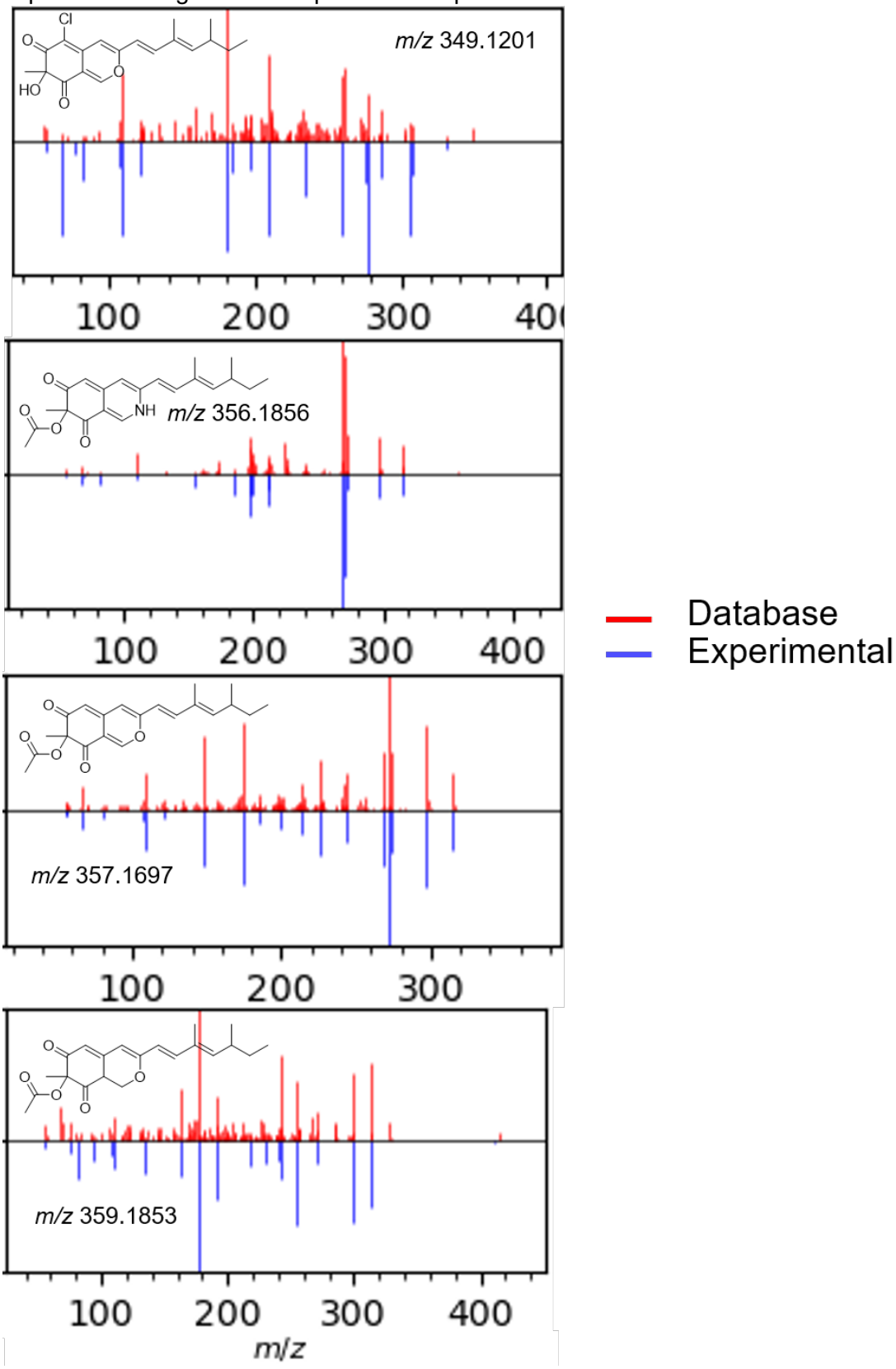
200
300
400
m/z
Database
Experimental

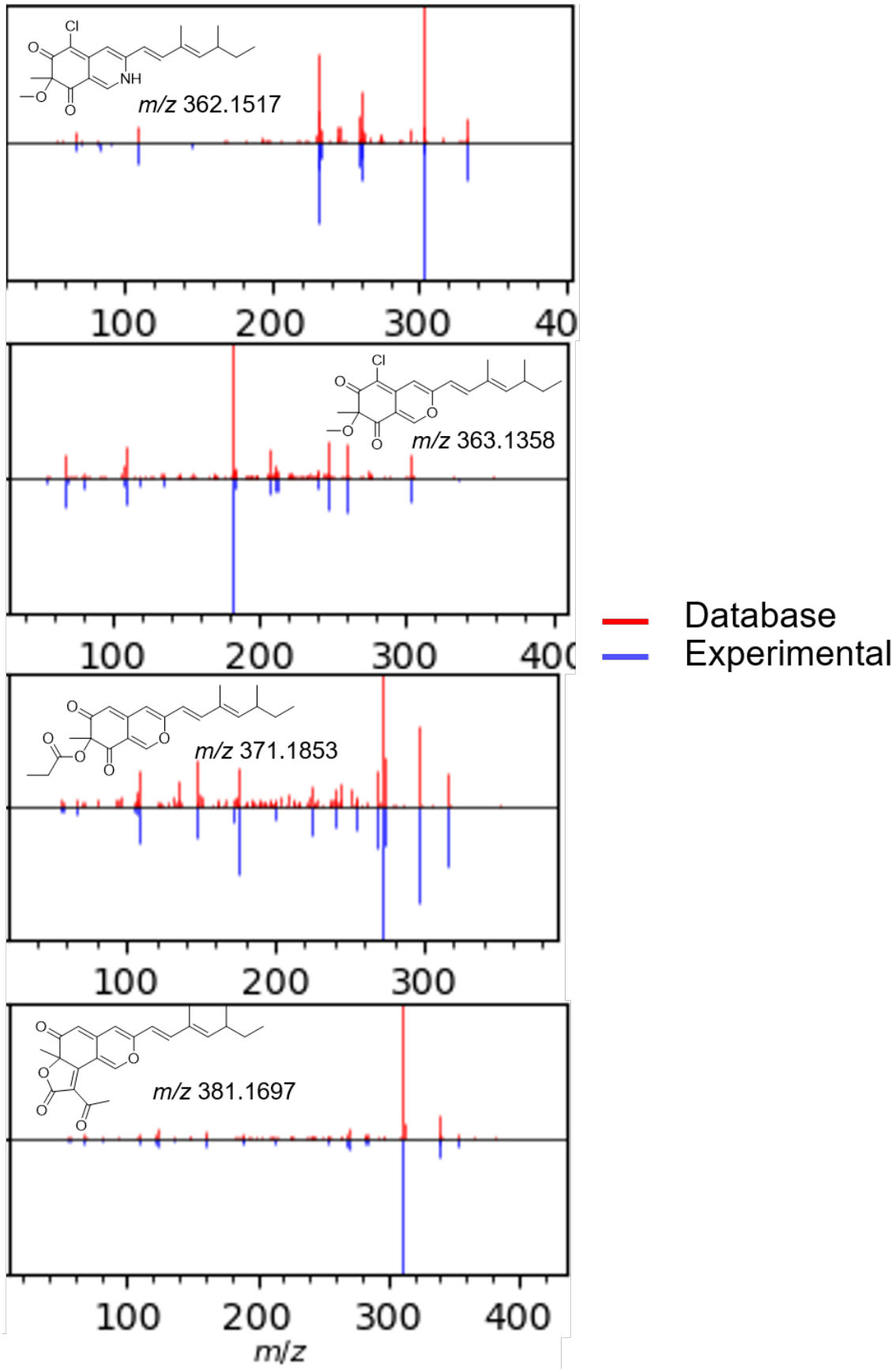
Table S2: Dereplicated azaphilones from *P. sclerotiorum* crude extract.

CN111							
$[M+H]^+$	Error (ppm)	Formula	Name	Database	Cosine score	Annotation*	Ref
349.1202	-0.3	$C_{19}H_{21}ClO_4$		In-House	0.65	Level 2	[1]
356.1853	1.0	$C_{21}H_{25}NO_4$		In-House	0.86	Level 2	[1]
357.1700	-1.0	$C_{21}H_{24}O_5$		In-House	0.95	Level 2	[1]
359.1857	-1.1	$C_{21}H_{26}O_5$		In-House	0.74	Level 2	[1]
362.1522	-1.3	$C_{20}H_{24}ClNO_3$		In-House	0.88	Level 2	[1]
363.1353	1.3	$C_{20}H_{24}ClO_4$		In-House	0.85	Level 2	[1]
371.1844	2.4	$C_{22}H_{26}O_5$		In-House	0.84	Level 2	[1]
381.1697	-0.1	$C_{23}H_{24}O_5$	Isorotiorin	In-House	0.96	Level 2	[2]
385.2007	0.7	$C_{23}H_{28}O_5$		In-House	0.92	Level 2	[1]
390.1474	-1.9	$C_{21}H_{24}ClNO_4$	Sclerotioramine	MONA	0.90	Level 1	[1, 2]
391.1309	-0.6	$C_{21}H_{23}ClO_5$	Sclerotiorine	MS-DIAL	0.82	Level 1	[1, 2]
391.2114	0.3	$C_{22}H_{30}O_6$		In-House	0.86	Level 2	[1]
399.2169	-0.8	$C_{24}H_{30}O_5$		In-House	0.92	Level 2	[1]
404.1627	-1.0	$C_{22}H_{26}ClNO_4$		In-House	0.96	Level 2	[1]
405.1468	-1.2	$C_{22}H_{25}ClO_5$	Sclerketide B	In-House	0.95	Level 2	[1, 3]
407.1256	0.0	$C_{21}H_{23}ClO_6$		In-House	0.86	Level 2	[1]
415.1299	1.9	$C_{23}H_{23}ClO_5$	5-Chloroisorotiorin	In-House	0.93	Level 1	[1, 2]
417.1902	1.4	$C_{23}H_{28}O_7$	Geumsanol B	In-House	0.86	Level 2	[4]
419.1617	0.7	$C_{23}H_{27}ClO_5$		In-House	0.94	Level 2	[1]
433.1773	0.8	$C_{24}H_{29}ClO_5$		In-House	0.69	Level 2	[1]
434.1731	-0.5	$C_{23}H_{28}ClNO_5$	Isochromophilone VI	MSDIAL	0.83	Level 1	[1,5]
451.1526	1.3	$C_{23}H_{27}ClO_7$	Chlorogeumsanol B	In-House	0.84	Level 1	[1]
500.1839	-0.9	$C_{27}H_{30}ClNO_6$		In-House	0.96	Level 2	[1]

*Level 0 corresponds annotations from isolated pure compounds; level 1 to annotations by comparison with standards; level 2 to putative annotations (e.g., MS/MS library comparison or tentative structure); level 3 to a chemical class assignment.

Figure S2: Dereplicated azaphilone structures with their theoretical exact masses and experimental fragmentation spectrum comparison with databases.

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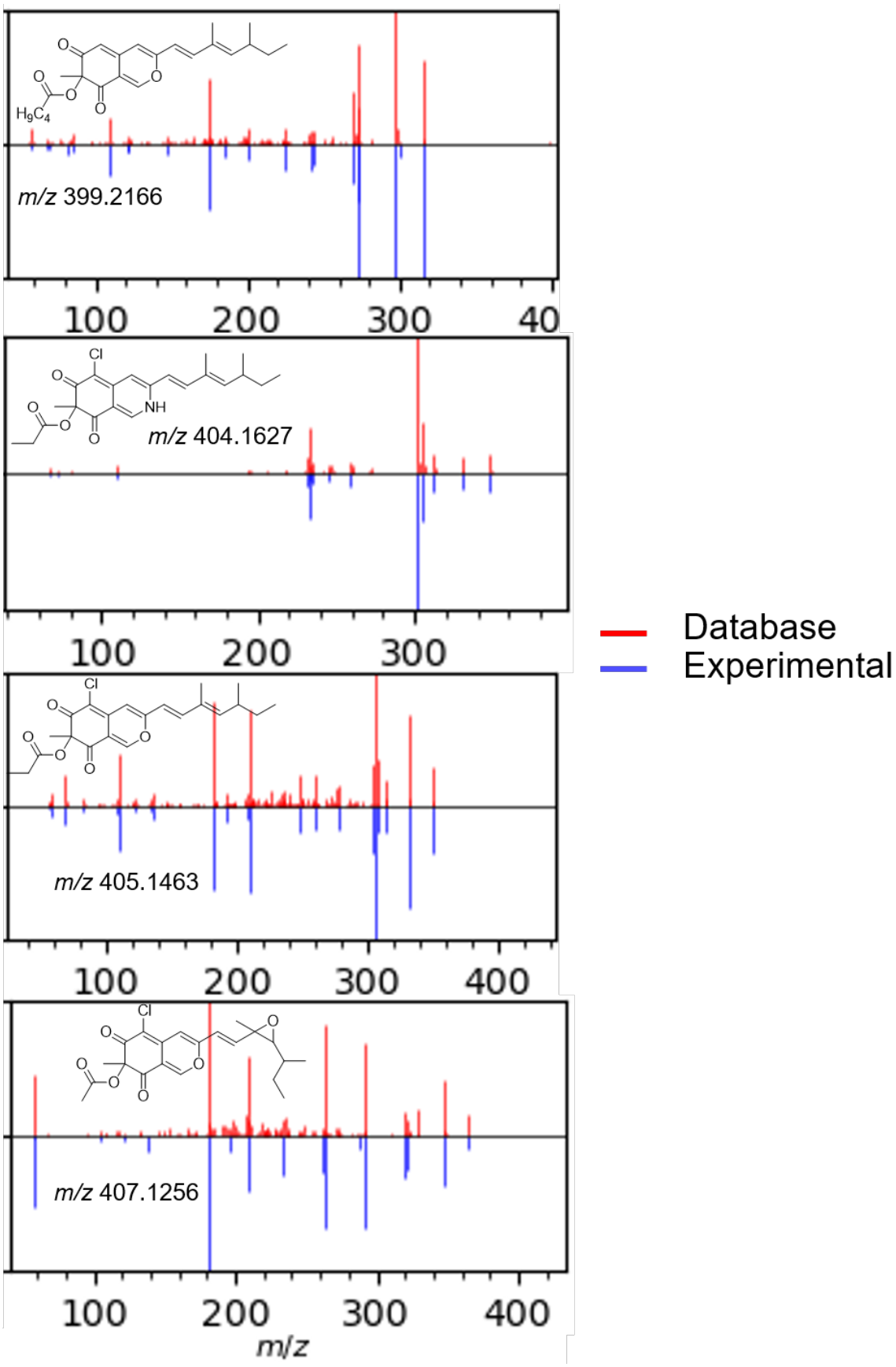
Experimental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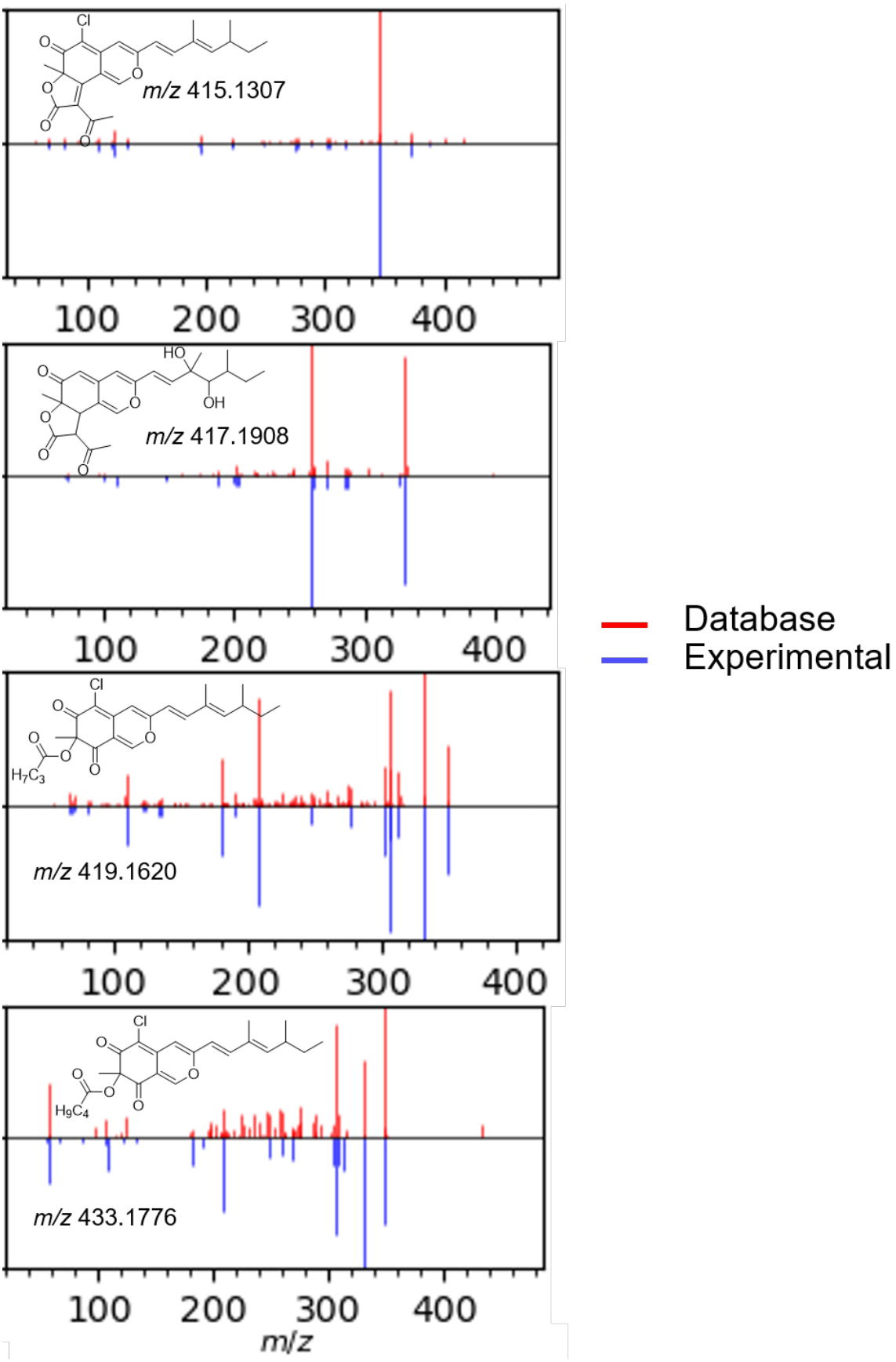
100
200
300
400
m/z
Database
Experimental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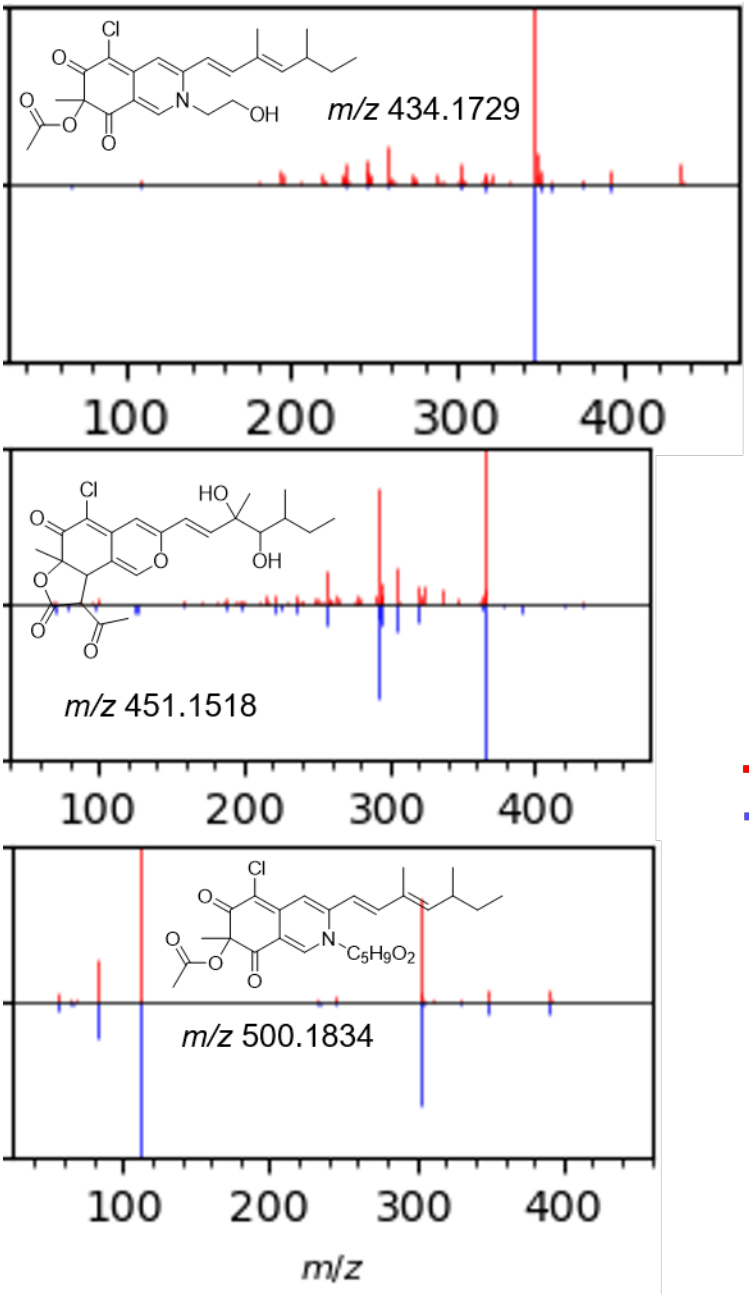
m/z
Database
Experimental



Database
Experimental

Table S3: Basic genomes statistics.

	Genome size (Mpb)	Contigs	N50 (Mpb)	N75 (Mpb)	L50 (contigs)	L75 (contigs)	%GC
Neonectria discophora	41.6	26	4.03	3.02	3	8	54.2
Penicillium sclerotiorum	34.7	10	4.34	3.95	4	6	48.3

Table S4: BUSCO score.

Gene	Complete		Fragmented (%)	Missing (%)
	Single copy (%)	Duplicated (%)		
Neonectria discophora	99.3	0.0	0.0	0.7
Penicillium sclerotiorum	97.6	0.7	0.0	1.7

Table S5: *Ndi_Ili* biosynthetic gene cluster annotation.

Gene name	Size (AA)	Best match Swissprot	Global alignement (I/S/G) %	Global alignement (I/S/G) AA	domain	_ID	Plausible function	Duplicated
Ndi_Ili_A	2014	A0A455R5P9	56/70/8	1201/1506/179	beta-ketoacyl synthase acyl transferase product template acyl carrier protein thioesterase	PF00109.26 PF00698.21 TIGR04532 PF00550.25 IPR001031	Non reducing polyketide synthase	N
Ndi_Ili_B	297	A0A455R413	49/59/16	167/203/55	prenyltransferase	PF01040.18	Prenyltransferase	N
Ndi_Ili_C	1065	A0A455R7E6	60/75/4	662/826/40	AMP-binding, NAD dependent epimerase/dehydratase	PF00501.28 PF01370.21	Non-canonical non-ribosomal peptide synthetase	N
Ndi_Ili_D	553	A0A455R7M0	68/79/5	387/447/31	tryptophan halogenase	PF04820.14	FAD dependant halogenase	N
Ndi_Ili_E	674	No reviewed protein			sugar (and other) transporter, major facilitator	PF00083.24 PF07690.16	Transporter	N
Ndi_Ili_F	451	No reviewed protein			ATPase family associated with various cellular activities	PF00004.29	ATPase	N
Ndi_Ili_G	532	No reviewed protein						N
Ndi_Ili_H	418	No reviewed protein						N
Ndi_Ili_I	442	No reviewed protein					unknown function	N
Ndi_Ili_J	782	No reviewed protein					unknown function	N

I: identity, S: similarity, G: gap

Table S6: *Ndi_WSC72* biosynthetic gene cluster annotation.

Gene name	Size (AA)	Best match Swissprot	Global alignement (I/S/G) %	Global alignement (I/S/G) AA	domain	_ID	Plausible function	Duplicated
Ndi_WSC72_A	1786	C5FM57	56/70/5	1030/1296/96	ketoacyl synthase acyl transferase product template acyl carrier protein	PF00109.26 PF00698.21 TIGR04532.6 PF00550.25	Non reducing polyketide synthase	N
Ndi_WSC72_B	556	Q2UPC7	57/72/5	322/408/31	tryptophan halogenase	PF04820.14	FAD dependant halogenase	N
Ndi_WSC72_C	220	No reviewed protein			glutathione S-transferase	IPR040079	Glutathione S-transferase	N
Ndi_WSC72_D	337	G3KLH5	54/71/3	174/227/11	β-lactamase	PF00753	β-lactamase	N
Ndi_WSC72_E	378	Q9P900	39/58/4	150/227/14	O-methyltransferase	PF00891	methyltransferase	N
Ndi_WSC72_F	516	No reviewed protein			copper oxidase	PF00394 PF07731	unknown function	N
Ndi_WSC72_G	261	A0A179G9G5	37/44/45	164/194/198	zinc finger	PF00172	Transcription factor	N
Ndi_WSC72_H	427	No reviewed protein			monooxygenases	PF01494	monooxygenase	N
Ndi_WSC72_I	532	No reviewed protein					unknown function	N
Ndi_WSC72_J	413	No reviewed protein					unknown function	N
Ndi_WSC72_K	292	No reviewed protein					unknown function	N
Ndi_WSC72_L	918	No reviewed protein					unknown function	N

Figure S3: Cluster comparison of azaphilone biosynthetic gene clusters described in literature with the putative azaphilone biosynthetic gene cluster of *Penicillium sclerotiorum* SNB-CN111. Only functions of tailoring enzyme related to *P. sclerotiorum* are annotated. BGC identifier are accession number from MIBIG repository. Clinker Software (https://github.com/gamcil/clinker).

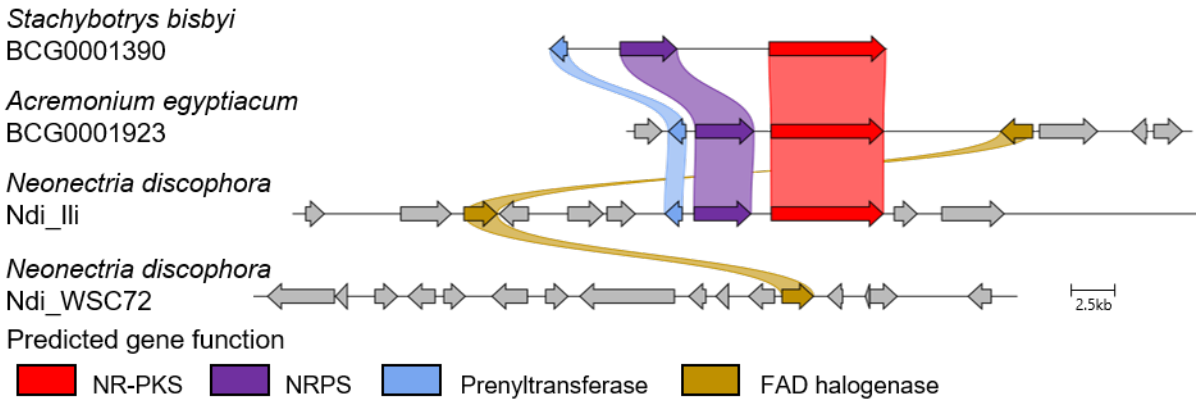


Table S7: Annotated ilicicolins from *N. discophora* crude extract, synthetized by *Ndi_Ili* biosynthetic pathway.

CN63					
ID	**$[M+H]^+$**	**Error (ppm)**	**Formula**	**Identification**	**Ref**
9	373.2364	2.5	$C_{23}H_{32}O_4$	Dereplication, standard	[8]
2	407.1985	1.1	$C_{23}H_{31}ClO_4$	Dereplication, standard	[8]
8	357.2422	0.6	$C_{23}H_{32}O_3$	Dereplication, *in silico*	[6]
1	391.2028	1.7	$C_{23}H_{31}ClO_3$	Dereplication, *in silico*	[9]

Table S8: *Psc_Aza* biosynthetic gene cluster annotation.

Gene name	Size (AA)	Best match Swissprot	Global alignement (I/S/G) %	Global alignement (I/S/G) AA	Pfam domain	PFAM_ID	Plausible_function	Duplicated
Psc_Aza_A	2584	Q0CF75	65/77/7	1744/2076/185	beta-ketoacyl synthase acyl transferase polyketide synthase dehydratase methyltransferase enoylreductase ketoreductase phosphopantetheine attachment site	PF00109.26 PF00698.21 PF14765.6 PF08242.12 IPR020843 PF00107.26 PF08659.10	Highly reducing polyketide synthase	N
Psc_Aza_B	2726	A0A0K0MCJ4	67/78/5	1870/2188/129	beta-ketoacyl synthase acyl transferase phosphopantetheine attachment site methyltransferase terminal domain	PF16073.5 PF00109.26 PF00698.21 PF00550.25 IPR013120	Non reducing polyketide synthase	N
Psc_Aza_C	352	Q0CF72	44/55/32	208/256/152	FAD-binding domain_3	PF01494.19	Monooxygenase FAD dependant	N
Psc_Aza_D	449	A0A084B9Z3	34/50/8	162/239/40	transferase	PF02458	Acyltransferase	N
Psc_Aza_E	471	Q0CF74	49/66/4	232/318/18	FAD binding domain	PF01565.23	FAD-linked oxidoreductase	N
Psc_Aza_F	341	No reviewed protein			zinc-binding dehydrogenase, alcohol dehydrogenase GroES-like	PF00107.26 PF08240.12	Dehydrogenase	N
Psc_Aza_G	385	No reviewed protein			alcohol dehydrogenase GroES-like	PF08240.12	Alcohol dehydrogenase	N
Psc_Aza_H	561	Q0CCX4	55/68/8	318/392/43	tryptophan halogenase	PF04820.14	FAD-dependant halogenase	N
Psc_Aza_I	590	No reviewed protein					Unknow function	N
Psc_Aza_J	227	No reviewed protein			amine oxidoreductase	PF01593.24	Amine oxidase	N
Psc_Aza_K	306	No reviewed protein			G protein-coupled glucose receptor	PF11970.8	G protein-coupled receptor	N
Psc_Aza_L	590	No reviewed protein			major facilitator	PF07690.16	Efflux pump	N
Psc_Aza_M	407	No reviewed protein					Unknow function	N

I: identity, S: similarity, G: gap

Figure S4: Cluster comparison of azaphilone biosynthetic gene clusters described in literature with the putative azaphilone biosynthetic gene cluster of *Penicillium sclerotiorum* SNB-CN111. Only functions of tailoring enzyme related to *P. sclerotiorum* are annotated. BGC identifier are accession number from MIBIG repository. Clinker Software (https://github.com/gamcil/clinker).

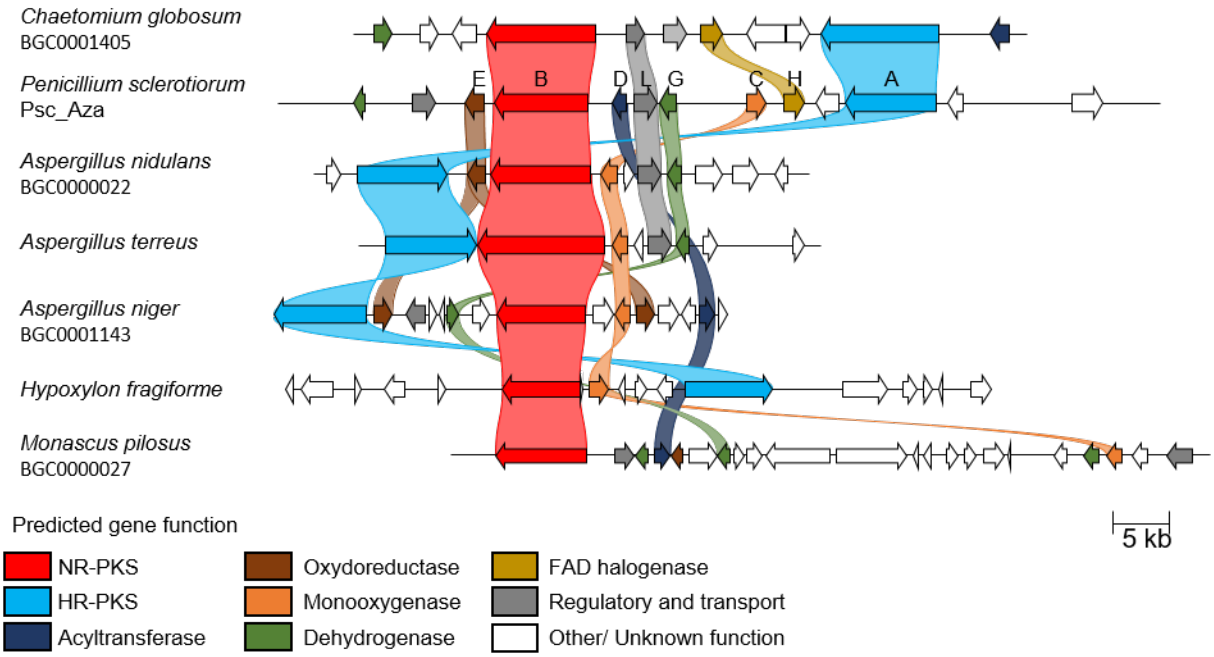


Figure S5: MS/MS spectra of [**10**+H]$^+$ and the corresponding fragmentation scheme.

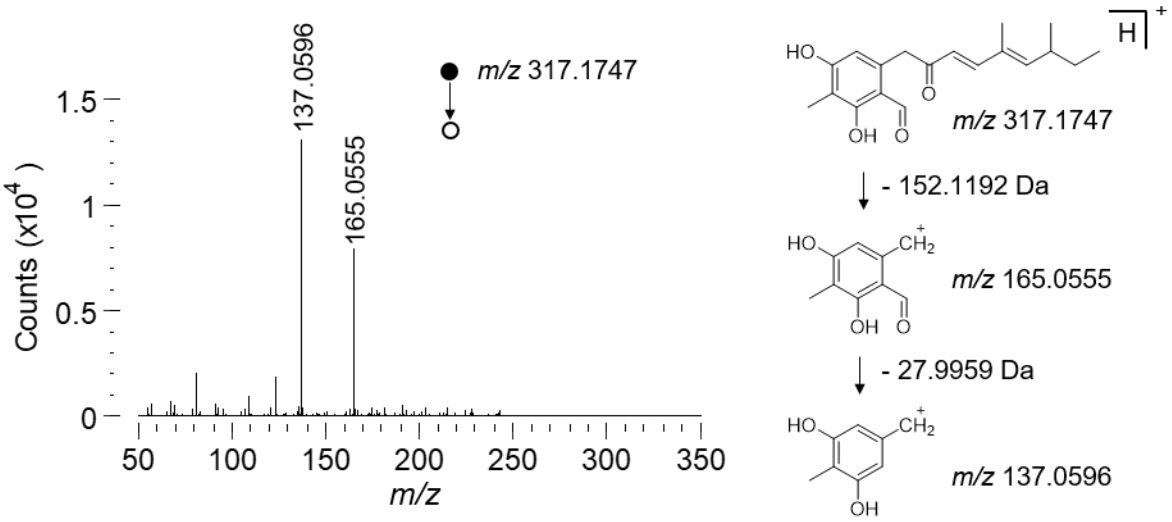


Figure S6: MS/MS spectra of [**11**+H]$^+$ and the corresponding fragmentation scheme.

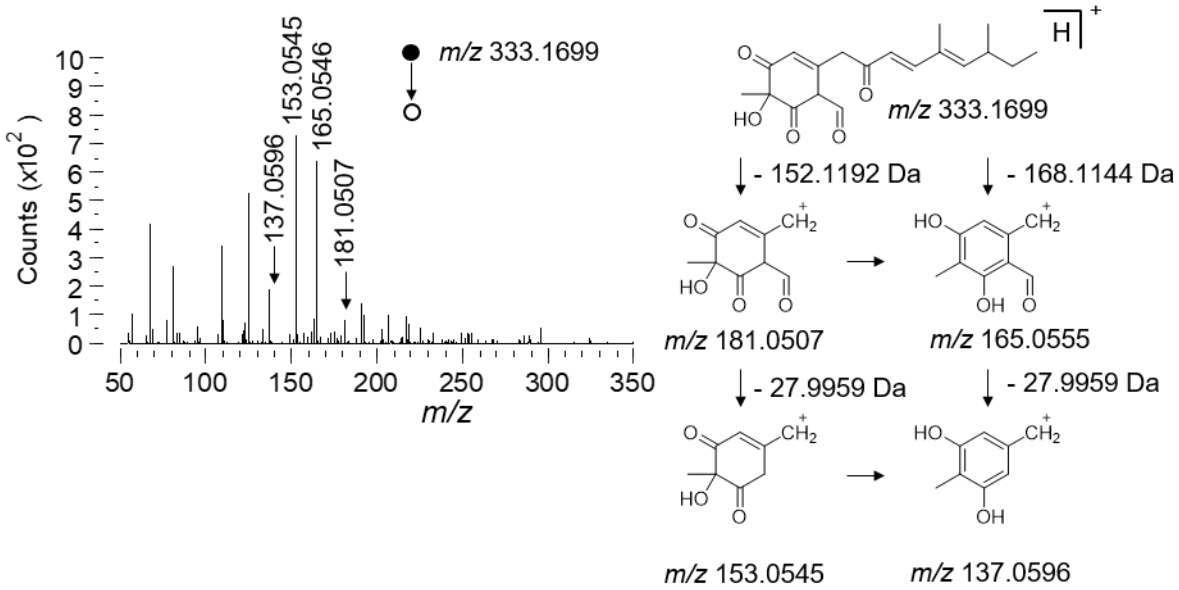


Figure S7: MS/MS spectra of [**12**+H]$^+$ and the corresponding fragmentation scheme.

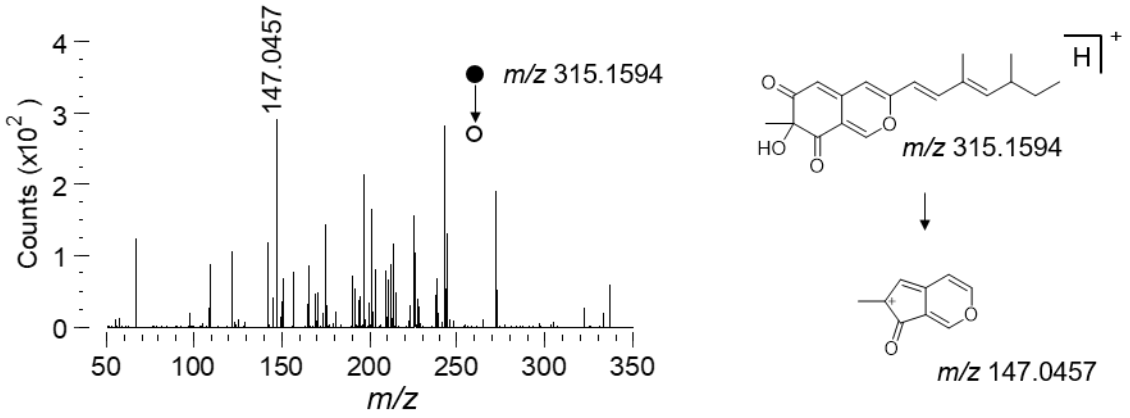


Figure S8: Comparison of intermediates **12** and **21** fragmentation spectra with their common neutral losses corresponding to H/Cl differences.

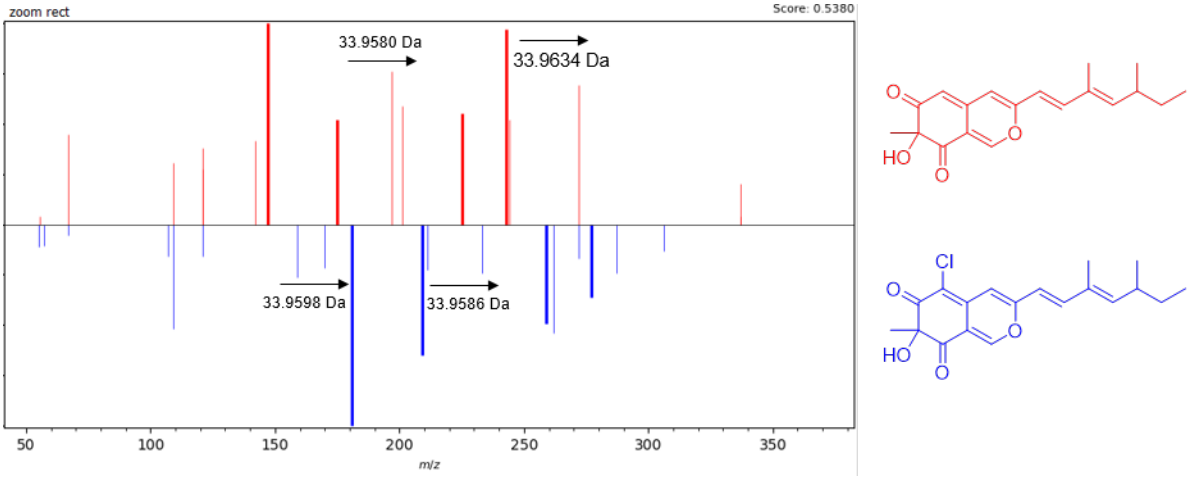


Table S9: Annotated azaphilones from *P. sclerotiorum* crude extract and fractions, synthetized by *Psc_Aza* biosynthetic pathway.

CN111					
ID	**$[M+H]^+$**	**Error (ppm)**	**Formula**	**Identification**	**Ref**
10	317.1747	0.1	$C_{19}H_{24}O_4$	Manual annotation, *in silico*	This study
11	333.1699	-0.8	$C_{19}H_{24}O_5$	Manual annotation, *in silico*	This study
12	315.1594	-1	$C_{19}H_{22}O_4$	Manual annotation, *in silico*	[12]
21	349.1205	-1.1	$C_{19}H_{21}ClO_4$	Dereplication, *in silico*	[1]
13	357.1705	-2.4	$C_{21}H_{24}O_5$	Dereplication, *in silico*	[1]
4	391.1309	-0.6	$C_{21}H_{23}ClO_5$	Dereplication, standard	[1, 2]
19	361.2021	-3.2	$C_{21}H_{28}O_5$	Manual annotation, *in silico*	This study
20	395.1626	-1.6	$C_{21}H_{27}Cl_{O5}$	Dereplication, *in silico*	[1, 2]
15	381.1691	1.5	$C_{23}H_{24}O_5$	Dereplication, *in silico*	[1, 2]
6	415.1312	-1.3	$C_{23}H_{23}ClO_5$	Dereplication, *standard*	[1, 2]
16	383.1859	-1.6	$C_{23}H_{26}O_5$	Dereplication, *in silico*	[1, 2]
17	417.1467	-0.9	$C_{23}H_{25}ClO_5$	Dereplication, *in silico*	[1, 2]

Figure S9: Extracted ion chromatogram of halogenated ilicicolins from scaffold A (H, Cl and Br) with their isotopic patterns.

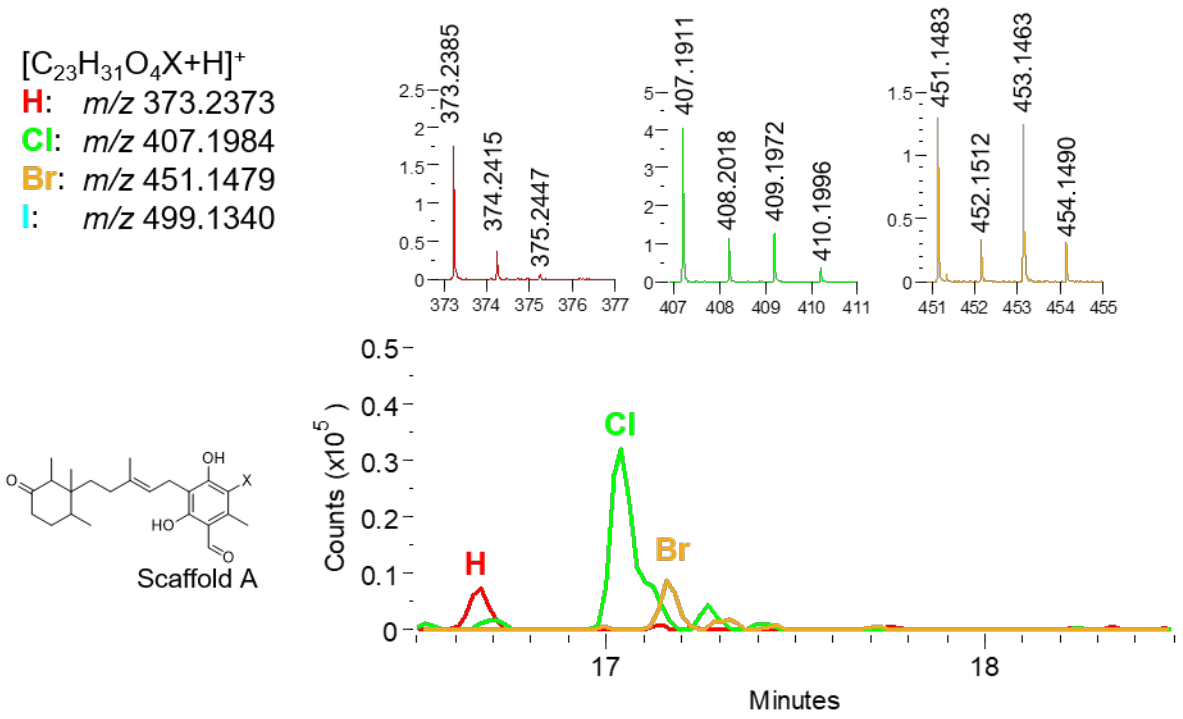


Figure S10: Extracted ion chromatogram of halogenated ilicicolins from scaffold B (H, Cl and Br) with their isotopic patterns.

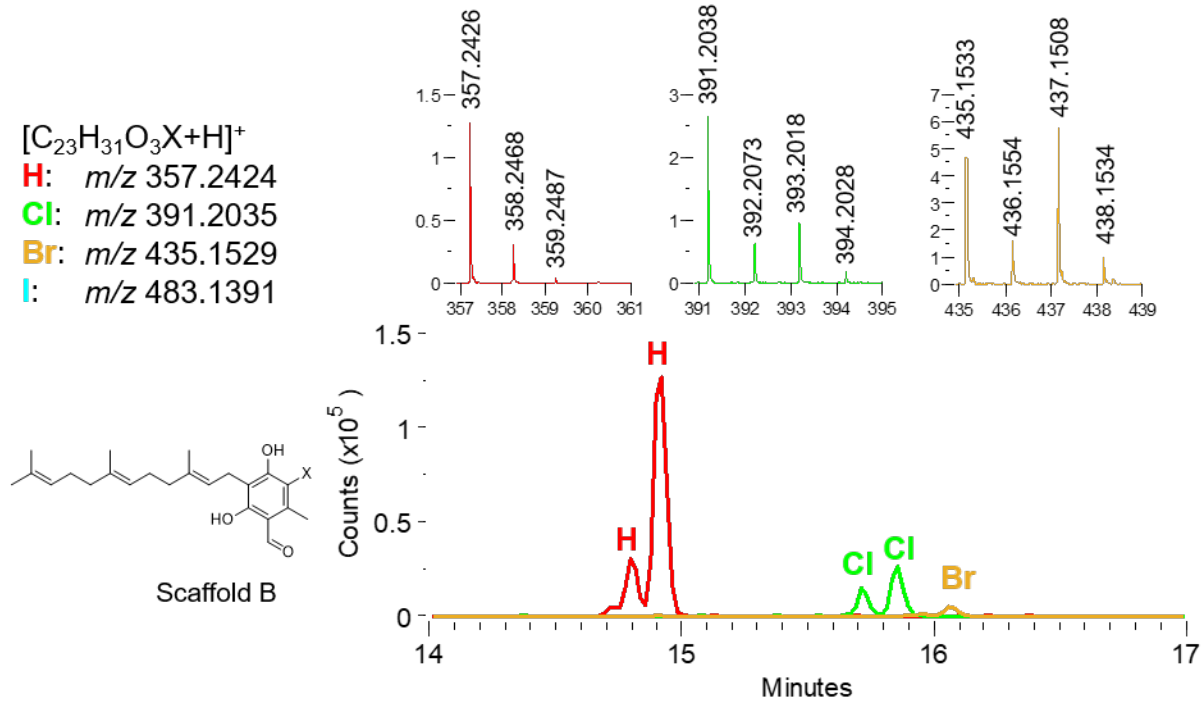


Figure S11: Extracted ion chromatogram of halogenated azaphilones from scaffold B' (H, Cl, Br and I) with their isotopic patterns.

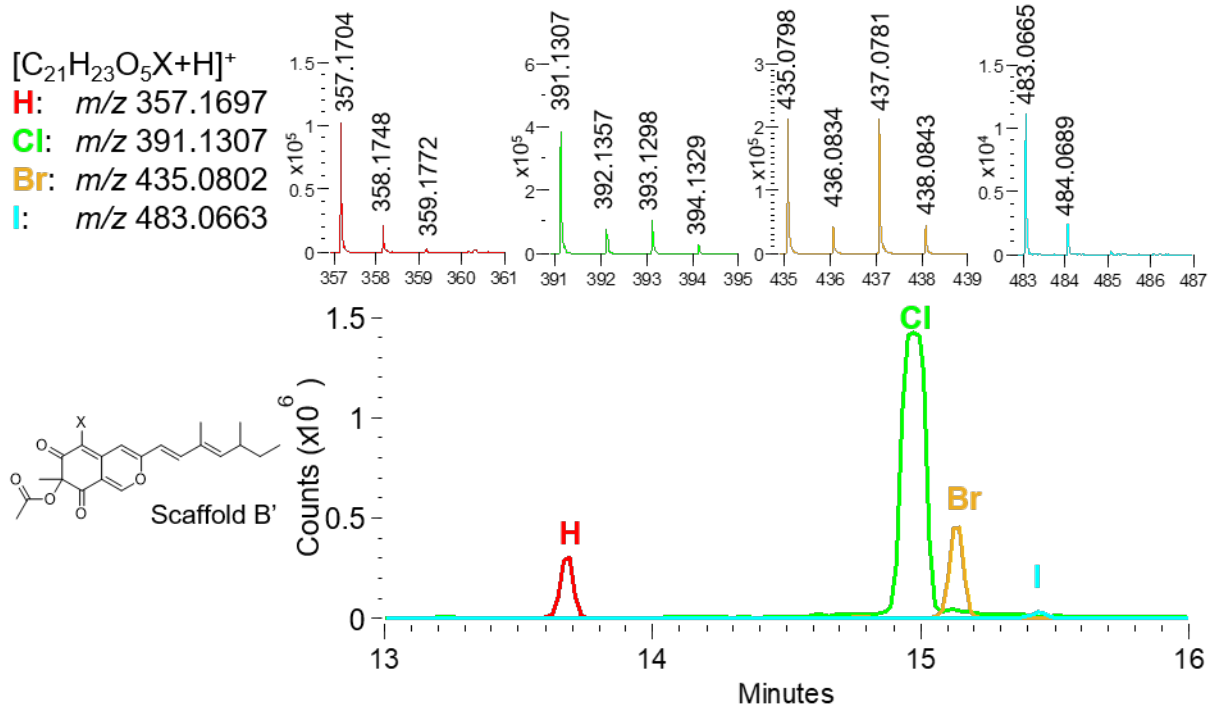


Figure S12: Extracted ion chromatogram of halogenated azaphilones from scaffold C' (H, Cl, Br and I) with their isotopic patterns.

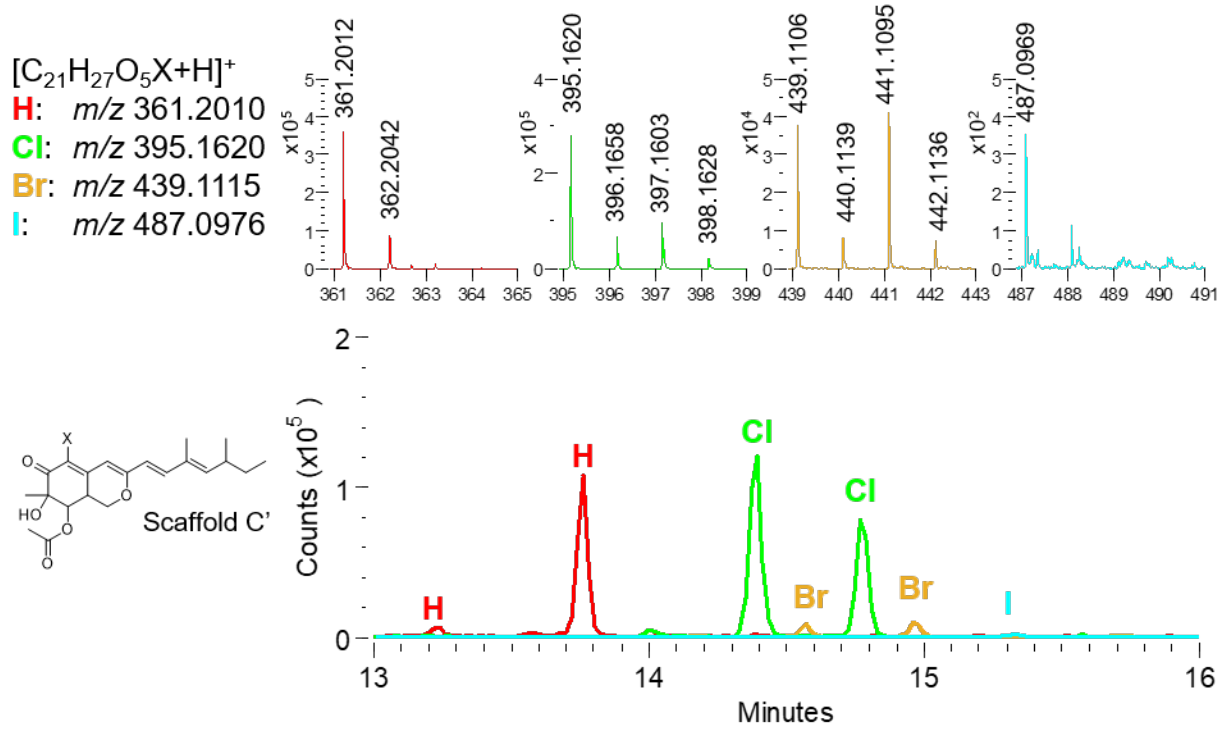


Figure S13: Extracted ion chromatogram of halogenated azaphilones from scaffold D' (H, Cl, Br and I) with their isotopic patterns.

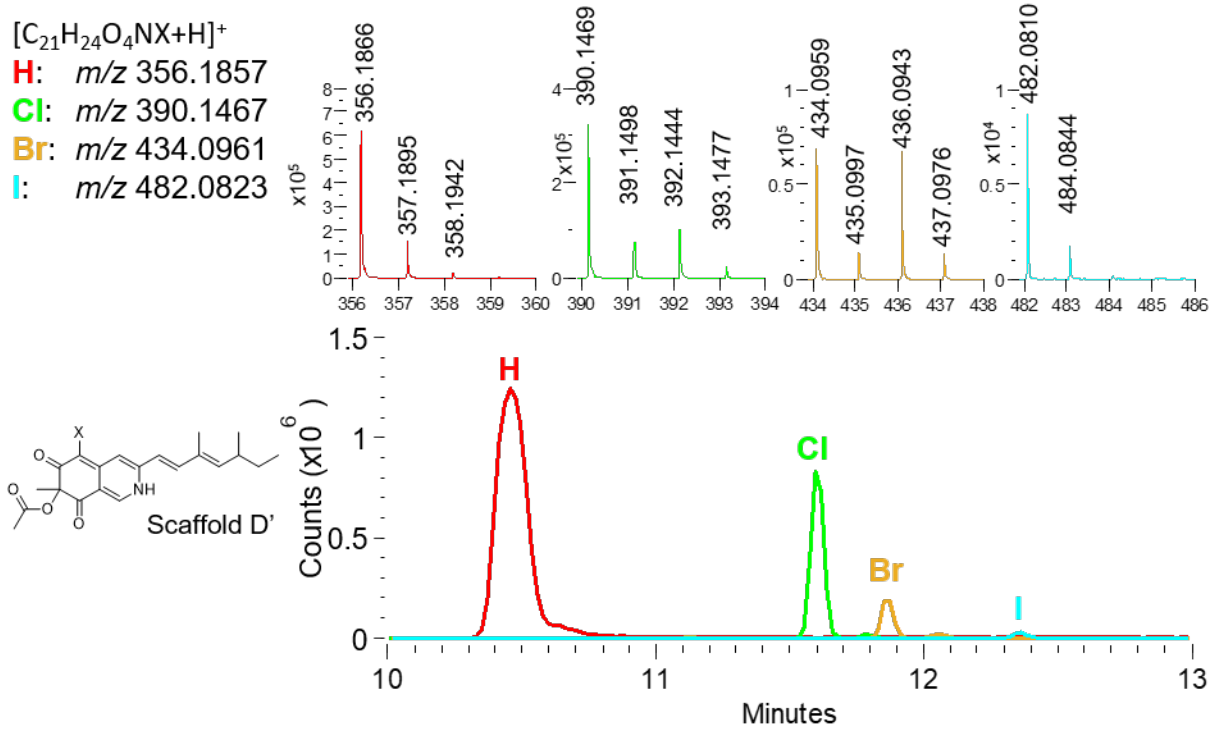


Figure S14: Extracted ion chromatogram of halogenated azaphilones from scaffold E' (H, Cl, Br and I) with their isotopic patterns.

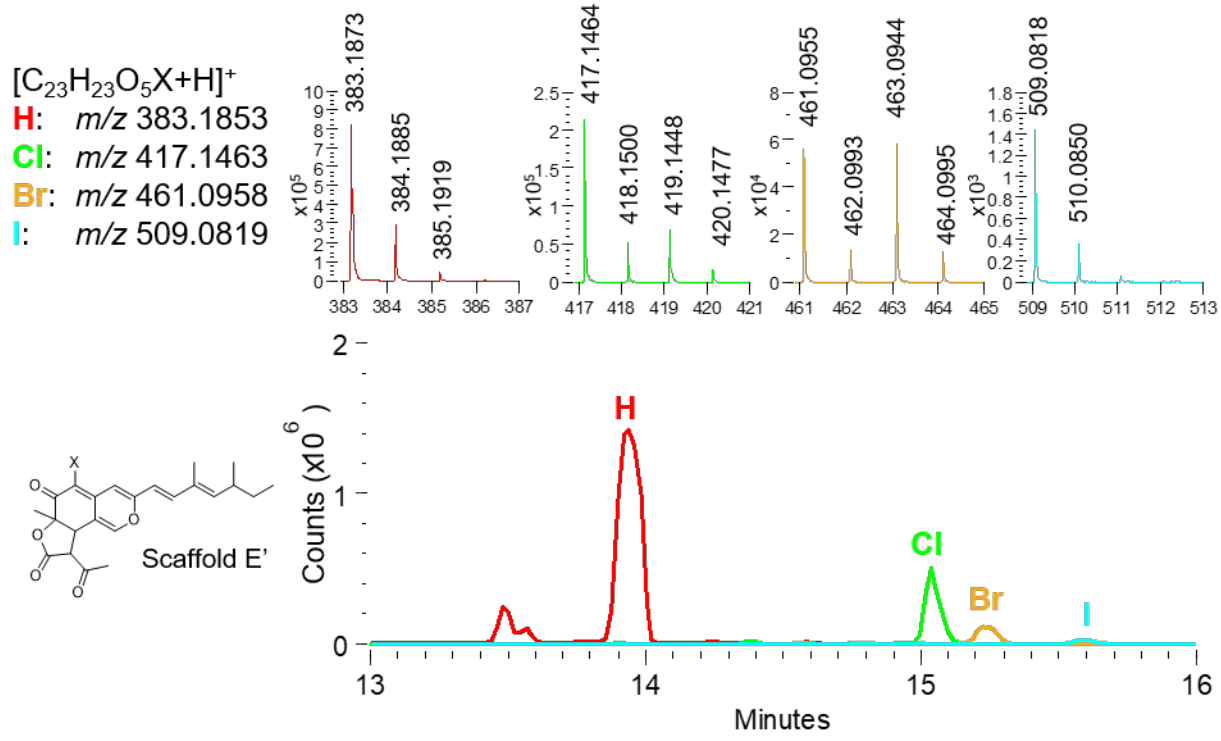


Figure S15: HRMS of compound **22.**

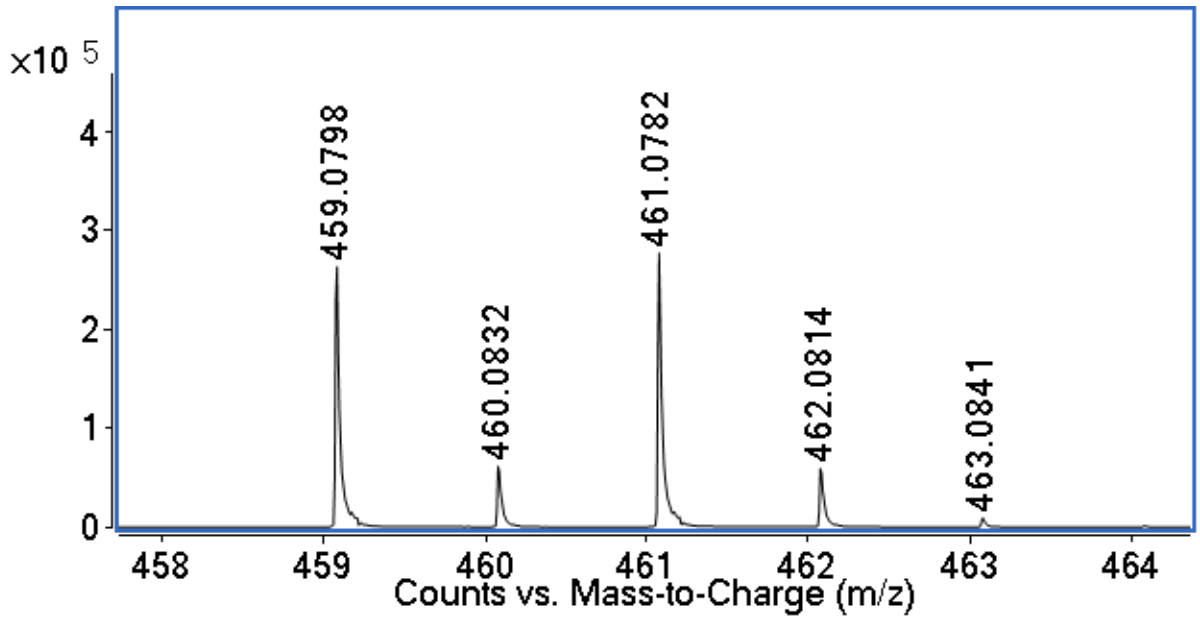


Table S10: Comparative ^{1}H NMR spectroscopic data for compounds **22** (isolated during this study), sclerotiorin (**4**) sclerotioramine (**5**) and 5-chloroisorotiorin (**6a**) isolated by our group (reference 20 of the manuscript) and **6b** and **15** (from literature)

Position

	22	**4**	**5**	**6a**	**6b**	**15**
	DMF-*d7*[a]	$CDCl_3$[b]	$CDCl_3$[b]	MeOD[c]	$CDCl_3$[b]	MeOD[c]
1	8.82, s	7.90, s	7.93, s	8.88, s	8.84, s	8.88, s
4	6.98, s	6.61, s	6.86, s	6.81, s	6.16, s	6.81, s
5					6.34, s	
9	6.78, d(16.1)	6.51, d(15.9)	6.13, d(16.1)	6.35, d(15.6)	5.97, d(15.8)	6.35, d(15.6)
10	7.26, d(16.2)	7.03, d(15.9)	7.04, d(16.7)	7.19, (16.0)	7.04, d(15.8)	7.19, d(16.0)
12	5.87, d(10.2)	5.67, d(10.0)	5.69, d(9.9)	5.77, d(9.8)	5.67, d(9.9)	5.77, d(9.8)
13	2.55, m	2.45, m	2.47, m	2.53,m	2.48, m	2.52, m
14	1.45/1.34, m	1.29/1.39, m	1.30/1.40, m	1.34/1.46, m	1.35, m	1.46/1.34, m
15	0.87, t(7.4)	0.83, t(7.5)	0.84, t(7.5)	0.89, t(7.6)	0.85, t(7.5)	0.89, t(7.6)
16	1.02, d(6.7)	0.98, d(6.8)	0.99, d(6.8)	1.03, d(6.6)	1.00, d(6.7)	1.03, d(6.6)
17	1.92, s	1.81, s	1.83, s	1.88, s	1.81, s	1.88, s
18	1.72, s	1.53, s	1.57, s	1.68, s	1.69, s	1.68, s
20		2.13, s	2.16, s			
5''	2.57, s			2.55, s	2.59, s	2.55, s

[a] DMF-*d7*, 700 MHz, this study
[b] $CDCl_3$, no indication[13]
[c] MeOD, 500 MHz[1]

Table S11: Comparative ^{13}C NMR spectroscopic data for compounds **22** (isolated during this study), sclerotiorin (**4**) and sclerotioramine (**5**) isolated by our group (reference 20 of the manuscript) and **6b** and **15** (from literature)

	22	**4**	**5**	**6b**	**15**
	DMF-*d7*[a]	$CDCl_3$[b]	$CDCl_3$[b]	$CDCl_3$[b]	$CDCl_3$[c]
1	153.2 CH	152.8 CH	138.4 CH	153.1, CH	151.9, CH
3	159.7, C	158.4, C	146.3, C	156.3, C	158.0, C
4	109.5, CH	110.8, CH	110.3, CH	108.2, CH	105.5, CH
4a	143.4, C	138.8, C	147.1, C	123.1, C	139.4, C
5	100.8, C	106.6, C	101.5, C	105.3, CH	108.7, C*
6	184.5, C	186.0, C	183.7, C	189.9, C	194.1, C
7	88.4, C	84.7, C	85.4, C	87.5, C	87.4, C
8	164.2, C	192.0, C	193.3, C	165.6, C	163.6, C
8a	112.1, C	114.8, C	114.2, C	110.7, C	110.2, C*
9	118.1, CH	115.9, CH	116.4, CH	115.4, CH	115.6, CH
10	143.6, CH	143.0, CH	142.9, CH	144.1, CH	143.5, CH
11	133.9, C	132.2, C	132.0, C	131.8, C	131.9, C
12	149.1, CH	149.0, CH	148.7, CH	148.3, CH	149.2, CH
13	35.9, CH	35.3, CH	35.1, CH	35.5, CH	35.1, CH
14	30.3, CH_2	30.2, CH_2	30.6, CH_2	30.0, CH_2	29.8, CH_2
15	12.5, CH_3	12.1, CH_3	12.0, CH_3	11.9, CH_3	11.8, CH_3
16	20.8, CH_3	20.2, CH_3	20.1, CH_3	20.1, CH_3	20.0, CH_3
17	12.9, CH_3	12.5, CH_3	12.4, CH_3	12.4, CH_3	12.2, CH_3
18	26.4, CH_3	22.7, CH_3	23.6, CH_3	26.4, CH_3	26.1, CH_3
19		170.3, C	170.9, C		
20		20.3, CH_3	20.6, CH_3		
2’	169.4, C			168.3, C	167.7, C
3'	124.8, C			142.3, C	123.7, C
4’	195.2, C			194.4, C	182.9, C
5'	30.5, CH_3			30.0, CH_3	29.9, CH_3

[a] DMF-*d7*, 700 MHz, this study.
[b] $CDCl_3$, no indication[13]
[c] $CDCl_3$, 100 MHz[14]
*Authors indicate that these assignments were interchangeable.

Figure S16: ^{1}H NMR spectrum (700 MHz, DMF-*d7*) of compound **22.**

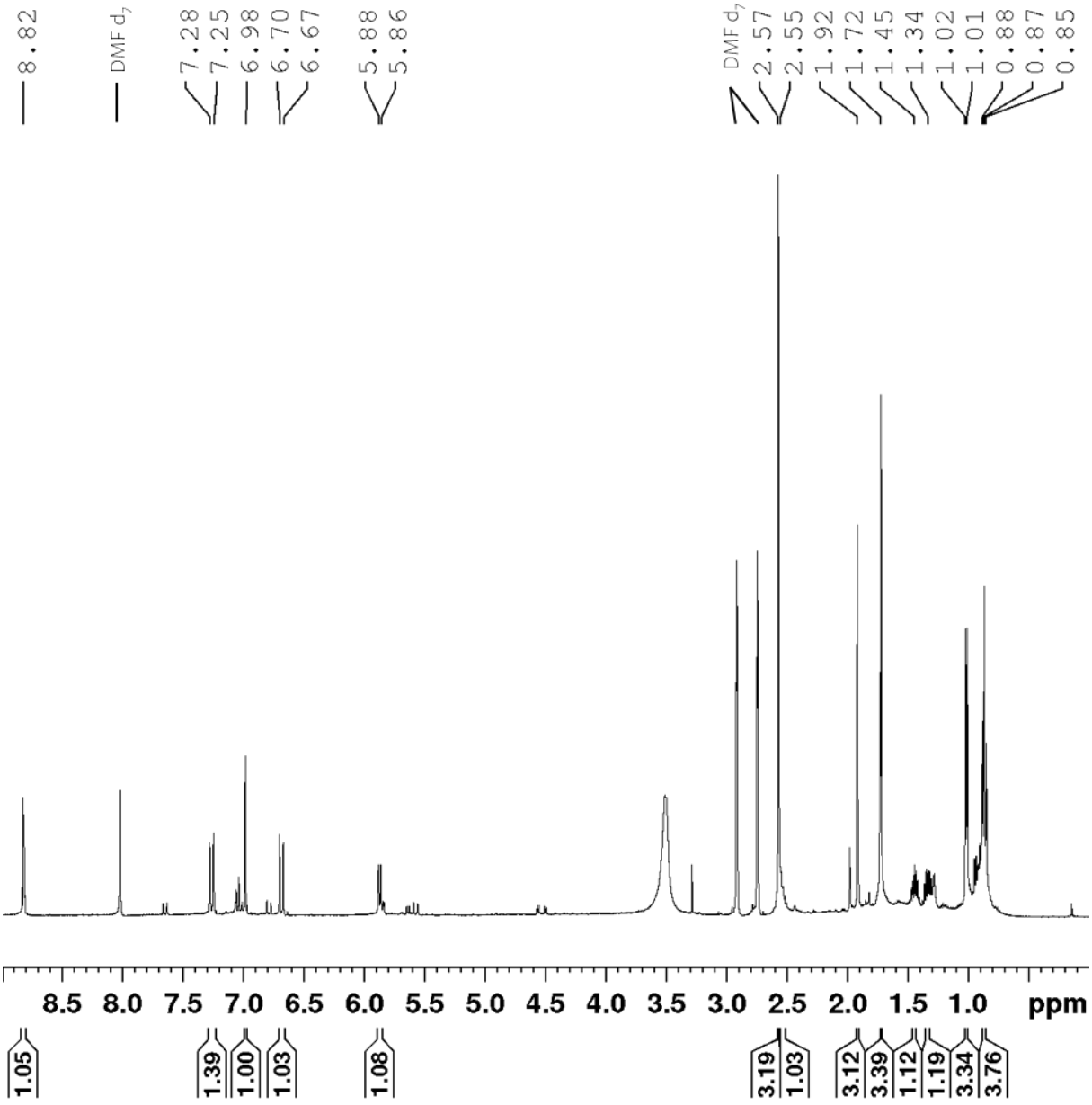


Figure S17: ^{13}C NMR spectrum (175 MHz, DMF-*d7*) of compound **22.**

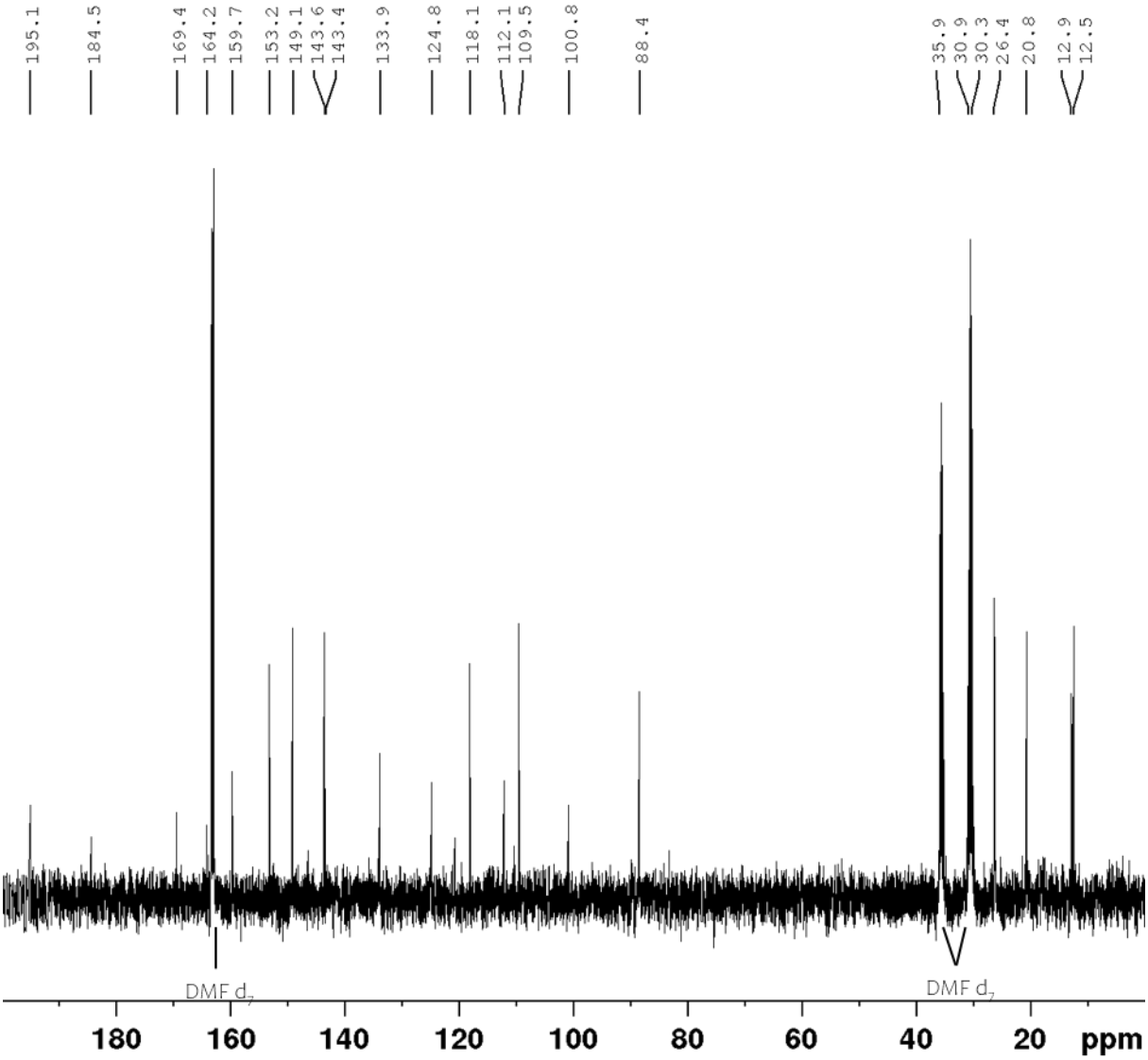


Figure S18: COSY NMR spectrum (700 MHz, DMF-*d7*) of compound **22.**

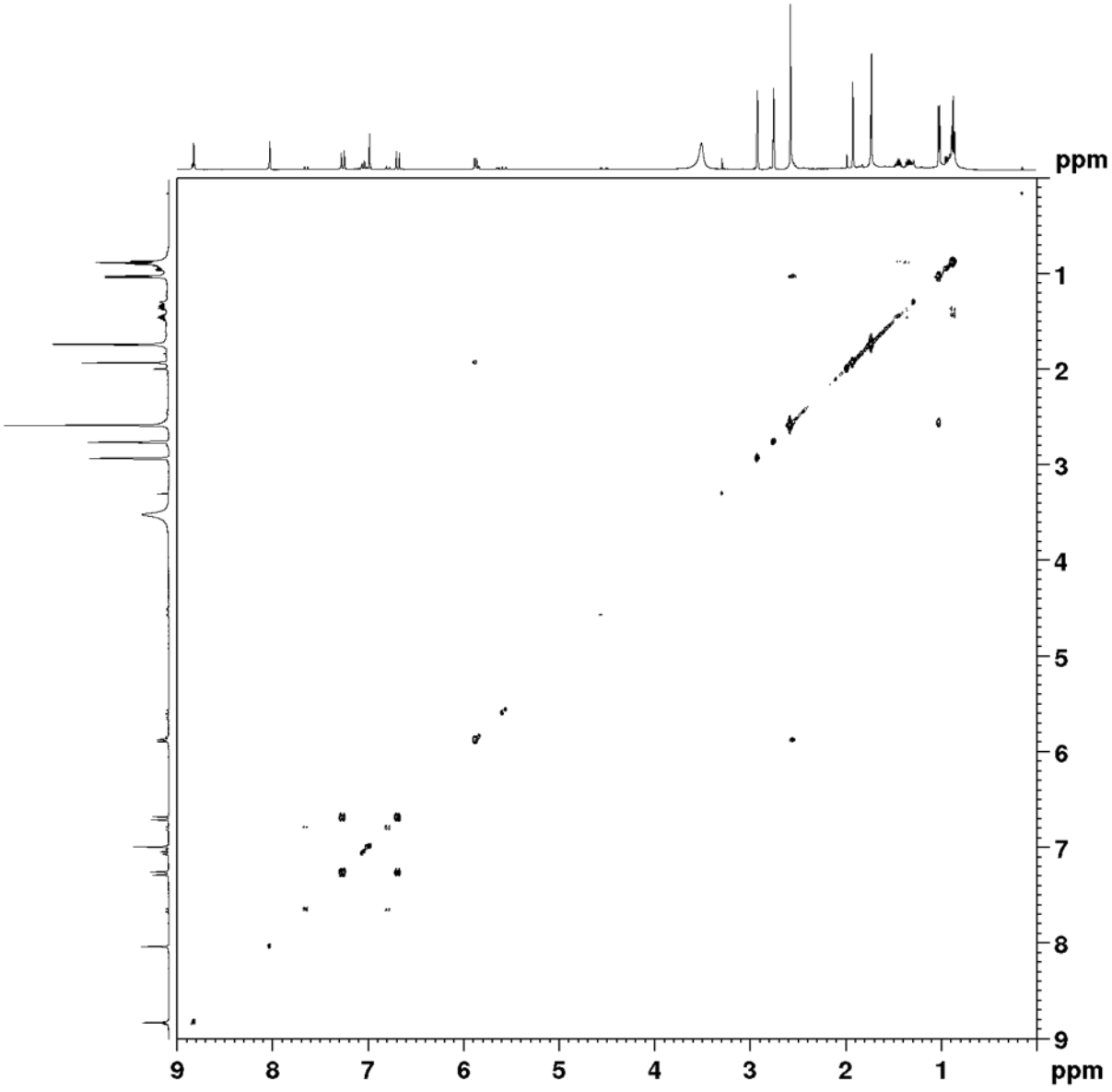


Figure S19: HSQC NMR spectrum (700 MHz, DMF-*d7*) of compound **22.**

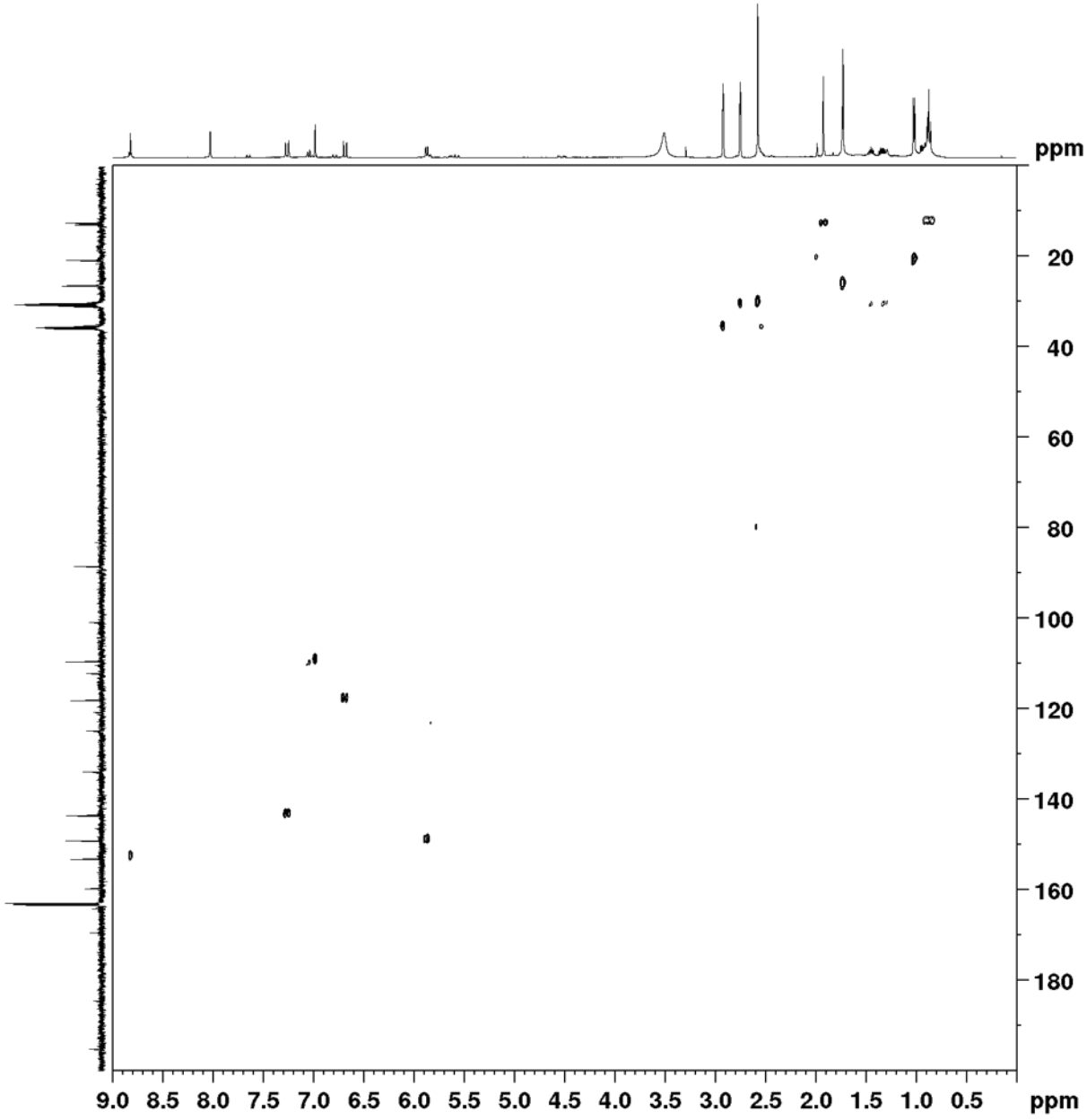


Figure S20: HMBC NMR spectrum (700 MHz, DMF-*d7*) of compound **22.**

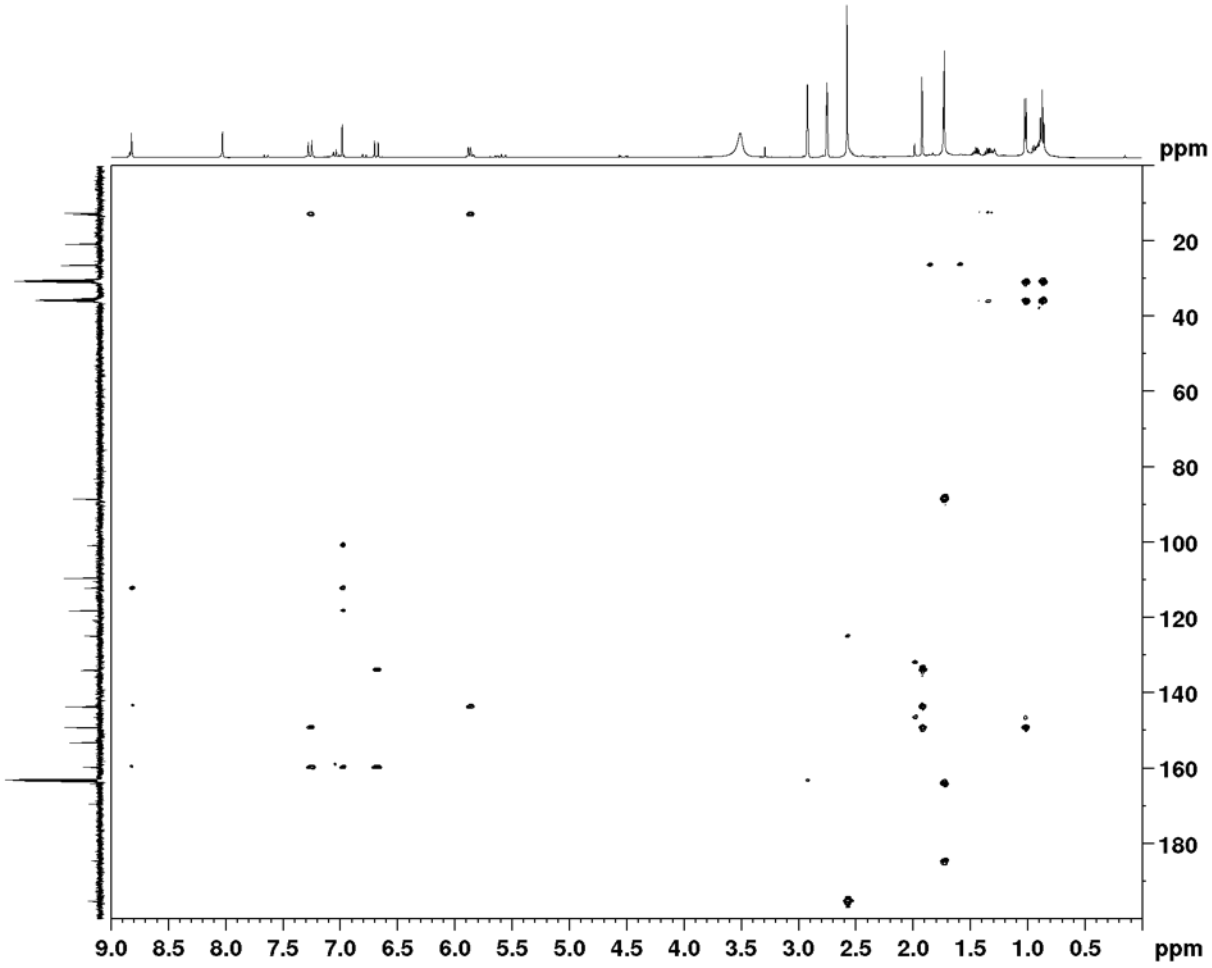
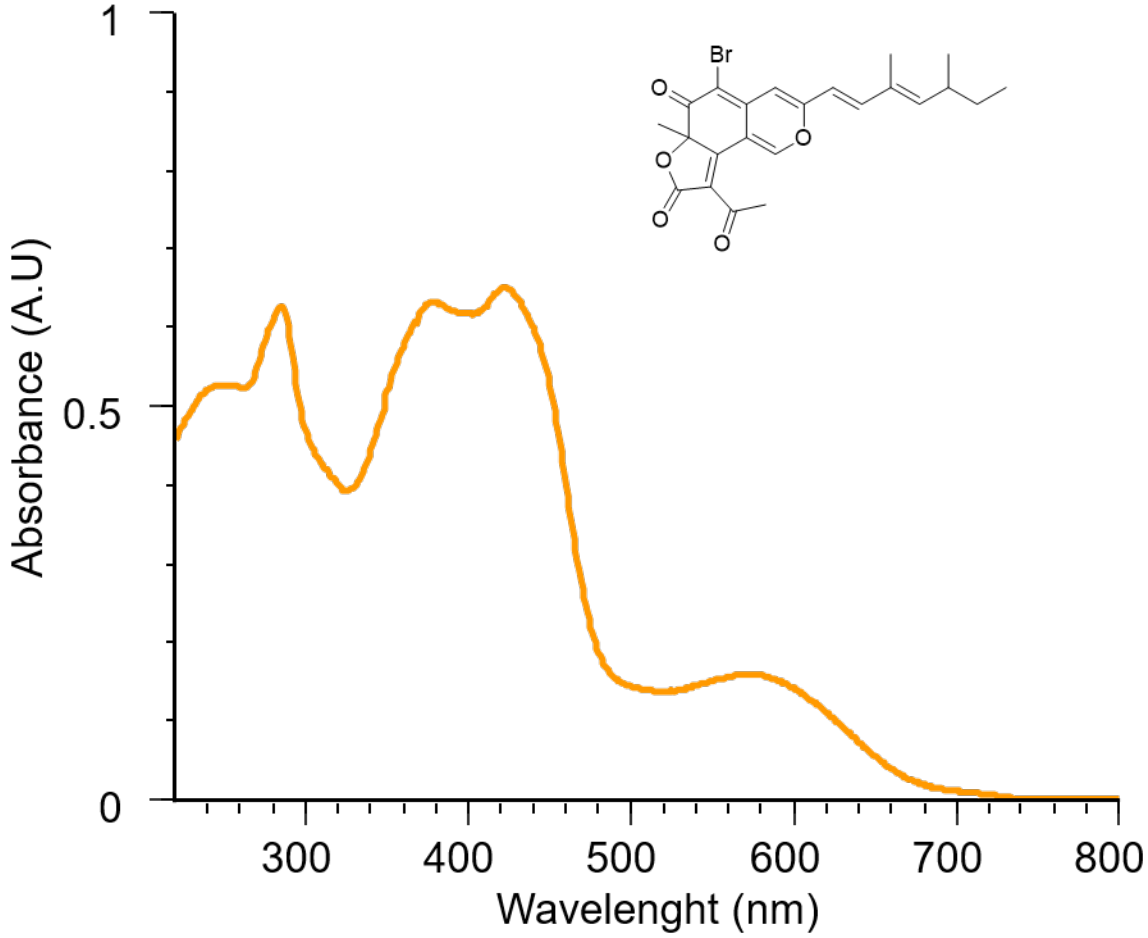


Figure S21: UV spectra in methanol of isolated azaphilone ***22***



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