Supplementary Information

Engineering the substrate specificity of a modular polyketide synthase for installation of consecutive non-natural extender units

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Table S1. High-Resolution LC-MS Retention Times, Calculated Masses, and Observed Masses for DEBS/Pik PKS-Catalyzed Reaction Products. N.D. = Not Detected. Products with more than one peak are likely due to different ring conformations of narbonolide products. Products **5a-d** and **6a-d** have identical masses and were not differentiated.

Compound & Retention Time (min)		Calculated Mass ($[M-H_2O+H]^+$)	Observed Mass ($[M-H_2O+H]^+$)	Calculated Mass ($[M+H]^+$)	Observed Mass ($[M+H]^+$)	Calculated Mass ($[M+Na]^+$)	Observed Mass ($[M+Na]^+$)
1	7.68	279.1955	279.1949	297.2060	297.2055	319.1880	319.1873
2	9.21/9.92	335.2217	335.2210	353.2323	353.2316	375.2142	375.2129
3a	8.65	303.1955	303.1949	321.2060	321.2053	343.1880	343.1877
3b	9.41	293.2111	293.2106	311.2217	311.2211	333.2036	333.2037
3c	8.13	305.2111	305.2104	323.2217	323.2210	345.2036	345.2058
3d	N.D.	321.2424	N.D.	339.2530	N.D.	361.2349	N.D.
4a	11.79	--	--	319.1904	319.1889	341.1723	341.1718
4b	12.42	--	--	309.2060	309.2054	331.1880	331.1872
4c	12.87	--	--	321.2060	321.2054	343.1880	343.1873
4d	14.55	--	--	337.2373	337.2363	359.2193	359.2182
5a **6a**	10.31/10.82	359.2217	359.2208	377.2323	377.2314	399.2142	399.2133

5b **6b**	10.40/10.71 11.46/11.80	349.2373	349.2364	367.2479	367.2471	389.2298	389.2288
5c **6c**	11.34/12.01	361.2373	361.2364	379.2479	379.2469	401.2298	401.2288
5d **6d**	13.19/13.98 14.42/15.48	377.2686	377.2677	395.2792	395.2777	417.2611	417.2618
7a	11.15/11.45	383.2217	383.2209	401.2323	401.2315	423.2142	423.2133
7b	11.42/12.49	363.2530	363.2519	381.2636	381.2626	403.2455	403.2447
7c	12.92/14.00	387.2530	387.2520	405.2636	405.2618	427.2455	427.2449
7d	N.D.	419.3156	N.D.	437.3262	N.D.	459.3081	N.D.

Table S2. High-resolution LC-MS peak areas for PKS-catalyzed reaction products with non-natural extenders. Extracted ion count peak areas from one replicate are shown for each reaction condition. N.D. = Not Detected. For products with multiple peaks, peak areas are summed. Products **5a-d** and **6a-d** have identical masses and were not differentiated. See Supplementary Table S1 for retention times and calculated and observed masses for each product.

Enzymes and Substrates		Representative EIC Peak Areas					
		1	**3a-d**	**4a-d**	**2**	**5a-d/6a-d**	**7a-d**
PikAIII WT PikAIV WT	**8/9a**	604,644,918	7,436,664	19,463,238	6,426,814,005	2,130,190,170	34,365,789
	8/9b	558,290,483	2,784,286	7,078,095	6,829,997,016	2,948,022,986	72,153,536
	8/9c	514,002,858	N.D.	N.D.	7,055,041,197	320,457,815	N.D.
	8/9d	353,277,555	N.D.	N.D.	628,694,156	N.D.	N.D.
PikAIII Y755V PikAIV Y753V	**8/9a**	306,251,903	207,133,468	16,446,420	231,423,866	502,212,154	109,769,795
	8/9b	763,629,093	69,743,628	15,336,705	976,106,367	1,528,345,552	564,559,230
	8/9c	275,694,862	8,959,070	11,751,681	611,588,065	153,840,570	30,925,719
	8/9d	311,277,819	N.D.	41,383,819	608,122,645	35,004,271	N.D.

Table S3. High-resolution LC-MS peak areas for PKS-catalyzed reaction products with substrates 7 and 8a. Extracted ion count peak areas from one replicate are shown for each reaction condition. N.D. = Not Detected. For products with multiple peaks, peak areas are summed. Products **5a** and **6a** have identical masses and were not differentiated. See Supplementary Table S1 for retention times and calculated and observed masses for each product.

	Representative EIC Peak Areas					
	1	**3a**	**4a**	**2**	**5a/6a**	**7a**
R1	99,267,500	1,283,686	2,951,168	1,418,188,126	409,872,173	5,118,193
R2	21,455,393	977,191	2,313,400	226,409,954	49,123,149	349,108
R3	79,186,380	1,335,707	160,599,190	933,317,508	570,558,286	10,970,635
R4	360,898,980	1,422,361	1,753,026	--	--	--
R5	N.D.	N.D.	N.D.	--	--	--
R6	660,712,285	29,785,869	28,612,559	--	--	--
R7	200,092,464	16,349,384	4,315,620	--	--	--
R8	N.D.	N.D.	N.D.	--	--	--
R9	247,579,476	1,042,640	1,144,330	--	--	--
R10	57,054,504	881,084	14,630,215	--	--	--
R11	N.D.	N.D.	N.D.	--	--	--
R12	290,068,634	125,867,179	93,320,119	--	--	--
R13	N.D.	N.D.	N.D.	--	--	--
R14	99,267,500	1,283,686	2,951,168	1,418,188,126	409,872,173	5,118,193
R15	799,983,835	46,534,105	21,451,594	10,281,410,822	2,836,675,028	107,026,112
R16	88,167,565	100,155,924	11,338,699	342,849,625	990,486,379	266,850,718
R17	72,284,698	2,134,500	1,235,595	62,733,768	223,361,437	3,860,401
R18	102,893,998	82,829,384	7,769,051	108,989,992	269,734,723	74,090,230
R19	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

Table S4. Product distributions catalyzed by mutant PikAIIITE. The percentage of each **1** and **3a** was determined by LC-MS analysis of the product mixtures and reported as an average of two independent measurements. See Supplementary Table S1 for retention times and calculated and observed masses for each product.

N.D., Not detected.

PikAIIITE Variant	% Product	
	1	**3a**
D624E	N.D.	N.D.
E658D	N.D.	N.D.
E658V	100.00	N.D.
I684L	100.00	N.D.
I684V	99.68	0.32 ± 0.006
I684G	98.84	1.16 ± 0.03
I684H	99.50	0.50
I684R	100.00	N.D.
L689V	99.34	0.66 ± 0.3
P752V	99.38	0.62
V753A	98.35	1.65 ± 0.04
V753I	100.00	N.D.
Y755R	N.D.	N.D.
Y755V	68.23	31.8 ± 0.5
S757P	99.73	0.27 ± 0.4
S757A	99.83	0.17 ± 0.01
S757G	100.00	N.D.
L808A	98.02	1.98 ± 1.9
L808G	100.00	N.D.
L808S	99.81	0.19 ± 0.05
L808V	99.68	0.32 ± 0.3

Table S5. Pik and DEBS PKS DNA FASTA sequences. The AT domains are in bolded (PikAT5 = red, PikAT6 = purple, EryAT6 = black) and include the linkers that were swapped during chimeragenesis. Pik docking domain is italicized.

Construct	Nucleotide Sequences
PikAIII WT	ATGGCGAACAACGAAGACAAGCTCCGCGACTACCTCAAGCGCGTCACCGC CGAGCTGCAGCAGAACACCAGGCGTCTGCGCGAGATCGAGGGACGCACGC ACGAGCCGGTGGCGATCGTGGGCATGGCCTGCCGCCTGCCGGGCGGTGTC GCCTCGCCCGAGGACCTGTGGCAGCTGGTGGCCGGGGACGGGGACGCGAT CTCGGAGTTCCCGCAGGACCGCGGCTGGGACGTGGAGGGGCTGTACGACC CCGACCCGGACGCGTCCGGCAGGACGTACTGCCGGTCCGGCGGATTCCTG CACGACGCCGGCGAGTTCGACGCCGACTTCTTCGGGATCTCGCCGCGCGA GGCCCTCGCCATGGACCCGCAGCAGCGACTGTCCCTCACCACCGCGTGGG AGGCGATCGAGAGCGCGGGCATCGACCCGACGGCCCTGAAGGGCAGCGGC CTCGGCGTCTTCGTCGGCGGCTGGCACACCGGCTACACCTCGGGGCAGAC CACCGCCGTGCAGTCGCCCGAGCTGGAGGGCCACCTGGTCAGCGGCGCGG CGCTGGGCTTCCTGTCCGGCCGTATCGCGTACGTCCTCGGTACGGACGGA CCGGCCCTGACCGTGGACACGGCCTGCTCGTCCTCGCTGGTCGCCCTGCA CCTCGCCGTGCAGGCCCTCCGCAAGGGCGAGTGCGACATGGCCCTCGCCG GTGGTGTCACGGTCATGCCCAACGCGGACCTGTTCGTGCAGTTCAGCCGG CAGCGCGGGCTGGCCGCGGACGGCCGGTCGAAGGCGTTCGCCACCTCGGC GGACGGCTTCGGCCCCGCGGAGGGCGCCGGAGTCCTGCTGGTGGAGCGCC TGTCGGACGCCCGCCGCAACGGACACCGGATCCTCGCGGTCGTCCGCGGC AGCGCGGTCAACCAGGACGGCGCCAGCAACGGCCTCACGGCTCCGCACGG GCCCTCCCAGCAGCGCGTCATCCGACGGGCCCTGGCGGACGCCCGGCTCG CGCCGGGTGACGTGGACGTCGTCGAGGCGCACGGCACGGGCACGCGGCTC GGCGACCCGATCGAGGCGCAGGCCCTCATCGCCACCTACGGCCAGGAGAA GAGCAGCGAACAGCCGCTGAGGCTGGGCGCGTTGAAGTCGAACATCGGGC ACACGCAGGCCGCGGCCGGTGTCGCAGGTGTCATCAAGATGGTCCAGGCG ATGCGCCACGGACTGCTGCCGAAGACGCTGCACGTCGACGAGCCCTCGGA CCAGATCGACTGGTCGGCGGGCACGGTGGAACTCCTCACCGAGGCCGTCG ACTGGCCGGAGAAGCAGGACGGCGGGCTGCGCCGCGCGGCTGTCTCCTCC **TTCGGCATCAGCGGGACGAACGCGCACGTCGTCCTGGAGGAGGCCCCGGC** **GGTCGAGGACTCCCCGGCCGTCGAGCCGCCGGCCGGTGGCGGTGTGGTGC** **CGTGGCCGGTGTCCGCGAAGACTCCGGCCGCGCTGGACGCCCAGATCGGG** **CAGCTCGCCGCGTACGCGGACGGTCGTACGGACGTGGATCCGGCGGTGGC** **CGCCCGCGCCCTGGTCGACAGCCGTACGGCGATGGAGCACCGCGCGGTCG** **CGGTCGGCGACAGCCGGGAGGCACTGCGGGACGCCCTGCGGATGCCGGAA** **GGACTGGTACGCGGCACGTCCTCGGACGTGGGCCGGGTGGCGTTCGTCTT** **CCCCGGCCAGGGCACGCAGTGGGCCGGCATGGGCGCCGAACTCCTTGACA** **GCTCACCGGAGTTCGCTGCCTCGATGGCCGAATGCGAGACCGCGCTCTCC** **CGCTACGTCGACTGGTCTCTTGAAGCCGTCGTCCGACAGGAACCCGGCGC** **ACCCACGCTCGACCGCGTCGACGTCGTCCAGCCCGTGACCTTCGCTGTCA** **TGGTCTCGCTGGCGAAGGTCTGGCAGCACCACGGCATCACCCCCCAGGCC** **GTCGTCGGCCACTCGCAGGGCGAGATCGCCGCCGCGTACGTCGCCGGTGC** **ACTCACCCTCGACGACGCCGCCCGCGTCGTCACCCTGCGCAGCAAGTCCA** **TCGCCGCCCACCTCGCCGGCAAGGGCGGCATGATCTCCCTCGCCCTCGAC** **GAGGCGGCCGTCCTGAAGCGACTGAGCGACTTCGACGGACTCTCCGTCGC** **CGCCGTCAACGGCCCCACCGCCACCGTCGTCTCCGGCGACCCGACCCAGA**

	TCGAGGAACTCGCCCGCACCTGCGAGGCCGACGGCGTCCGTGCGCGGATC **ATCCCGGTCGACTACGCCTCCCACAGCCGGCAGGTCGAGATCATCGAGAA** **GGAGCTGGCCGAGGTCCTCGCCGGACTCGCCCCGCAGGCTCCGCACGTGC** **CGTTCTTCTCCACCCTCGAAGGCACCTGGATCACCGAGCCGGTGCTCGAC** **GGCACCTACTGGTACCGCAACCTGCGCCATCGCGTGGGCTTCGCCCCCGC** **CGTGGAGACCTTGGCGGTTGACGGCTTCACCCACTTCATCGAGGTCAGCG** **CCCACCCCGTCCTCACCATGACCCTCCCCGAGACCGTCACCGGCCTCGGC** **ACCCTCCGCCGCGAACAGGGAGGCCAGGAGCGTCTGGTCACCTCACTCGC** **CGAAGCCTGGGCCAACGGCCTCACCATCGACTGGGCGCCCATCCTCCCCA** **CCGCAACCGGCCACCACCCCGAG**CTCCCCACCTACGCCTTCCAGACCGAG CGCTTCTGGCTGCAGAGCTCCGCGCCCACCAGCGCCGCCGACGACTGGCG TTACCGCGTCGAGTGGAAGCCGCTGACGGCCTCCGGCCAGGCGGACCTGT CCGGGCGGTGGATCGTCGCCGTCGGGAGCGAGCCAGAAGCCGAGCTGCTG GGCGCGCTGAAGGCCGCGGGAGCGGAGGTCGACGTACTGGAAGCCGGGGC GGACGACGACCGTGAGGCCCTCGCCGCCCGGCTCACCGCACTGACGACCG GCGACGGCTTCACCGGCGTGGTCTCGCTCCTCGACGACCTCGTGCCACAG GTCGCCTGGGTGCAGGCACTCGGCGACGCCGGAATCAAGGCGCCCCTGTG GTCCGTCACCCAGGGCGCGGTCTCCGTCGGACGTCTCGACACCCCCGCCG ACCCCGACCGGGCCATGCTCTGGGGCCTCGGCCGCGTCGTCGCCCTTGAG CACCCCGAACGCTGGGCCGGCCTCGTCGACCTCCCCGCCCAGCCCGATGC CGCCGCCCTCGCCCACCTCGTCACCGCACTCTCCGGCGCCACCGGCGAGG ACCAGATCGCCATCCGCACCACCGGACTCCACGCCCGCCGCCTCGCCCGC GCACCCCTCCACGGACGTCGGCCCACCCGCGACTGGCAGCCCCACGGCAC CGTCCTCATCACCGGCGGCACCGGAGCCCTCGGCAGCCACGCCGCACGCT GGATGGCCCACCACGGAGCCGAACACCTCCTCCTCGTCAGCCGCAGCGGC GAACAAGCCCCCGGAGCCACCCAACTCACCGCCGAACTCACCGCATCGGG CGCCCGCGTCACCATCGCCGCCTGCGACGTCGCCGACCCCCACGCCATGC GCACCCTCCTCGACGCCATCCCCGCCGAGACGCCCCTCACCGCCGTCGTC CACACCGCCGGCGCACCGGGCGGCGATCCGCTGGACGTCACCGGCCCGGA GGACATCGCCCGCATCCTGGGCGCGAAGACGAGCGGCGCCGAGGTCCTCG ACGACCTGCTCCGCGGCACTCCGCTGGACGCCTTCGTCCTCTACTCCTCG AACGCCGGGGTCTGGGGCAGCGGCAGCCAGGGCGTCTACGCGGCGGCCAA CGCCCACCTCGACGCGCTCGCCGCCCGGCGCCGCGCCCGGGGCGAGACGG CGACCTCGGTCGCCTGGGGCCTCTGGGCCGGCGACGGCATGGGCCGGGGC GCCGACGACGCGTACTGGCAGCGTCGCGGCATCCGTCCGATGAGCCCCGA CCGCGCCCTGGACGAACTGGCCAAGGCCCTGAGCCACGACGAGACCTTCG TCGCCGTGGCCGATGTCGACTGGGAGCGGTTCGCGCCCGCGTTCACGGTG TCCCGTCCCAGCCTTCTGCTCGACGGCGTCCCGGAGGCCCGGCAGGCGCT CGCCGCACCCGTCGGTGCCCCGGCTCCCGGCGACGCCGCCGTGGCGCCGA CCGGGCAGTCGTCGGCGCTGGCCGCGATCACCGCGCTCCCCGAGCCCGAG CGCCGGCCGGCGCTCCTCACCCTCGTCCGTACCCACGCGGCGGCCGTACT CGGCCATTCCTCCCCCGACCGGGTGGCCCCCGGCCGTGCCTTCACCGAGC TCGGCTTCGACTCGCTGACGGCCGTGCAGCTCCGCAACCAGCTCTCCACG GTGGTCGGCAACAGGCTCCCCGCCACCACGGTCTTCGACCACCCGACGCC CGCCGCACTCGCCGCGCACCTCCACGAGGCGTACCTCGCACCGGCCGAGC CGGCCCCGACGGACTGGGAGGGGCGGGTGCGCCGGGCCCTGGCCGAACTG CCCCTCGACCGGCTGCGGGACGCGGGGGTCCTCGACACCGTCCTGCGCCT CACCGGCATCGAGCCCGAGCCGGGTTCCGGCGGTTCGGACGGCGGCGCCG CCGACCCTGGTGCGGAGCCGGAGGCGTCGATCGACGACCTGGACGCCGAG GCCCTGATCCGGATGGCTCTCGGCCCCCGTAACACCTGA
PikAIV WT	ATGACGAGTTCCAACGAACAGTTGGTGGACGCTCTGCGCGCCTCTCTCAA GGAGAACGAAGAACTCCGGAAAGAGAGCCGTCGCCGGGCCGACCGTCGGC

	AGGAGCCCATGGCGATCGTCGGCATGAGCTGCCGGTTCGCGGGCGGAATC CGGTCCCCCGAGGACCTCTGGGACGCCGTCGCCGCGGGCAAGGACCTGGT CTCCGAGGTACCGGAGGAGCGCGGCTGGGACATCGACTCCCTCTACGACC CGGTGCCCGGGCGCAAGGGCACGACGTACGTCCGCAACGCCGCGTTCCTC GACGACGCCGCCGGATTCGACGCGGCCTTCTTCGGGATCTCGCCGCGCGA GGCCCTCGCCATGGACCCGCAGCAGCGGCAGCTCCTCGAAGCCTCCTGGG AGGTCTTCGAGCGGGCCGGCATCGACCCCGCGTCGGTCCGCGGCACCGAC GTCGGCGTGTACGTGGGCTGTGGCTACCAGGACTACGCGCCGGACATCCG GGTCGCCCCCGAAGGCACCGGCGGTTACGTCGTCACCGGCAACTCCTCCG CCGTGGCCTCCGGGCGCATCGCGTACTCCCTCGGCCTGGAGGGACCCGCC GTGACCGTGGACACGGCGTGCTCCTCTTCGCTCGTCGCCCTGCACCTCGC CCTGAAGGGCCTGCGGAACGGCGACTGCTCGACGGCACTCGTGGGCGGCG TGGCCGTCCTCGCGACGCCGGGCGCGTTCATCGAGTTCAGCAGCCAGCAG GCCATGGCCGCCGACGGCCGGACCAAGGGCTTCGCCTCGGCGGCGGACGG CCTCGCCTGGGGCGAGGGCGTCGCCGTACTCCTCCTCGAACGGCTCTCCG ACGCGCGGCGCAAGGGCCACCGGGTCCTGGCCGTCGTGCGCGGCAGCGCC ATCAACCAGGACGGCGCGAGCAACGGCCTCACGGCTCCGCACGGGCCCTC CCAGCAGCGCCTGATCCGCCAGGCCCTGGCCGACGCGCGGCTCACGTCGA GCGACGTGGACGTCGTGGAGGGCCACGGCACGGGGACCCGTCTCGGCGAC CCGATCGAGGCGCAGGCGCTGCTCGCCACGTACGGGCAGGGGCGCGCCCC GGGGCAGCCGCTGCGGCTGGGGACGCTGAAGTCGAACATCGGGCACACGC AGGCCGCTTCGGGTGTCGCCGGTGTCATCAAGATGGTGCAGGCGCTGCGC CACGGGGTGCTGCCGAAGACCCTGCACGTGGACGAGCCGACGGACCAGGT CGACTGGTCGGCCGGTTCGGTCGAGCTGCTCACCGAGGCCGTGGACTGGC CGGAGCGGCCGGGCCGGCTCCGCCGGGCGGGCGTCTCCGCGTTCGGCGTG GGC**GGGACGAACGCGCACGTCGTCCTGGAGGAGGCCCCGGCGGTCGAGGA** **GTCCCCTGCCGTCGAGCCGCCGGCCGGTGGCGGCGTGGTGCCGTGGCCGG** **TGTCCGCGAAGACCTCGGCCGCACTGGACGCCCAGATCGGGCAGCTCGCC** **GCATACGCGGAAGACCGCACGGACGTGGATCCGGCGGTGGCCGCCCGCGC** **CCTGGTCGACAGCCGTACGGCGATGGAGCACCGCGCGGTCGCGGTCGGCG** **ACAGCCGGGAGGCACTGCGGGACGCCCTGCGGATGCCGGAAGGACTGGTA** **CGGGGCACGGTCACCGATCCGGGCCGGGTGGCGTTCGTCTTCCCCGGCCA** **GGGCACGCAGTGGGCCGGCATGGGCGCCGAACTCCTCGACAGCTCACCCG** **AATTCGCCGCCGCCATGGCCGAATGCGAGACCGCACTCTCCCCGTACGTC** **GACTGGTCTCTCGAAGCCGTCGTCCGACAGGCTCCCAGCGCACCGACACT** **CGACCGCGTCGACGTCGTCCAGCCCGTCACCTTCGCCGTCATGGTCTCCC** **TCGCCAAGGTCTGGCAGCACCACGGCATCACCCCCGAGGCCGTCATCGGC** **CACTCCCAGGGCGAGATCGCCGCCGCGTACGTCGCCGGTGCCCTCACCCT** **CGACGACGCCGCTCGTGTCGTGACCCTCCGCAGCAAGTCCATCGCCGCCC** **ACCTCGCCGGCAAGGGCGGCATGATCTCCCTCGCCCTCAGCGAGGAAGCC** **ACCCGGCAGCGCATCGAGAACCTCCACGGACTGTCGATCGCCGCCGTCAA** **CGGGCCTACCGCCACCGTGGTTTCGGGCGACCCCACCCAGATCCAAGAAC** **TTGCTCAGGCGTGTGAGGCCGACGGCATCCGCGCACGGATCATCCCCGTC** **GACTACGCCTCCCACAGCGCCCACGTCGAGACCATCGAGAACGAACTCGC** **CGACGTCCTGGCGGGGTTGTCCCCCCAGACACCCCAGGTCCCCTTCTTCT** **CCACCCTCGAAGGCACCTGGATCACCGAACCCGCCCTCGACGGCGGCTAC** **TGGTACCGCAACCTCCGCCATCGTGTGGGCTTCGCCCCGGCCGTCGAGAC** **CCTCGCCACCGACGAAGGCTTCACCCACTTCATCGAGGTCAGCGCCCACC** **CCGTCCTCACCATGACCCTCCCCGACAAGGTCACCGGCCTGGCCACCCTC** **CGACGCGAGGACGGCGGACAGCACCGCCTCACCACCTCCCTTGCCGAGGC** **CTGGGCCAACGGCCTCGCCCTCGACTGGGCCTCCCTCCTGCCCGCCACGG** **GCGCCCTCAGCCCCGCCGTCCCCGAC**CTCCCGACGTACGCCTTCCAGCAC

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Ery6TE WT	*ATGACGAGTTCCAACGAACAGTTGGTGGACGCTCTGCGCGCCTCTCTCAA* *GGAGAACGAAGAACTCCGGAAAGAGAGCCGTCGCCGGGCCGACCGTCGGC* *AGGAGGAG*ATCGCGATCGTCGGCATGGCCTGCCGCTTCCCCGGCGGCGTG CACAACCCCGGTGAGCTGTGGGAGTTCATCGTCGGCGGCGGAGACGCCGT GACGGAGATGCCCACCGACCGCGGCTGGGACCTCGACGCGCTGTTCGACC CCGACCCGCAGCGCCACGGAACCAGCTACTCGCGACACGGCGCGTTCCTC GACGGGGCCGCCGACTTCGACGCGGCGTTCTTCGGGATCTCGCCGCGCGA GGCGCTGGCGATGGACCCGCAGCAGCGCCAGGTCCTGGAAACGACGTGGG AGCTGTTCGAGAACGCCGGCATCGACCCGCACTCGCTGCGGGGCAGCGAC ACCGGCGTCTTCCTCGGCGCCGCGTACCAGGGCTACGGCCAGGACGCGGT GGTGCCCGAGGACAGCGAGGGCTACCTGCTCACCGGCAACTCCTCCGCCG TGGTGTCCGGCCGGGTCGCCTACGTGCTGGGGCTGGAAGGCCCCGCGGTC ACGGTGGACACGGCGTGTTCGTCGTCGTTGGTGGCCTTGCATTCGGCGTG TGGGTCGTTGCGTGACGGTGACTGCGGTCTTGCGGTGGCCGGTGGTGTGT CGGTGATGGCGGGCCCGGAGGTGTTCACCGAGTTCTCCCGCCAGGGCGGC TTGGCCGTGGACGGGCGCTGCAAGGCGTTCTCCGCGGAGGCCGACGGCTT CGGTTTCGCCGAGGGCGTCGCGGTGGTCCTGCTCCAGCGGTTGTCCGACG CCCGCAGGGCGGGTCGCCAGGTGCTCGGCGTGGTCGCGGGCTCGGCGATC AACCAGGACGGCGCGAGCAACGGTCTCGCGGCGCCGAGCGGCGTCGCCCA GCAGCGCGTGATCCGCAAGGCGTGGGCGCGTGCGGGGATCACGGGCGCGG ATGTGGCCGTGGTGGAGGCGCATGGGACCGGTACGCGGCTGGGCGATCCG GTGGAGGCGTCGGCGTTGCTGGCTACTTACGGCAAGTCGCGCGGGTCGTC GGGCCCGGTGCTGCTGGGTTCGGTGAAGTCGAACATCGGTCACGCGCAGG CGGCCGCGGGTGTCGCGGGCGTGATCAAGGTGGTCCTGGGGTTGAACCGC GGCCTGGTGCCGCCGATGCTCTGCCGCGGCGAGCGGTCGCCGCTGATCGA

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CGTTGCGTGCGCTGGCGGGCAAGGGCGGCATGGTCTCGTTGGCGGCTCCC
GGTGAACGCGCCCGCGCGCTGATCGCACCGTGGGAGGACCGGATCTCCGT
CGCGGCGGTCAACTCCCCGTCCTCGGTCGTGGTCTCCGGCGATCCGGAGG
CGCTGGCCGAACTCGTCGCACGTTGCGAGGACGAGGGCGTGCGCGCCAAG
ACGCTCCCGGTGGACTACGCCTCGCACTCCCGCCACGTCGAGGAGATCCG
CGAGACGATCCTCGCCGACCTCGACGGCATCTCCGCGCGGCGTGCCGCCA
TCCCGCTCTACTCCACGCTGCACGGCGAACGGCGCGACGGCGCCGACATG
GGTCCGCGGTACTGGTACGACAACCTGCGCTCCCAGGTGCGCTTCGACGA
GGCGGTCTCGGCCGCCGTCGCCGACGGTCACGCCACCTTCGTCGAGATGA
GCCCGCACCCGGTGCTCACCGCGGCGGTGCAGGAGATCGCCGCGGACGCC
GTGGCCATCGGGTCGCTGCACCGCGACACCGCGGAGGAGCACCTGATCGC
CGAGCTCGCCCGGGCGCACGTGCACGGCGTGGCCGTGGACTGGCGGAACG
TCTTCCCGGCGGCACCTCCGGTGGCGCTGCCCAACTACCCGTTCGAGCCC
CAGCGGTACTGGCTCGCGCCGGAGGTGTCCGACCAGCTCGCCGACAGCCG
CTACCGCGTCGACTGGCGACCGCTGGCCACCACGCCGGTGGACCTGGAAG
GCGGCTTCCTGGTCCACGGGTCCGCACCGGAGTCGCTGACCAGCGCAGTC
GAGAAGGCCGGAGGCCGCGTCGTGCCGGTCGCCTCGGCCGACCGCGAAGC
GCTCGCGGCGGCCCTGCGGGAGGTGCCGGGCGAGGTCGCCGGCGTGCTCT
CGGTCCACACCGGCGCCGCAACGCACCTCGCCCTGCACCAGTCGCTGGGT
GAGGCCGGCGTGCGGGCCCCGCTCTGGCTGGTCACCAGCCGAGCGGTCGC
GCTCGGGGAGTCCGAGCCGGTCGATCCCGAGCAGGCGATGGTGTGGGGTC
TCGGGCGCGTCATGGGCCTGGAGACCCCGGAACGGTGGGGCGGTCTGGTG
GACCTGCCCGCCGAACCCGCGCCGGGGGACGGCGAGGCGTTCGTCGCCTG
CCTCGGCGCGGACGGCCACGAGGACCAGGTCGCGATCCGTGACCACGCCC
GCTACGGCCGCCGCCTCGTCCGCGCCCCGCTGGGCACCCGCGAGTCGAGC
TGGGAGCCGGCGGGCACGGCGCTGGTCACCGGCGGCACCGGTGCGCTCGG
CGGCCACGTCGCCCGCCACCTCGCCAGGTGCGGGGTGGAGGACCTGGTGC
TGGTCAGCAGGCGCGGCGTCGACGCTCCCGGCGCGGCCGAGCTGGAAGCC
GAACTGGTCGCCCTCGGCGCGAAGACGACCATCACCGCCTGCGACGTGGC
CGACCGCGAGCAGCTCTCCAAGCTGCTGGAAGAACTGCGCGGGCAGGGAC
GTCCGGTGCGGACCGTCGTGCACACCGCCGGGGTGCCCGAATCGAGGCCG
CTGCACGAGATCGGCGAGCTGGAGTCGGTCTGCGCGGCGAAGGTGACCGG
GGCCCGGCTGCTCGACGAGCTGTGCCCGGACGCCGAGACCTTCGTCCTGT
TCTCGTCCGGAGCGGGGGTGTGGGGCAGTGCGAACCTCGGCGCCTACTCC
GCGGCCAACGCCTACCTCGACGCGCTGGCCCACCGCCGCCGTGCGGAAGG
CCGTGCGGCGACGTCCGTCGCGTGGGGCGCCTGGGCGGGCGAGGGCATGG

	CCACCGGCGACCTCGAGGGGCTCACCCGGCGCGGCCTGCGCCCGATGGCG CCCGAGCGCGCGATCCGCGCGCTGCACCAGGCGCTGGACAACGGCGACAC GTGCGTTTCGATCGCCGACGTCGACTGGGAGCGCTTCGCGGTCGGCTTCA CCGCCGCCCGGCCGCGTCCGCTGCTGGACGAGCTCGTCACGCCGGCGGTG GGGGCCGTCCCCGCGGTGCAGGCGGCCCCGGCGCGGGAGATGACGTCGCA GGAGTTGCTGGAGTTCACGCACTCGCACGTCGCGGCGATCCTCGGGCATT CCAGCCCGGACGCGGTCGGGCAGGACCAGCCGTTCACCGAGCTCGGCTTC GACTCGCTGACCGCGGTCGGGCTGCGCAACCAGCTCCAGCAGGCCACCGG GCTCGCGCTGCCCGCGACCCTGGTGTTCGAGCACCCCACGGTCCGCAGGT TGGCCGACCACATAGGACAGCAGCTCGACAGCGGGACTCCCGCCCGGGAA GCGAGCAGCGCTCTTCGCGACGGCTACCGGCAGGCGGGCGTGTCGGGCAG GGTCCGGTCCTACCTCGACCTGCTGGCGGGGCTGTCGGACTTCCGCGAGC ACTTCGACGGCTCCGACGGGTTCTCCCTCGATCTCGTGGACATGGCCGAC GGTCCCGGAGAGGTCACGGTGATCTGCTGCGCGGGAACGGCGGCGATCTC CGGTCCGCACGAGTTCACCCGGCTCGCCGGGGCGCTGCGCGGAATCGCTC CGGTTCGGGCCGTGCCCCAGCCCGGCTACGAGGAGGGCGAACCTCTGCCG TCGTCGATGGCGGCGGTGGCGGCGGTGCAGGCCGATGCGGTCATCAGGAC ACAGGGGGACAAGCCGTTCGTGGTGGCCGGTCACTCCGCGGGGGCACTGA TGGCCTACGCGCTGGCGACCGAACTGCTCGATCGCGGGCACCCGCCACGC GGTGTCGTCCTGATCGACGTCTACCCGCCCGGTCACCAGGACGCGATGAA CGCCTGGCTGGAGGAGCTGACCGCCACGCTGTTCGACCGCGAGACGGTGC GGATGGACGACACCAGGCTCACCGCCCTGGGCGCCTACGACCGCCTCACC GGTCAGTGGCGACCCCGGGAAACCGGGCTGCCGACGCTGCTGGTCAGCGC CGGCGAGCCGATGGGTCCGTGGCCCGACGACAGCTGGAAGCCGACGTGGC CCTTCGAGCACGACACCGTCGCCGTCCCCGGCGACCACTTCACGATGGTG CAGGAACACGCCGACGCGATCGCGCGGCACATCGACGCCTGGCTGGGCGG AGGGAATTCAAGA

Table S6. Primers for Pik and DEBS PKS construction and mutagenesis.

Primer Name	5'→3' Primer Sequence	Primer Function
PikAT5_PikAIV.Gib1	CGTGGGCGGGACGAACGCG	Amplifies PikAT5 for GA with PikAIV
PikAT5_PikAIV.Gib2	GAGCGGTGCTGGAAGGCGTAGGTGGGG	
PikAIV_PikAT5.Gib1	CTTCCAGCACCGCTCGTAC	Amplifies PikAIV for GA with PikAT5
PikAIV_PikAT5.Gib2	TTCGTCCCGCCCACGCCGAA	
PikAT6_PikAIII.Gib1	CATCAGCGGGACGAACGCG	Amplifies PikAT6 for GA with PikAIII
PikAT6_PikAIII.Gib2	CGCTCGGTCTGGAAGGCGTACGTCGGGAG	
PikAIII_PikAT6.Gib1	CTTCCAGACCGAGCGCTTC	Amplifies PikAIII for GA with PikAT6
PikAIII_PikAT6.Gib2	TTCGTCCCGCTGATGCCGAA	
PikAIII_V753X.FOR	ATCATCCCGDNTGACTACGCCTCCCACAGCCGGCA GGTCGAGATCA	Partial saturation of PikAIII Val753
PikAIII_V753X.REV	GGCGTAGTCANHCGGGATGATCCGCGCACGGACG CCGTCGGCCTCG	
PikAIII_Y755V.FOR	GTCGCCTCCCACAGCCGG	Introduces Y755V mutation in PikAIII
PikAIII_Y755V.REV	GTCGACCGGGATGATCCGC	
PikAIV_Y753X.FOR	NNKGCCTCCCACAGCGCCC	Full saturation of PikAIV Tyr753
PikAIV_Y753X.REV	GTCGACGGGGATGATCCGTGC	
Ery6-AT.Gib3	CTGCCCAACTACCCGTTCG	Amplifies Ery6TE for GA with Pik ATs
Ery6-AT.Gib2	GCTCACCCCGAACGCC	
PikAT6_Ery6.Gib1	GCGTTCGGGGTGAGCGGGACGAACGCG	Amplifies PikAT6 for GA with Ery6TE
PikAT6_Ery6.Gib3	GGGTAGTTGGGCAGGTCGGGGACGGCGG	
PikAT5_Ery6.Gib1	GCGTTCGGGGTGAGCGGGACGAACGC	Amplifies PiKAT5 for GA with Ery6TE
PikAT5_Ery6.Gib3	GGGTAGTTGGGCAGCTCGGGGTGGTGGCC	

Table S7. High-resolution LC-MS parameters and gradient.

HESI Source Parameters	
Spray Voltage	3.5 kV
Capillary Temperature	350 °C
Heater Temperature	300 °C
S Lens RF Level	70 V
Sheath Gas Flow Rate	60 au
Resolution	70,000 FWHM
Scan Range	100-1000 m/z

LC Gradient	
Time (min)	% B
0.0	25.0
1.0	25.0
10.0	59.0
11.0	85.0
12.0	85.0
12.5	25.0
16.5	25.0

Figure S1. Amino Acid Alignment of EryAT2, EryAT6, PikAT5, and PikAT6. Residues targeted for mutagenesis are boxed in red. Numbering does not include the N-terminal hexhistidine tag.

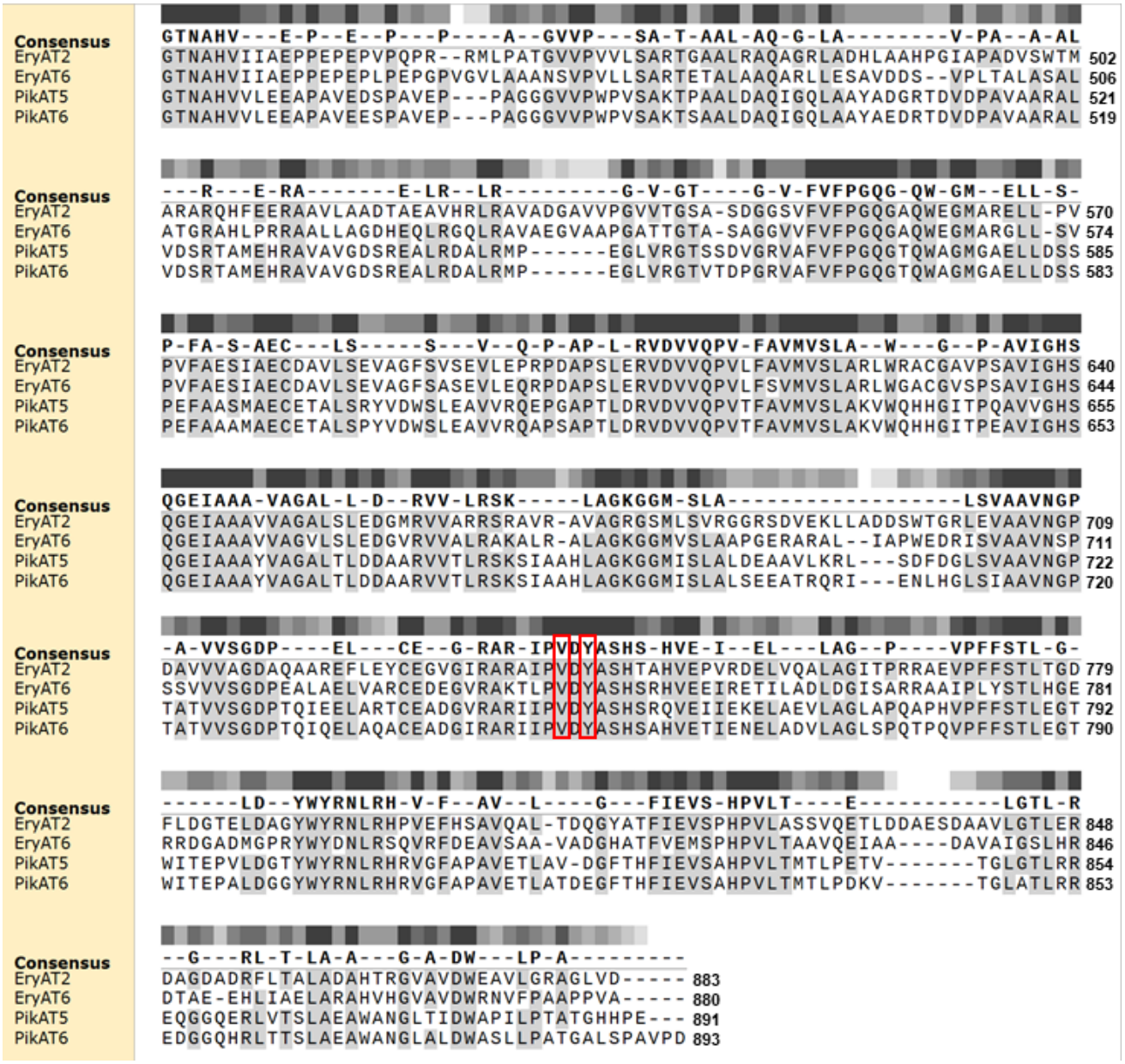
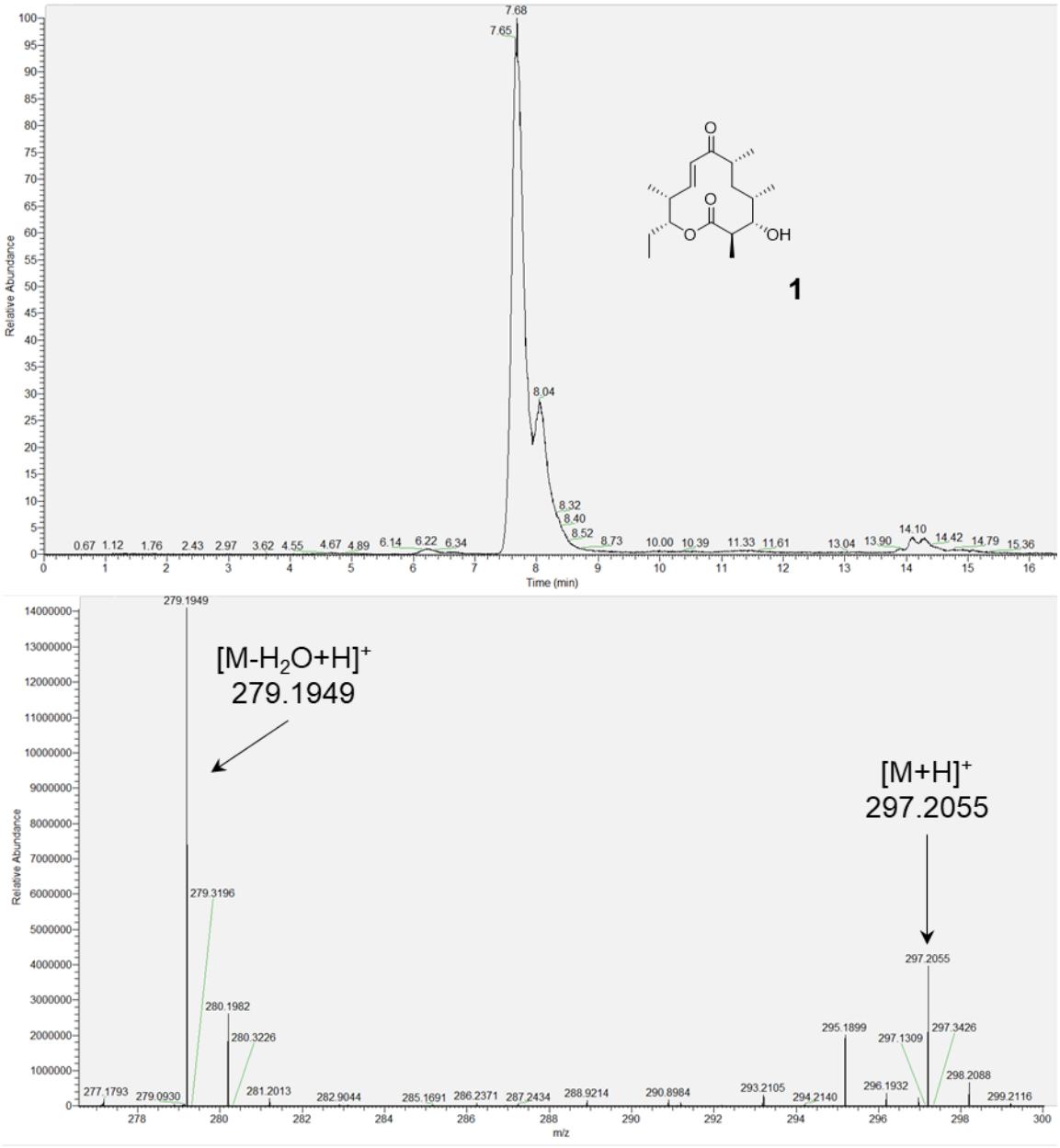
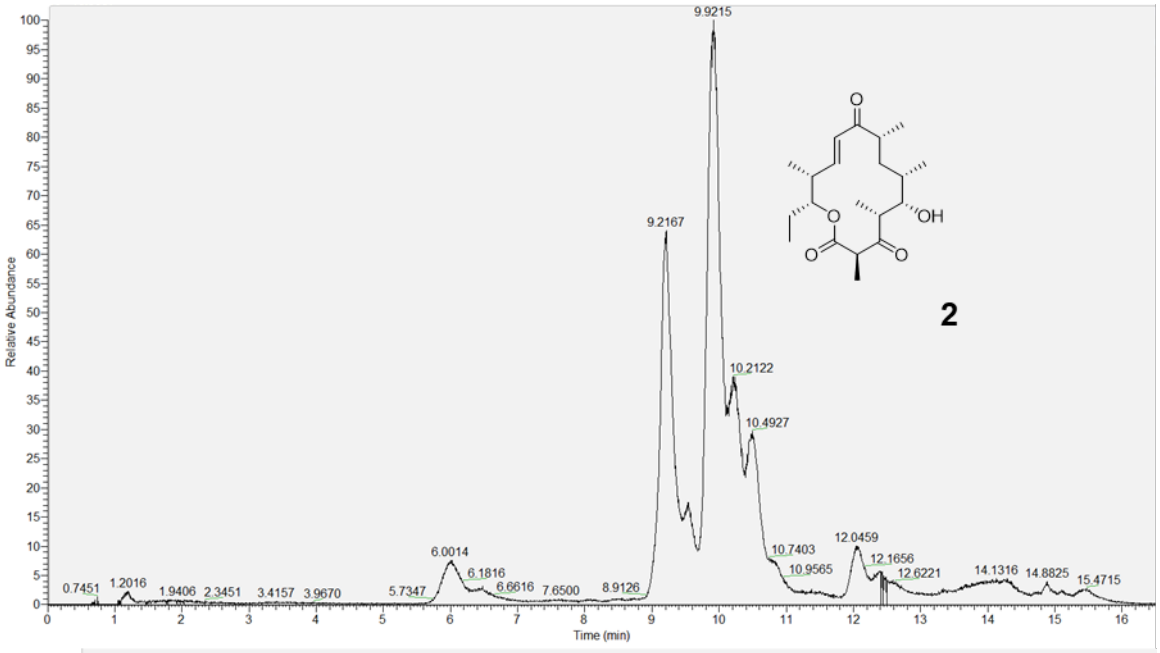


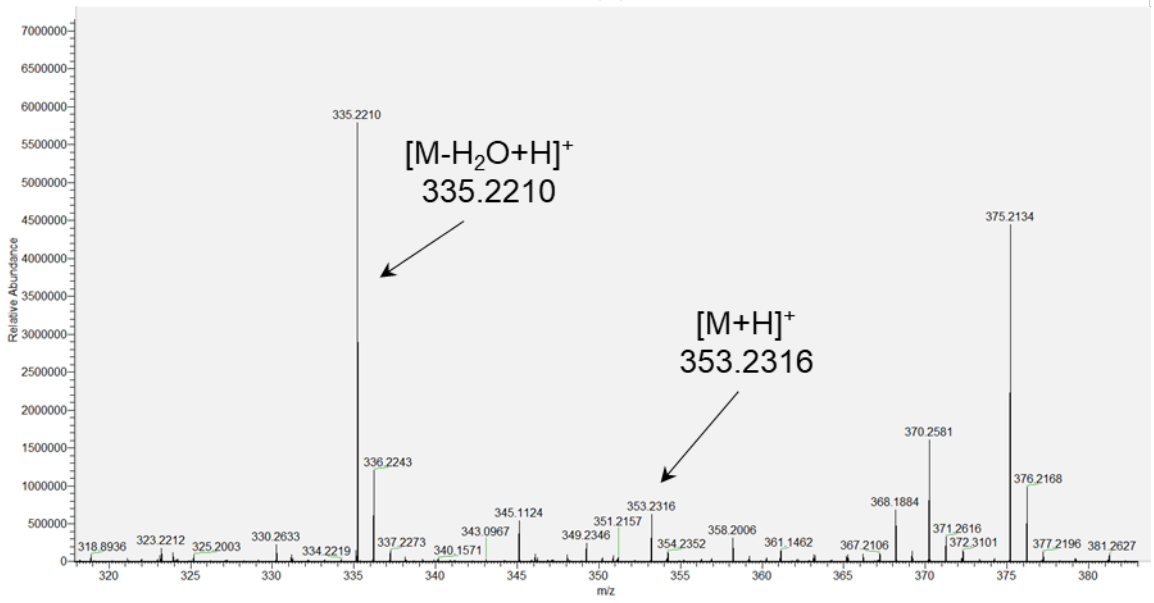
Figure S2. Representative LC-MS chromatograms of products from lysate module-catalyzed reactions using 8 and 9a. Top panel for each compound shows the extracted ion chromatogram. Bottom panel shows the total ion spectra.

(A)

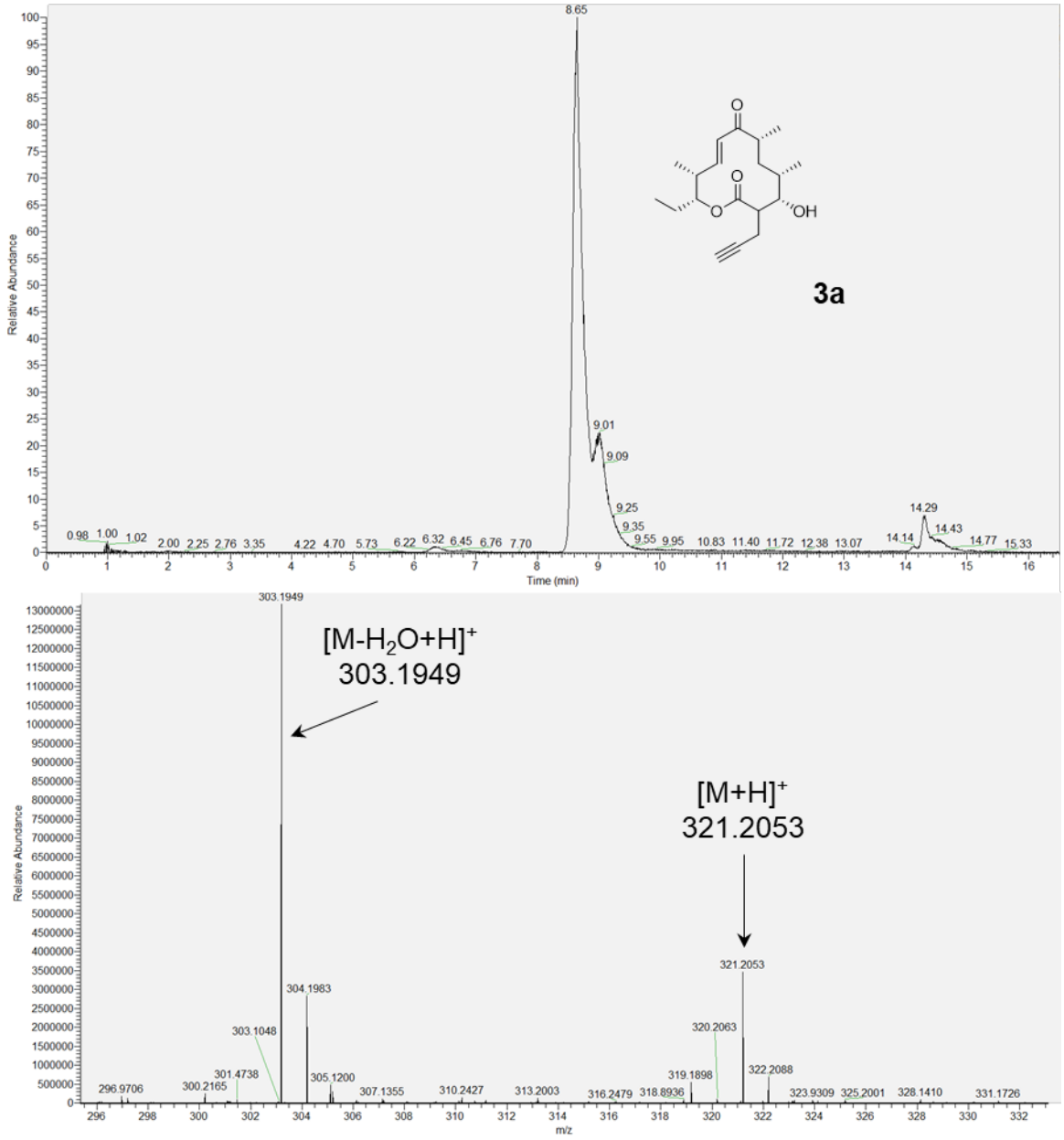


(B)

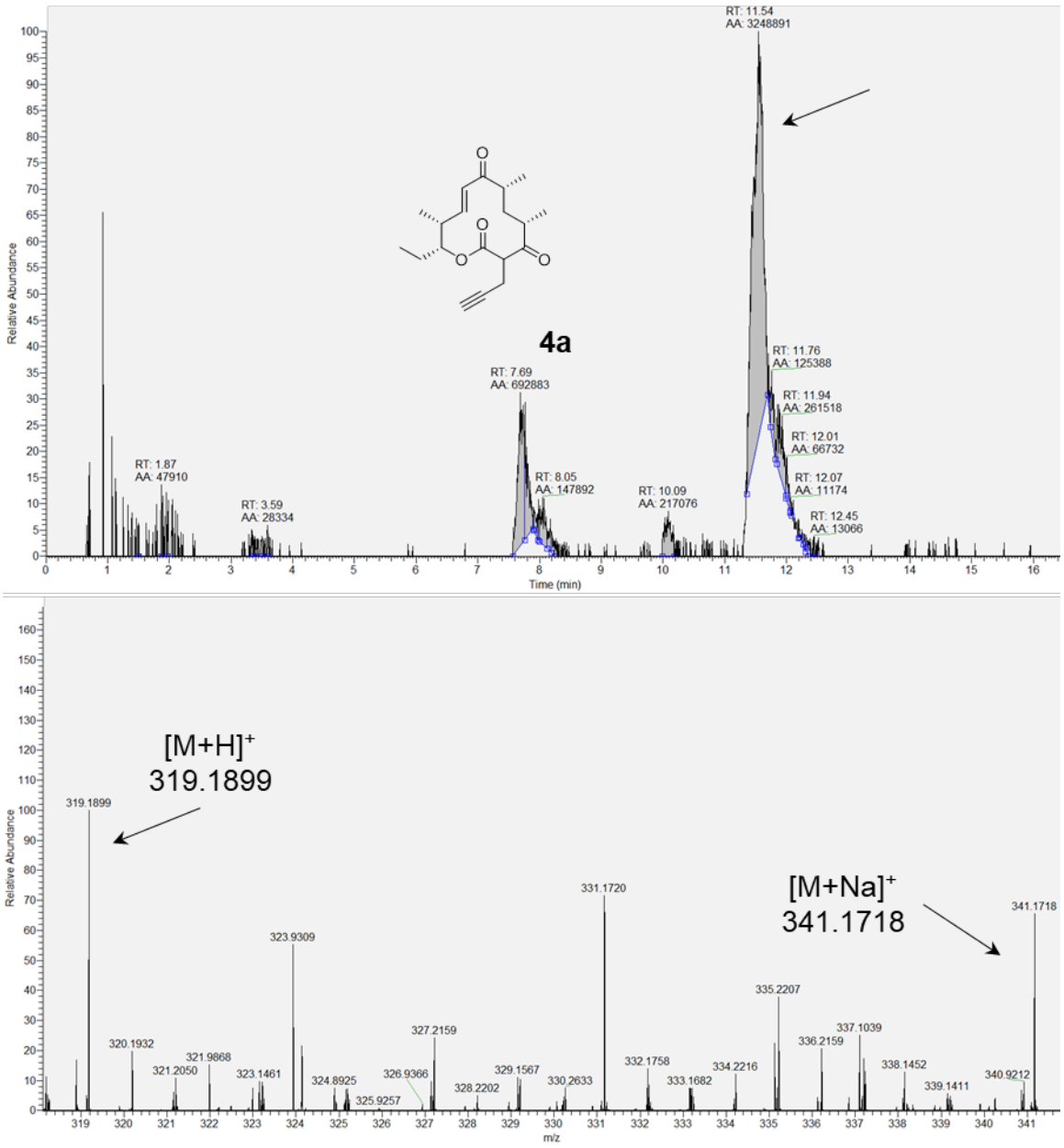

9.9215
9.2167
10.2122
10.4927
6.0014
6.1816
6.6616
7.6500
8.9126
10.7403
10.9565
12.0459
12.1656
12.6221
14.1316
14.8825
15.4715
0.7451
1.2016
1.9406
2.3451
3.4157
3.9670
5.7347
Relative Abundance
Time (min)
O
OH
2


335.2210
[M-H2O+H]+
335.2210
[M+H]+
353.2316
375.2134
370.2581
376.2168
336.2243
368.1884
371.2616
372.3101
345.1124
353.2316
351.2157
343.0967
349.2346
358.2006
354.2352
361.1462
367.2106
377.2196
381.2627
318.8936
323.2212
325.2003
330.2633
334.2219
337.2273
340.1571
Relative Abundance
m/z

(C)


8.65
9.01
9.09
9.25
9.35
9.55
9.95
10.83
11.40
11.72
12.38
13.07
14.14
14.29
14.43
14.77
15.33
0.98
1.00
1.02
2.00
2.25
2.76
3.35
4.22
4.70
5.73
6.22
6.32
6.45
6.76
7.70
Relative Abundance
Time (min)
O
O
O
OH
3a
303.1949
[M-H2O+H]+
303.1949
[M+H]+
321.2053
321.2053
304.1983
303.1048
320.2063
322.2088
301.4738
305.1200
319.1898
296.9706
300.2165
307.1355
310.2427
313.2003
316.2479
318.8936
323.9309
325.2001
328.1410
331.1726
Relative Abundance
m/z

(D)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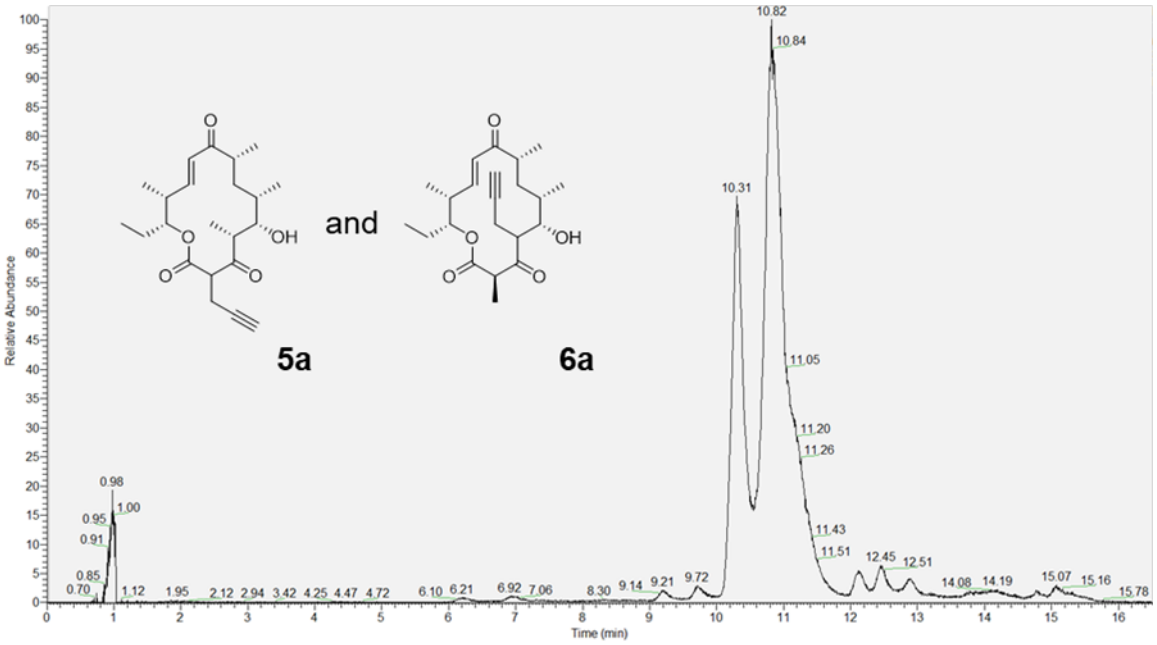
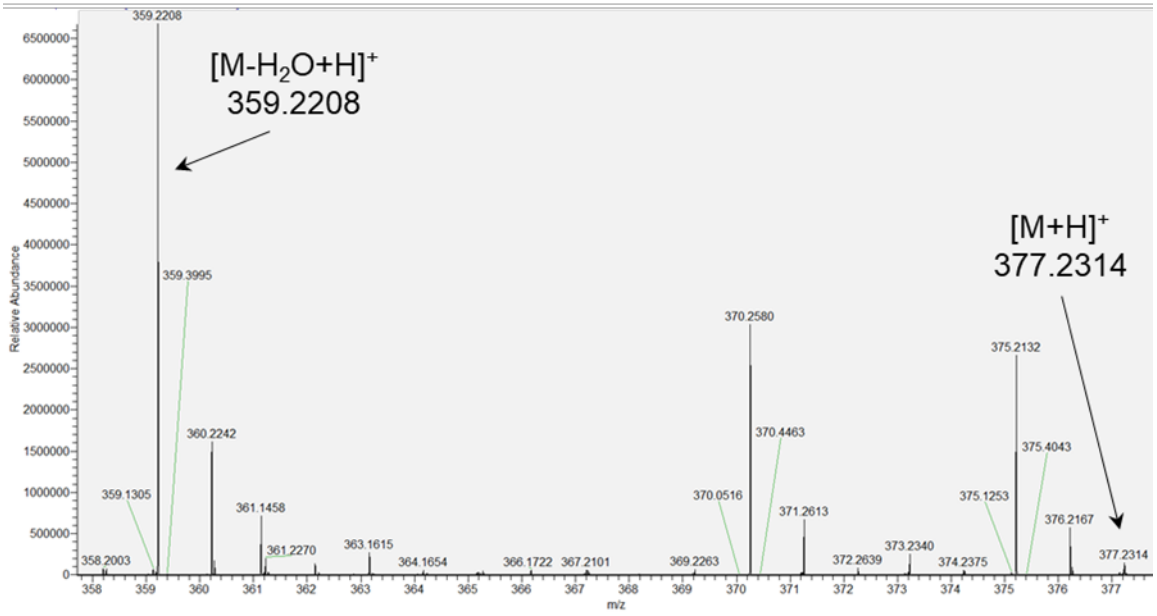
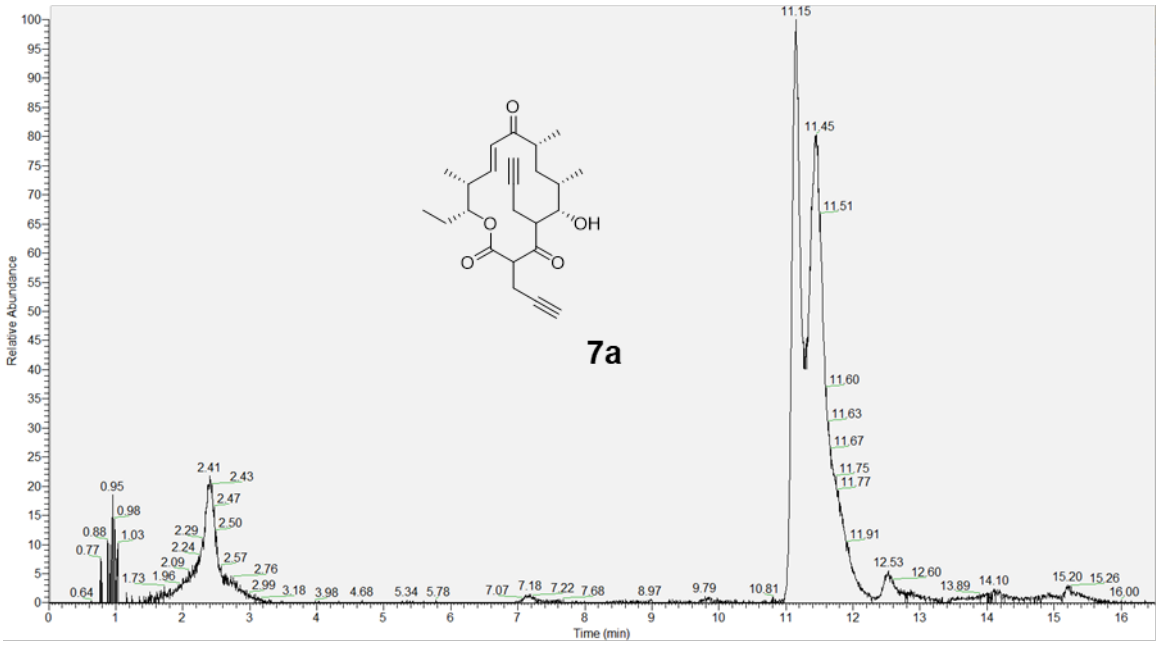
AA: 3248891
4a
RT: 7.69
AA: 692883
RT: 11.76
AA: 125388
RT: 11.94
AA: 261518
RT: 12.01
AA: 66732
RT: 12.07
AA: 11174
RT: 12.45
AA: 13066
RT: 1.87
AA: 47910
RT: 3.59
AA: 28334
RT: 8.05
AA: 147892
RT: 10.09
AA: 217076
Relative Abundance
Time (min)
[M+H]+
319.1899
[M+Na]+
341.1718
319.1899
323.9309
331.1720
341.1718
335.2207
327.2159
320.1932
321.9868
321.2050
323.1461
324.8925
326.9366
325.9257
328.2202
329.1567
330.2633
332.1758
333.1682
334.2216
336.2159
337.1039
338.1452
339.1411
340.9212
m/z

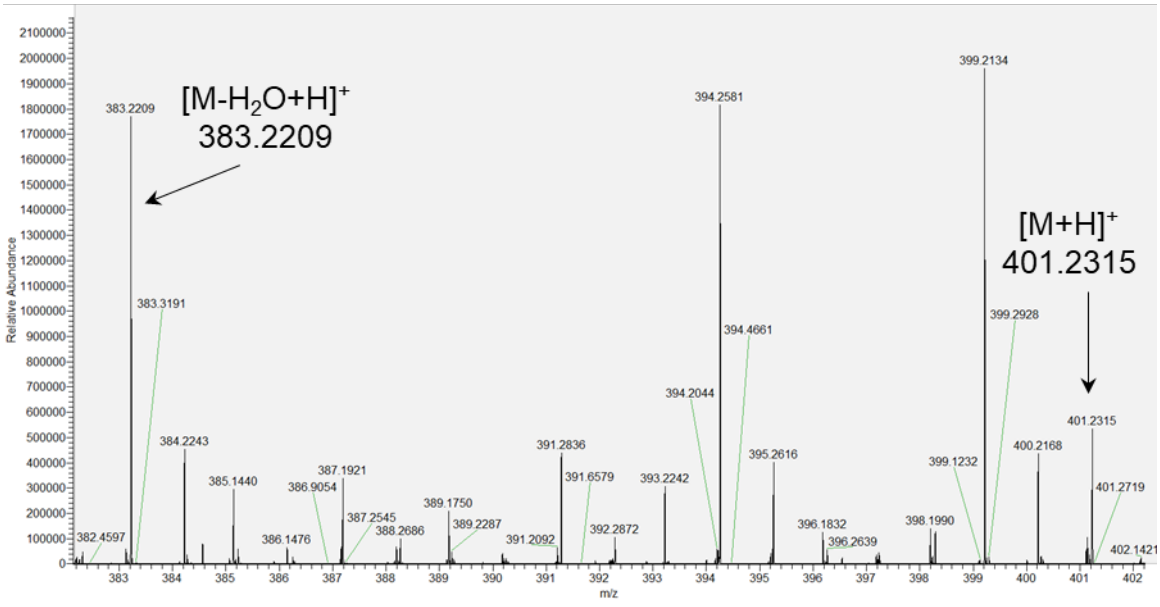
(E)


and
5a
6a
Relative Abundance
Time (min)

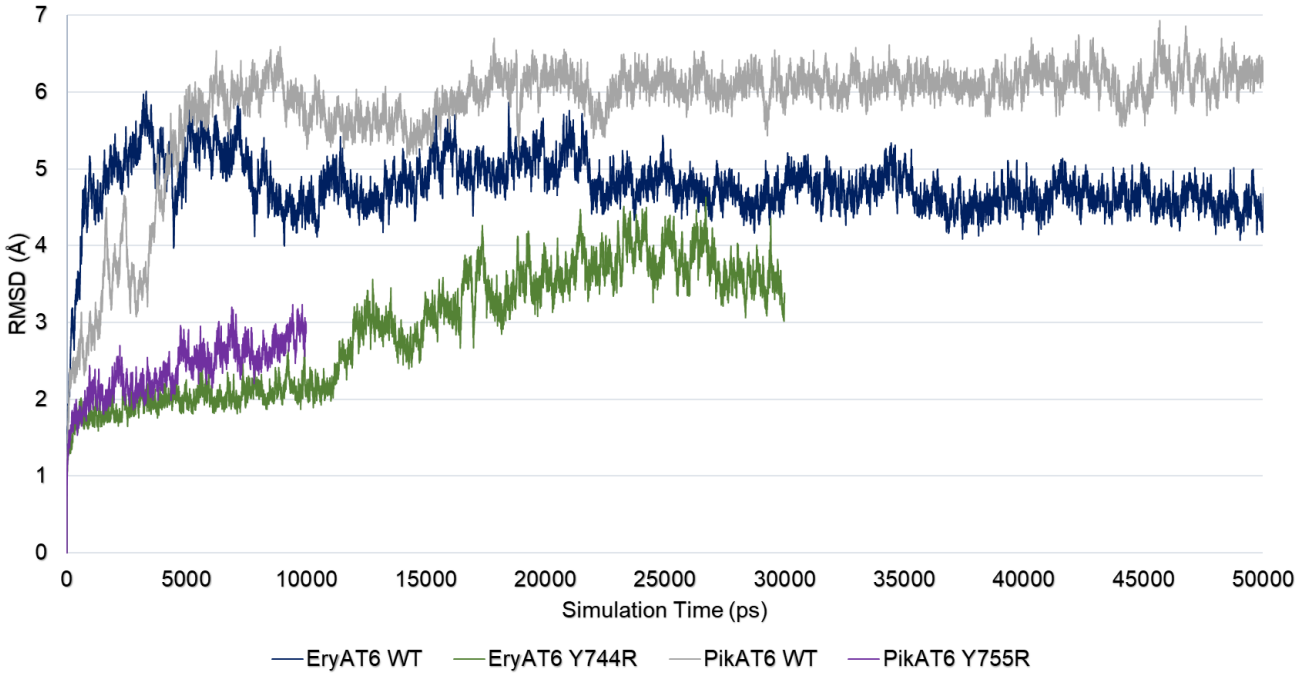

[M-H2O+H]+
359.2208
[M+H]+
377.2314
Relative Abundance
m/z

(F)


Relative Abundance
Time (min)
OH
7a
11.15
11.45
11.51
11.60
11.63
11.67
11.75
11.77
11.91
12.53
12.60
13.89
14.10
15.20
15.26
16.00
2.41
2.43
2.47
2.50
2.57
2.76
2.99
3.18
3.98
4.68
5.34
5.78
7.07
7.18
7.22
7.68
8.97
9.79
10.81
0.64
0.77
0.88
0.95
0.98
1.03
1.73
1.96
2.09
2.24
2.29


[M-H2O+H]+
383.2209
[M+H]+
401.2315
Relative Abundance
m/z
383.2209
383.3191
384.2243
385.1440
386.9054
387.1921
387.2545
388.2686
389.1750
389.2287
391.2092
391.2836
391.6579
392.2872
393.2242
394.2044
394.2581
394.4661
395.2616
396.1832
396.2639
398.1990
399.1232
399.2134
399.2928
400.2168
401.2315
401.2719
402.1421
382.4597
386.1476

Supplementary Figure S3. RMSDs for EryAT6 and PikAT6 MD simulations. Root mean square deviations (RMSDs) for the MD simulations of: EryAT6 wild-type over 50 ns (blue); PikAT6 wild-type over 50 ns (grey); EryAT6 Y744R over 30 ns (green); PikAT6 Y755R over 10 ns (purple). The simulations of the mutant enzymes were derived from the previously-stabilized simulations of the wild-type enzymes. Both mutant enzymes showed some instability over time relative to their wild-type counterparts.



Supplementary Figure S4. Comparison of PikAT5 and PikAT6 active sites. (A) Homology model of PikAT5 active site with the YASH motif and other important residues shown. **(B)** Homology model of PikAT6 active site after a 60 ns MD simulation. The YASH motif and other important residues are shown.

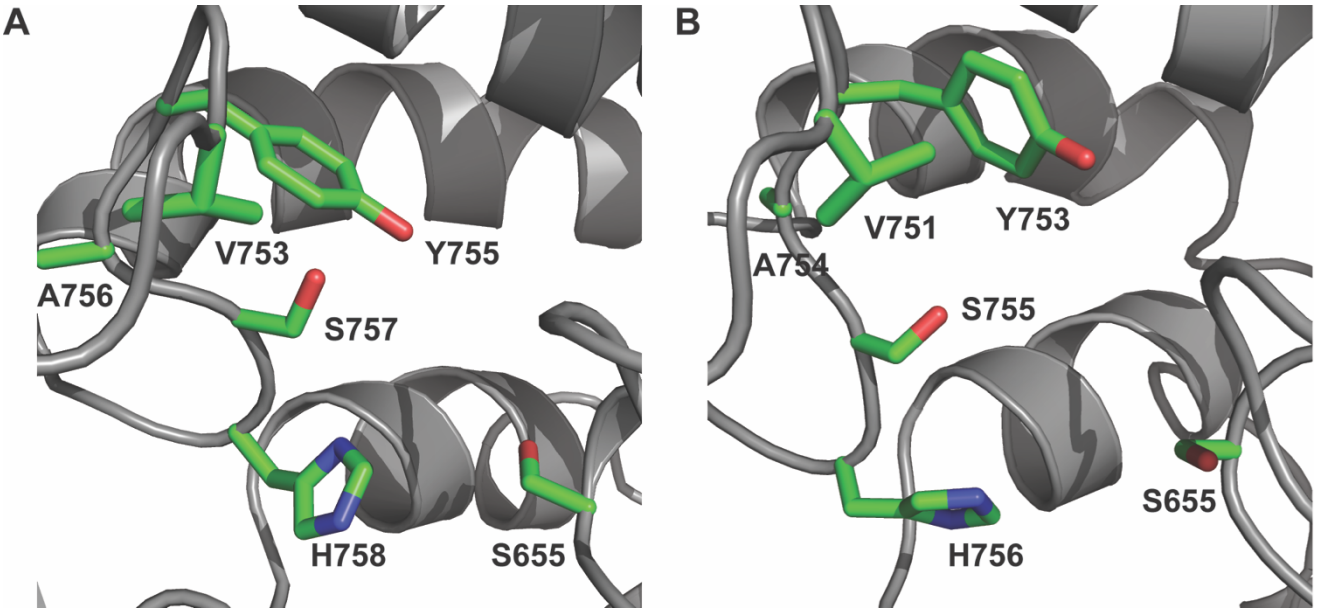
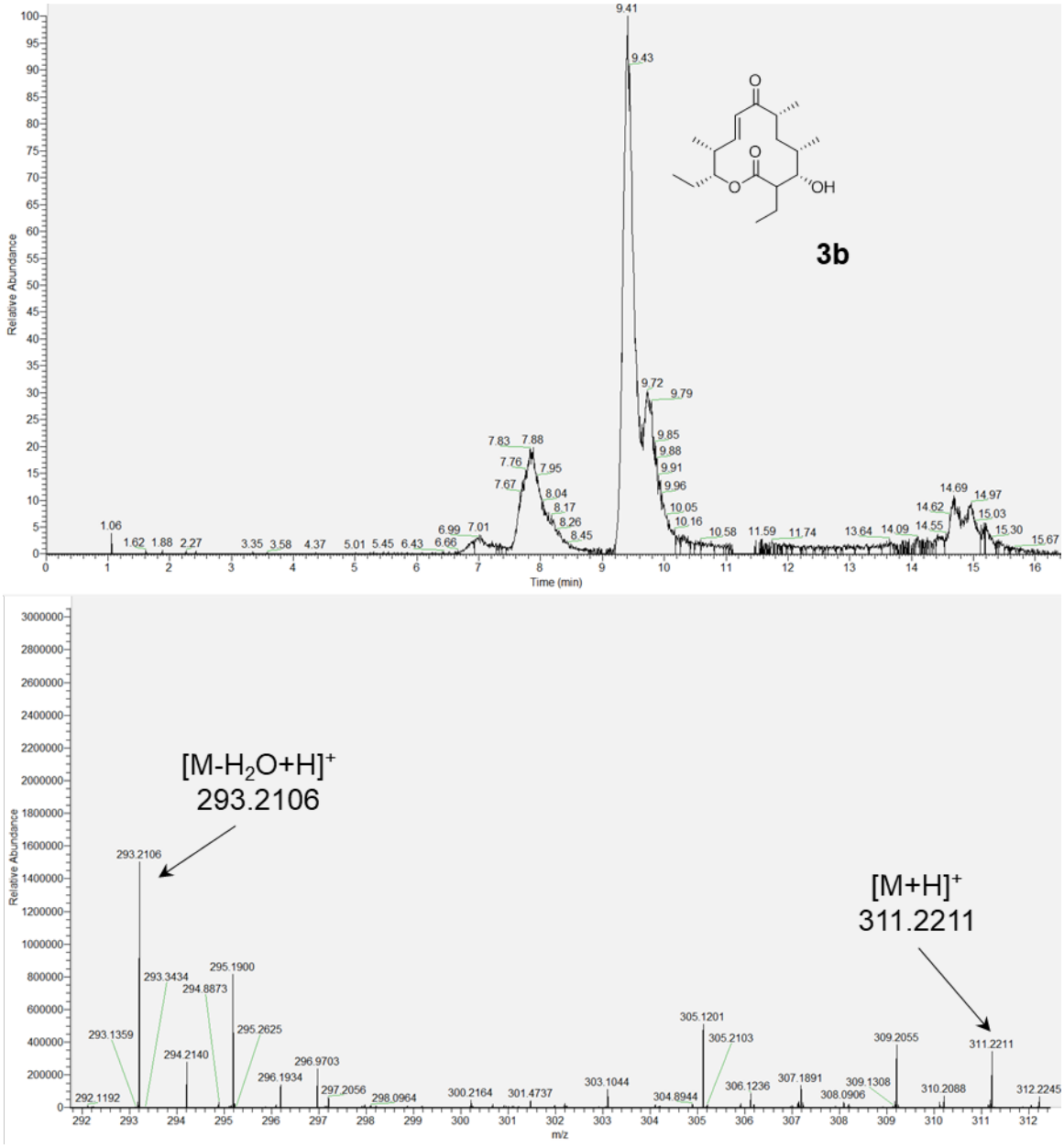
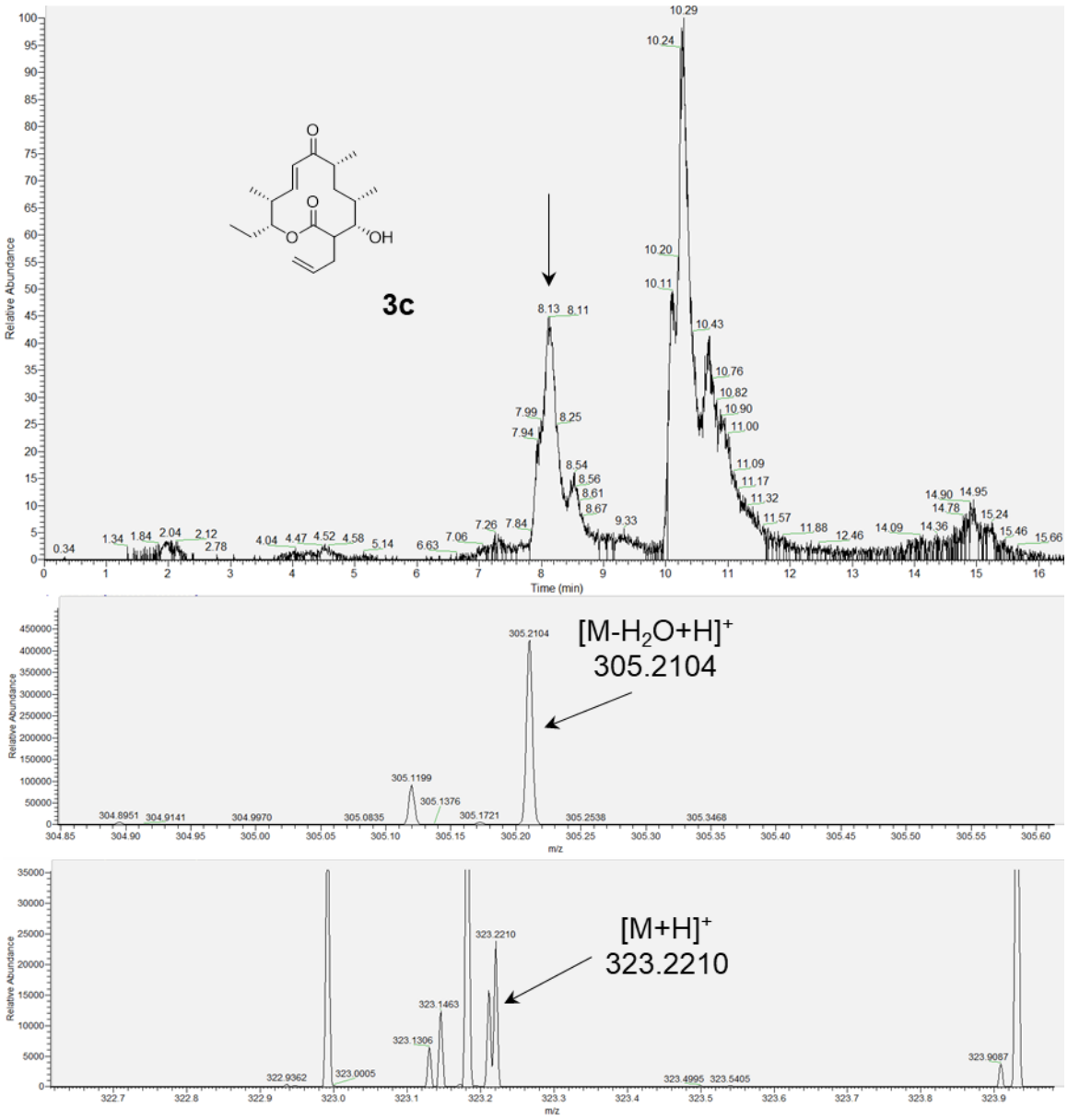


Figure S5. Representative LC-MS chromatograms of products from lysate module-catalyzed reactions using 8 and 9b-d. Top panel for each compound shows the extracted ion chromatogram. Bottom panel shows the total ion spectra.

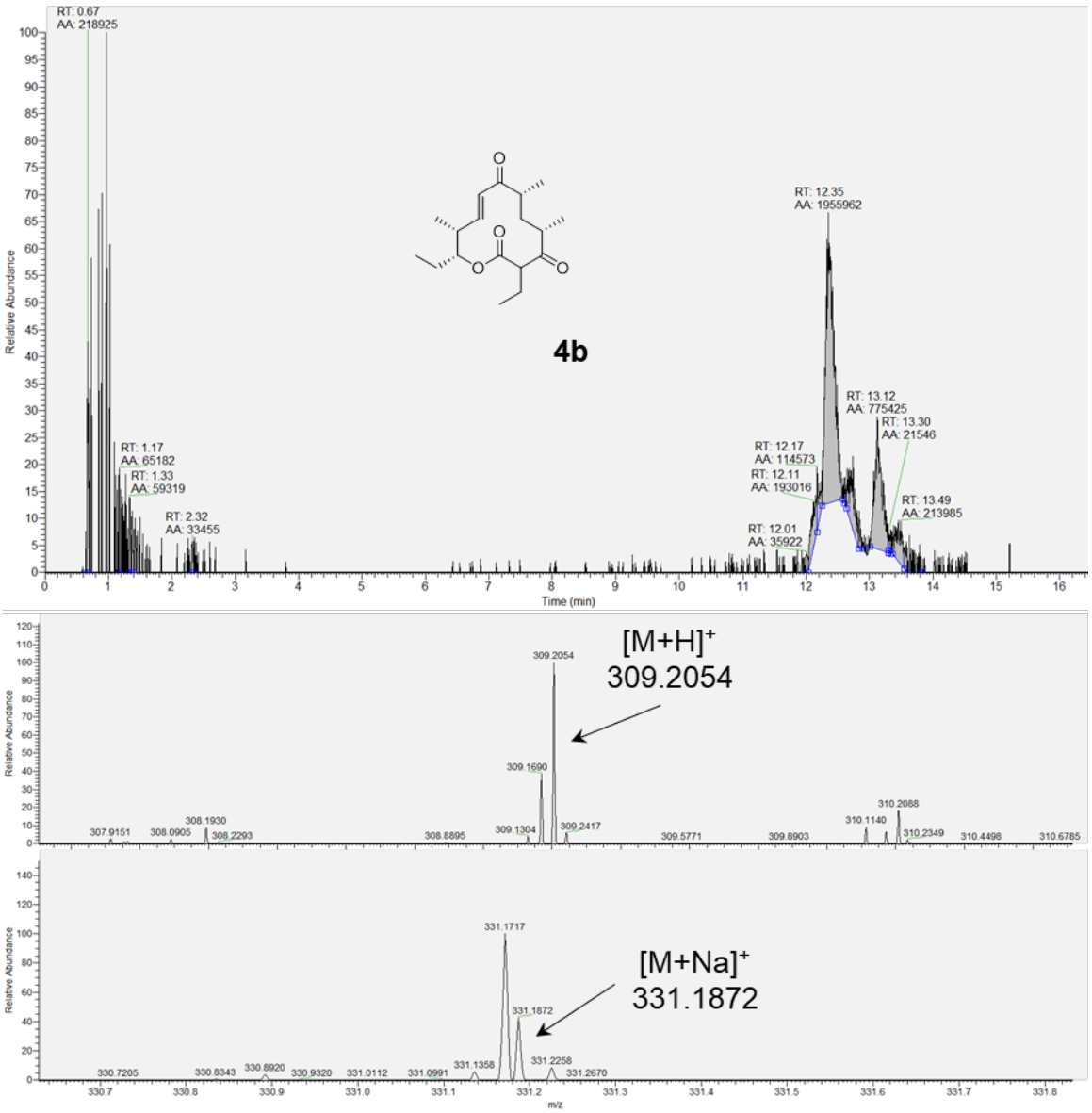
(A)



(B)


3c
OH
Relative Abundance
Time (min)
[M-H2O+H]+
305.2104
m/z
[M+H]+
323.2210
m/z

(C)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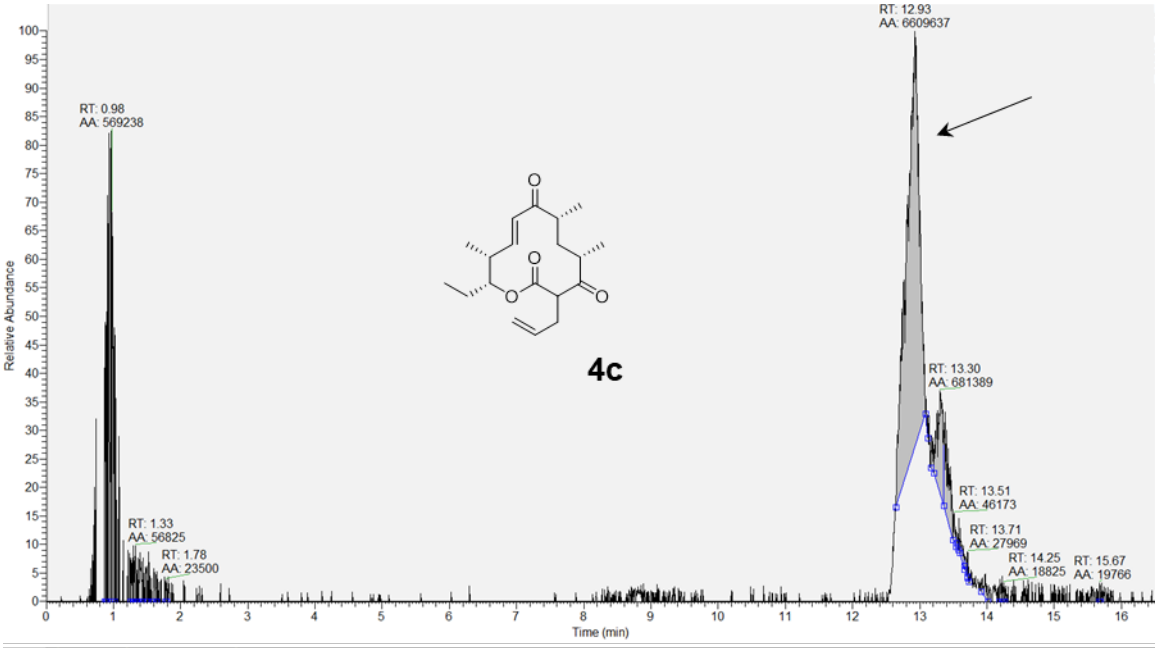
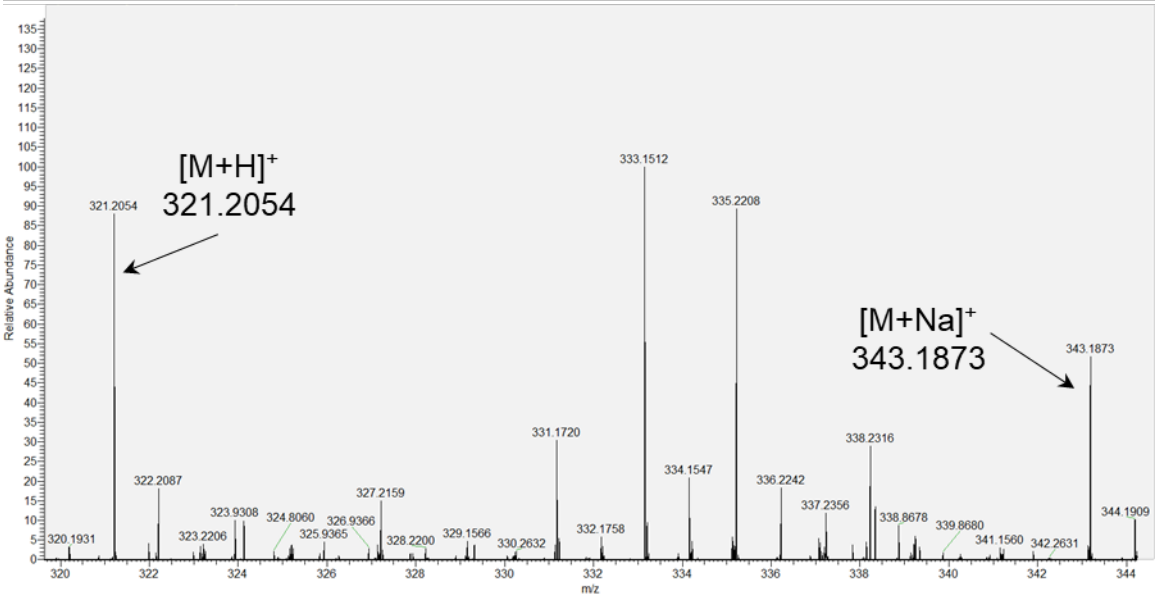
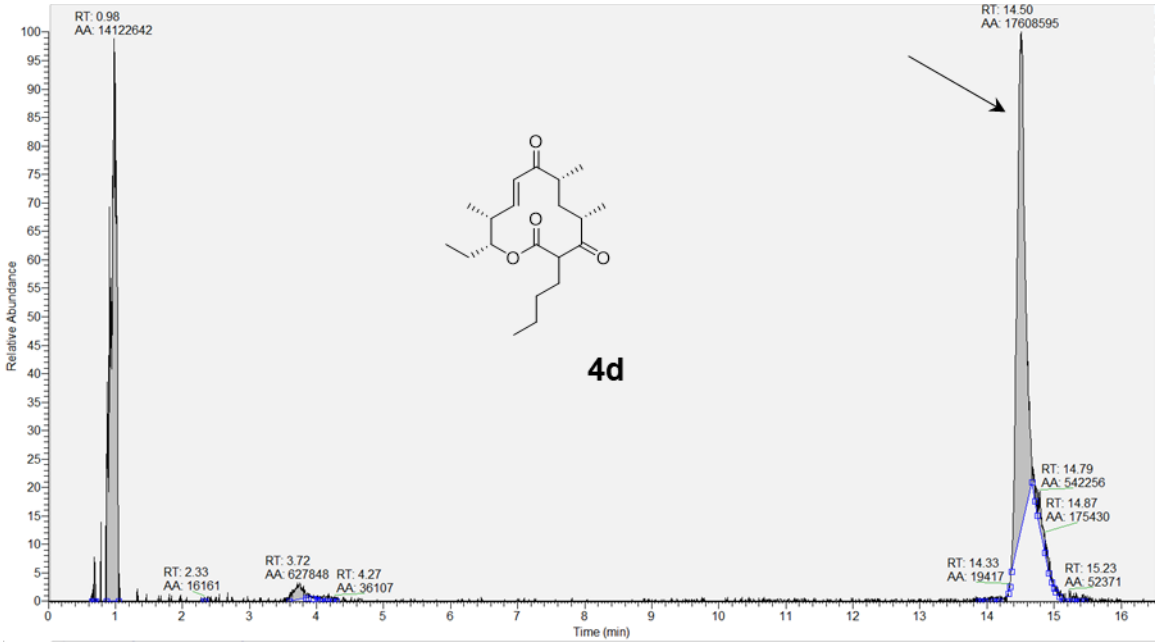
AA: 218925
RT: 1.17
AA: 65182
RT: 1.33
AA: 59319
RT: 2.32
AA: 33455
RT: 12.35
AA: 1955962
RT: 13.12
AA: 775425
RT: 13.30
AA: 21546
RT: 12.17
AA: 114573
RT: 12.11
AA: 193016
RT: 13.49
AA: 213985
RT: 12.01
AA: 35922
4b
Relative Abundance
Time (min)
[M+H]+
309.2054
309.2054
309.1690
308.1930
307.9151
308.0905
308.2293
308.8895
309.1304
309.2417
309.5771
309.8903
310.2088
310.1140
310.2349
310.4498
310.6785
[M+Na]+
331.1872
331.1717
331.1872
330.7205
330.8343
330.8920
330.9320
331.0112
331.0991
331.1358
331.2258
331.2670
m/z

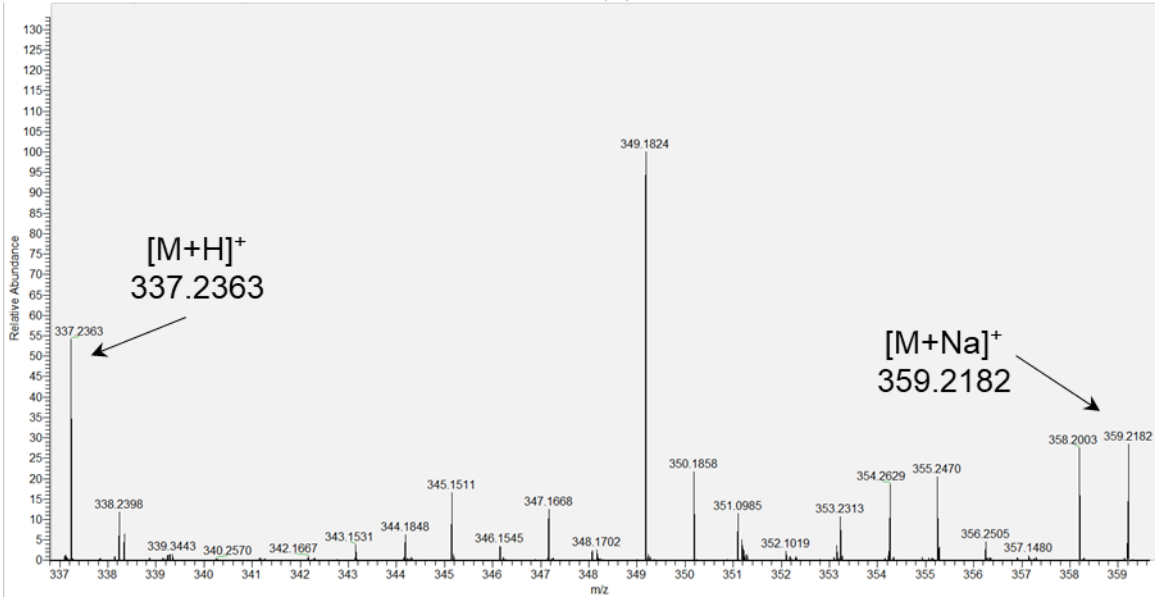
(D)


RT: 12.93
AA: 6609637
RT: 0.98
AA: 569238
4c
RT: 13.30
AA: 681389
RT: 13.51
AA: 46173
RT: 13.71
AA: 27969
RT: 14.25
AA: 18825
RT: 15.67
AA: 19766
RT: 1.33
AA: 56825
RT: 1.78
AA: 23500
Relative Abundance
Time (min)

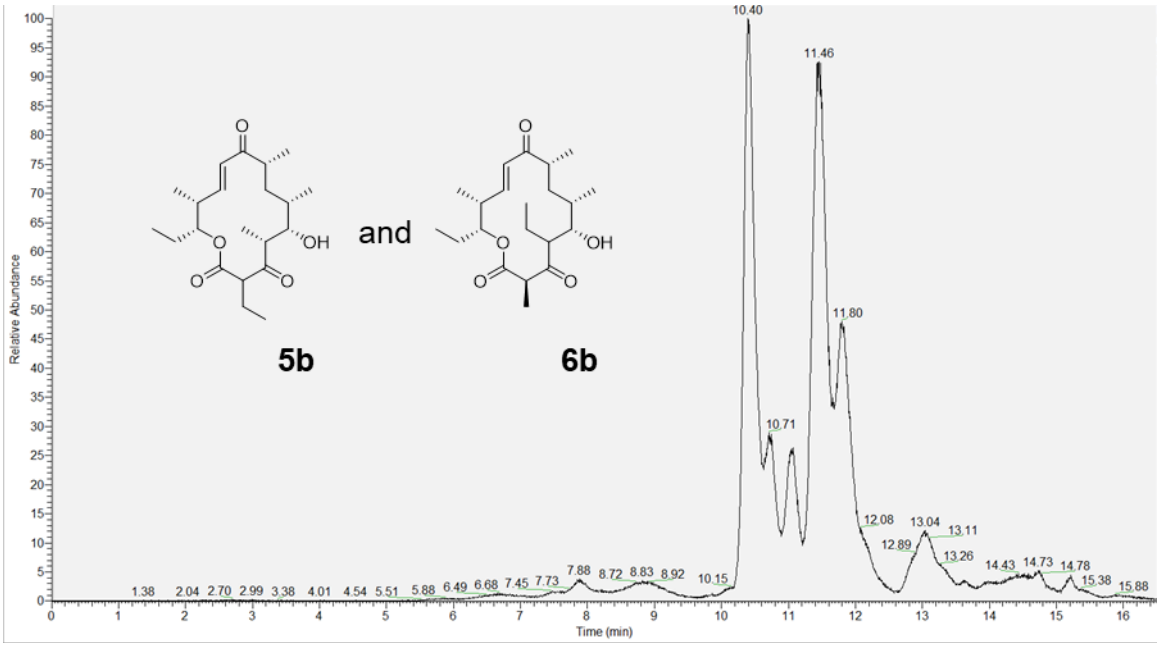

[M+H]+
321.2054
[M+Na]+
343.1873
Relative Abundance
m/z

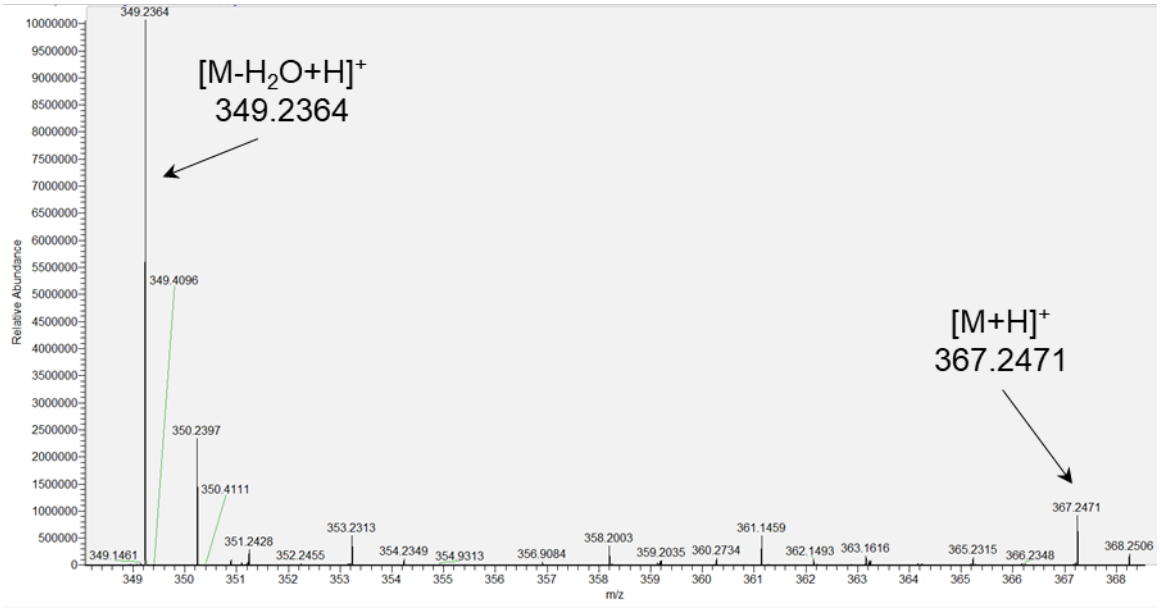
(E)


RT: 0.98
AA: 14122642
RT: 14.50
AA: 17608595
4d
RT: 14.79
AA: 542256
RT: 14.87
AA: 175430
RT: 2.33
AA: 16161
RT: 3.72
AA: 627848
RT: 4.27
AA: 36107
RT: 14.33
AA: 19417
RT: 15.23
AA: 52371
Relative Abundance
Time (min)

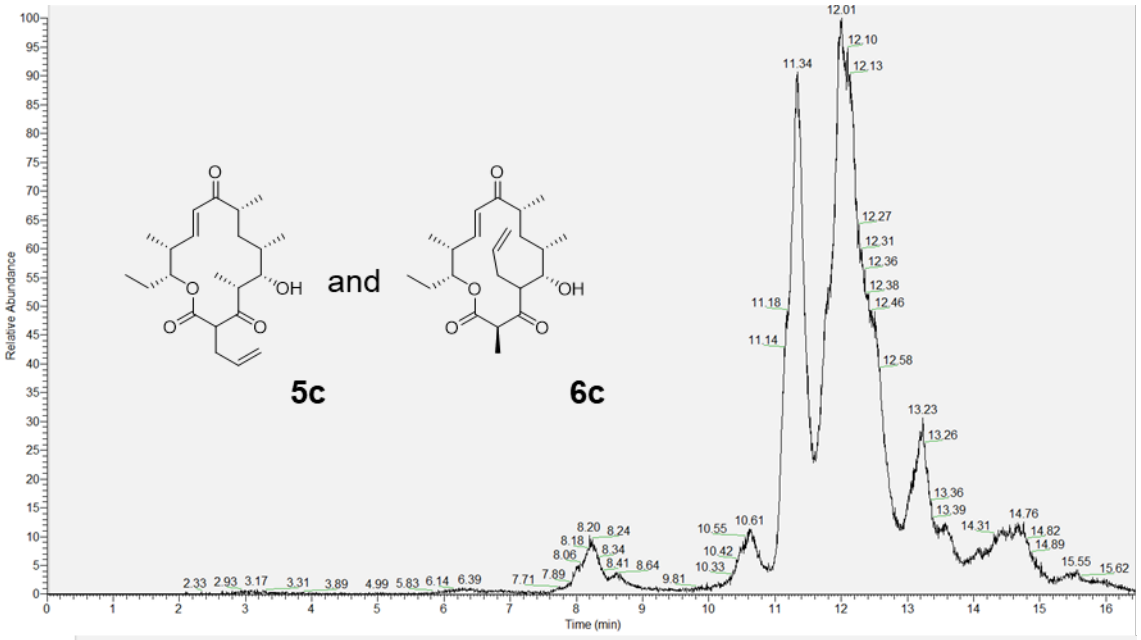

[M+H]+
337.2363
[M+Na]+
359.2182
349.1824
337.2363
358.2003
359.2182
350.1858
354.2629
355.2470
345.1511
347.1668
338.2398
351.0985
353.2313
344.1848
343.1531
346.1545
348.1702
339.3443
340.2570
342.1667
352.1019
356.2505
357.1480
Relative Abundance
m/z

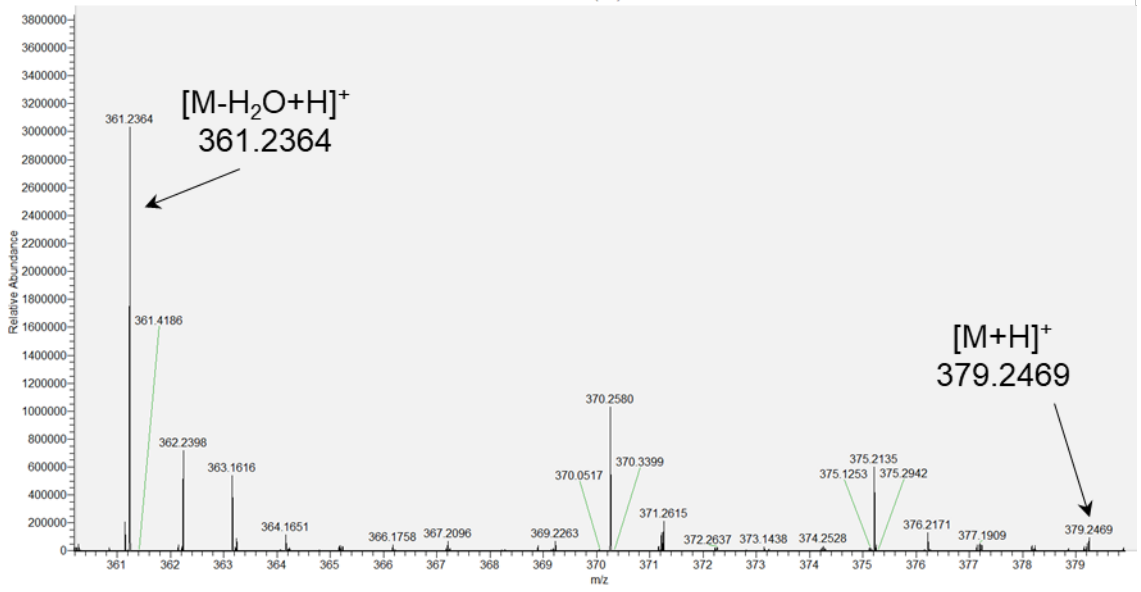
(F)


and
5b
6b
Relative Abundance
Time (min)
10.40
11.46
11.80
10.71
12.08
13.04
13.11
12.89
13.26
14.43
14.73
14.78
15.38
15.88
7.88
8.72
8.83
8.92
10.15
1.38
2.04
2.70
2.99
3.38
4.01
4.54
5.51
5.88
6.49
6.68
7.45
7.73

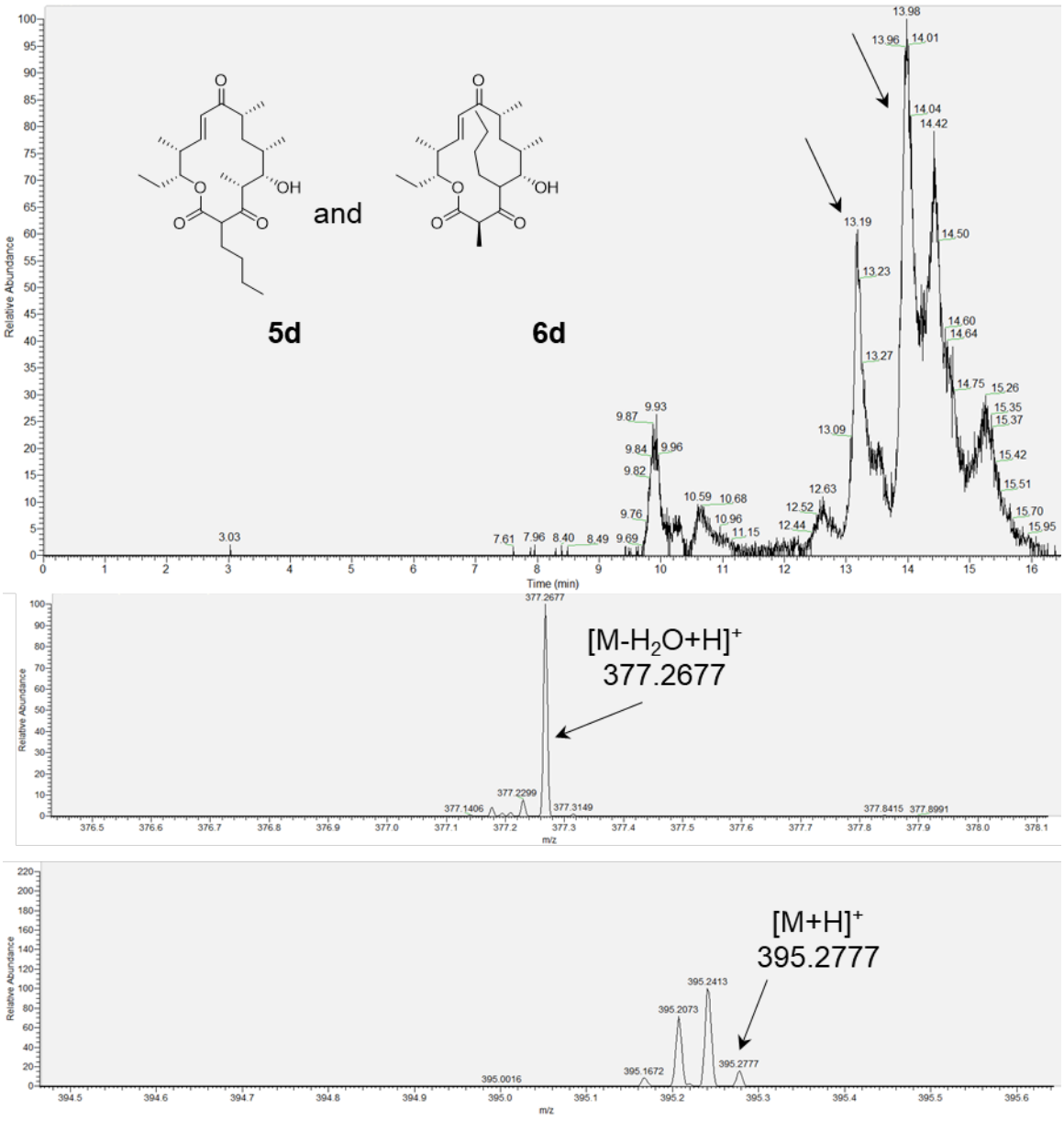

[M-H2O+H]+
349.2364
[M+H]+
367.2471
Relative Abundance
m/z
349.2364
349.4096
350.2397
350.4111
351.2428
353.2313
352.2455
354.2349
354.9313
356.9084
358.2003
359.2035
360.2734
361.1459
362.1493
363.1616
365.2315
366.2348
367.2471
368.2506
349.1461

(G)

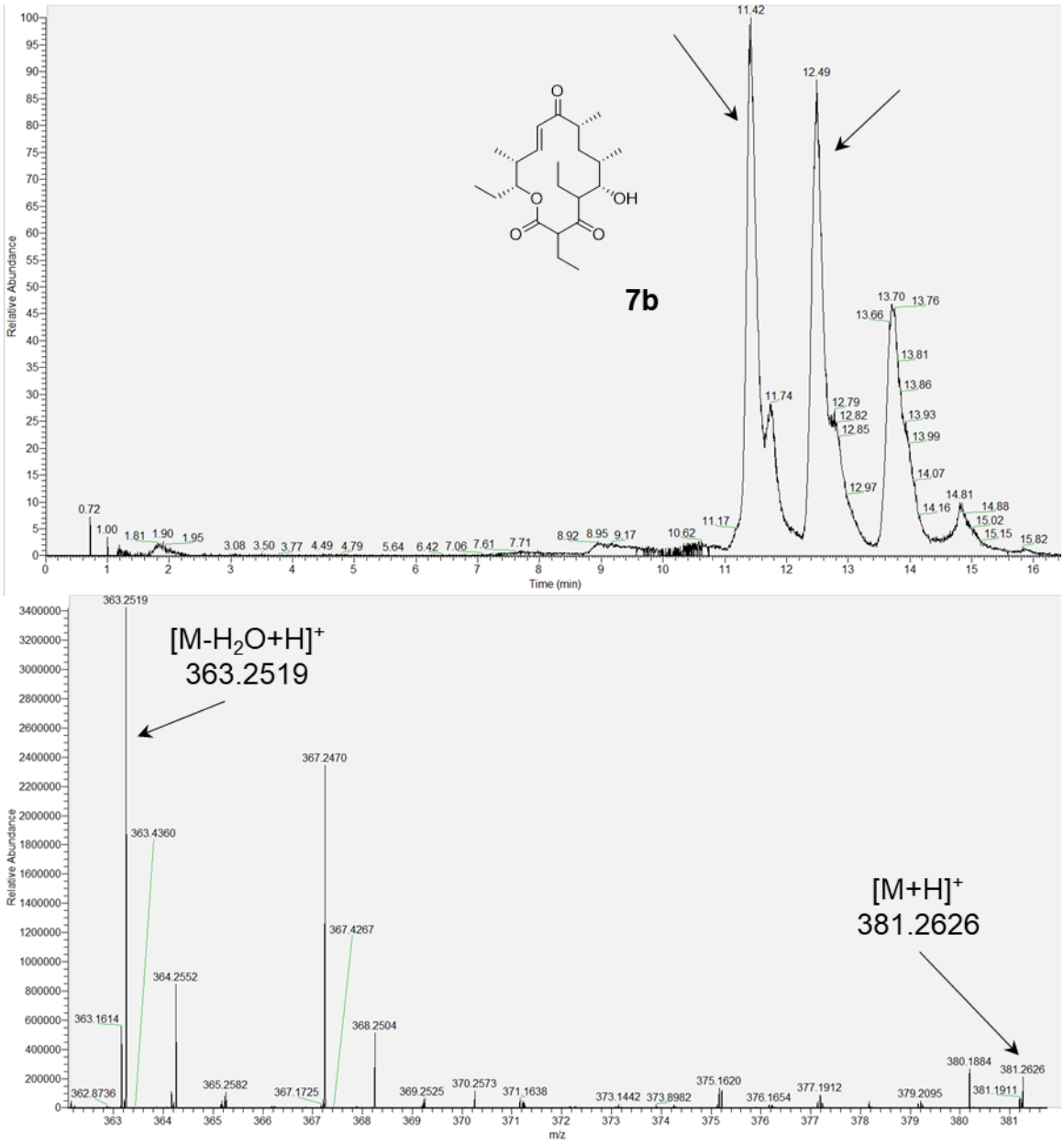

5c
and
6c
Relative Abundance
Time (min)


[M-H2O+H]+
361.2364
[M+H]+
379.2469
Relative Abundance
m/z

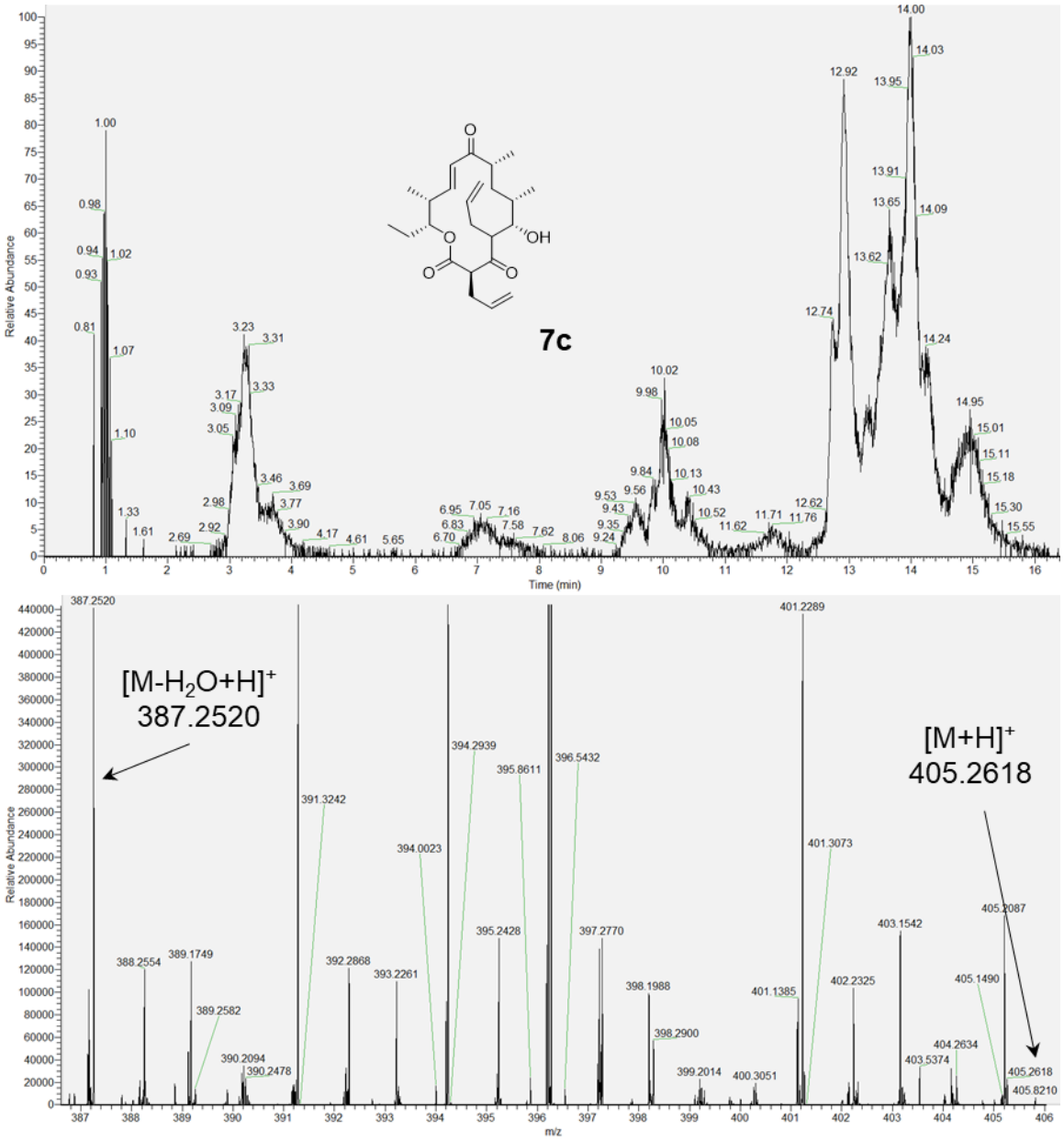
(H)


and
5d
6d
Relative Abundance
Time (min)
[M-H2O+H]+
377.2677
[M+H]+
395.2777
m/z

(I)


11.42
12.49
OH
7b
13.70
13.76
13.66
13.81
13.86
11.74
12.79
12.82
12.85
13.93
13.99
14.07
12.97
14.81
14.16
14.88
15.02
15.15
15.82
11.17
10.62
8.92
8.95
9.17
0.72
1.00
1.81
1.90
1.95
3.08
3.50
3.77
4.49
4.79
5.64
6.42
7.06
7.61
7.71
Relative Abundance
Time (min)
363.2519
[M-H2O+H]+
363.2519
367.2470
363.4360
367.4267
364.2552
363.1614
368.2504
[M+H]+
381.2626
380.1884
381.2626
381.1911
362.8736
365.2582
367.1725
369.2525
370.2573
371.1638
373.1442
373.8982
375.1620
376.1654
377.1912
379.2095
Relative Abundance
m/z

(J)



Supplemental Methods

Site-directed mutagenesis of modules

All PikAIII and PikAIV site-directed mutants were constructed by 'round-the-horn' mutagenesis[1] using the templates from **Supplementary Table S1** and and the oligonucleotide sequences described **Supplementary Table S2**. Each PCR contained: 5x GC Phusion DNA polymerase buffer (4 µL), DNAse free water (11.8 µL), forward/reverse primer mix (2 µL, 10 µM), template DNA (1 µL, 50 ng/mL), dNTP (0.4 µL, 2.5 mM each dNTP), DMSO (0.6 µl), and Phusion High Fidelity DNA Polymerase (0.2 µL) (New England Biolabs, NEB, Ipswich, MA). PCR cycling parameters: step 1) 98 °C, 30 s; step 2) 29x [a) 98 °C, 10 s; b) 72 °C, 5 min]; step 3) 72 °C, 5 min. Next, PCR mixture was subjected to restriction enzyme digest with *Dpn*I to remove any remaining template DNA. The *Dpn*I reaction mixture contained: 10x CutSmart Buffer (2 µL), the PCR mixture (17 µL), and *Dpn*I (1 µL). The mixture was vortexed, centrifuged, and incubated for 3 h at 37 °C. The digested reaction mixture was then purified by agarose gel electrophoresis, with total 8 µl of DNA eluted at the final step. The PCR product was then ligated using T4 DNA ligase in a reaction containing: 1 µl of the 10x T4 DNA ligase buffer, 8 µl of the *Dpn*I-treated and purified PCR product, and 1 µl of the T4 DNA ligase (overnight incubation at 16 °C). Each subsequent ligation reaction was transformed directly into *E. cloni* 10G electrocompetent cells (Lucigen). Individual transformants from each library were sequenced to identify incorporation of mutant codons.

Construction of PikAIII and PikAIV chimeras

AT swaps of PikAIII AT and PikAIV AT were constructed using Gibson assembly (NEB) and boundaries defined by the Keasling group.[2-3] Each AT domain was PCR amplified using primers listed in Table S6 to have 15 bp overlaps with the PCR-amplified module-AT. The Gibson assembly mixtures were used to transform *E. coli* DH5α competent cells. Mutant sequences were confirmed by DNA sequencing and were transformed into BAP1 competent cells with the pRARE plasmid (Novagen) and plated onto LB agar plates (50 µg/mL kanamycin and 100 µg/mL spectinomycin).

Expression and purification of wild-type and mutant MatB

The expression and purification of MatB T207G/M306I has been previously described[4-6]. Briefly, *E. coli* BL21(DE3) pLysS competent cells were transformed with plasmid and positive transformants were selected on LB agar supplemented with 30 µg/mL kanamycin. A single colony was transferred to LB (3 mL) supplemented with kanamycin (30 µg/mL) and grown at 37 °C and 250 rpm overnight. The culture was used to inoculate LB media (1 L) supplemented with kanamycin (30 µg/mL). One liter culture was incubated at 37 °C and 250 rpm to an OD_{600} of 0.6, at which time protein synthesis was induced by the addition of IPTG to a final concentration of

1 mM. After incubation at 18 °C and 200 rpm for 18 h, cells were collected by centrifugation at 5,000 *g* for 20 min, and resuspended in 100 mM Tris-HCl pH 8.0 (20 mL) containing NaCl (300 mM) and then lysed by sonication. Following centrifugation at 10,000 *g*, the soluble extract was loaded onto a 1 mL HisTrap HP column (GE Healthcare, Piscataway, NJ) and purified by fast protein liquid chromatography using the following buffers: wash buffer [20 mM phosphate (pH 7.4) containing 0.5 M NaCl and 20 mM imidazole] and elution buffer [20 mM phosphate (pH 7.4) containing 0.5 M NaCl and 200 mM imidazole]. The purified protein was concentrated using an Amicon Ultra 30 kDa MWCO centrifugal filter (Millipore Corp., Billerica, MA) and stored as 10% glycerol stocks at -80 °C. Protein purity was verified by SDS-PAGE. Protein quantification was carried out using the Bradford Protein Assay Kit from Bio-Rad.

Synthesis of acyl-CoAs by MatB

The MatB-catalyzed synthesis of extender units **8** and **9a**-**d** has been previously described[4-6]. Briefly, reactions were performed in a 50 µL reaction mixture containing 100 mM sodium phosphate (pH 7), $MgCl_2$ (2 mM), ATP (12 mM), coenzyme A (8 mM), malonate or corresponding analog (16 mM) and wild-type or mutant MatB (10 µg) at 25 °C. Aliquots were removed after 3 h incubation, and quenched with an equal volume of ice-cold methanol, centrifuged at 10,000 *g* for 10 min, and cleared supernatants used for HPLC analysis on a Varian ProStar HPLC system. A series of linear gradients was developed from 0.1% TFA (A) in water to methanol (HPLC grade, B) using the following protocol: 0-32 min, 80% B; 32-35 min, 100% A. The flow rate was 1 mL/min, and the absorbance was monitored at 254 nm using Pursuit XRs C18 column (250 x 4.6 mm, Varian Inc.). To ensure complete conversion, the malonate analog and the acyl-CoA product HPLC peak areas were integrated, and the conversion (%) calculated as a percent of the total peak area. Product elution times and LC-MS data were in complete agreement with that previous described[4-5].

Supplemental References

1. Moore, S. 'Round-the-Horn Site-Directed Mutagenesis. https://openwetware.org/wiki/'Round-the-horn_site-directed_mutagenesis.
2. Yuzawa, S.; Deng, K.; Wang, G.; Baidoo, E. E.; Northen, T. R.; Adams, P. D.; Katz, L.; Keasling, J. D., Comprehensive in Vitro Analysis of Acyltransferase Domain Exchanges in Modular Polyketide Synthases and Its Application for Short-Chain Ketone Production. *ACS Synth. Biol.* **2017,** *6* (1), 139-147.
3. Gibson, D. G.; Young, L.; Chuang, R. Y.; Venter, J. C.; Hutchison, C. A., 3rd; Smith, H. O., Enzymatic Assembly of DNA Molecules up to Several Hundred Kilobases. *Nat. Methods* **2009,** *6* (5), 343-345.
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5. Koryakina, I.; McArthur, J. B.; Draelos, M. M.; Williams, G. J., Promiscuity of a Modular Polyketide Synthase Towards Natural and Non-Natural Extender Units. *Org. Biomol. Chem.* **2013,** *11*, 4449-4458.
6. Koryakina, I.; Williams, G. J., Mutant Malonyl-CoA Synthetases with Altered Specificity for Polyketide Synthase Extender Unit Generation. *ChemBioChem* **2011,** *12* (15), 2289-2293.