Supplementary Information

Sterol methyltransferases in uncultured bacteria complicate eukaryotic biomarker interpretations

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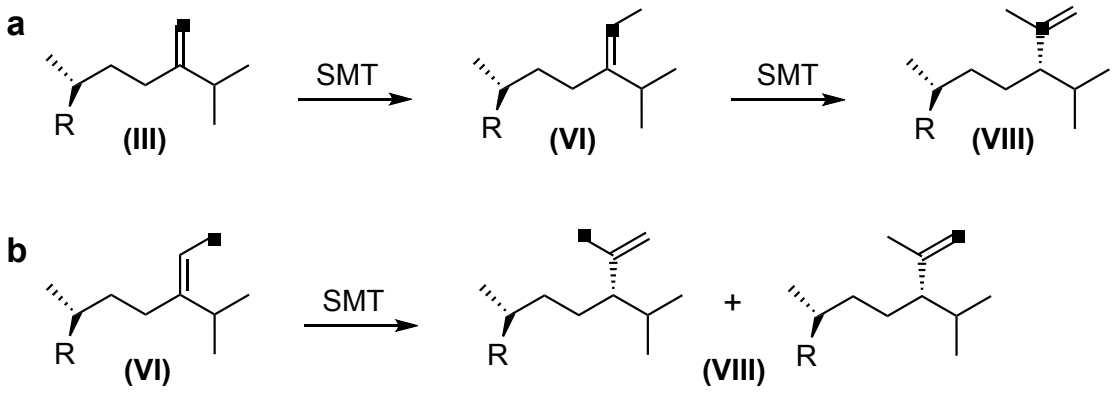
Contains:

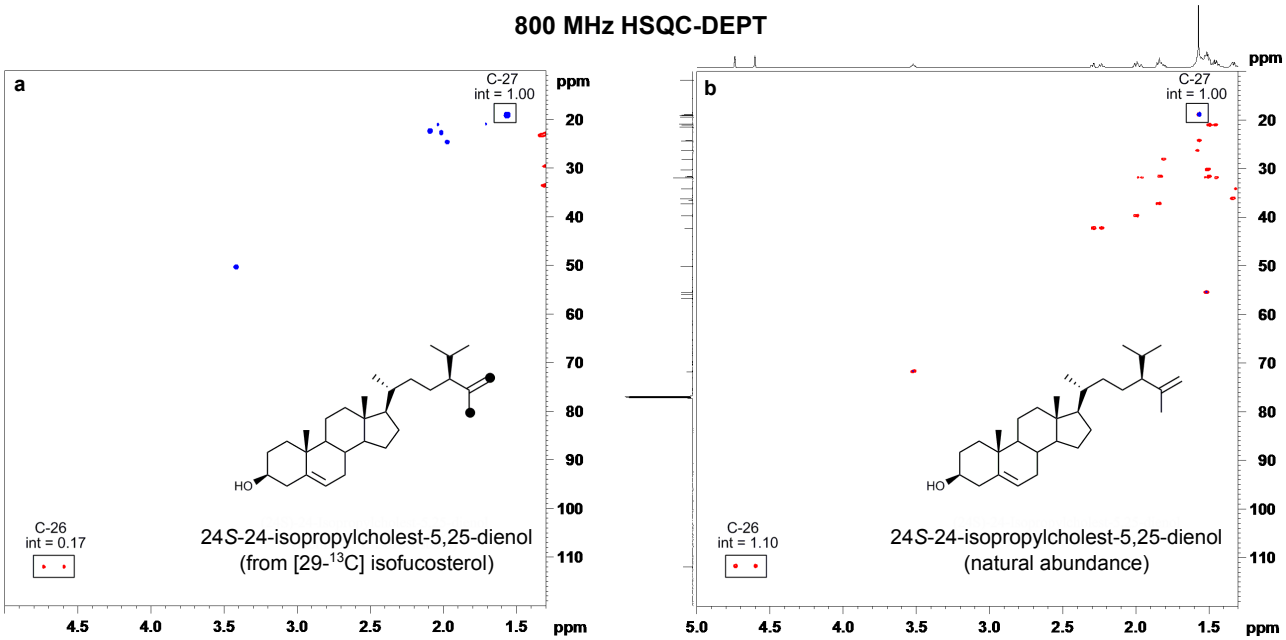
Supplementary Figures 1-7

Supplementary Tables 1-5

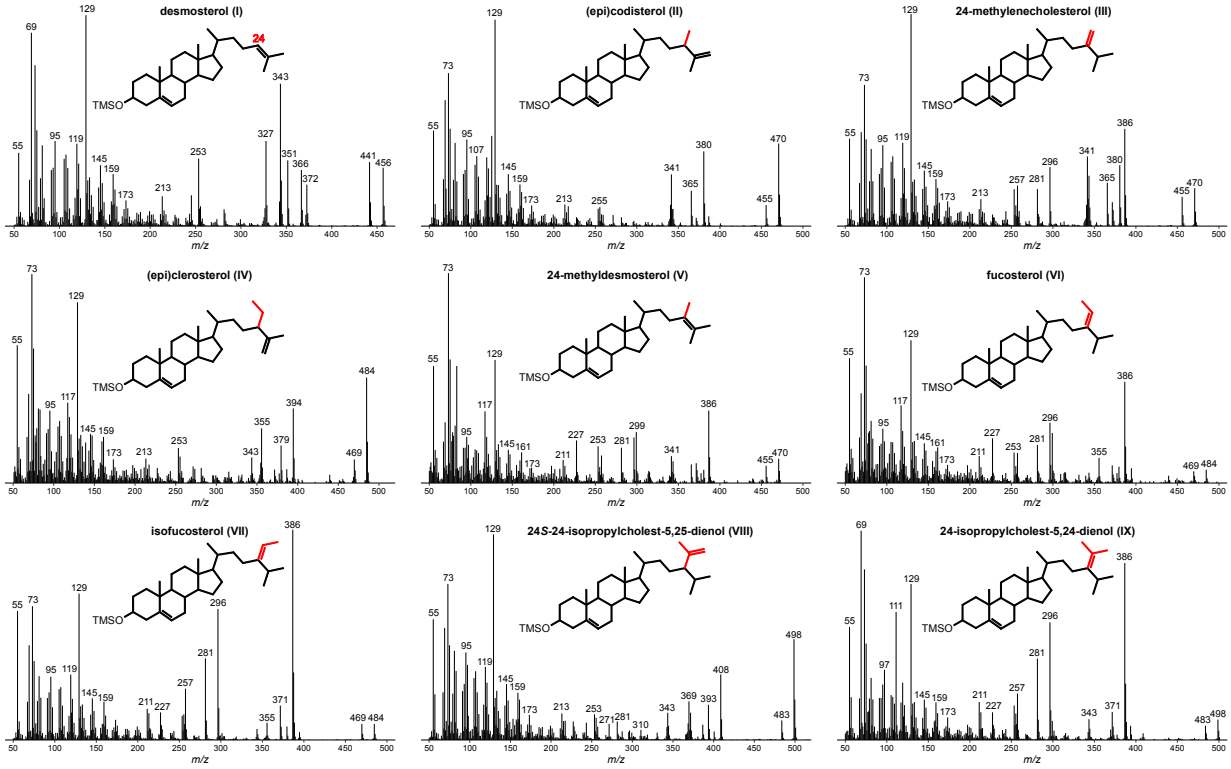
Supplementary References



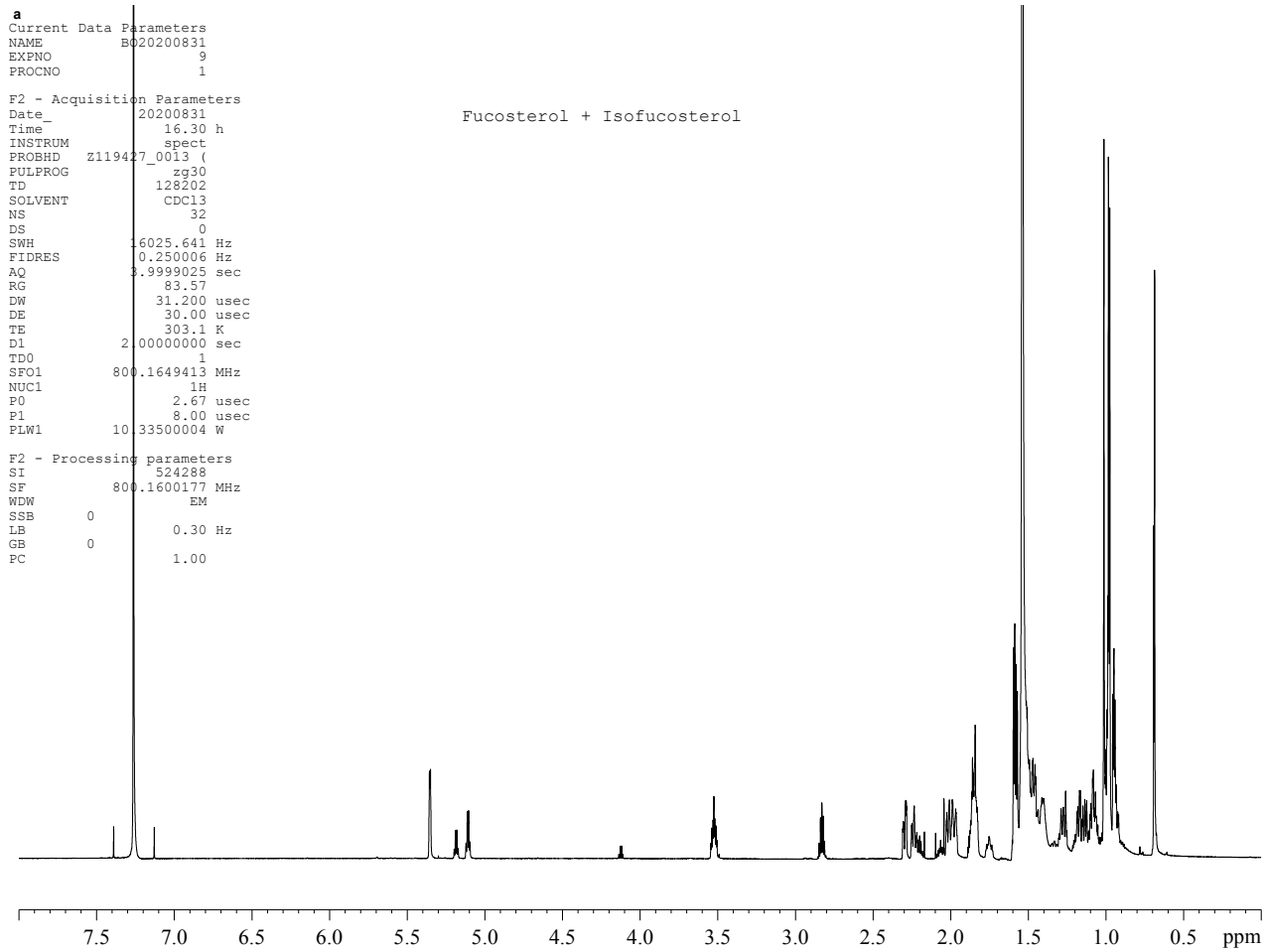
Supplementary Figure 1. **^{13}C-labeling experiments with propylating SMTs from an *Aplysina aerophoba* symbiont and a Chlamydiae sp.** Positions of the ^{13}C labels are shown for the predominant propylation pathway proceeding from 24-methylenecholesterol (III) to isofucosterol (VI) to 24S-24-isopropylcholest-5,25,dienol (VIII). **a** Experiments with [28-^{13}C] 24-methylenecholsterol as the substrate. **b** Experiments with [29-^{13}C] isofucosterol as the substrate.



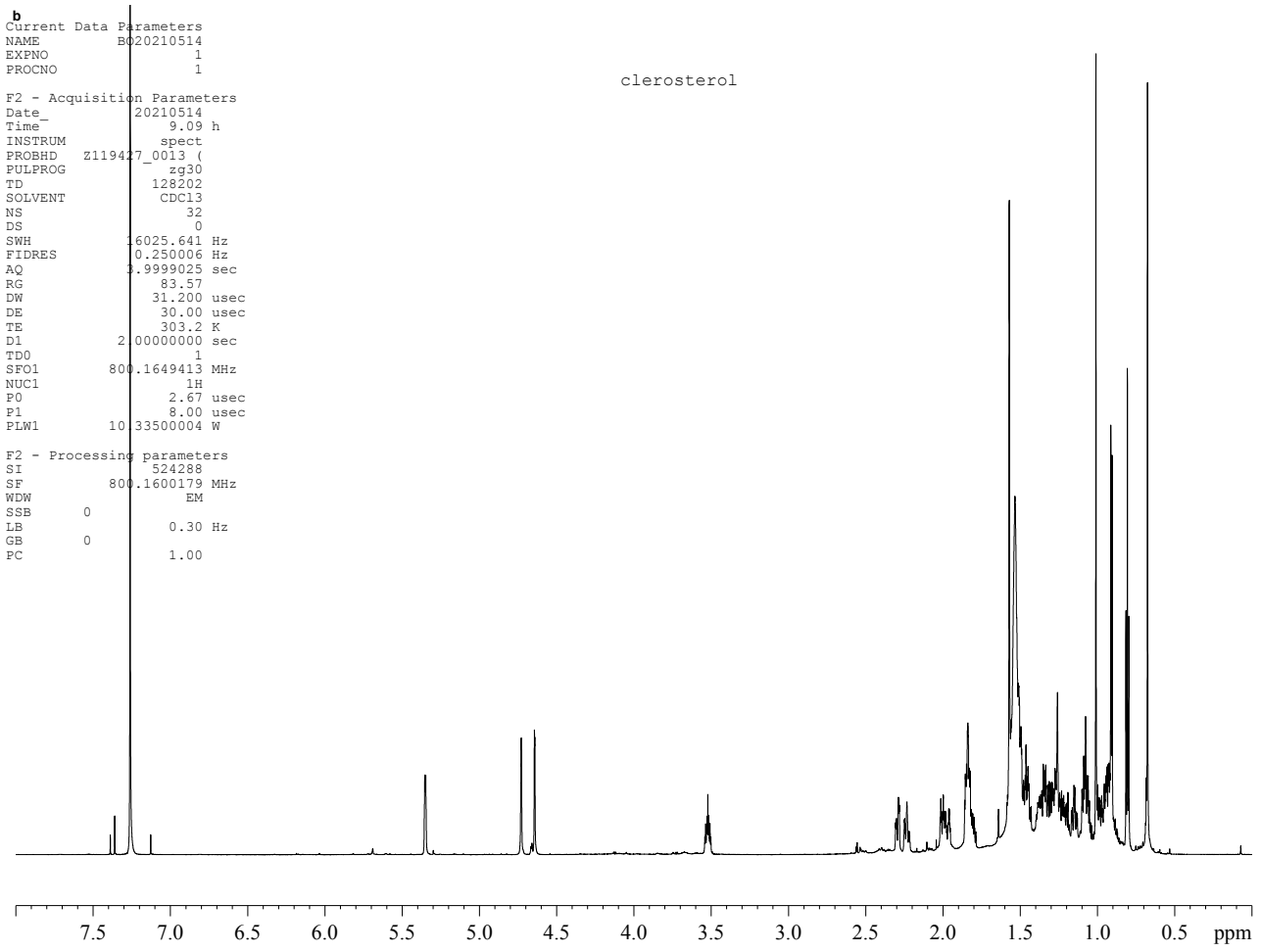
Supplementary Figure 2. **2D NMR (HSQC). a** 24*S*-24-isopropylcholest-5,25,dienol (VIII) from an in vitro reaction performed with [29-^{13}C] isofucosterol and a Chlamydiae sp. MAG SMT and **b** naturally abundant 24*S*-24-isopropylcholest-5,25,dienol (VIII). Additional NMR spectra are shown in Supplementary Figures 4-7 and are summarized in Supplementary Table 3.



Supplementary Figure 3. **Representative mass spectra of sterols identified in the main text.** Data are from in vitro reactions performed with the "Chlamydiae sp., drinking water 1 of 2" SMT **(I, V, VI, VII, VIII, IX)** and the "*Nitrospira* sp., coral reef 1 of 2" SMT (**II**) with desmosterol as the substrate, or the "*Chondrilla nucula*" SMT with 24-methylenecholesterol as the substrate (**III, IV**). Total lipid extracts were derivatized to trimethylsilyl ethers. The raw data used to generate this figure are provided in Supplementary Data File 3. Detailed GC-MS methods can be found in the Methods section of the main text.



Supplementary Figure 4. **Raw 800 MHz ^{1}H-NMR spectra of the following sterols: a** fucosterol plus isofucosterol, **b** clerosterol, **c** epiclerosterol, **d** 24*S*-24-isopropylcholesta-5,25-dienol, **e** 24*R*-24-isopropylcholesta-5,25-dienol, **f** [26, 27, 29, 30-^{13}C] 24-isopropylcholesta-5,24-dienol, and **g** [26, 27, 29, 30-^{13}C] 24-isopropylcholesta-5,23-dienol plus 24*S*-24-isopropylcholesta-5,25-dienol. Chemical shifts are summarized in Supplementary Table 3.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1.0
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c

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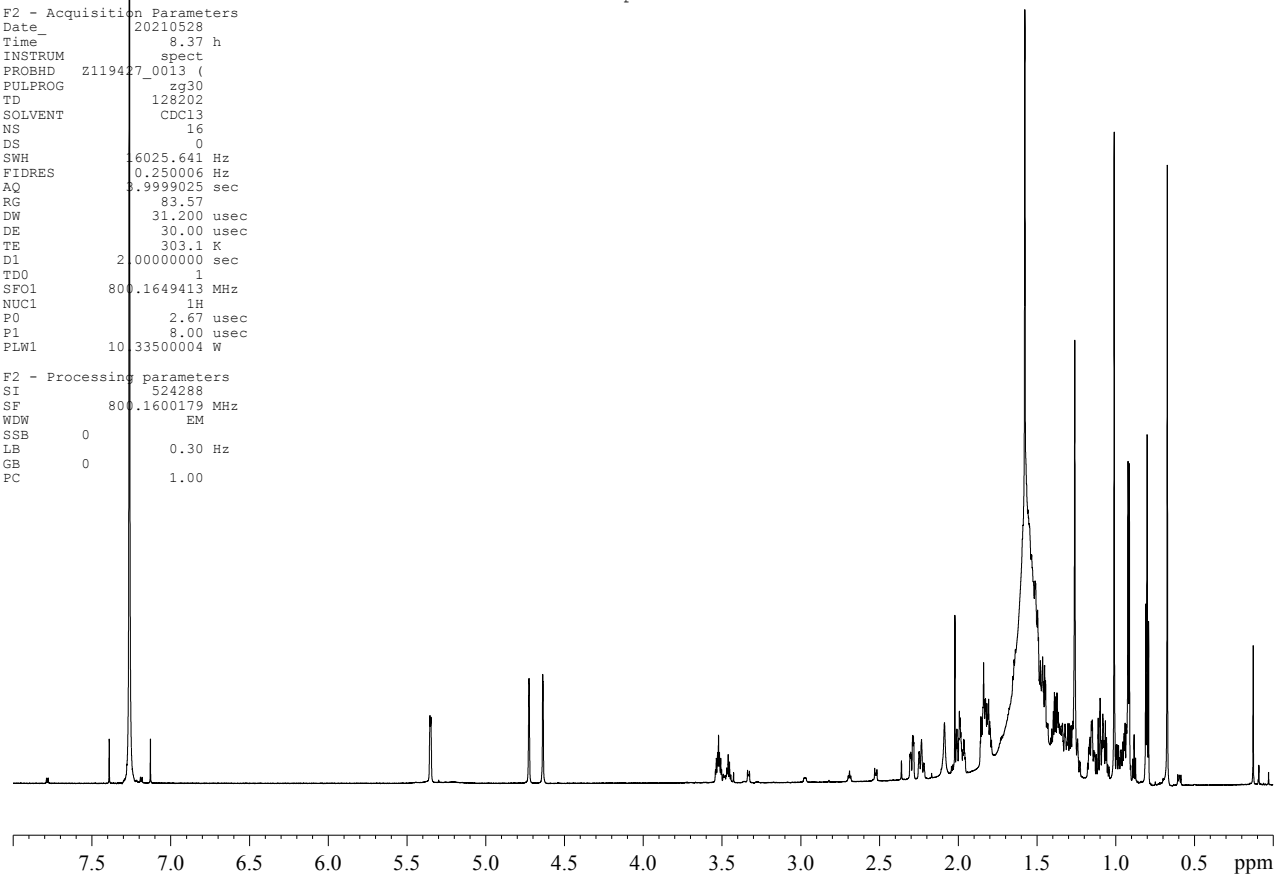

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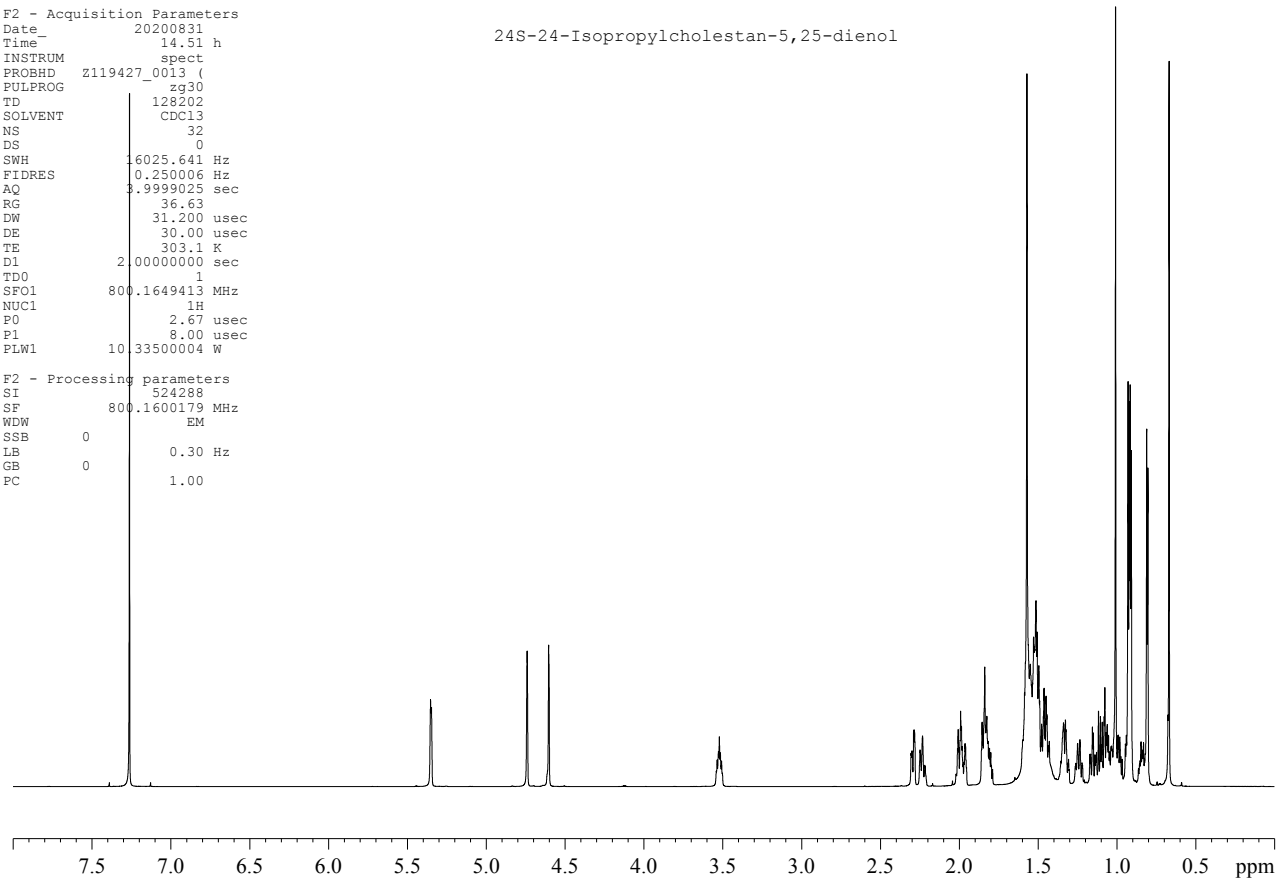
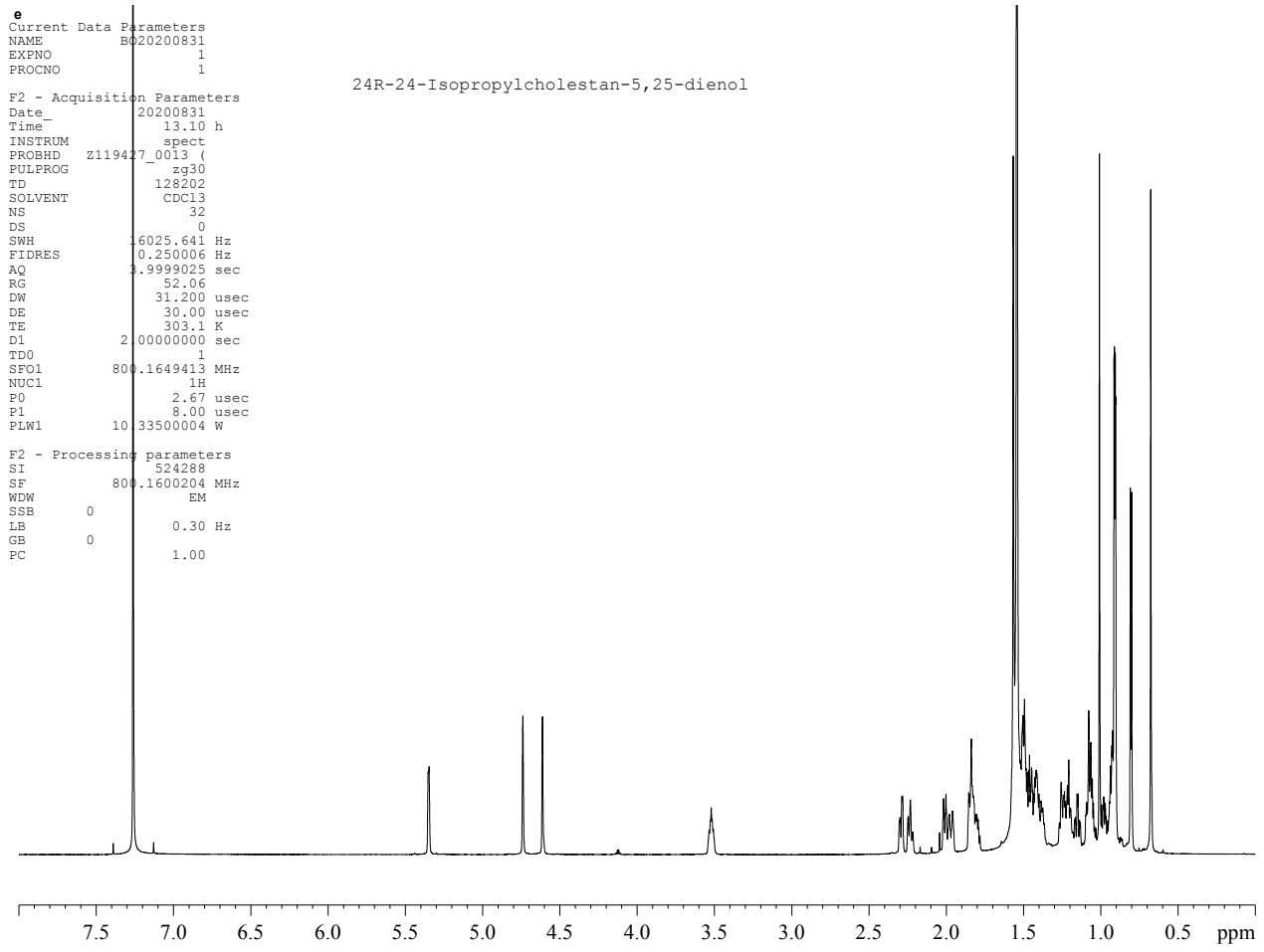
24S-24-Isopropylcholestan-5,25-dienol

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3.5
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ppm

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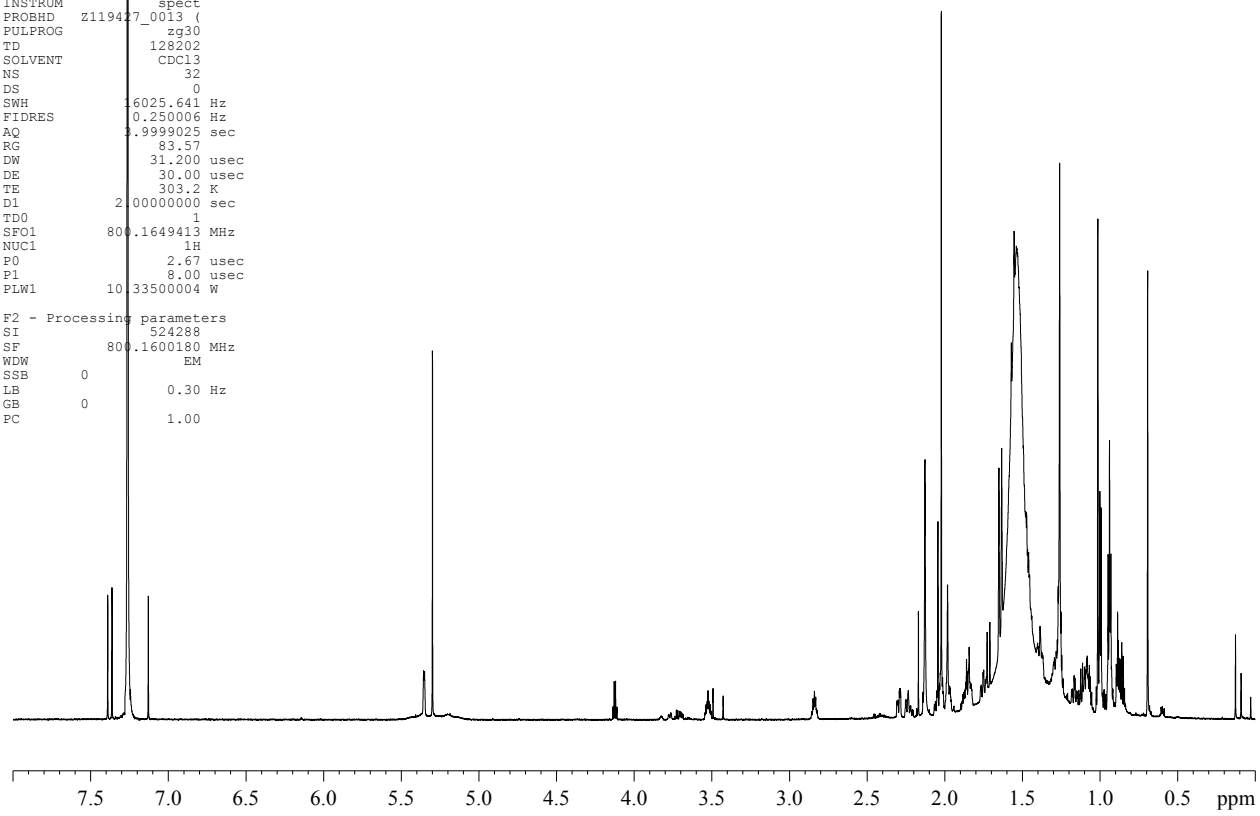

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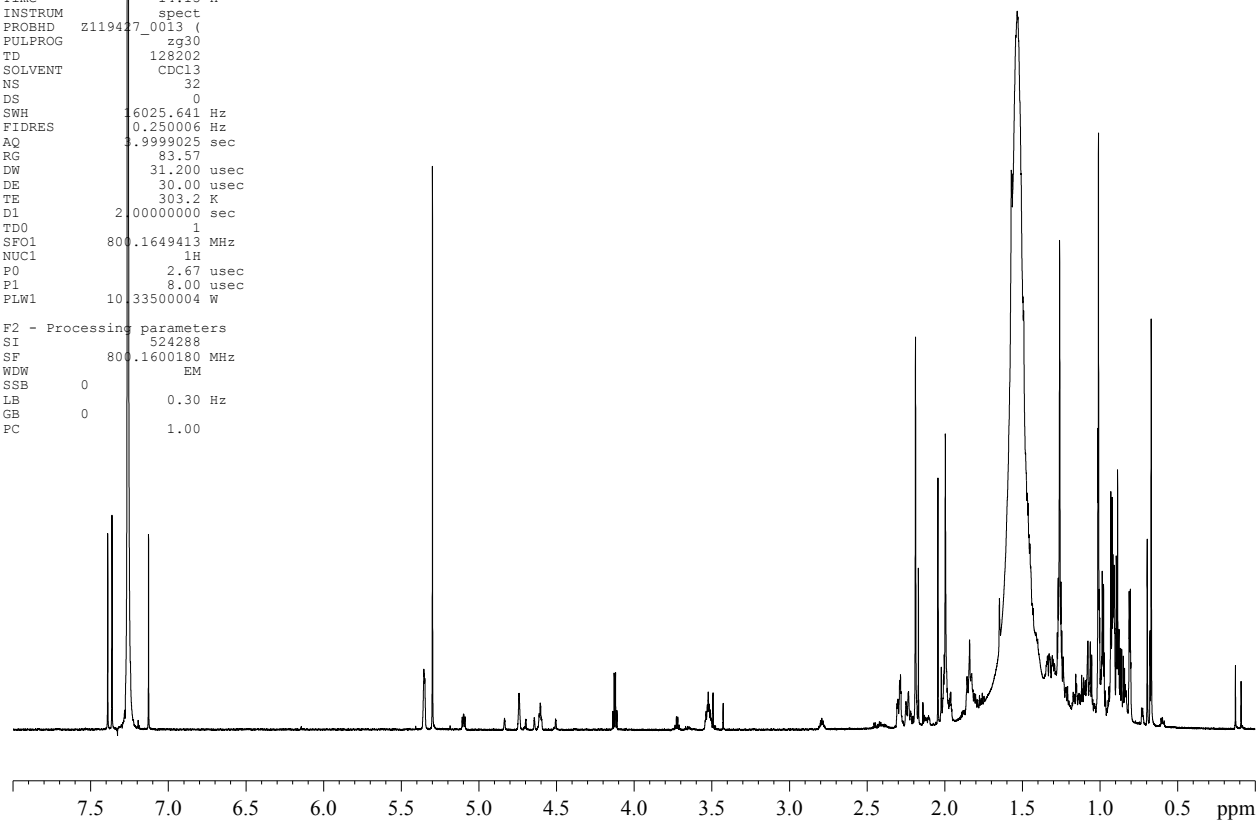

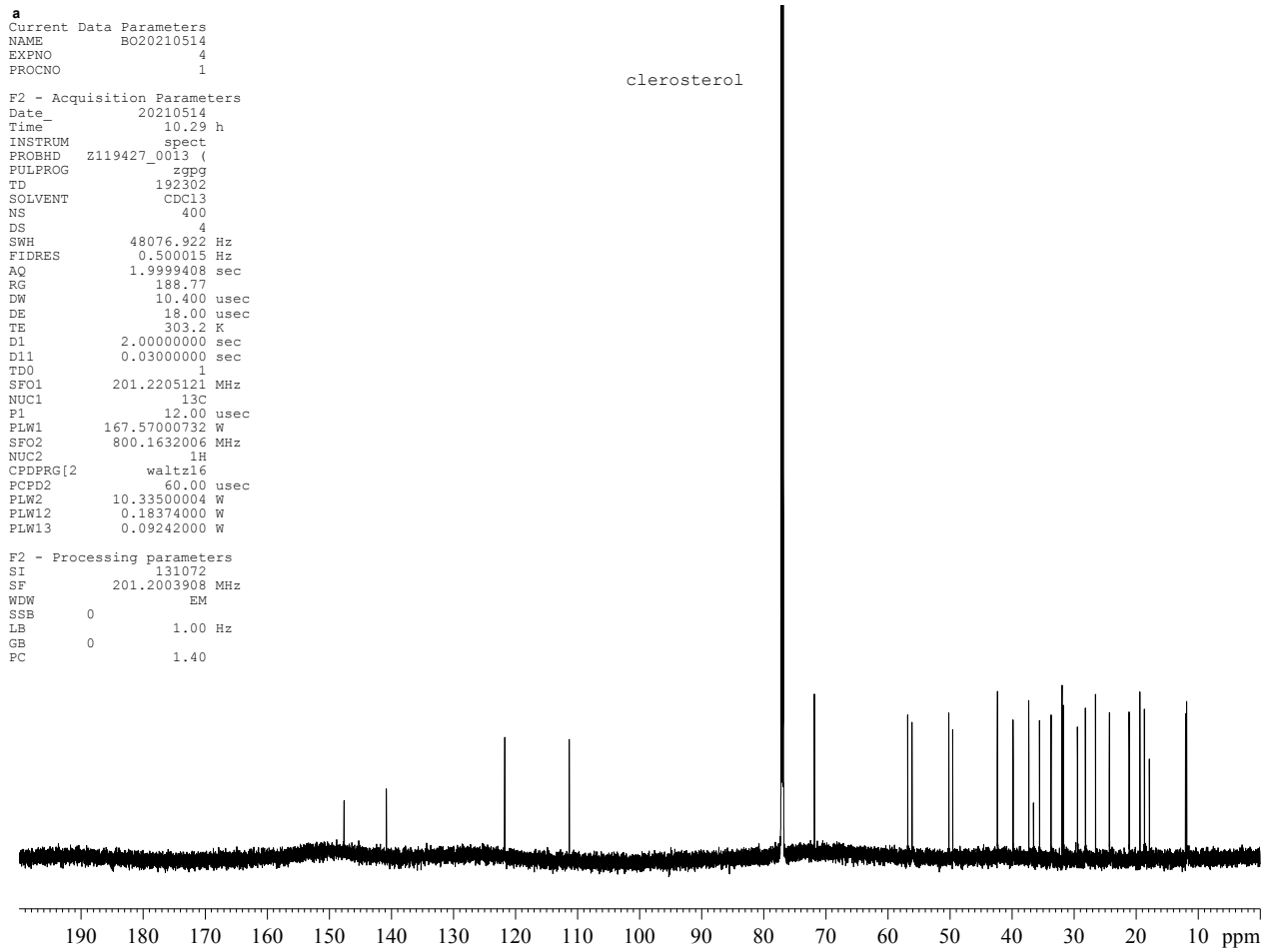

Supplementary Figure 5. **Raw 201 MHz ^{13}C-NMR spectra of the following sterols: a** clerosterol, **b** epiclerosterol, **c** 24*S*-24-isopropylcholesta-5,25-dienol **d** 24*R*-24-isopropylcholesta-5,25-dienol, **e** [26, 27, 29, 30-^{13}C] 24-isopropylcholesta-5,24-dienol, and **f** [26, 27, 29, 30-^{13}C] 24-isopropylcholesta-5,23-dienol plus 24*S*-24-isopropylcholesta-5,25-dienol. Chemical shifts are summarized in Supplementary Table 3.

b

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SFO2         800.1632006 MHz
NUC2                  1H
CPDPRG[2         waltz16
PCPD2              60.00 usec
PLW2         10.33500004 W
PLW12         0.18374000 W
PLW13         0.09242000 W

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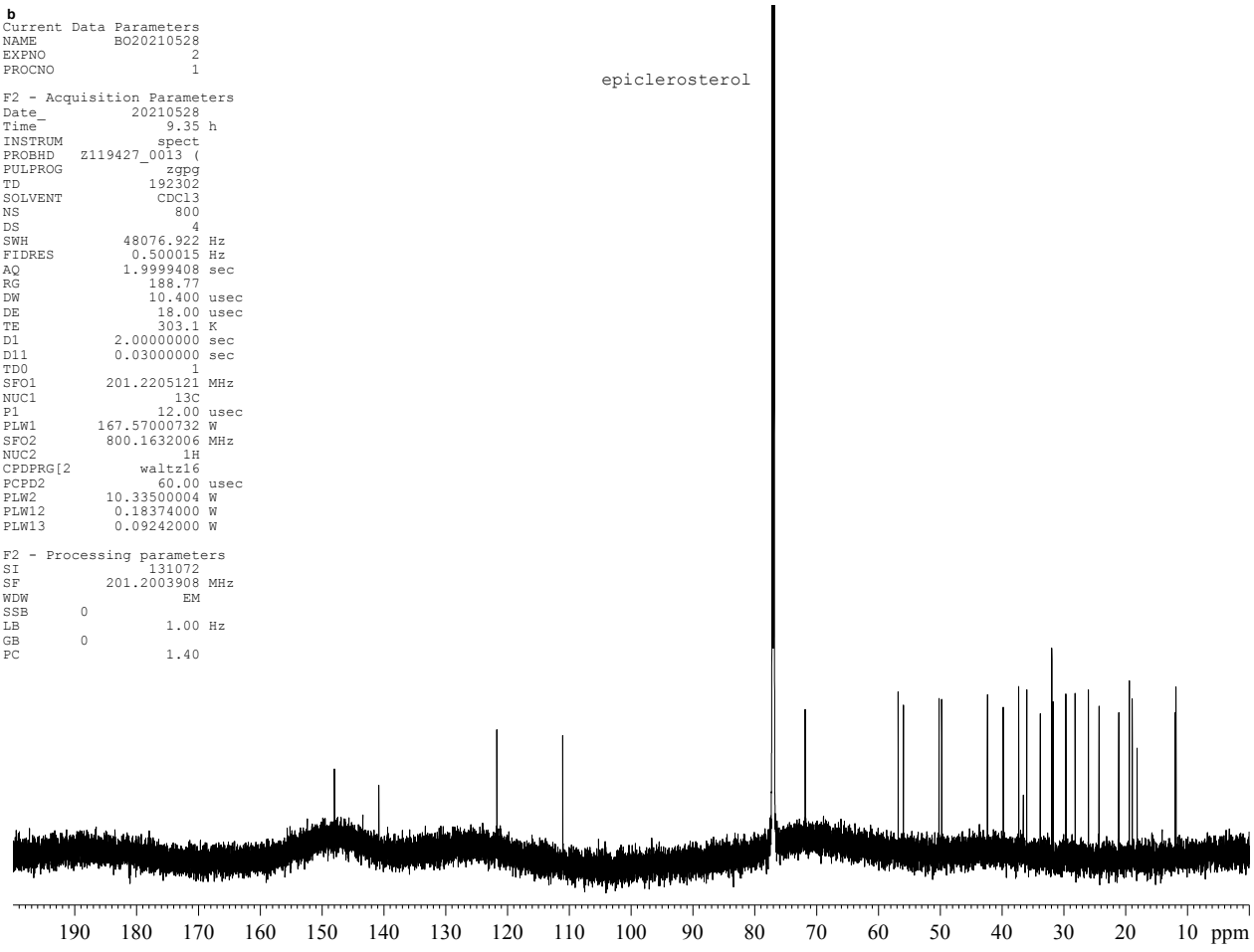

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NUC1                  13C
P1                 12.00 usec
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SFO2         800.1632006 MHz
NUC2                  1H
CPDPRG[2          waltz16
PCPD2                60.00 usec
PLW2         10.33500004 W
PLW12         0.18374000 W
PLW13         0.09242000 W

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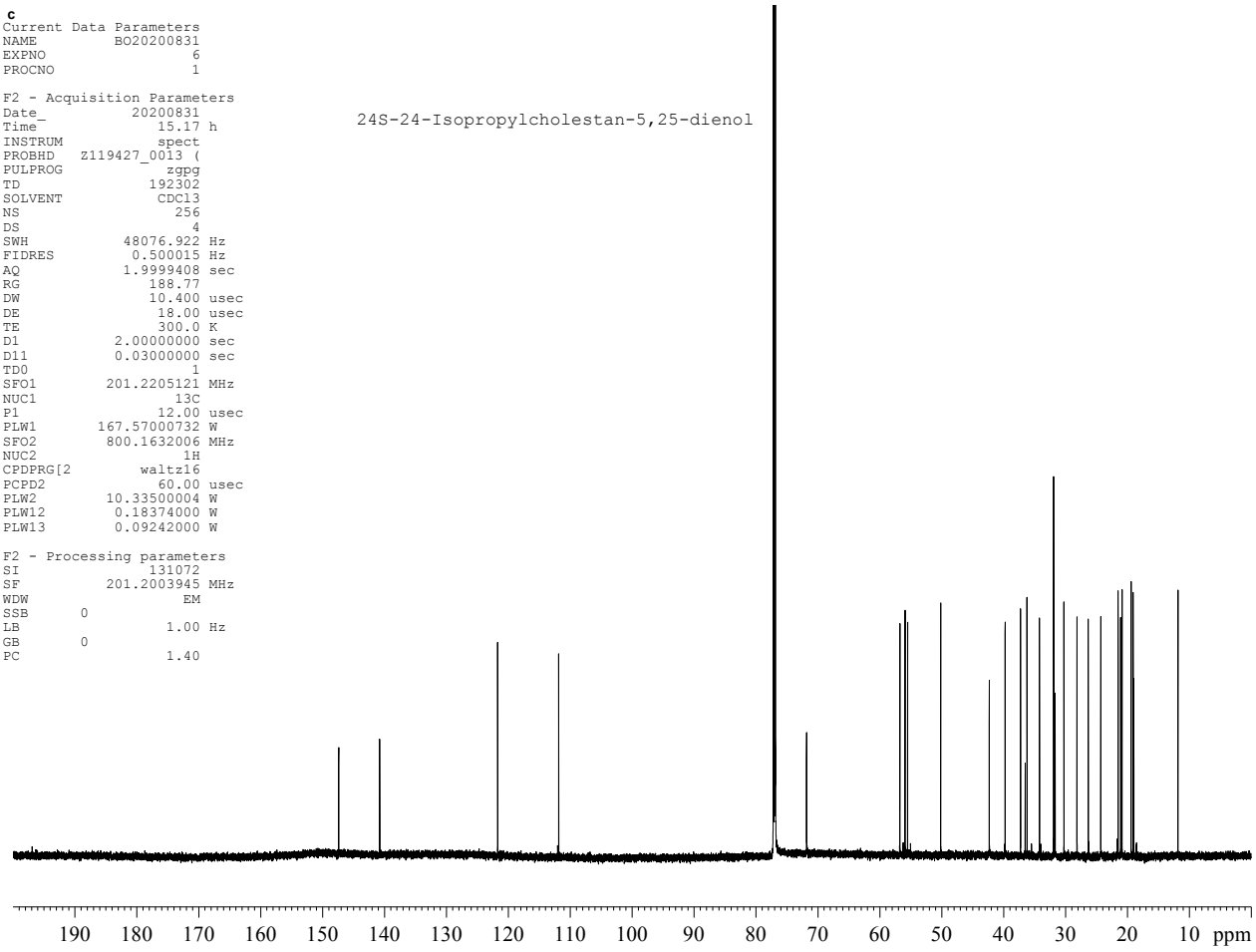
24S-24-Isopropylcholestan-5,25-dienol



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PLW13         0.09242000 W

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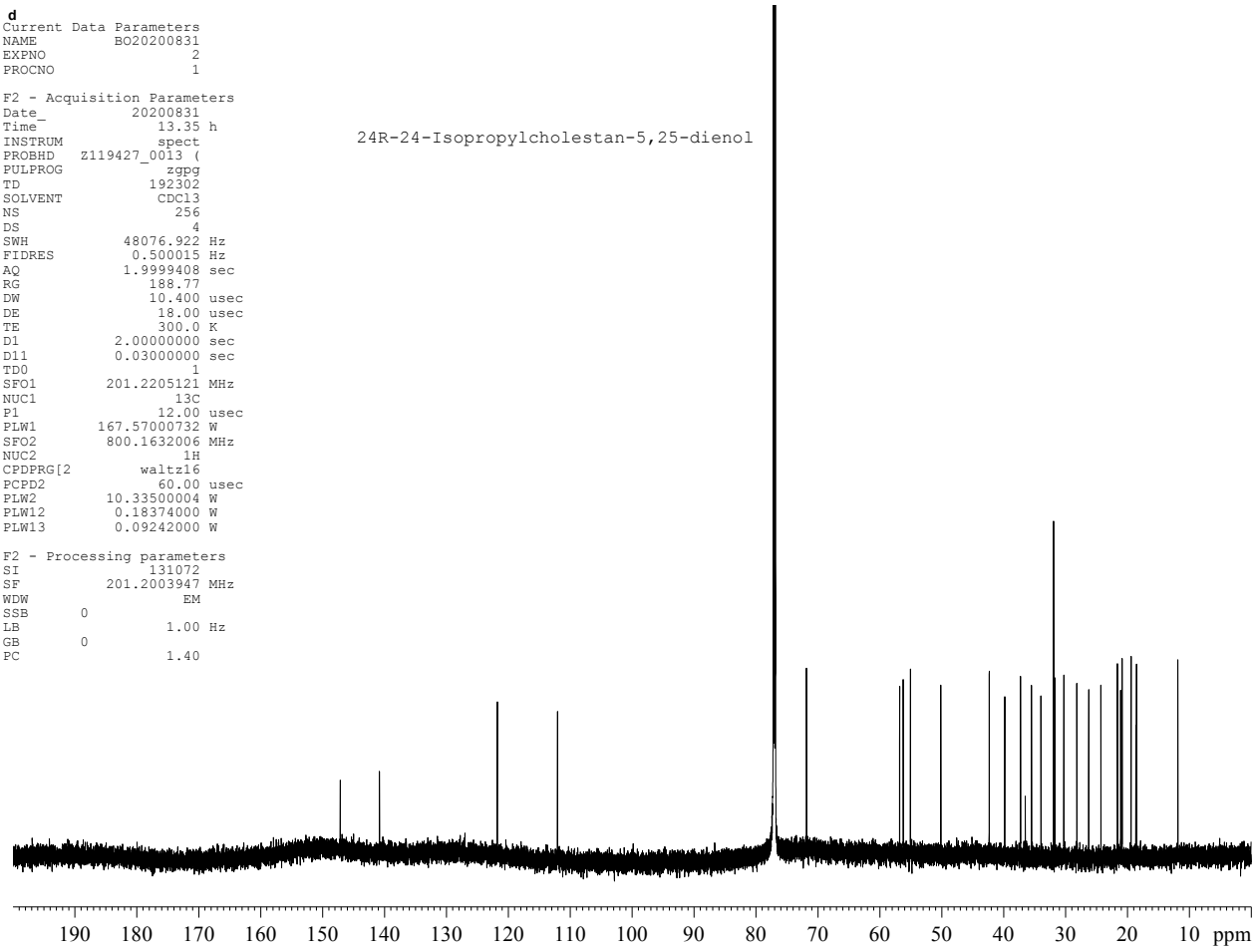
24R-24-Isopropylcholestan-5,25-dienol



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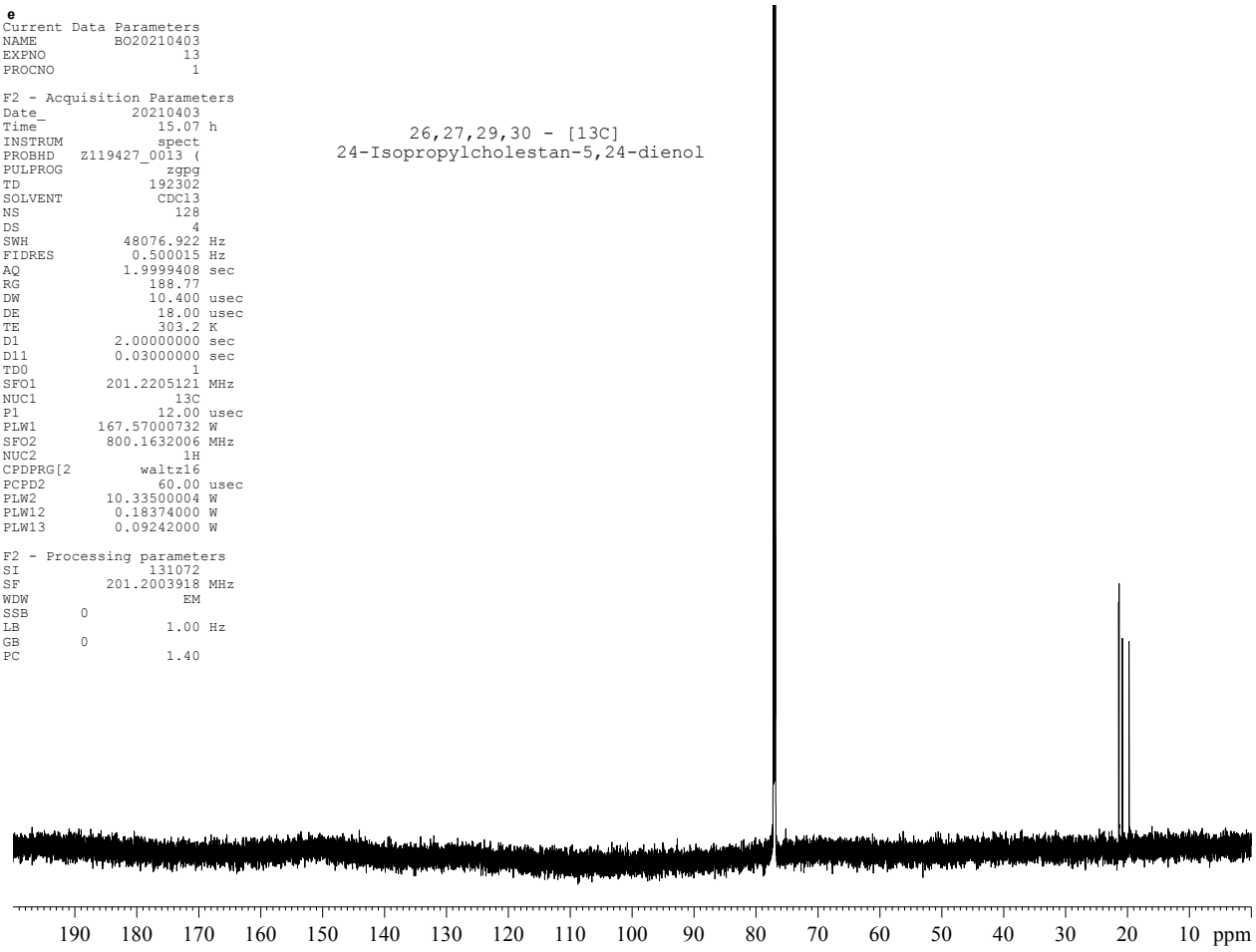
26,27,29,30 - [13C]
24-Isopropylcholestan-5,24-dienol



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PCPD2              60.00 usec
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PLW13         0.09242000 W

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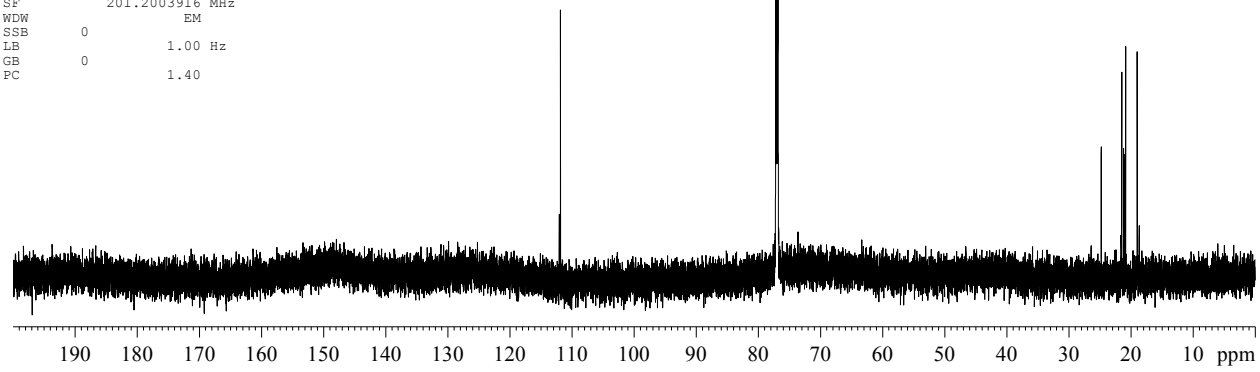
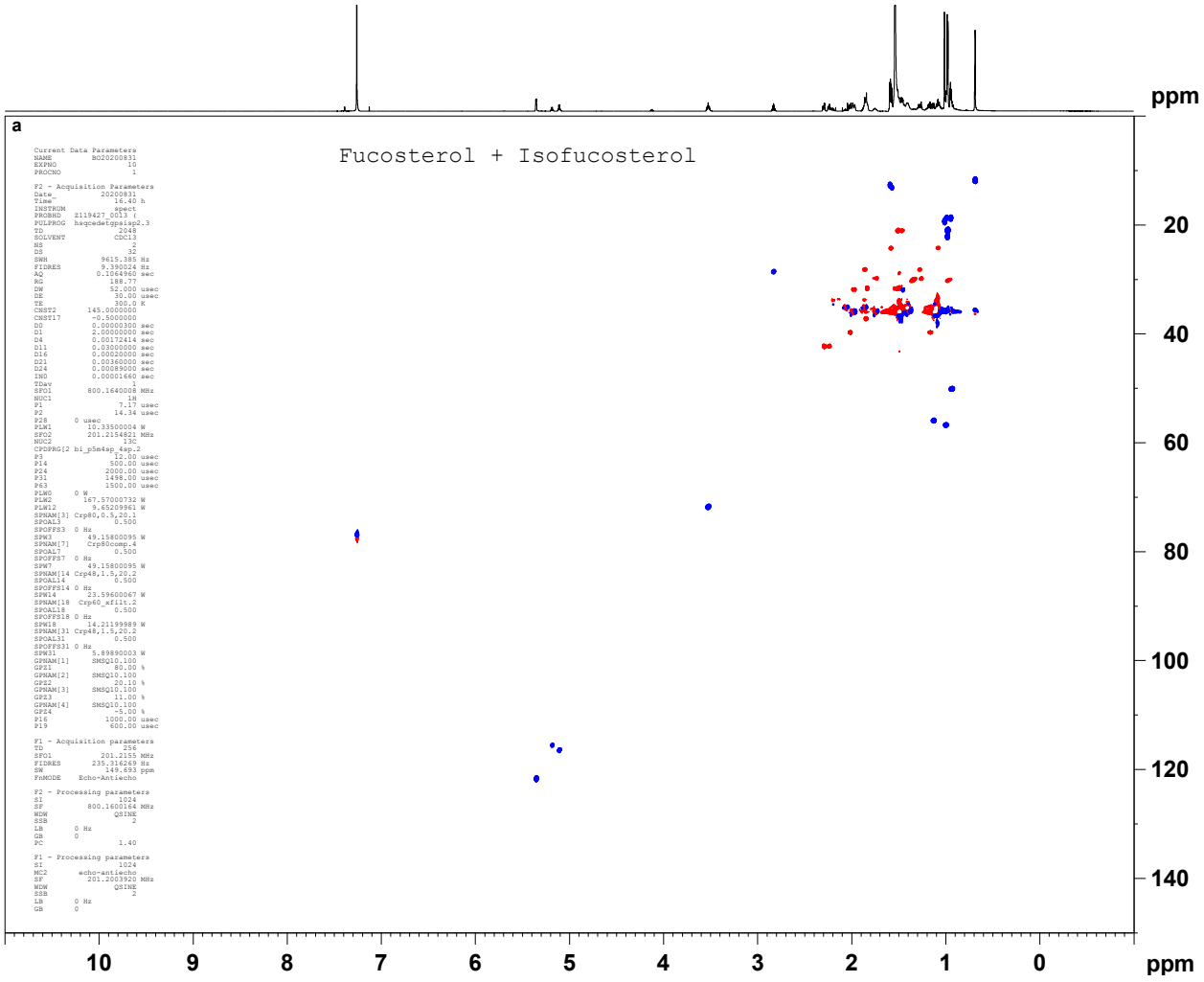
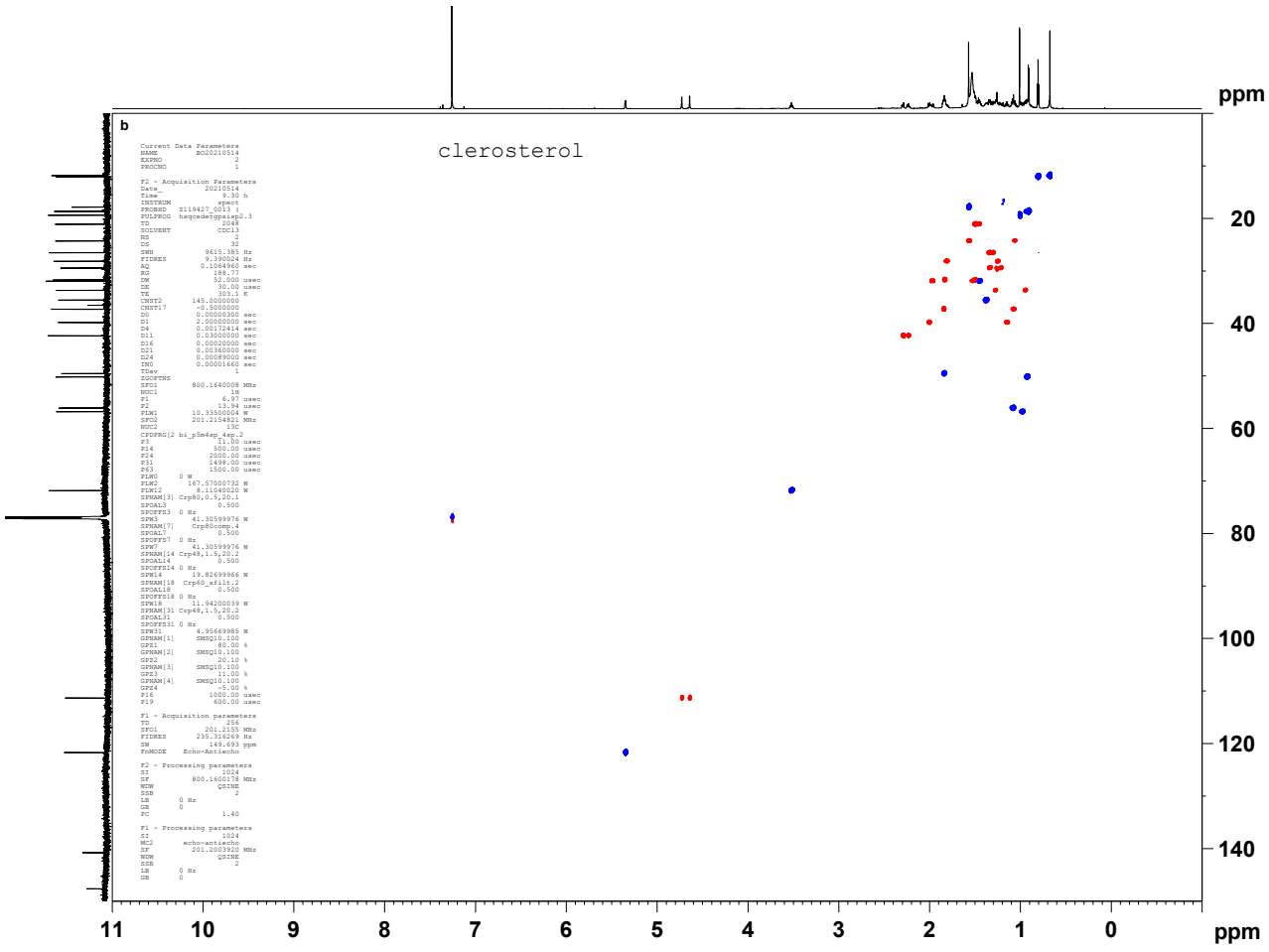
26,27,29,30 - [13C]
24-Isopropylcholestan-5,23-dienol
+ 24S-24-Isopropylcholestan-5,25-dienol





Supplementary Figure 6. **Raw 2D NMR (HSQC) spectra of the following sterols: a** fucosterol plus isofucosterol, **b** clerosterol, **c** epiclerosterol, **d** 24*S*-24-isopropylcholesta-5,25-dienol, **e** 24*R*-24-isopropylcholesta-5,25-dienol, **f** [26, 27, 29, 30-^{13}C] 24-isopropylcholesta-5,24-dienol, and **g** [26, 27, 29, 30-^{13}C] 24-isopropylcholest-5,23-dienol plus 24*S*-24-isopropylcholesta-5,25-dienol. Chemical shifts are summarized in Supplementary Table 3.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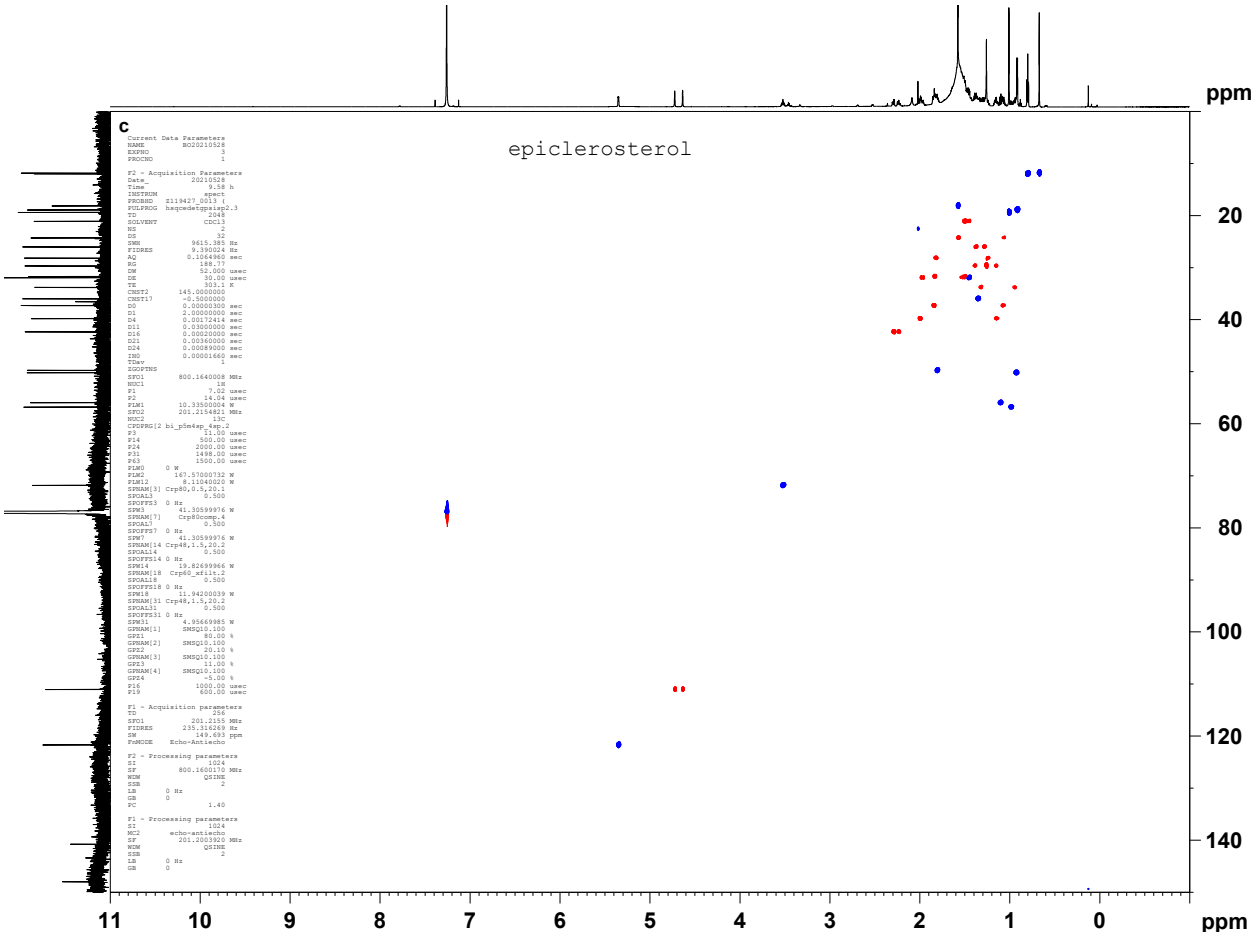
ppm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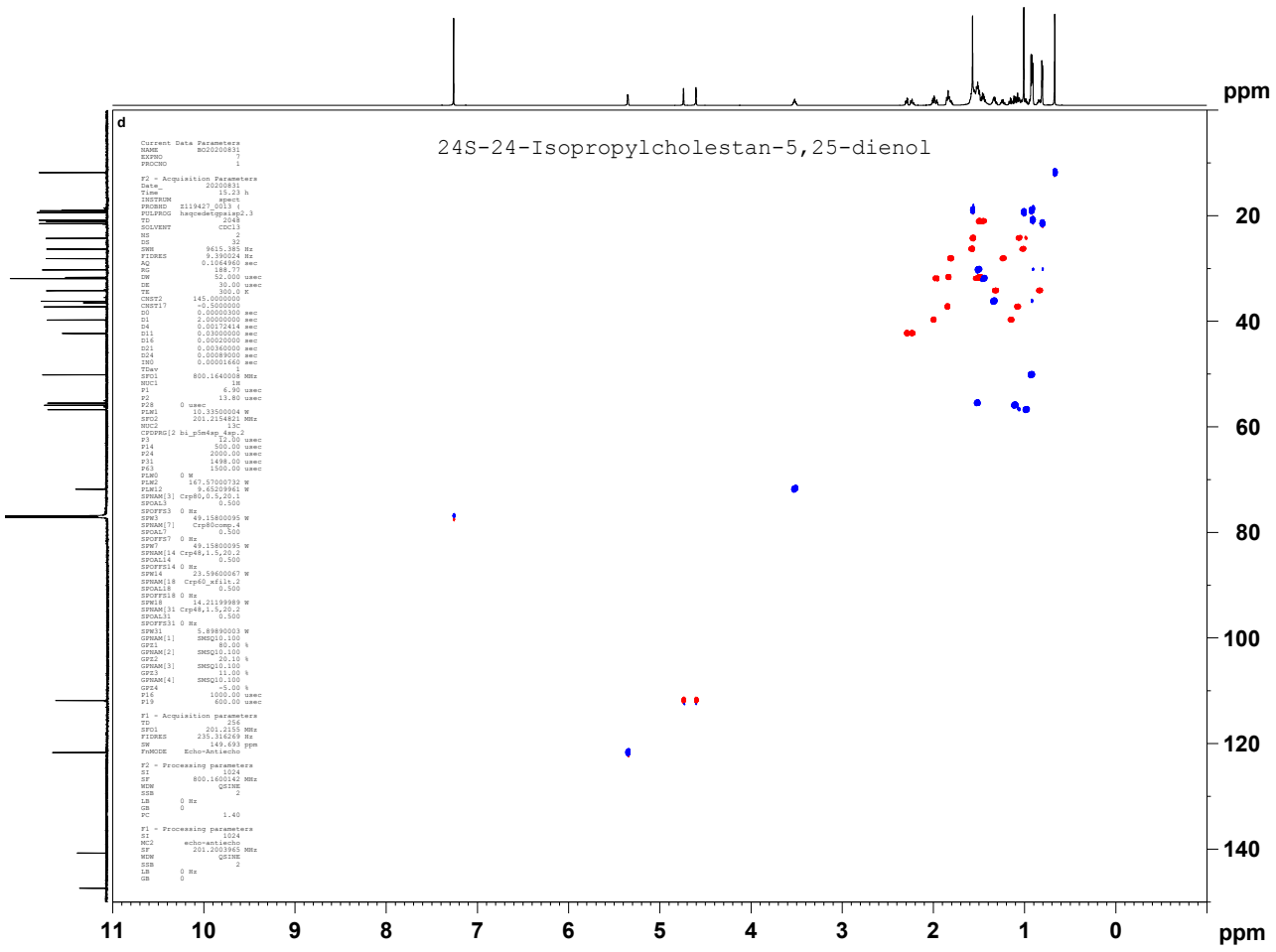
24S-24-Isopropylcholestan-5,25-dienol
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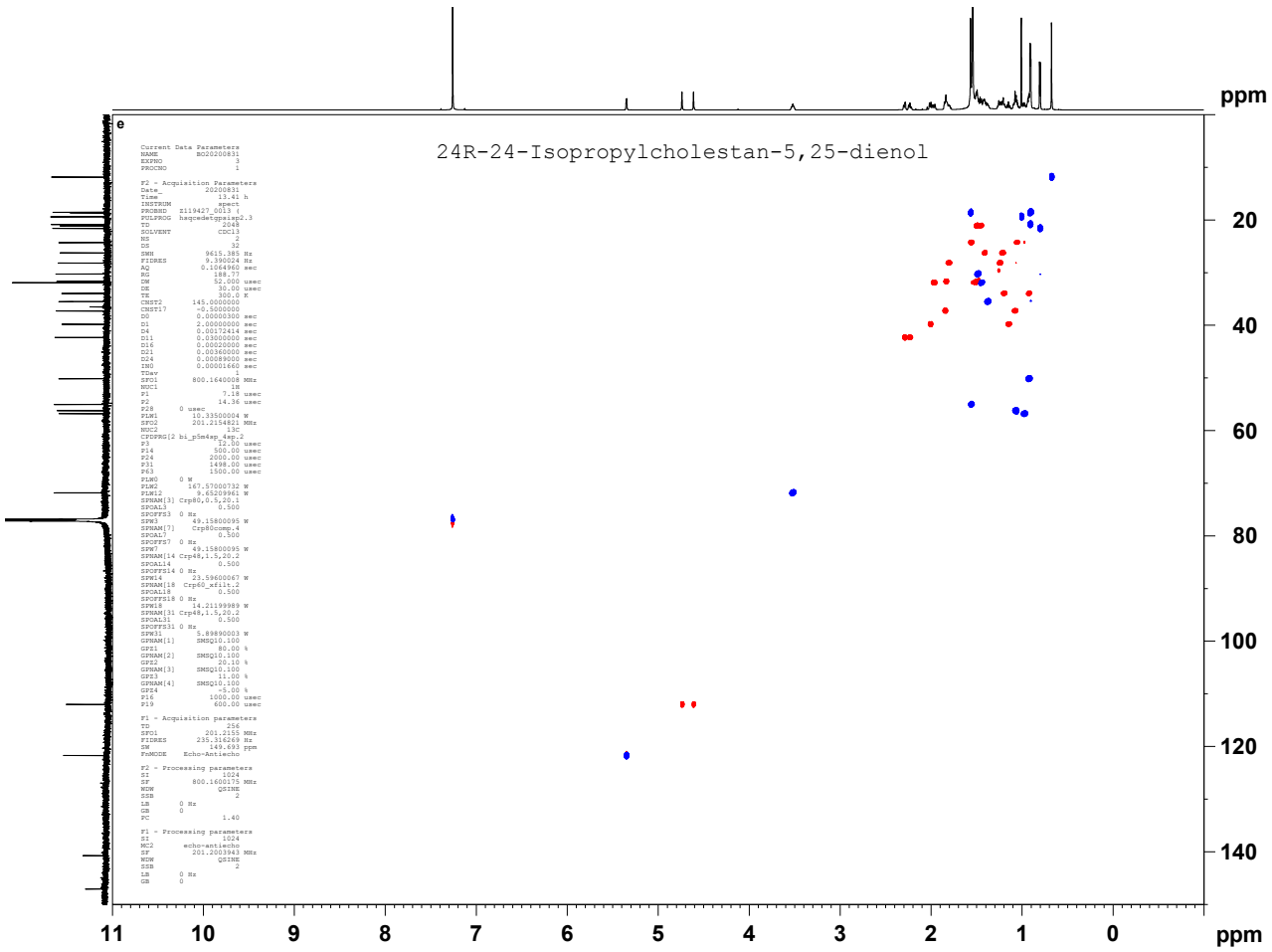
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24R-24-Isopropylcholestan-5,25-dienol
ppm
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10
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8
7
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5
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3
2
1
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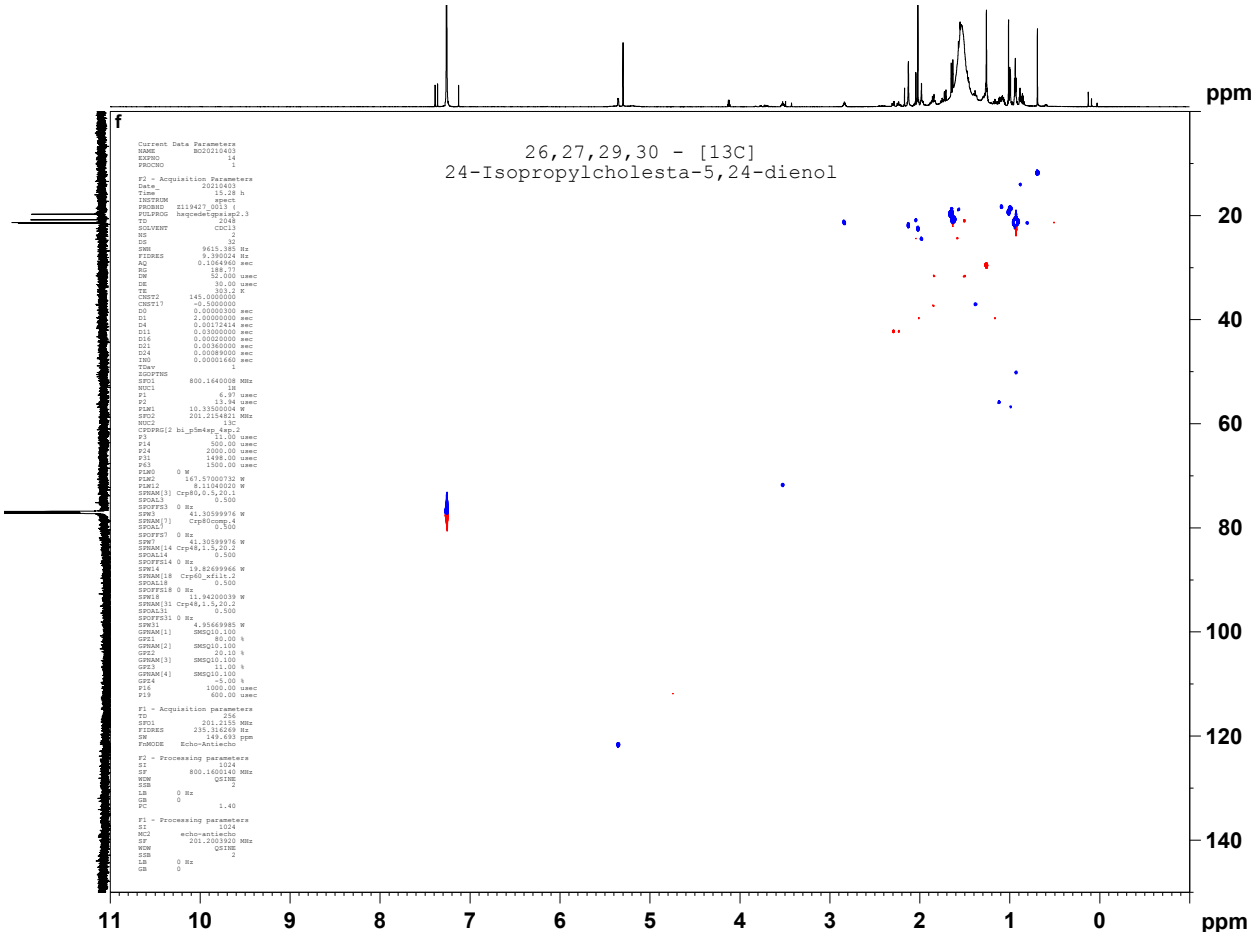
20
40
60
80
100
120
140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26,27,29,30 - [13C]
24-Isopropylcholesta-5,24-dienol
ppm
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8
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60
80
100
120
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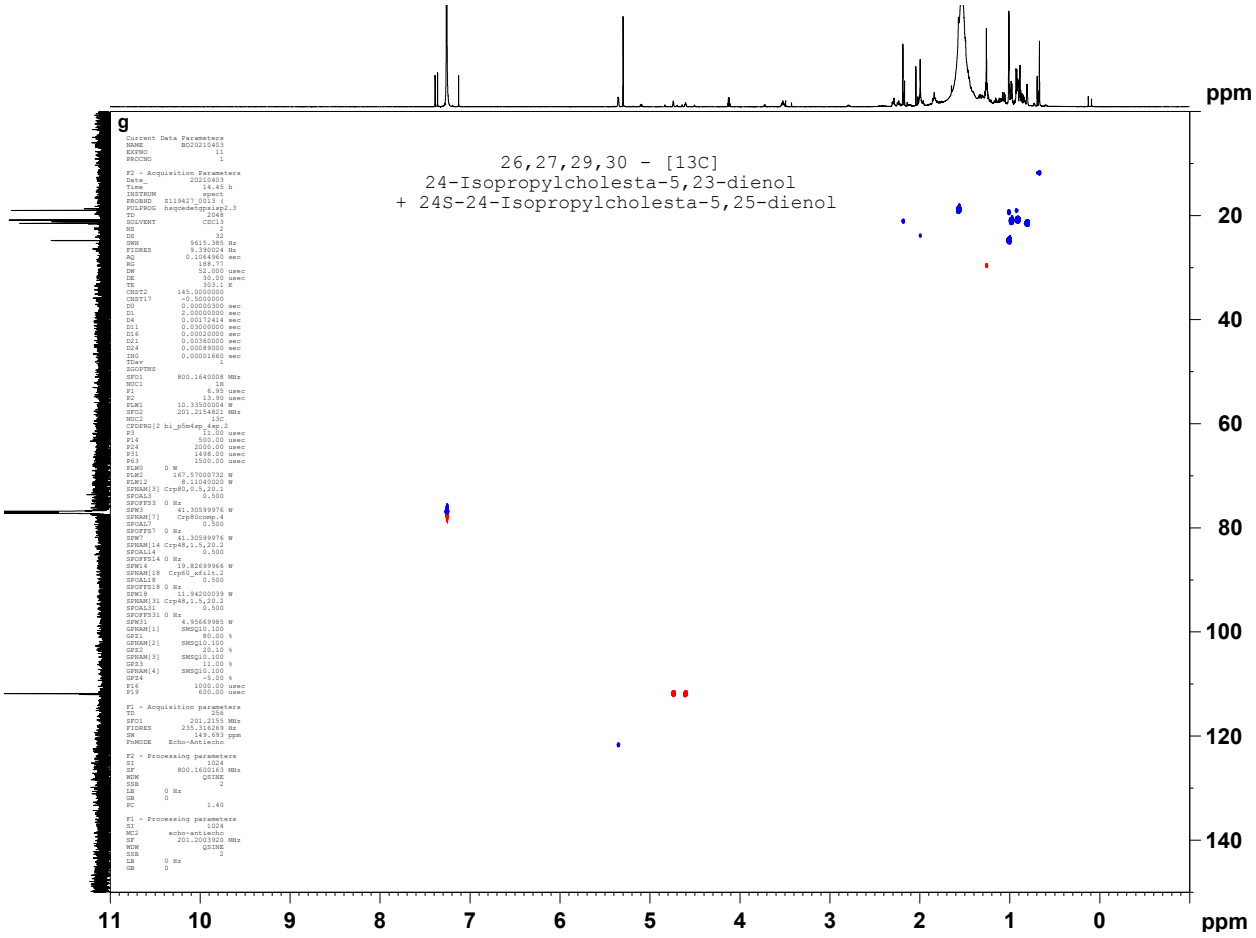
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+ 24S-24-Isopropylcholesta-5,25-dienol
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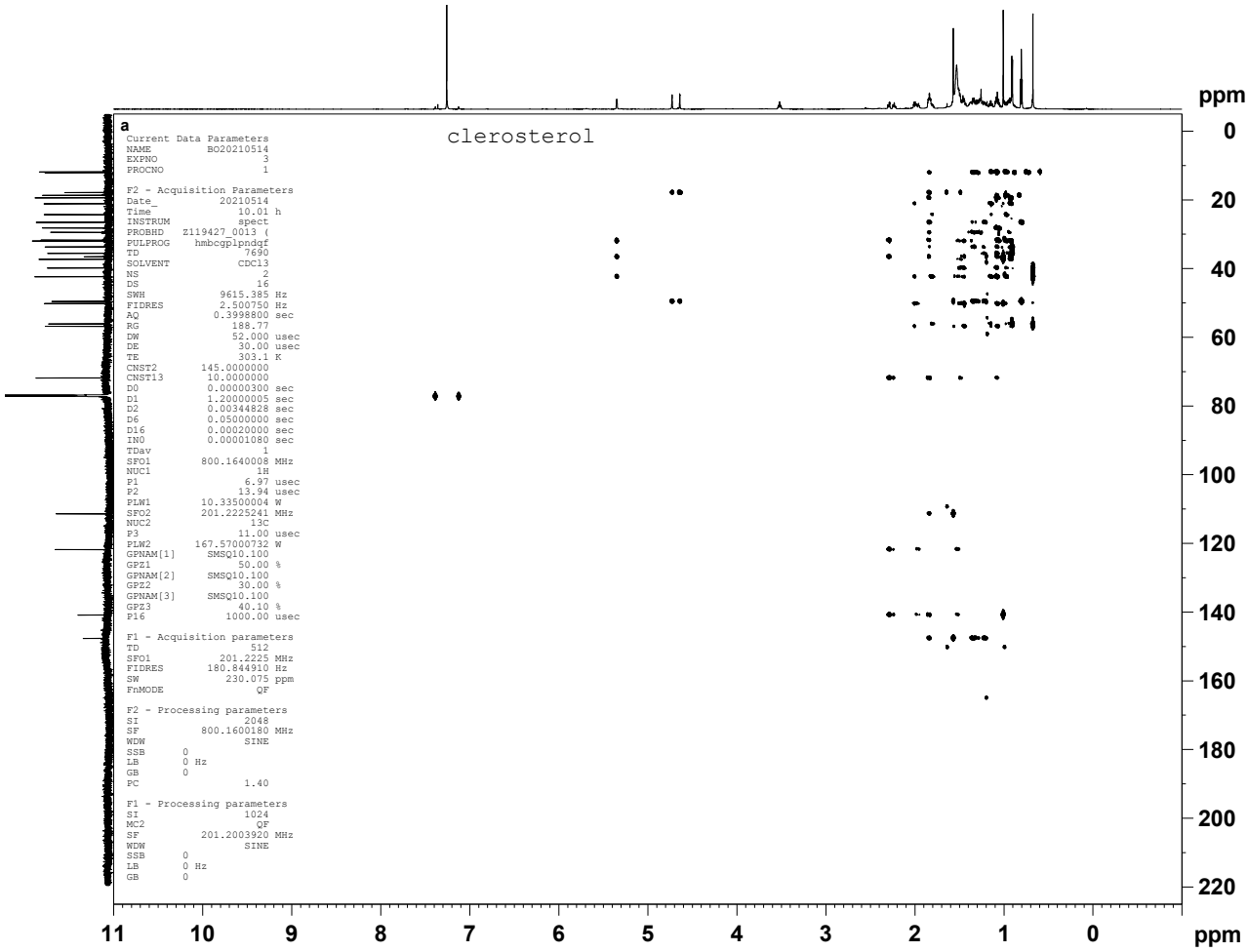
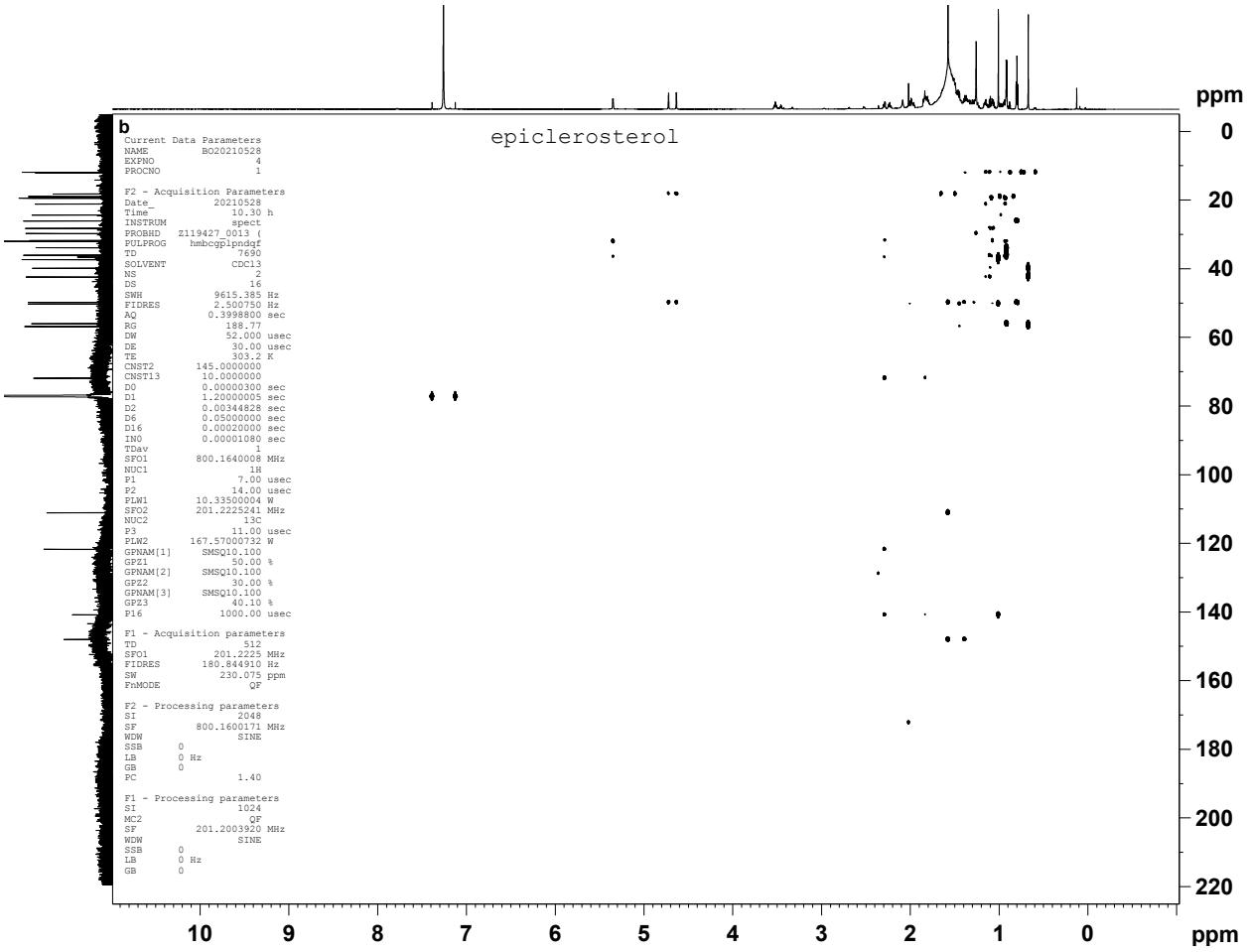


Supplementary Figure 7. **Raw 2D NMR (HMBC) spectra of the following sterols: a** clerosterol, **b** epiclerosterol, **c** 24*S*-24-isopropylcholesta-5,25-dienol, **d** 24*R*-24-isopropylcholesta-5,25-dienol, and **e** [26, 27, 29, 30-^{13}C] 24-isopropylcholesta-5,24-dienol. Chemical shifts are summarized in Supplementary Table 3.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DE 30.00 usec
TE 303.2 K
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CNST13 10.0000000
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D1 1.20000005 sec
D2 0.00344828 sec
D6 0.05000000 sec
D16 0.00020000 sec
IN0 0.00001080 sec
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NUC1 1H
P1 7.00 usec
P2 14.00 usec
PLW1 10.33500004 W
SFO2 201.2225241 MHz
NUC2 13C
P3 11.00 usec
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SW 230.075 ppm
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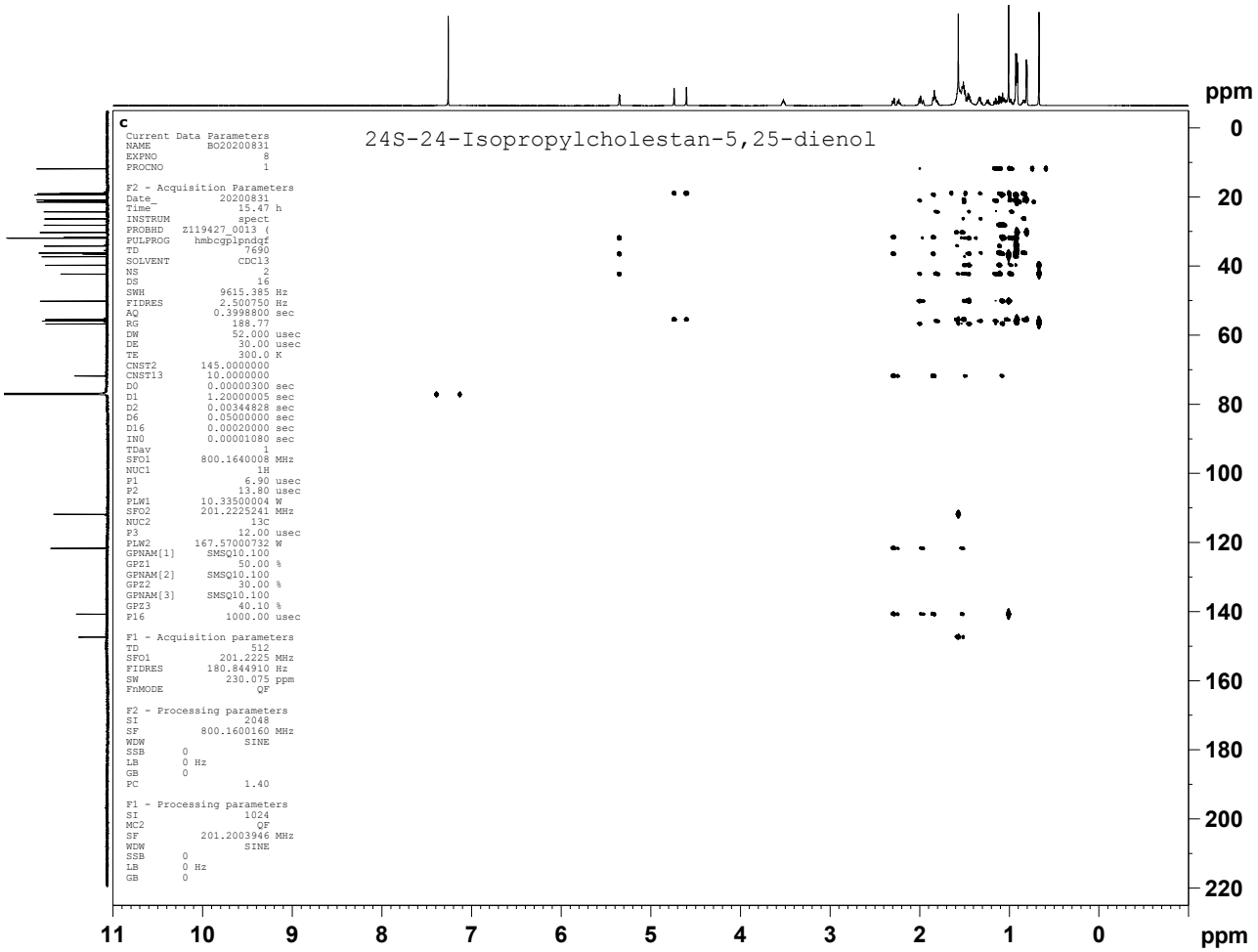
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DE 30.00 usec
TE 300.0 K
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CNST13 10.0000000
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D6 0.05000000 sec
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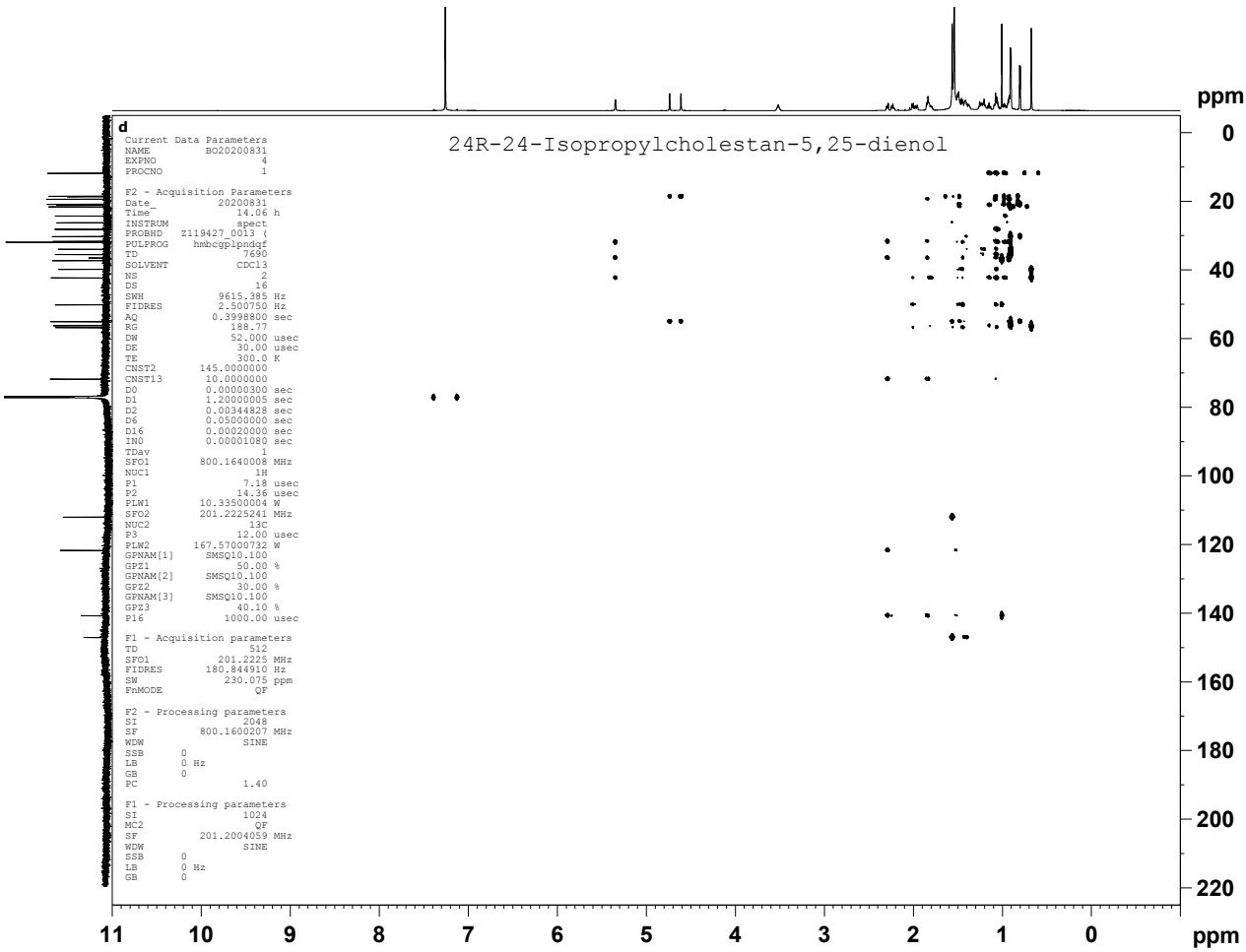
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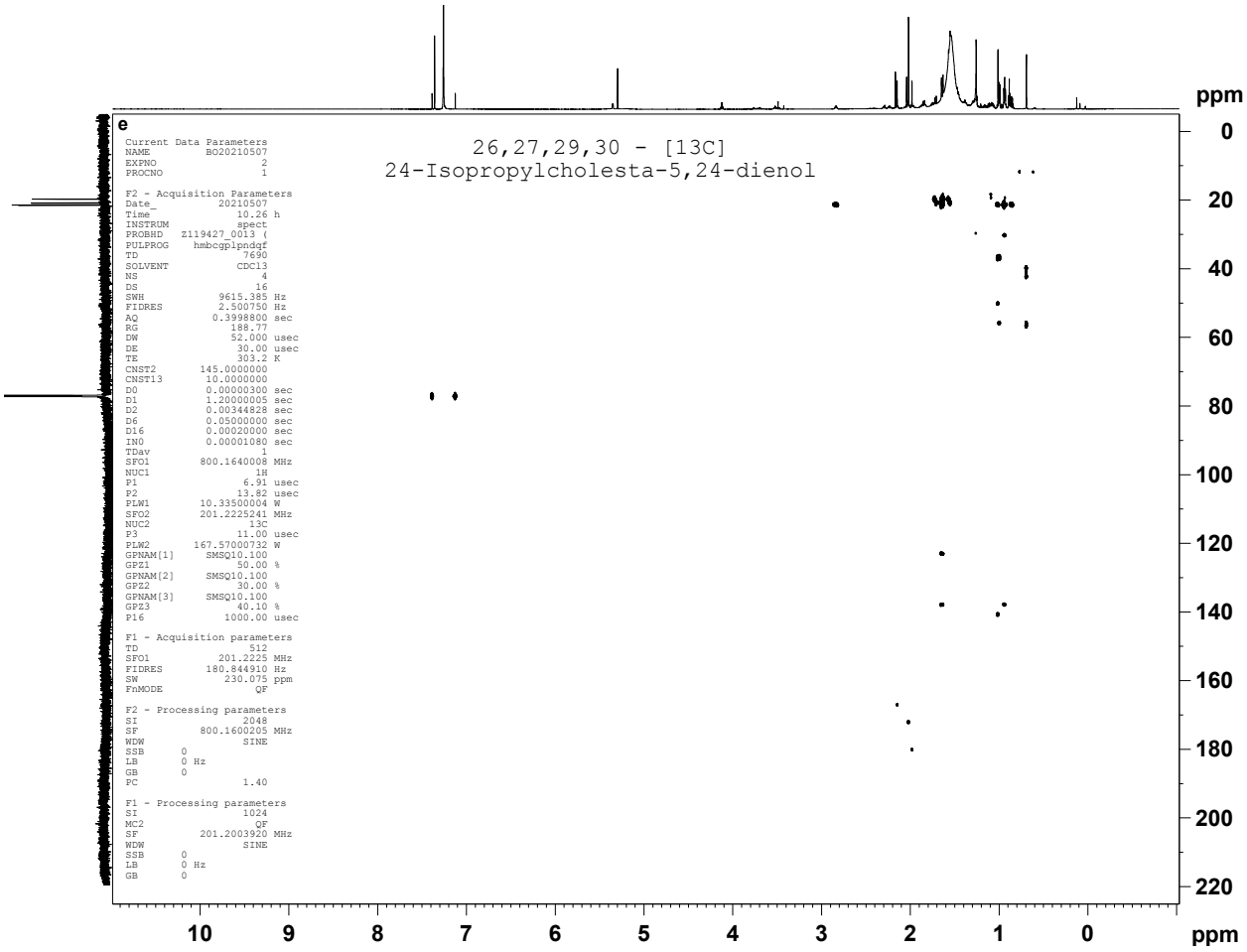
ppm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D6 0.05000000 sec
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SFO2 201.2225241 MHz
NUC2 13C
P3 11.00 usec
PLW2 167.57000732 W
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GPZ1 50.00 %
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GPZ2 30.00 %
GPNAM[3] SMSQ10.100
GPZ3 40.10 %
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SW 230.075 ppm
FnMODE QF
F2 - Processing parameters
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SF 800.1600205 MHz
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LB 0 Hz
GB 0
PC 1.40
F1 - Processing parameters
SI 1024
MC2 QF
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ppm
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20
40
60
80
100
120
140
160
180
200
220
10
9
8
7
6
5
4
3
2
1
0
ppm

Supplementary Table 1. **Sterols previously identified in sponges relevant to this study.** The major sterol and the presence and/or percentages of C_{27}-C_{30} sterols for each species are given when available.

Sponge species	Major sterol	C_{27}	C_{28}	C_{29}	C_{30}	Reference
DEMOSPONGIAE						
Amphimedon queenslandica	cholesterol	40	24	36		Gold et al., 2017[1]
Chondrilla nucula	cholestanol	yes	yes	yes		Bergmann and McTigue, 1948[2]
Sarcotragus fasciculatus			yes	yes		Venkateswarlu et al., 1996[3]
Haliclona amboinensis	fucosterol	19	24	56		Fromont et al., 1994[4]
Aplysina aerophoba	aplysterol	13	9	77	1*	Nechev et al., 2002[5] Zumberge et al., 2018[6]
Petrosia ficiformis	petrosterol	>2	>6	>60	yes	Khalil and Djerassi, 1980[7]
Theonella swinhoei	theonellasterol	yes	yes	yes		Kho 1980[8]
HOMOSCLEROMORPHA						
Oscarella lobularis		yes	yes	yes		Aiello et al., 1991[9]
CALCAREA						
Sycon sp. 1	cholesterol	33	28	34		Bergquist et al., 1986[10]
Sycon sp. 2	cholesterol	54	25	21		Bergquist et al., 1986[10]

*Nechev et al., 2002[5] reports 1.2% 24-propyl-cholest-5,24(28)-dien-3β-ol in *A. aerophoba*. Zumberge et al., 2018[6] reports 0.5% 24-npc and 0.4% 24-ipc after catalytic hydropyrolysis of *A. aerophoba* biomass. 24-isopropyl sterols have not been reported as components of *A. aerophoba*'s native sterols, to our knowledge.

Supplementary Table 2. **Whokaryote predicts all metagenomic contigs containing sterol methyltransferases analyzed in this study as prokaryotic.**

SMT	contig	contig length	nr genes	ratio same orientation	ID general avg	ID general std	gene density	gene length	ID Q1	ID Q3	rbs ratio	tiara pred	predicted
Aplysina aerophoba JGIcombinedJ30088_10000835	JGIcombinedJ30088_10000835	32339	23	0.68	128.14	183.70	0.91	1285.09	3.75	181.00	0.96	1.0	prokaryote
Aplysina aerophoba Ga0209021_1000146	Ga0209021_1000146	97163	103	0.68	115.34	117.55	0.88	829.83	31.00	173.75	0.90	1.0	prokaryote
Petrosia ficiformis LXNJ01014139	LXNJ01014139.1	3313	4	0.00	100.67	62.53	0.91	752.00	56.50	124.50	0.25	1.0	prokaryote
Petrosia ficiformis LXNJ01003202	LXNJ01003202.1	8597	8	0.57	115.57	167.57	0.91	975.50	0.00	131.00	0.88	1.0	prokaryote
Petrosia ficiformis LXNJ01000389	LXNJ01000389.1	31336	22	0.67	79.90	78.14	0.94	1341.91	4.00	147.00	0.77	1.0	prokaryote
Petrosia ficiformis LXNJ01004423	LXNJ01004423.1	7072	6	0.60	102.20	58.00	0.93	1093.00	63.00	126.00	0.67	1.0	prokaryote
Sarcotragus foetidus	LXNI01004480.1	7225	5	0.50	105.25	156.88	0.94	1359.20	6.75	121.00	0.80	1.0	prokaryote
Theonella swinhoei associated Chromatiales sp. 1 of 2	2690316284	444506	427	0.74	74.66	122.32	0.93	968.13	1.00	104.25	0.68	1.0	prokaryote
Theonella swinhoei associated Chromatiales sp. 2 of 2	2690316289	117771	97	0.83	74.25	116.07	0.94	1142.96	0.00	97.50	0.65	1.0	prokaryote
Marine sediment, Gulf of Thailand	Ga0118733_100012602	21160	19	0.89	72.06	133.71	0.94	1042.53	0.00	65.50	0.84	1.0	prokaryote
Marine sediment, Helgoland, North Sea	Ga0055584_100171735	2192	3	0.50	80.50	75.50	0.93	676.00	42.75	118.25	0.00	1.0	prokaryote
Western Arctic Ocean	Ga0133547_10420286	2727	3	0.00	147.00	126.00	0.89	810.00	84.00	210.00	0.33	1.0	prokaryote
Freshwater, Lake Fryxell, Antarctica	Ga0105048_10034691	7428	9	0.63	86.00	80.31	0.88	722.33	13.50	104.75	0.44	1.0	prokaryote
Lake sediment, Walker Lake, Nevada	Ga0114946_10008497	6500	5	0.50	77.25	71.98	0.93	1212.80	22.50	103.75	0.40	1.0	prokaryote
Hot spring, Beatty, Nevada	Ga0114945_10021081	3480	3	0.00	63.00	7.00	0.96	1108.00	59.50	66.50	0.33	1.0	prokaryote
Hot spring sediment, Dewer Creek, BC	Ga0073932_1004097	18020	12	0.82	164.73	132.22	0.89	1342.75	67.00	269.00	0.92	1.0	prokaryote
Chlamydiae sp., drinking water 1 and 2	2619626156	1146800	1105	0.68	85.18	128.28	0.92	956.10	0.00	118.00	0.42	1.0	prokaryote
Nitrospira sp., coral reef 1 and 2	2681831735	70077	63	0.74	147.08	145.66	0.87	962.71	27.00	196.00	0.89	1.0	prokaryote
Sandaracinus sp., marine 1 and 2	2775515747	24518	17	0.88	112.25	106.29	0.89	1282.47	22.50	163.50	0.76	1.0	prokaryote
Spirochaetes sp., soil	2708772188	70818	55	0.54	151.69	167.86	0.89	1142.60	6.25	242.50	0.24	1.0	prokaryote

Supplementary Table 3. **800 MHz ^{1}H and 201 MHz ^{13}C NMR assignments of selected sterol side-chain positions at 30 °C.** ^{13}C data are in boldface. The assignments were determined by HSQC-DEPT, HMBC, and COSY 2D-NMR experiments. ^{1}H and ^{13}C chemical shifts are given to three and two decimal places, respectively, if directly observed, two and one, respectively, if determined from 2D spectra. Diastereotopic signals are not assigned. The raw spectra used to generate this table are provided in Supplementary Fig. 4-7.

	C-24	C-25	C-26	C-27	C-28	C-29	C-30
Fucosterol	**145.6**	2.199 **34.7**			5.183 **115.58**	1.574 **13.16**	
Isofucosterol	**147.1**	2.829 **28.6**			5.108 **116.48**	1.591 **12.76**	
Clerosterol*	1.838 **49.54**	**147.61**	4.730, 4.642 **111.33**	1.570 **17.85**	1.305, 1.341 **26.53**	0.805 **12.04**	
Epiclerosterol	1.81 **49.75**	**147.98**	4.725, 4.636 **111.06**	1.576 **18.13**	1.290, 1.380 **26.01**	0.800 **11.96**	
24*R*-24-Isopropylcholest-5,25-dienol*	1.56 **55.03**	**147.29**	4.613, 4.738 **112.02**	1.567 **18.63**	1.49 **30.25**	0.911 **20.85**	0.803 **21.60**
24*S*-24-Isopropylcholest-5,25-dienol	1.52 **55.50**	**147.38**	4.602 4.740 **111.85**	1.570 **18.95**	1.51 **30.24**	0.913 **20.85**	0.807 **21.49**
24-Isopropylcholest-5,23-dienol			1.013 **24.77**	1.013 **24.79**		0.980 **20.96**	0.980 **21.18**
24-Isopropylcholest-5,24-dienol	**137.0**	**123.1**	1.633 **20.81**	1.651 **19.73**		0.938 **21.34**	0.938 **21.41**

*Clerosterol and 24*R*-24-isopropylcholest-5,25-dienol were not detected as products of the *A. aerophoba* symbiont and Chlamydiae SMTs but are shown for comparative purposes.

Supplementary Table 4. **Sources of sterol methyltransferases analyzed in this study.**

SMT	Source	Genome ID/ BioProject	Gene ID/Locus
DEMOSPONGIAE			
Amphimedon queenslandica	JGI IMG	2507525020	2508051218
Chondrilla nucula	([11])		
Sarcotragus fasciculatus	([11])		
Haliclona amboinensis	Compagen		
Haliclona tubifera	Compagen		
HOMOSCLEROMORPHA			
Oscarella pearsei	Compagen		
Oscarella carmela	Compagen		
CALCAREA			
Sycon ciliatum	Compagen		
DEMOSPONGE METAGENOME			
Aplysina aerophoba JGIcombinedJ30088_10000835 1 of 2	JGI IMG	3300002448	JGIcombinedJ30088_1000083511
Aplysina aerophoba JGIcombinedJ30088_10000835 2 of 2	JGI IMG	3300002448	JGIcombinedJ30088_100008359
Aplysina aerophoba Ga0209021_1000146	JGI IMG	3300027386	Ga0209021_100014628
Petrosia ficiformis LXNJ01014139	GenBank	PRJNA318959	LXNJ01014139.1
Petrosia ficiformis LXNJ01003202	GenBank	PRJNA318959	LXNJ01003202.1
Petrosia ficiformis LXNJ01000389	GenBank	PRJNA318959	LXNJ01000389.1
Petrosia ficiformis LXNJ01004423	GenBank	PRJNA318959	LXNJ01004423.1
Sarcotragus foetidus	GenBank	PRJNA318959	LXNI01004480.1
DEMOSPONGE METAGENOME-ASSEMBLED GENOME			
Theonella swinhoei associated Chromatiales sp. 1 of 2	JGI IMG	2690315631	2690455922
Theonella swinhoei associated Chromatiales sp. 2 of 2	JGI IMG	2690315631	2690456335
OTHER METAGENOME			
Marine sediment, Gulf of Thailand	JGI IMG	3300010430	Ga0118733_1000126028
Marine sediment, Helgoland, North Sea	JGI IMG	3300004097	Ga0055584_1001717352
Western Arctic Ocean	JGI IMG	3300010883	Ga0133547_104202862
Freshwater, Lake Fryxell, Antarctica	JGI IMG	3300009032	Ga0105048_100346915
Lake sediment, Walker Lake, Nevada	JGI IMG	3300009504	Ga0114946_100084975
Hot spring, Beatty, Nevada	JGI IMG	3300009444	Ga0114945_100210811
Hot spring sediment, Dewer Creek, BC	JGI IMG	3300007072	Ga0073932_10040971
OTHER METAGENOME-ASSEMBLED GENOME			
Chlamydiae sp., drinking water 1 of 2	JGI IMG	2619618861	2619789262
Chlamydiae sp., drinking water 2 of 2	JGI IMG	2619618861	2619789261
Nitrospira sp., coral reef 1 of 2	JGI IMG	2681812944	2682229274
Nitrospira sp., coral reef 2 of 2	JGI IMG	2681812944	2682229270
Sandaracinus sp., marine 1 of 2	JGI IMG	2775506781	2775843484
Sandaracinus sp., marine 2 of 2	JGI IMG	2775506781	2775843485
Spirochaetes sp., soil	JGI IMG	2708742746	2709604172

Supplementary Table 5. **Best amino acid substitution models, gamma shape parameters, and invariable sites proportions under the Bayesian Information Criterion and Akaike Information Criterion using ModelTest-NG on XSEDE for generation of sterol methyltransferase and oxidosqualene cyclase maximum-likelihood trees.**

Protein	Substitution Model	Gamma Shape Parameter	Invariable Sites Proportion
sterol methyltransferase	LG+I+G	1.260	0.020
oxidosqualene cyclase	LG+I+G	1.212	0.022

SUPPLEMENTARY REFERENCES

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2. Bergmann, W. & McTigue, F. H. Contributions to the study of marine products. XXI. Chondrillasterol. *J Org Chem* **13**, 738–741 (1948).
3. Venkateswarlu, Y., Reddy, M. V. R. & Rao, M. R. A new epoxy sterol from the sponge *Ircinia fasciculata*. *J Nat Prod* **59**, 876–877 (1996).
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