Supplementary information for:

Enzymatic synthesis of polybrominated dioxins from the marine environment

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Recombinant protein purification and *in vitro* biochemical assays: *M. mediterranea* MMB-1 Bmp5 was amplified by PCR and ligated into the multiple cloning site-2 (MCS-2) of pRSF-Duet vector double digested with NdeI and XhoI restriction enzymes. Positive clones were verified by DNA sequencing as before and transformed into BL21Gold(DE3) cells for protein expression. Bmp5 was expressed in a manner identical to as described before. The culture was harvested by centrifugation, and the cells were resuspended in 20 mM Tris-HCl (pH 8.0) 500 mM NaCl buffer supplemented with 0.25 mg/mL DNaseI and lysed by sonication. The cell lysate was centrifuged at 16000×g for 45 min. The supernatant was collected, and solid streptomycin sulfate was added to a final concentration of 2% (w/v) with gentle stirring at 4 °C. The solution was centrifuged at 16000×g for 15 min, and to the supernatant thus obtained; solid ammonium sulfate was added with gentle stirring at 4 °C to a final concentration of 0.75 M. The solution was again centrifuged at 16000×g for 15 min and additional solid ammonium sulfate was added to a final concentration of 1.5 M. After centrifugation at 16000×g for 45 min, the supernatant was loaded onto an Amersham 16/60 liquid chromatography column packed with 10 mL phenyl sepharose matrix. The column had been pre-equilibrated in 20 mM Tris-HCl (pH 8.0) 1.8 M $(NH_4)_2SO_4$ buffer. The flow through was collected, and the column washed with additional equilibration buffer. As determined by SDS-PAGE, Bmp5 protein was majorly obtained in the flow through and wash fractions, while majority of the contaminant proteins were obtained in the elution fraction with 20 mM Tris-HCl (pH 8.0) used as the elution buffer. Bmp5 was extensively dialyzed in 20 mM Tris-HCl (pH 8.0) 100 mM KCl 20% glycerol buffer for 24 hours at 4 °C using a 10000 Da - 12000 Da molecular weight cutoff dialysis bag. The protein after dialysis was injected to ion exchange Q-ff, ion exchange SP-ff and Heparin protein purification columns connected in tandem. As judged by SDS-PAGE, Bmp5 was obtained in the flow through fractions. The protein was extensively dialyzed in to 20 mM Tris-HCl (pH 8.0) 20% glycerol buffer. Protein concentration was measured by Bradford assay. Analytical scale assays using

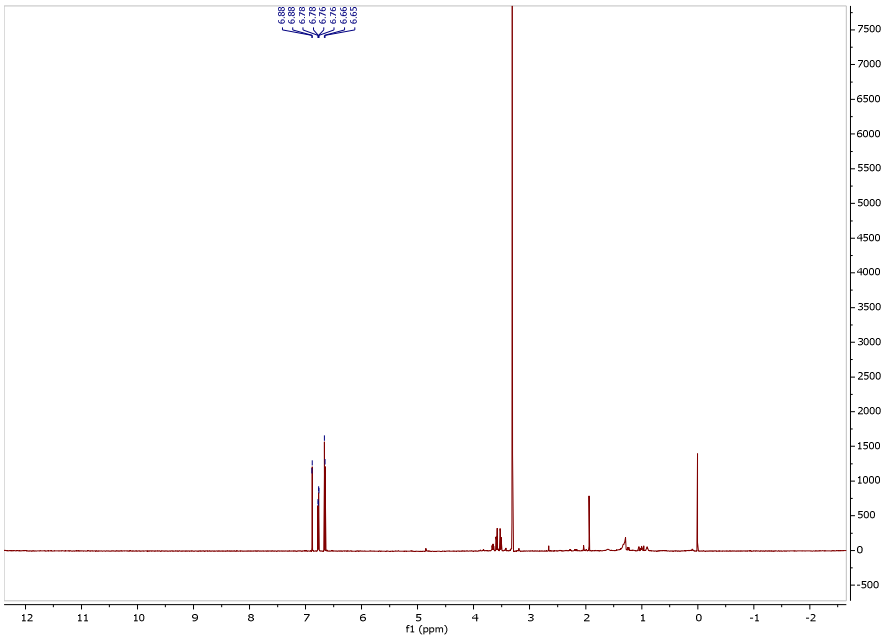
Bmp5 thus obtained were conducted in a manner identical to that described previously.[1] Assays for preparative scale isolation of **4** and **5** relied on a phosphite dehydrogenase dependent NADH regeneration system.[2]

M. mediterranea MMB-1 Bmp7 was expressed, purified and assayed in a manner identical to that described for *Pseudoalteromonas luteoviolacea* 2ta16 Bmp7.[1] Preparative scale assays using **5** as the substrate relied on a phosphite dehydrogenase dependent NADH regeneration system.[2]

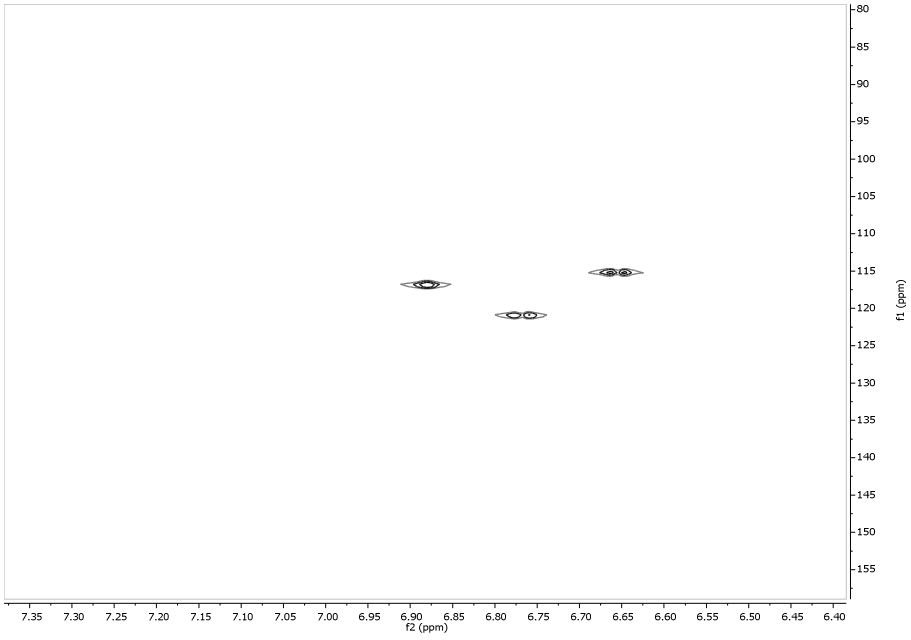
***In vivo* assays for the biosynthesis and coupling of bromophenols and bromocatechols:** *M. mediteranea* MMB-1 Bmp5 was cloned in the MCS2 site of pRSF-Duet vector using NdeI and XhoI restriction sites that were introduced using appropriately designed PCR primers. *m3553* and *M. mediterranea* Bmp5 were cloned into MCS1 and MCS2 sites respectively of the pRSF-Duet vector using NcoI-HindIII and NdeI-XhoI restriction sites. *M. mediterranea* MMB-1 Bmp7 was cloned in the MCS2 site of pCDF-Duet vector using NdeI and XhoI restriction sites. All vectors were confirmed by sequencing. The vectors, either individually (pRSF-Bmp5; pRSF-*bmp5+m3553*), or together (pRSF-*bmp5+m3553* and pCDF-*bmp7*) were transformed into *E. coli* BL21Gold(DE3) cells for protein expression. Cultures were grown in LB media supplemented with 50 μg/mL kanamycin and 50 μg/mL streptomycin as required, and 1 g/L KBr. The cultures were spiked with 0.5 mM final concentration of **1** or **3** as required. Cultures were grown at 30 °C and protein expression was induced by the addition of 50 μM IPTG when the optical density reached 0.6. After induction, growth was allowed to proceed at 30 °C for 2 days. 5 mL culture volume was extracted twice with equal volumes of ethyl acetate. The organic solvent was collected after brief centrifugation and removed by rotavap. The residue obtained was resuspended in 100 μL MeOH. For experiments described in, and related to **Figure 2d–2f**, 30 μL of the analyte was injected onto a Phenomenex Luna C18 5μ (4.6 × 100 mm) analytical column operating on an Agilent 1260 HPLC in tandem to an Agilent 6530 Accurate Mass Q-TOF mass spectrometer at room temperature and analyzed using Elution gradient I (see Methods section for manuscript). TFA in the solvents was replaced with formic acid for all LC-MS/MS experiments. Extracts of cultures grown in the presence of Bmp7 were analyzed using Elution gradient II as follows: 10% buffer B for 5 min, linear gradient to 70% buffer B across 10 min, linear gradient to 90% buffer B across 20 min, step increase to 100% buffer B, 100% buffer B for 10 min, linear decrease to 10% buffer B across 2 min, 10% buffer B for 5 min.

Synthesis of 5–7: 5 was synthesized by H_2O_2 catalyzed oxidation of 3,5-dibromosalicylaldehyde (Sigma-Aldrich 122130) in the presence of NaOH as has been described in literature[3] and the product was purified by preparative HPLC. ^{1}H-NMR characterization for **5** is provided in **Supplementary Figure 7**. A mixture of **6–7** were then synthesized by bromination of **5** with one equivalent of bromine as described in literature.[4] Specifically, 142 mg **5** was dissolved in 1 mL acetic acid. To this, 30 µL bromine (Sigma-Aldrich 207888) dissolved in 1 mL acetic acid was added at room temperature. The reaction was allowed to proceed at room temperature for 12 h. Excess bromine was quenched by the addition of sodium thiosulfate and the reaction was extracted twice with ethyl acetate. The extract was dried over $MgSO_4$ and the solvent was removed by rotavap. Products were separated by preparative HPLC.

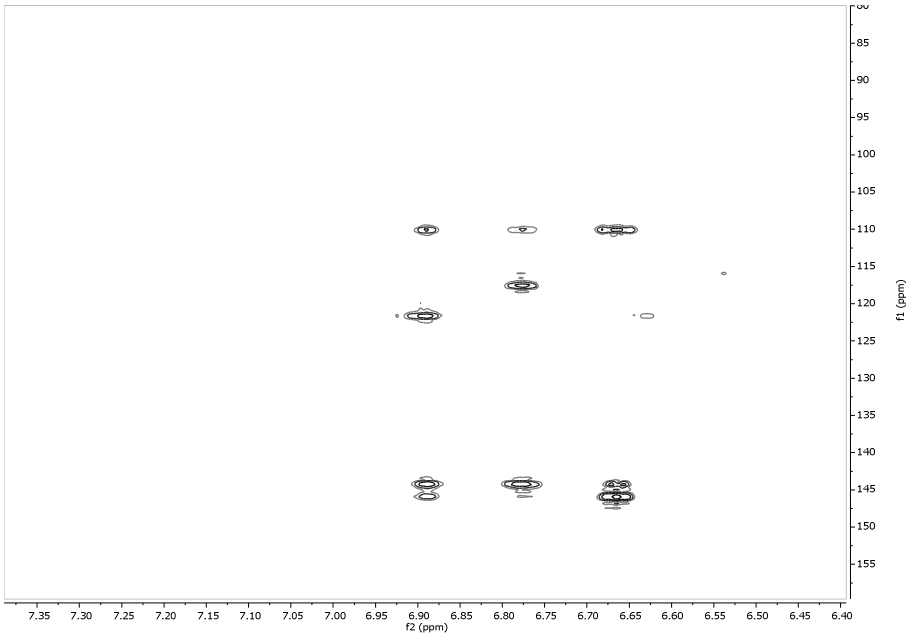
Supplementary figures:



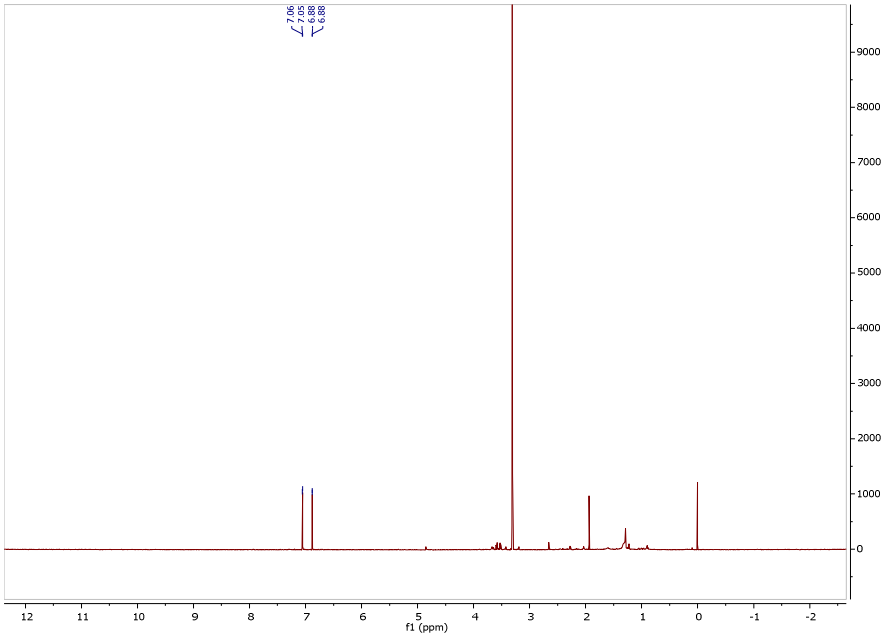
Supplementary Figure 1: ^{1}H NMR spectrum for **4** isolated from preparative scale incubation of **3** with Bmp5.
^{1}H NMR (600 MHz, Methanol-d_4) δ 6.88 (d, J = 2.3 Hz, 1H), 6.77 (dd, J = 8.4, 2.4 Hz, 1H), 6.66 (d, J = 8.4 Hz, 1H).



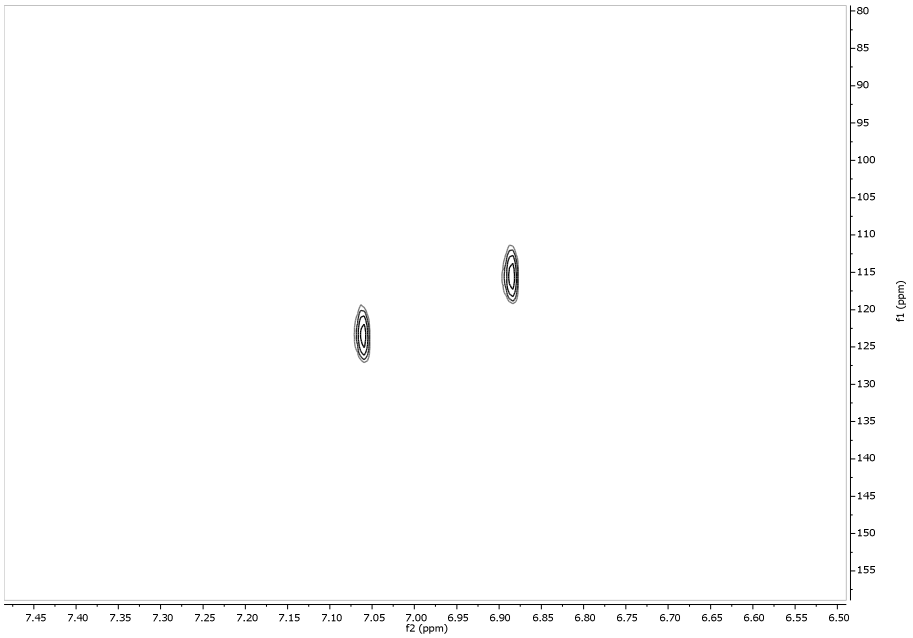
Supplementary Figure 2: HSQC spectrum for **4** isolated from preparative scale incubation of **3** with Bmp5.



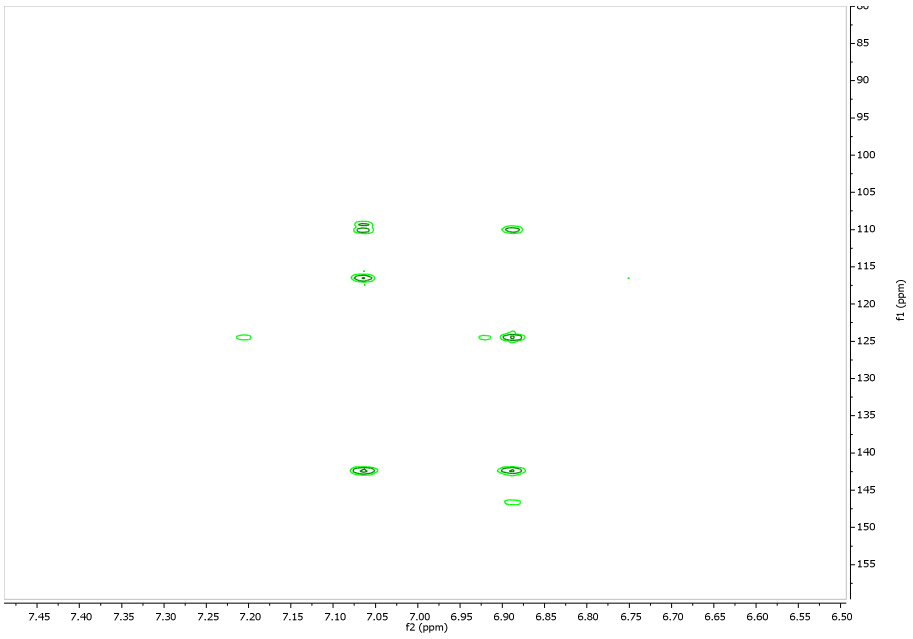
Supplementary Figure 3: HMBC spectrum for **4** isolated from preparative scale incubation of **3** with Bmp5.



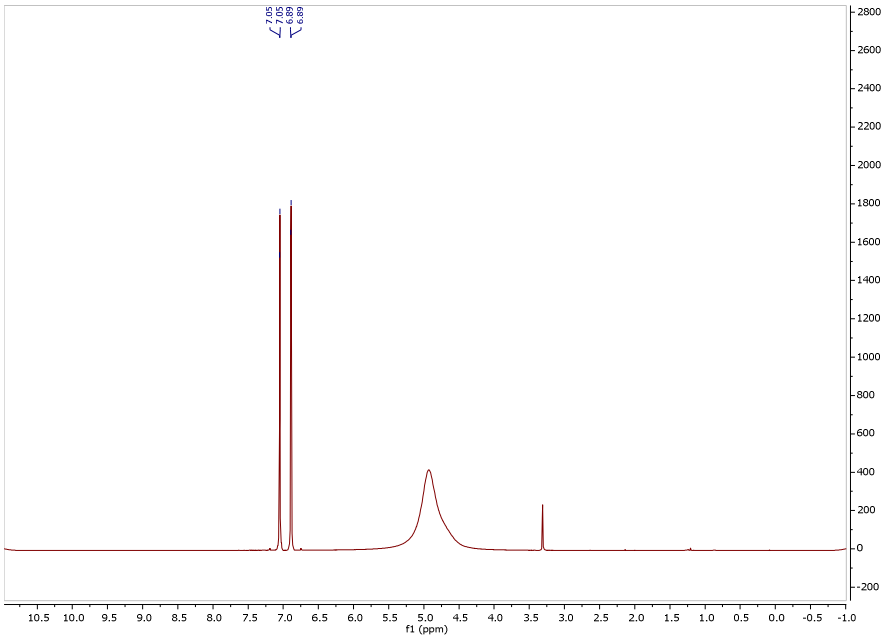
Supplementary Figure 4: ^{1}H NMR spectrum for **5** isolated from preparative scale incubation of **3** with Bmp5.
^{1}H NMR (600 MHz, Methanol-d_4) δ 7.06 (d, J = 2.3 Hz, 1H), 6.88 (d, J = 2.3 Hz, 1H).



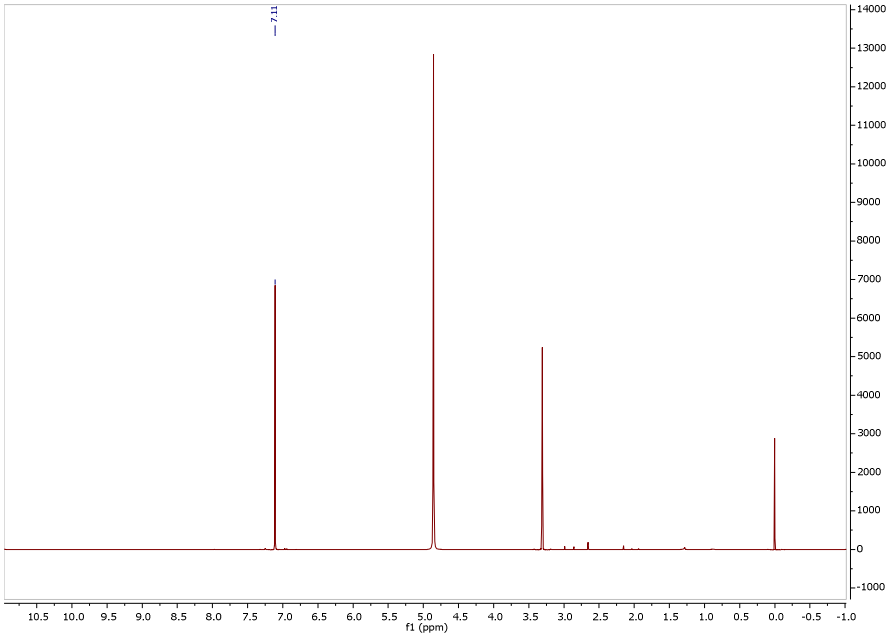
Supplementary Figure 5: HSQC spectrum for **5** isolated from preparative scale incubation of **3** with Bmp5.



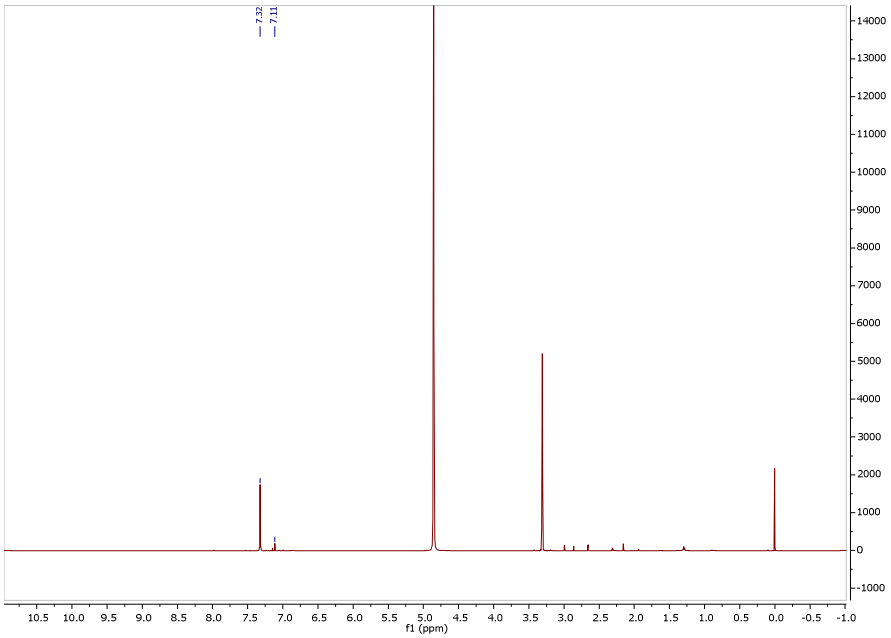
Supplementary Figure 6: HMBC spectrum for **5** isolated from preparative scale incubation of **3** with Bmp5.



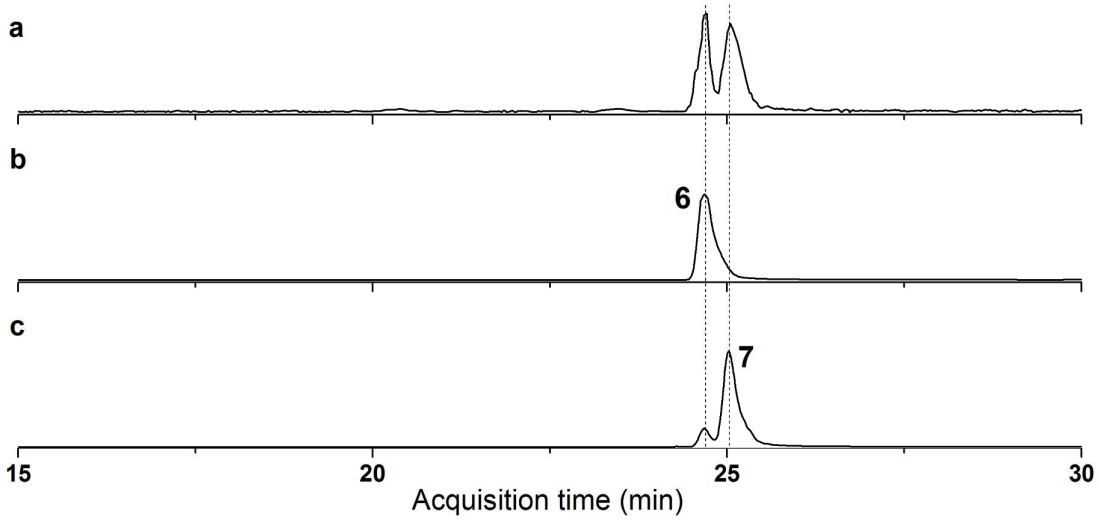
Supplementary Figure 7: ^{1}H NMR spectrum for synthetic standard for **5**.
^{1}H NMR (600 MHz, Methanol-d_4) δ 7.05 (d, J = 2.3 Hz, 1H), 6.89 (d, J = 2.3 Hz, 1H).
Synthetic standard for **4** was obtained commercially from Fisher-Scientific: 50-014-22890.



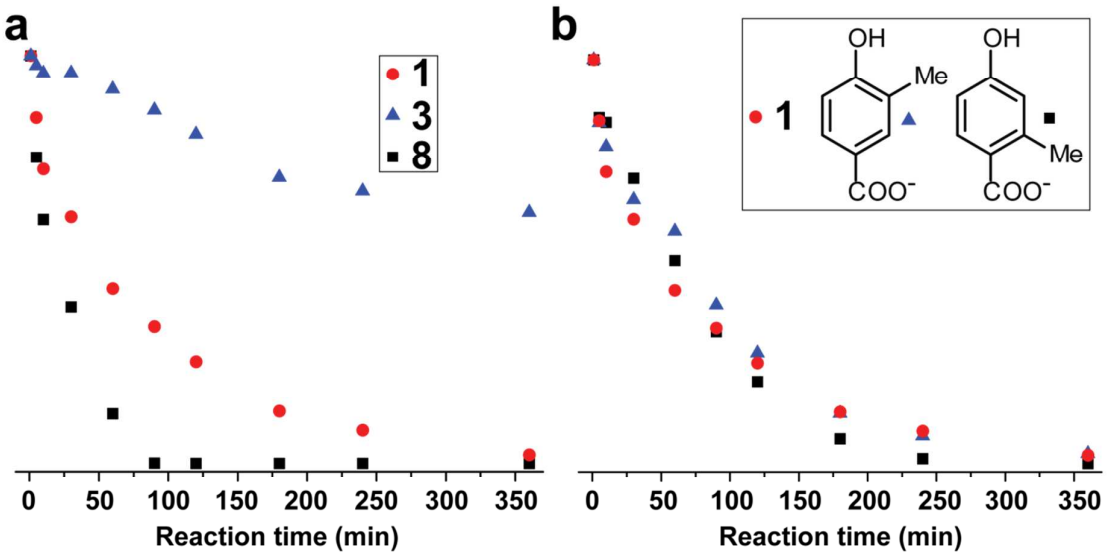
Supplementary Figure 8: ^{1}H NMR spectrum for synthetic standard for **6**.
^{1}H NMR (599 MHz, Methanol-d_4) δ 7.11 (s, 1H).



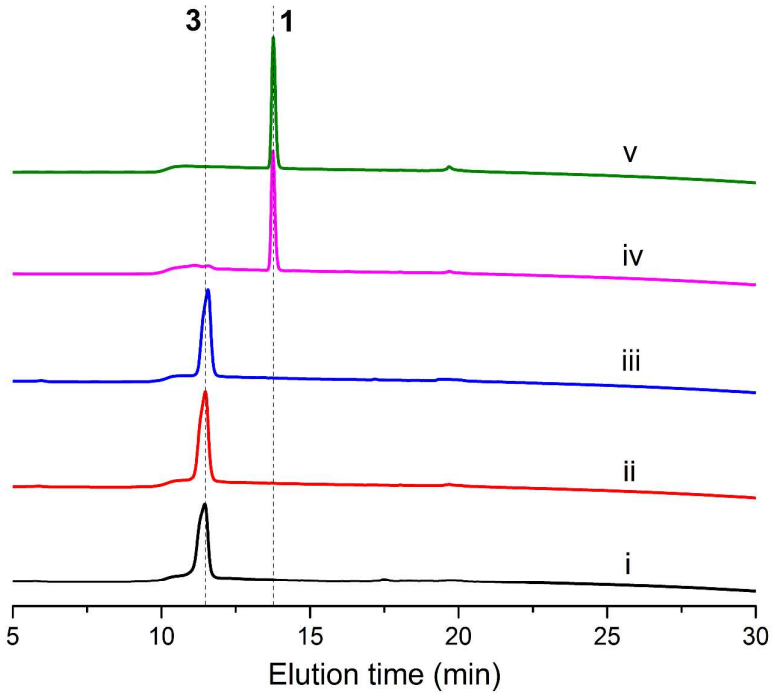
Supplementary Figure 9: ^{1}H NMR spectrum for synthetic standard for **7**.
^{1}H NMR (599 MHz, Methanol-d_4) δ 7.32 (s, 1H).
Note that the singlet at 7.11 ppm corresponds to **6** that could not be resolved from **7** during preparative HPLC. This is also shown in **Supplementary Figure 10**.



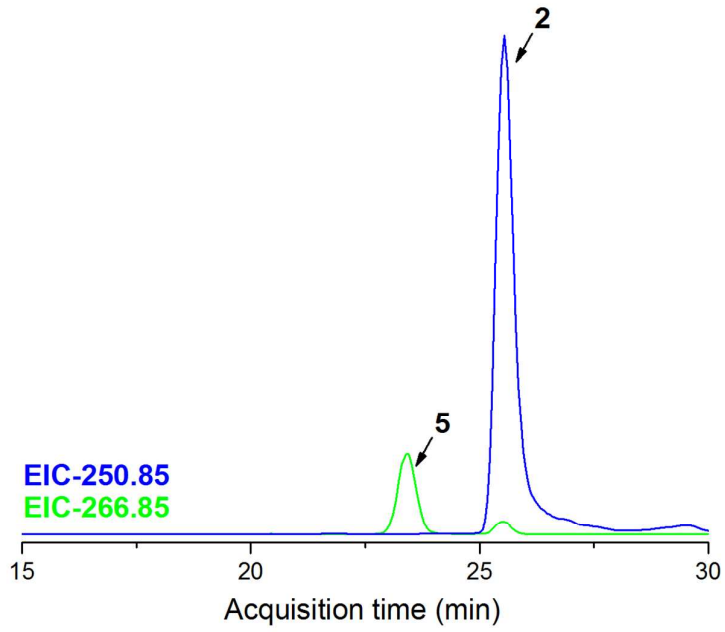
Supplementary Figure 10: Comparison of retention times for tribromocatechol products (**a**) generated by Bmp5 reaction with **5** against synthetic standards for (**b**) **6** and (**c**) **7**. All curves represent EICs for MS1 $[M-H]^-$ ions m/z=342.76. Note that the standard for **7** also possess a small amount **6** which also manifests itself as a singlet at 7.11 ppm in **Supplementary Figure 9**.



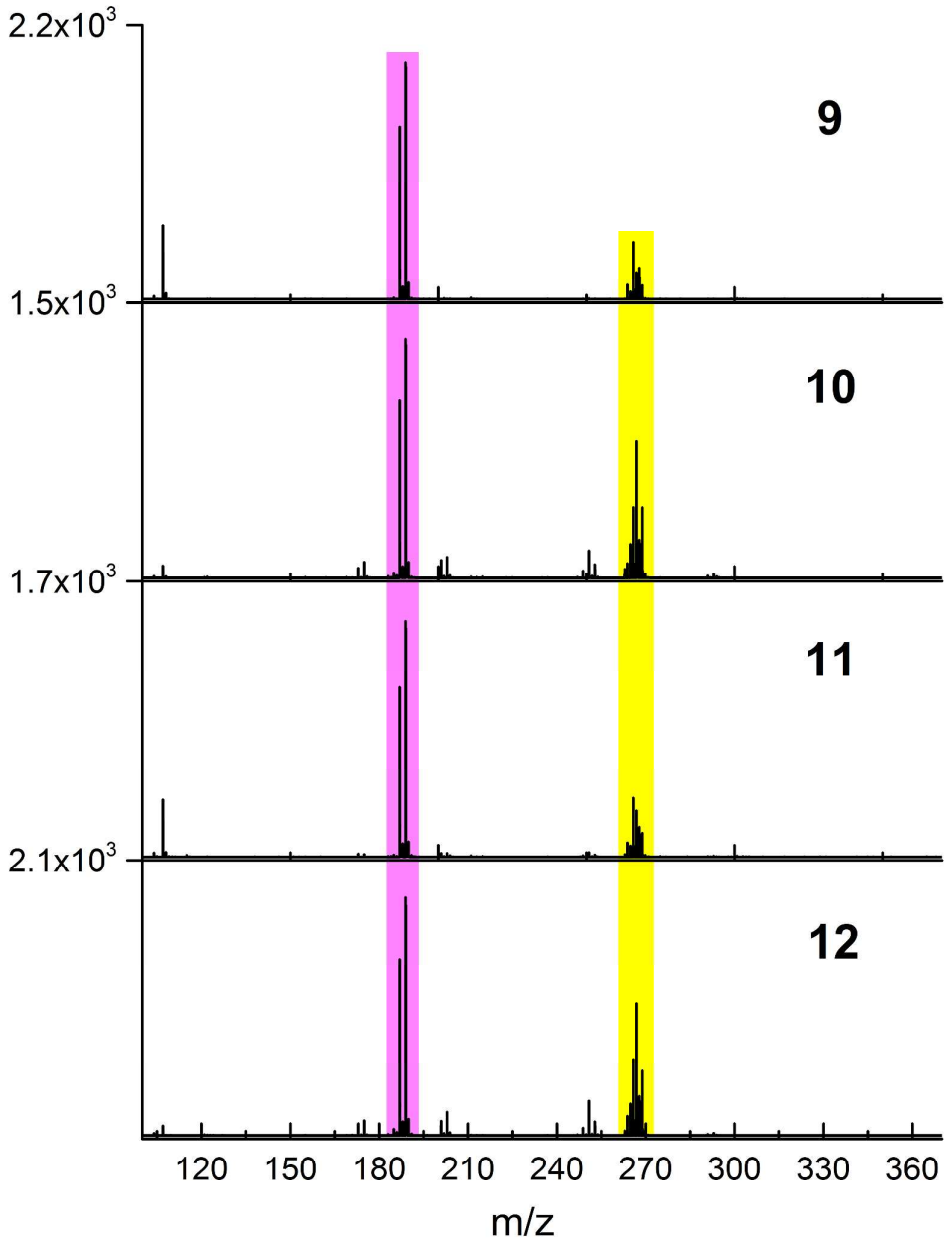
Supplementary Figure 11: Rate of substrate disappearance for (**a**) **1** (Sigma-Aldrich: 240141), **3** (Sigma-Aldrich: 37580) and **8** (Sigma-Aldrich: D109401), and (**b**) **1**, 3-methyl-4-hydroxybenzoic acid (Sigma-Aldrich: 659282) and 2-methyl-4-hydroxybenzoic acid (Sigma-Aldrich: 653160) upon reaction with Bmp5. Data points represents means value from three independent experiments. Each plot is individually normalized.



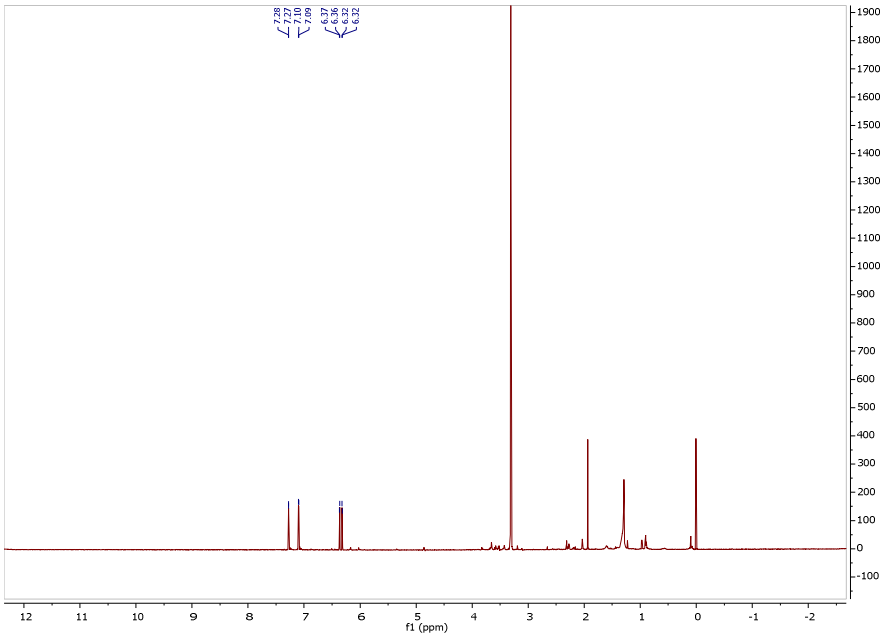
Supplementary Figure 12. Enzymatic assay for m3553 conducted and analyzed (**i**) in a manner identical to as shown in Figure 2c, with the addition of (**ii**) 7.5 units of catalase, or (**iii**) 10 units of superoxide dismutase showing conversion of substrate **1** to product **3**. Negative controls without the addition of (**iv**) enzyme or (**v**) NADPH show no conversion of **1** to **3**.



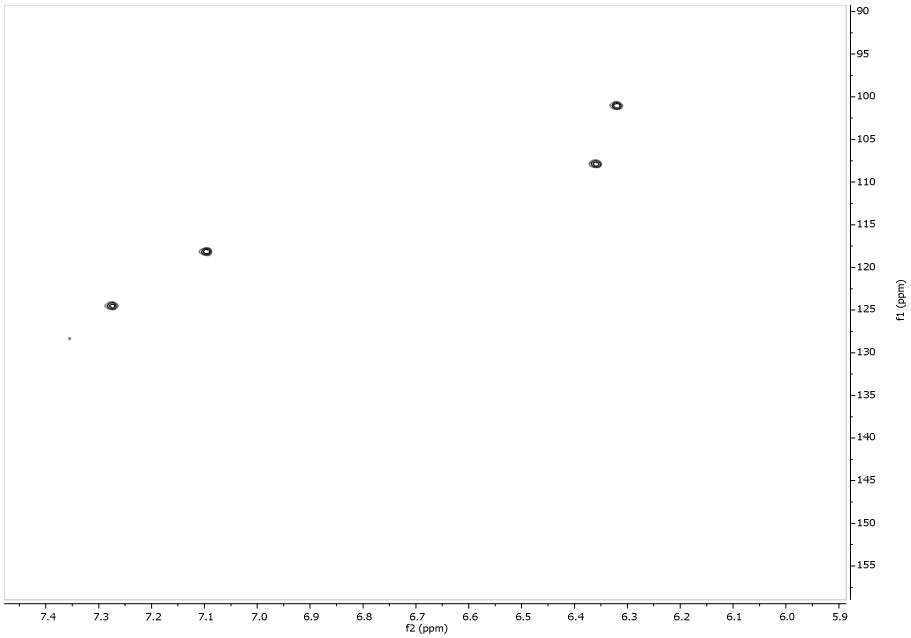
Supplementary Figure 13. Detection of **2** and **5** present in a methanol extract of Dysidea spp. sponge collected in Fiji. A 4 g wet weight sample of the sponge was frozen at -80 °C and lyophilized for 48 h. The sample was soaked in 5 mL MeOH for 4 h at room temperature. The organic solvent was collected by filtration, and the sponge was soaked in additional 5 mL MeOH for 48 h. The solvent was removed by filtration and combined with the previous fraction. 30 µL of the organic extract was directly injected to a C18 HPLC column and analyzed by LC-MS/MS using elution gradient I as described earlier. The EICs are generated in an identical manner as for **Figures 2d–2f.**



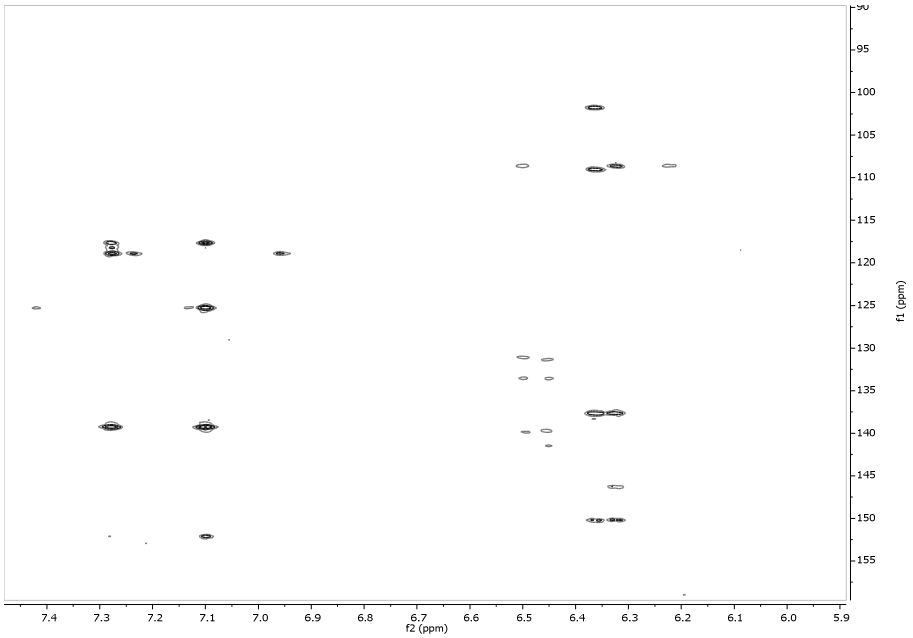
Supplementary Figure 14: MS2 profiles for **9–12**. Note that there are two major MS2 ions observed for each of the four species. The ion shaded in pink corresponds to monobromobenzenediol, while the one shaded yellow corresponds to dibromobenezediol. These ions are generated by scission of the ether linkage for OH-BDEs. Also note that polybrominated biphenyls are isomeric to OH-BDEs, but show successive losses of bromine atoms in a MS/MS experiment, thus differentiating the two classes of molecules.



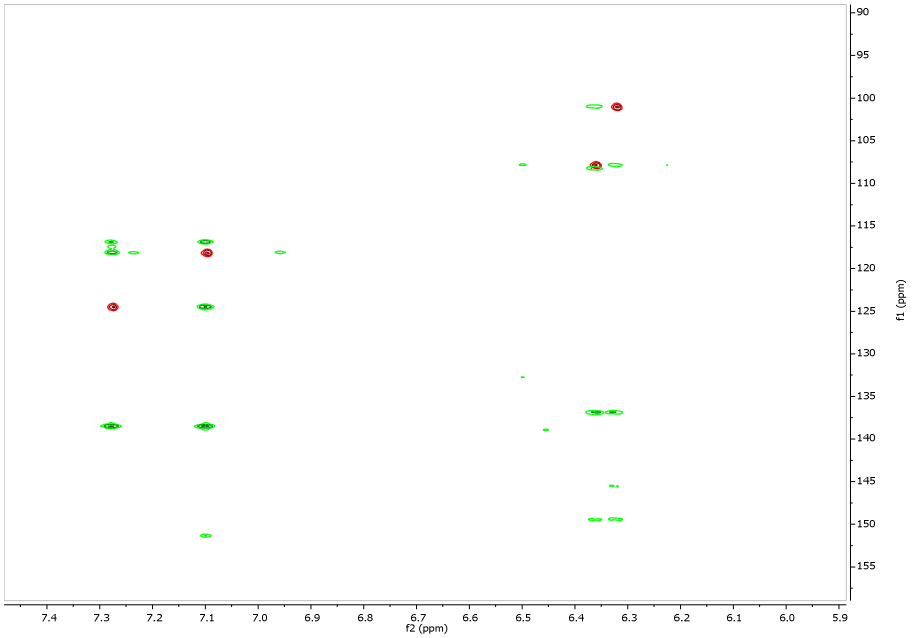
Supplementary Figure 15: ^{1}H NMR spectrum for **9**.
^{1}H NMR (600 MHz, Methanol-d_4) δ 7.28 (d, J = 2.3 Hz, 1H), 7.10 (d, J = 2.3 Hz, 1H), 6.37 (d, J = 2.8 Hz, 1H), 6.32 (d, J = 2.8 Hz, 1H). Note that the molecular formula for **9**, as predicted by MS is $C_{12}H_7Br_3O_4$, while only four distinct proton peaks can be observed by NMR. This implies that of the seven protons, three are likely exchangeable with the solvent and are hence not observed. This then leads to the proposal for three free hydroxyl moieties.



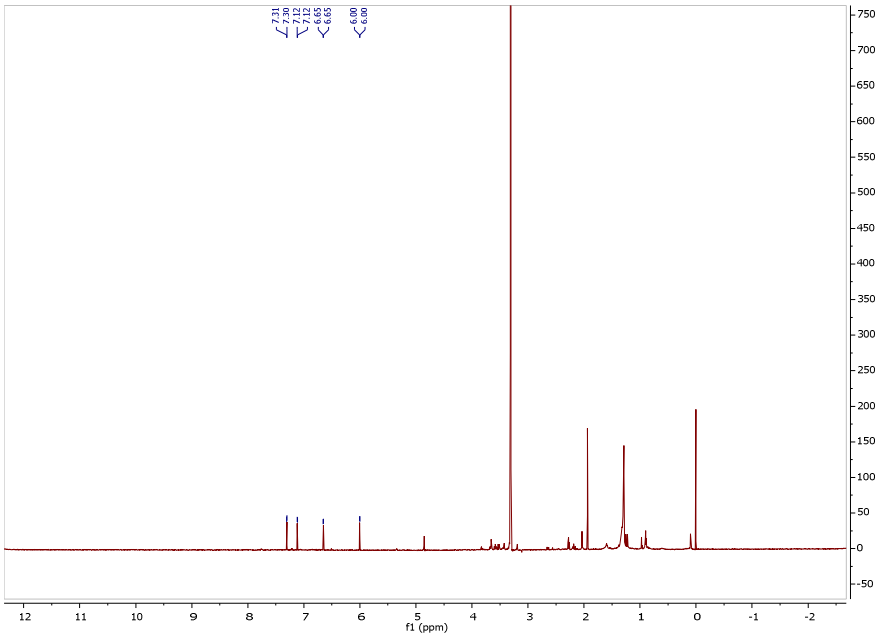
Supplementary Figure 16: HSQC spectrum for **9**.



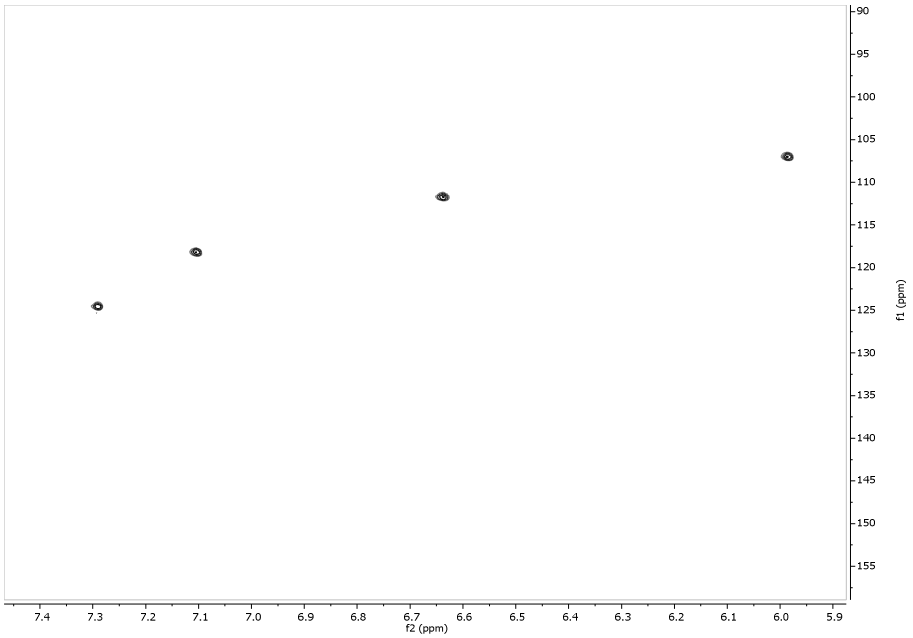
Supplementary Figure 17: HMBC spectrum for **9**.



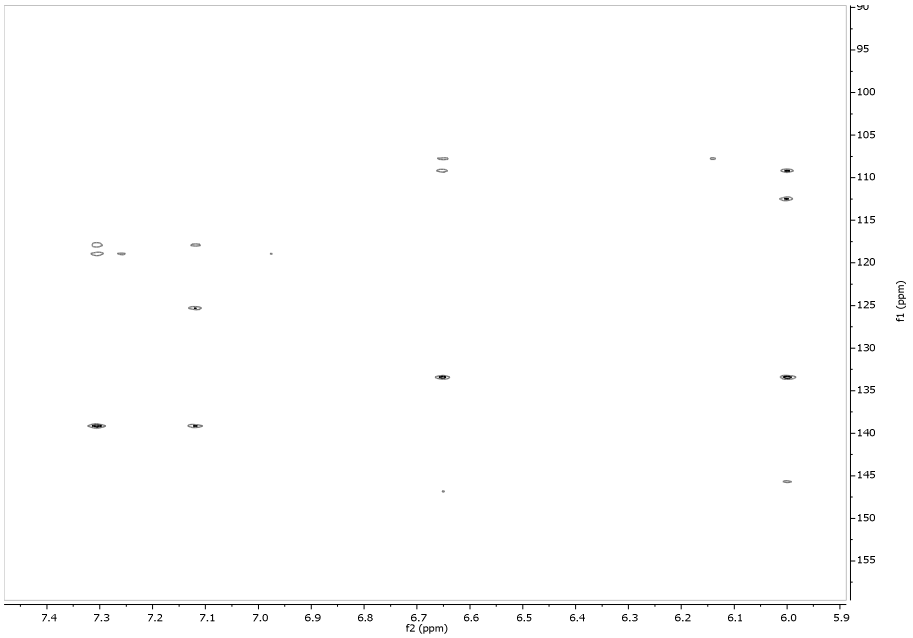
Supplementary Figure 18: Aligned HSQC (in red) and HMBC (in green) spectra for **9**.



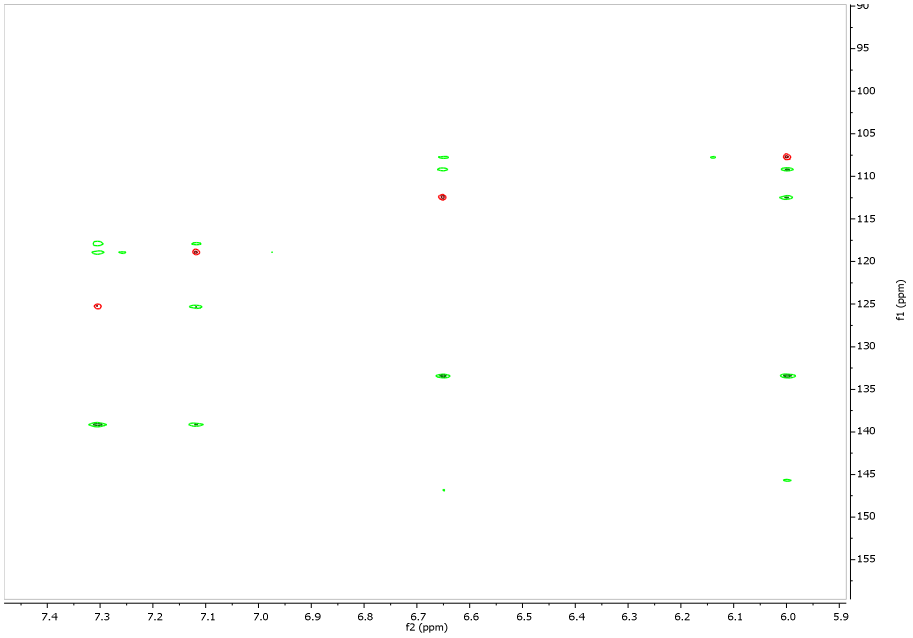
Supplementary Figure 19: ^{1}H NMR spectrum for **10**.
^{1}H NMR (600 MHz, Methanol-d_4) δ 7.30 (d, J = 2.2 Hz, 1H), 7.12 (d, J = 2.2 Hz, 1H), 6.65 (d, J = 2.2 Hz, 1H), 6.00 (d, J = 2.2 Hz, 1H).



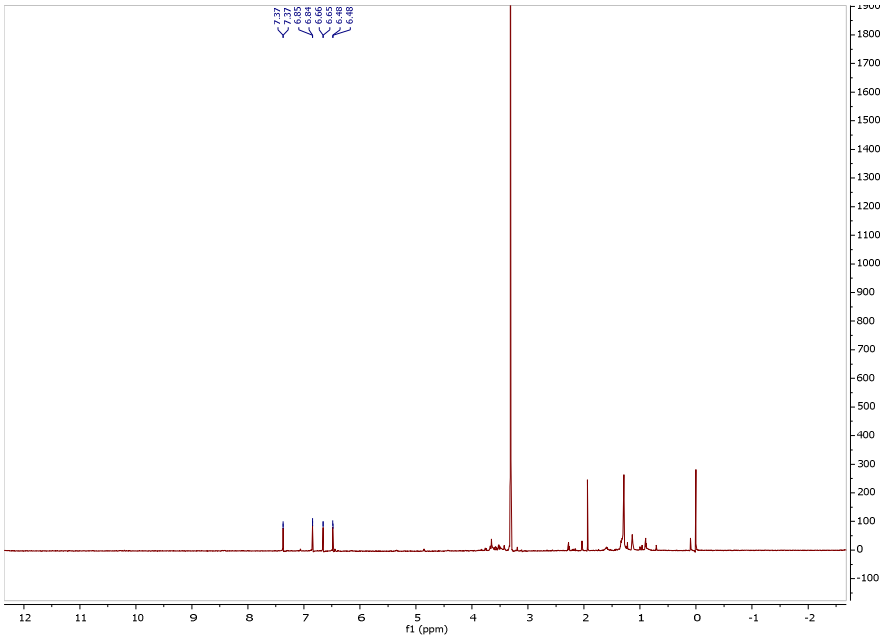
Supplementary Figure 20: HSQC spectrum for **10**.



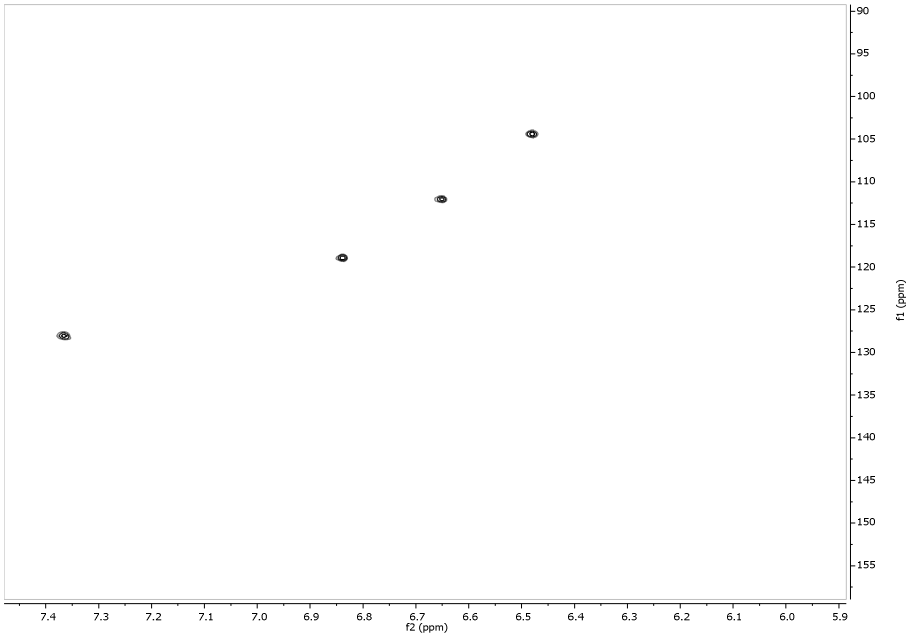
Supplementary Figure 21: HMBC spectrum for **10**.



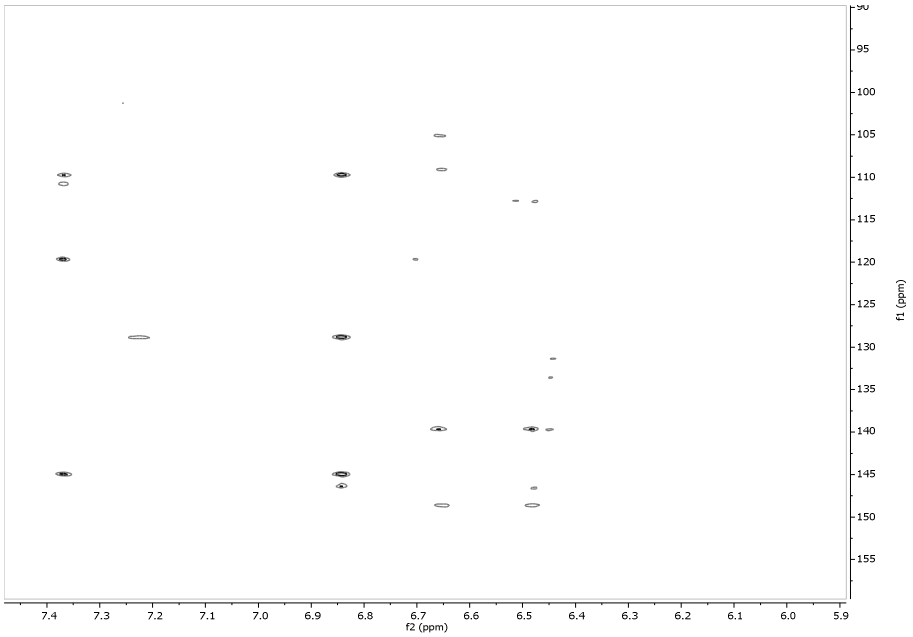
Supplementary Figure 22: Aligned HSQC (in red) and HMBC (in green) spectra for **10**.



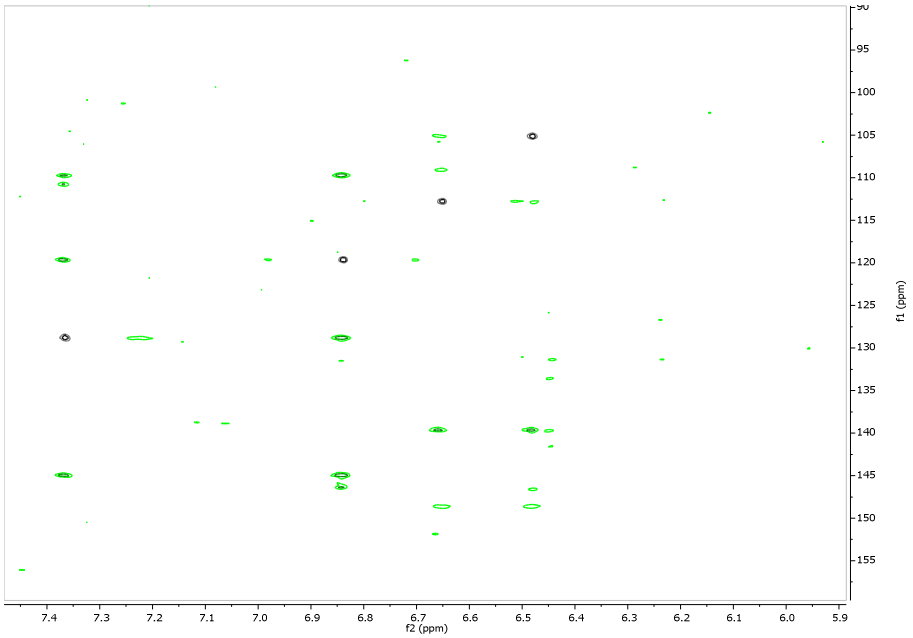
Supplementary Figure 23: ^{1}H NMR spectrum for **11**.
^{1}H NMR (600 MHz, Methanol-d_4) δ 7.37 (d, J = 2.3 Hz, 1H), 6.84 (d, J = 2.2 Hz, 1H), 6.66 (d, J = 2.8 Hz, 1H), 6.48 (d, J = 2.8 Hz, 1H).



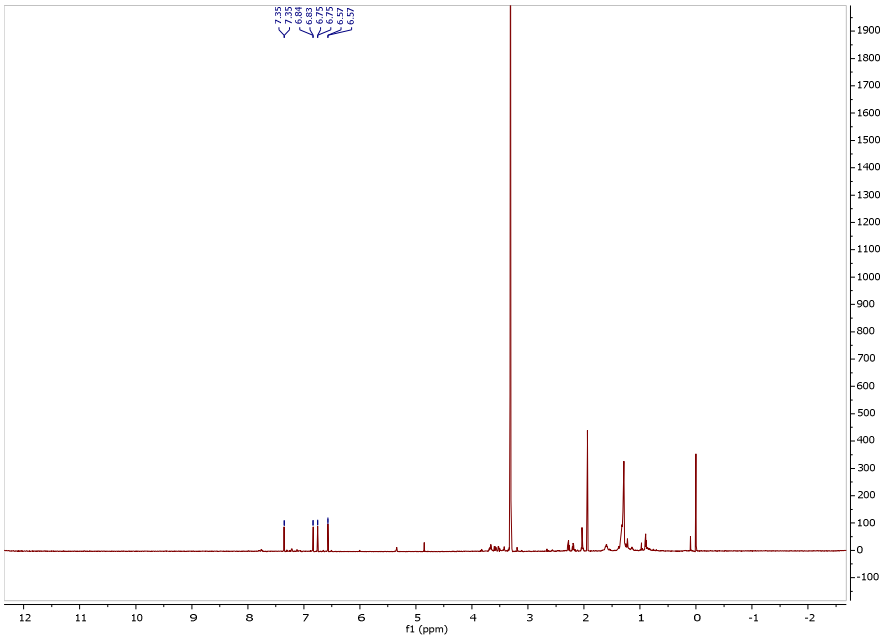
Supplementary Figure 24: HSQC spectrum for **11**.



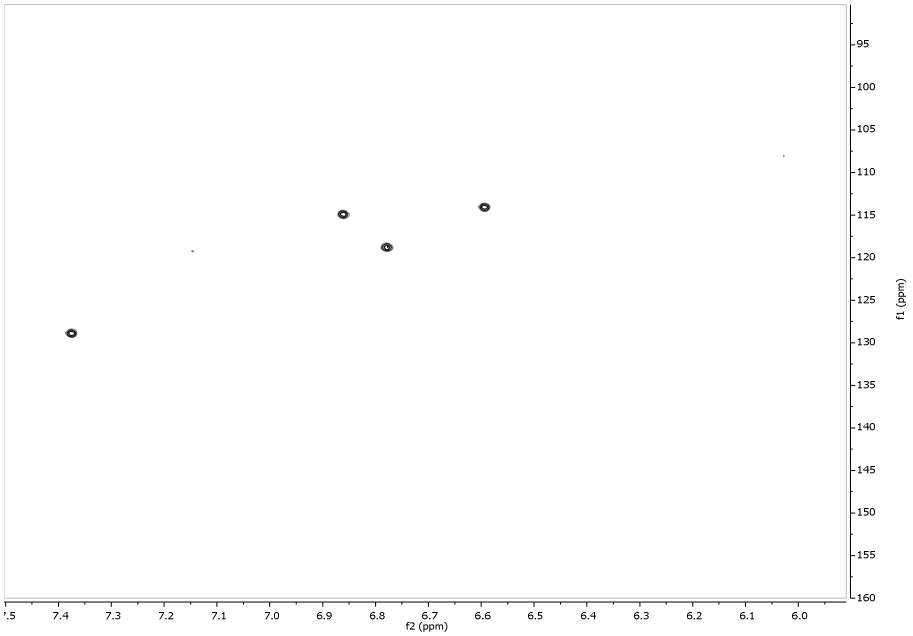
Supplementary Figure 25: HMBC spectrum for **11**.



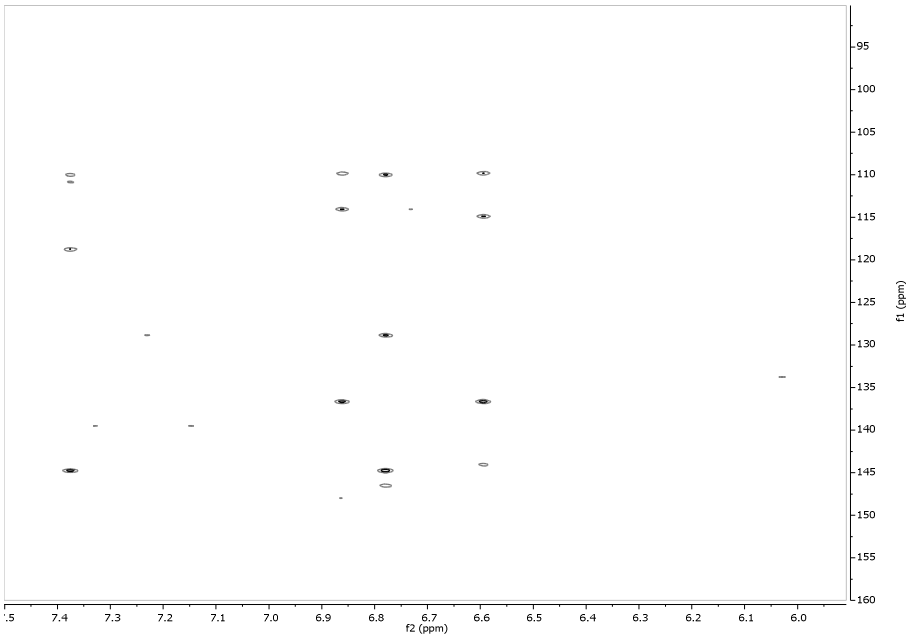
Supplementary Figure 26: Aligned HSQC (in black) and HMBC (in green) spectra for **11**.



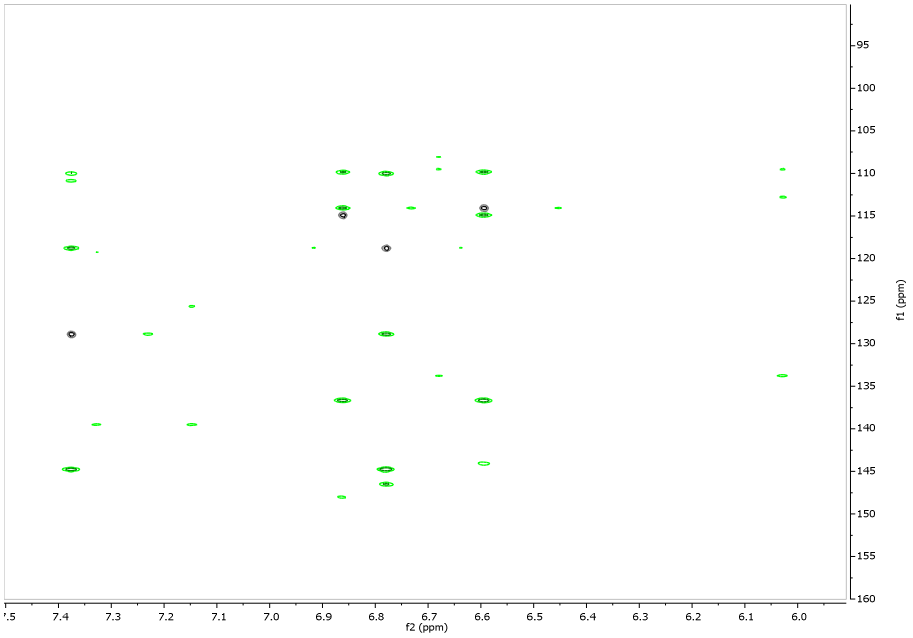
Supplementary Figure 27: ^{1}H NMR spectrum for **12**.
^{1}H NMR (600 MHz, Methanol-d_4) δ 7.35 (d, J = 2.3 Hz, 1H), 6.83 (d, J = 2.2 Hz, 1H), 6.75 (d, J = 2.2 Hz, 1H), 6.57 (d, J = 2.3 Hz, 1H).



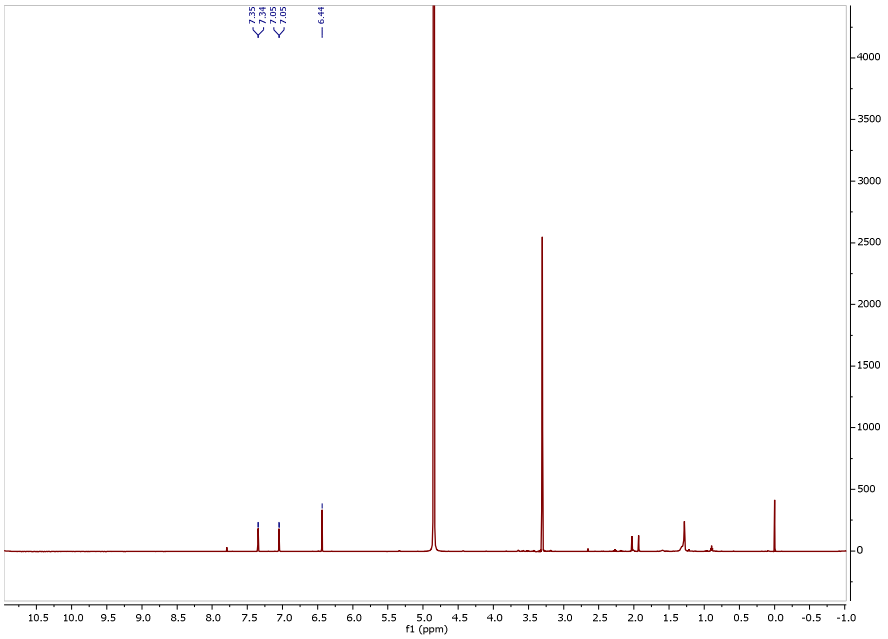
Supplementary Figure 28: HSQC spectrum for **12**.



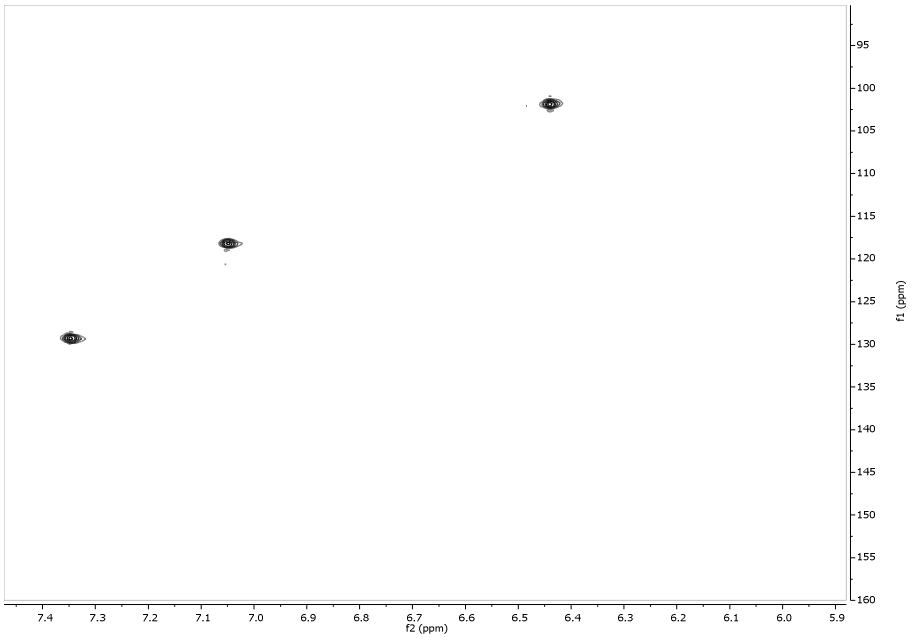
Supplementary Figure 29: HMBC spectrum for **12**.



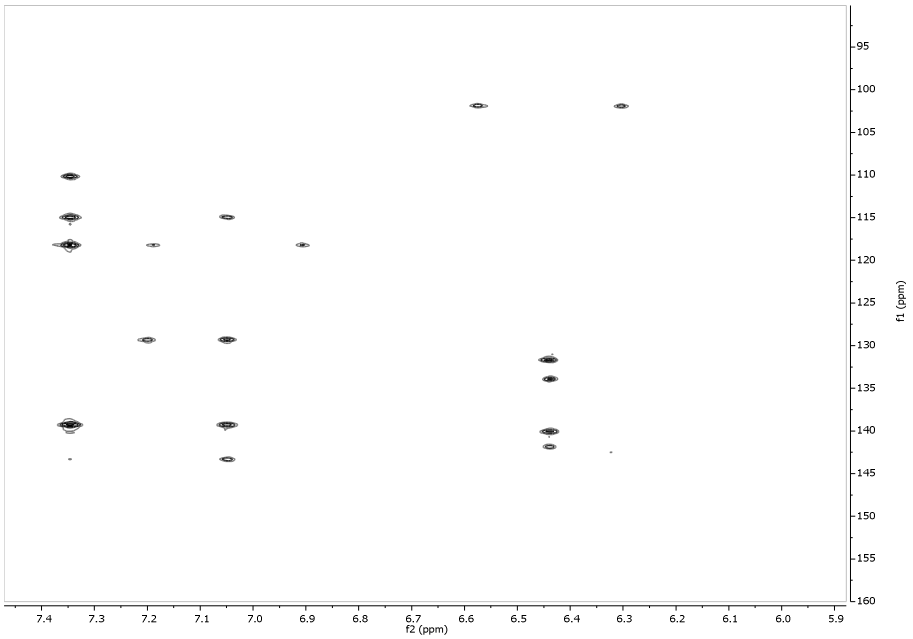
Supplementary Figure 30: Aligned HSQC (in black) and HMBC (in green) spectra for **12**.



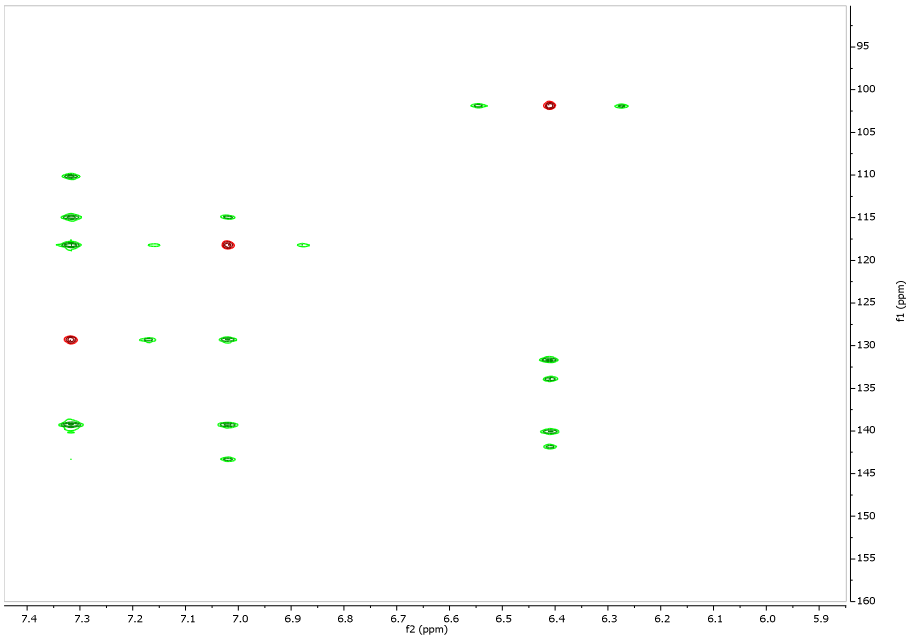
Supplementary Figure 31: ^{1}H NMR spectrum for **13**.
^{1}H NMR (599 MHz, Methanol-d_4) δ 7.35 (d, J = 2.2 Hz, 1H), 7.05 (d, J = 2.2 Hz, 1H), 6.44 (s, 1H). Note that the molecular formula for **13**, as predicted by MS is $C_{12}H_5Br_3O_4$, while only three distinct proton peaks can be observed by NMR. This implies that of the five protons, two are likely exchangeable with the solvent and are hence not observed. This then leads to the proposal for two free hydroxyl moieties. This is also supported by TMS derivatization of **13–14** and GC-MS analyses as described in **Supplementary Figure 40**.



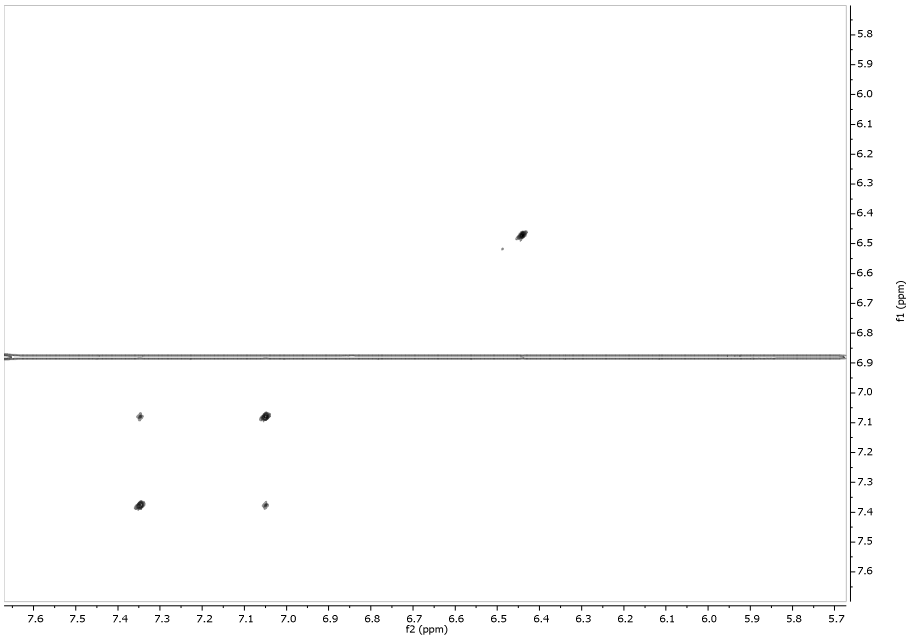
Supplementary Figure 32: HSQC spectrum for **13**.



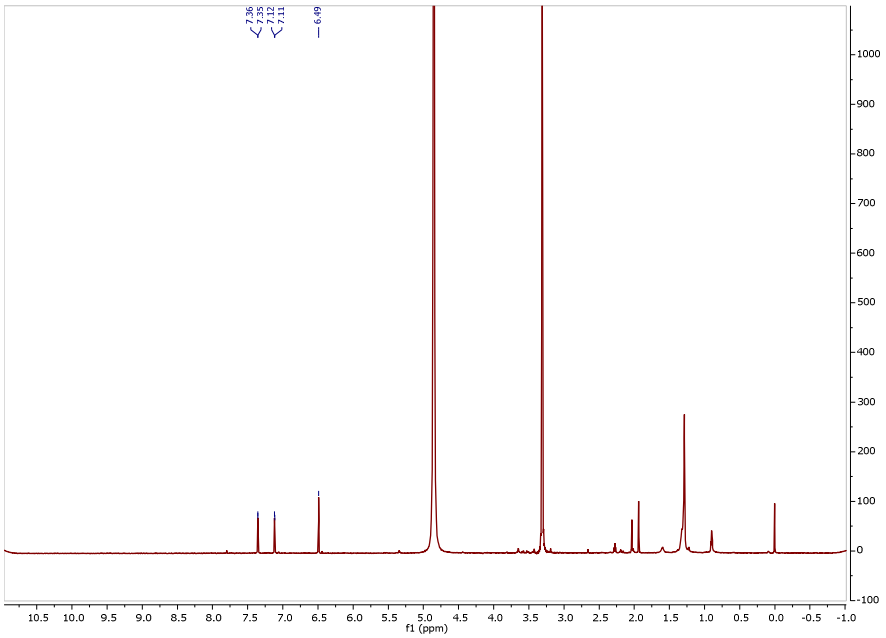
Supplementary Figure 33: HMBC spectrum for **13**.



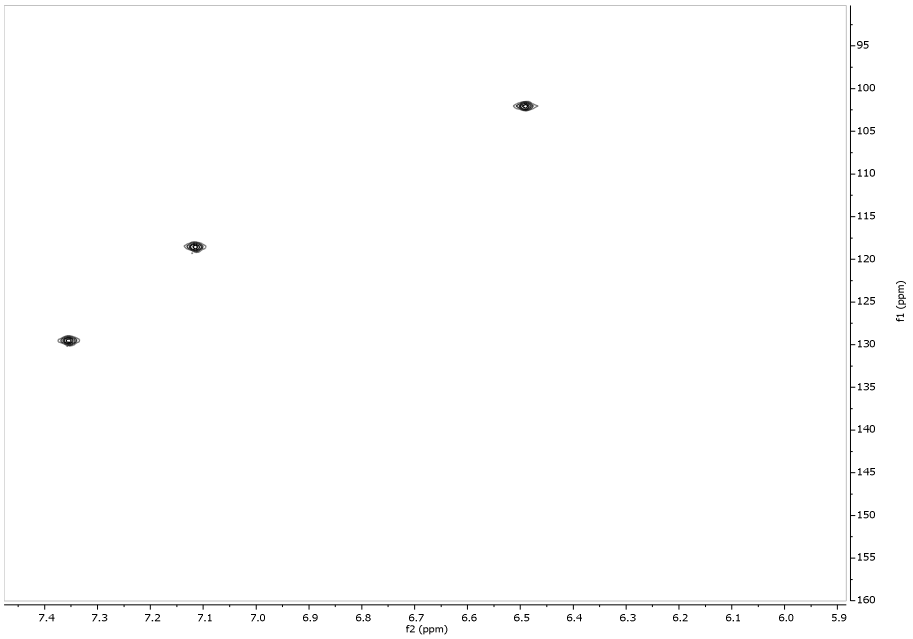
Supplementary Figure 34: Aligned HSQC (in red) and HMBC (in green) spectra for **13**.



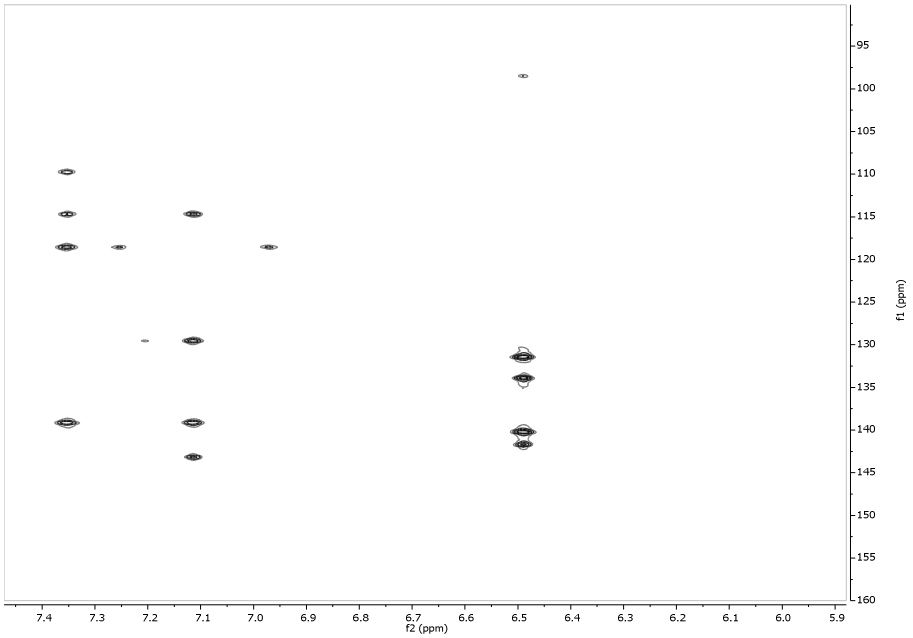
Supplementary Figure 35: 2D-NOESY spectrum for **13**.



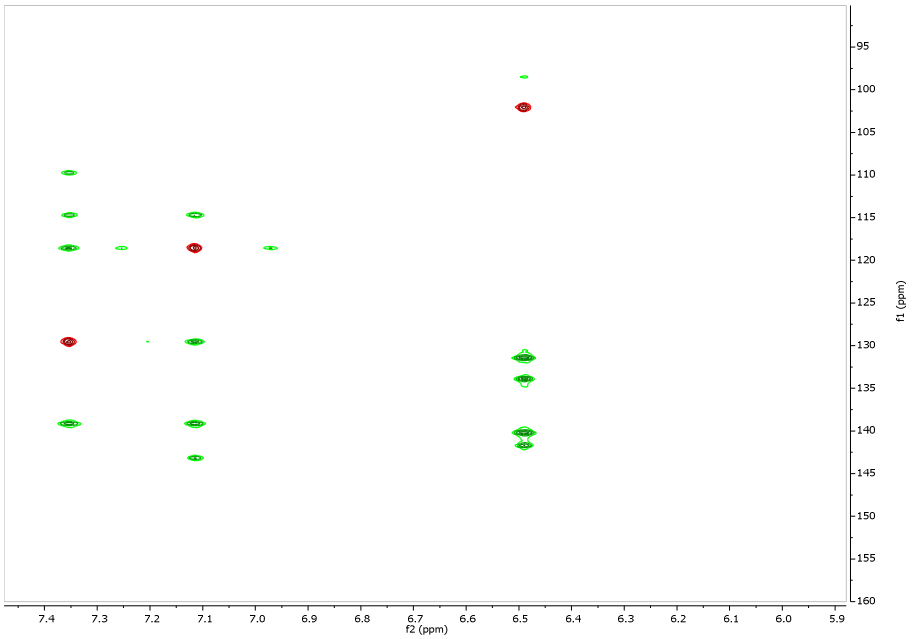
Supplementary Figure 36: HMBC spectrum for **14**.
^{1}H NMR (599 MHz, Methanol-d_4) δ 7.35 (d, J = 2.1 Hz, 1H), 7.12 (d, J = 2.1 Hz, 1H), 6.49 (s, 1H).



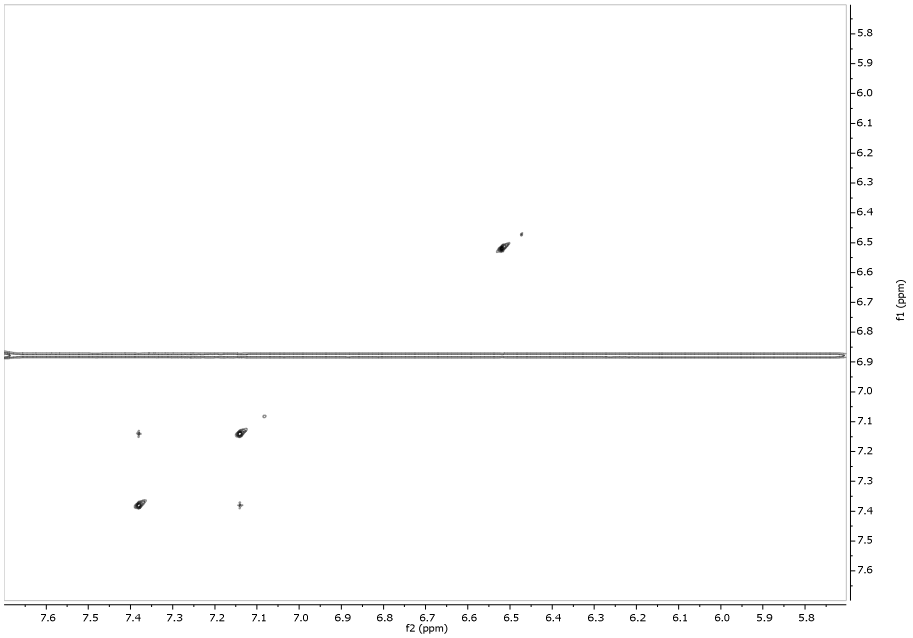
Supplementary Figure 37: HSQC spectrum for **14**.



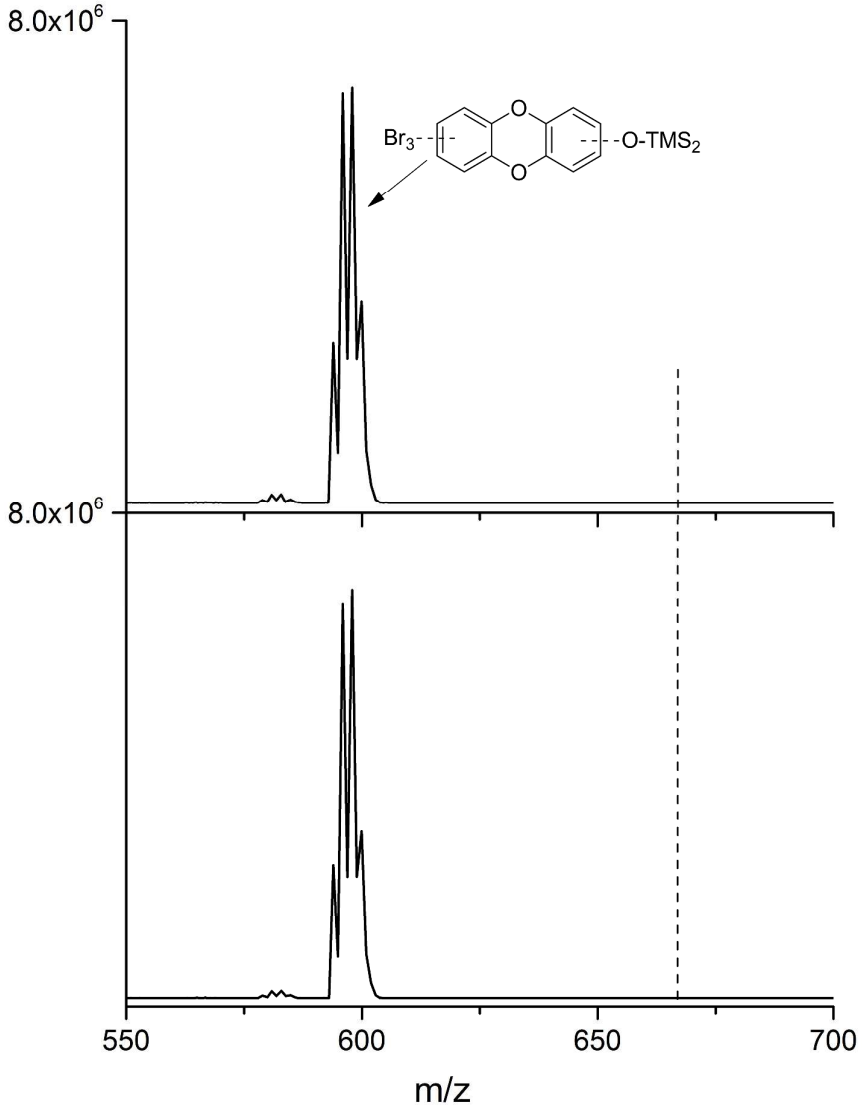
Supplementary Figure 38: HMBC spectrum for **14**.



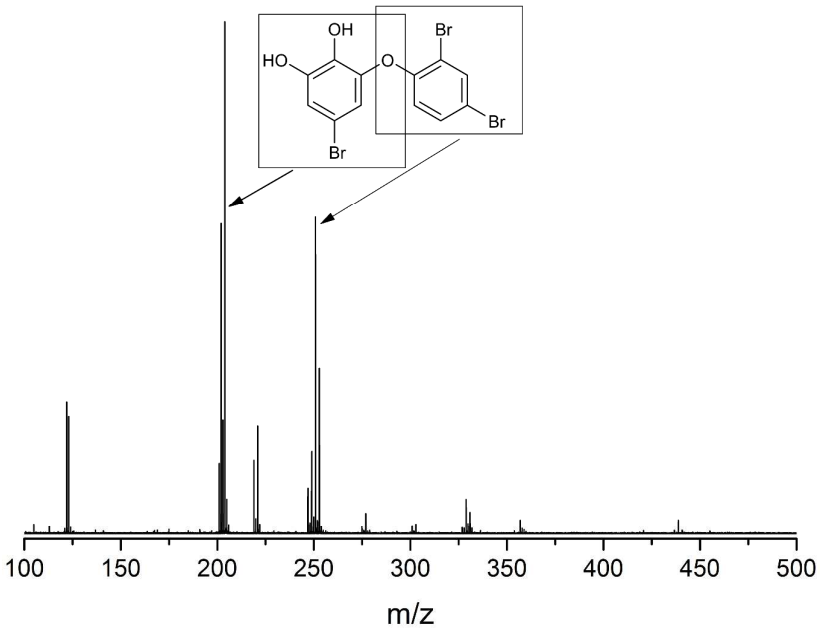
Supplementary Figure 39: Aligned HSQC (in red) and HMBC spectra (in green) for **14**.



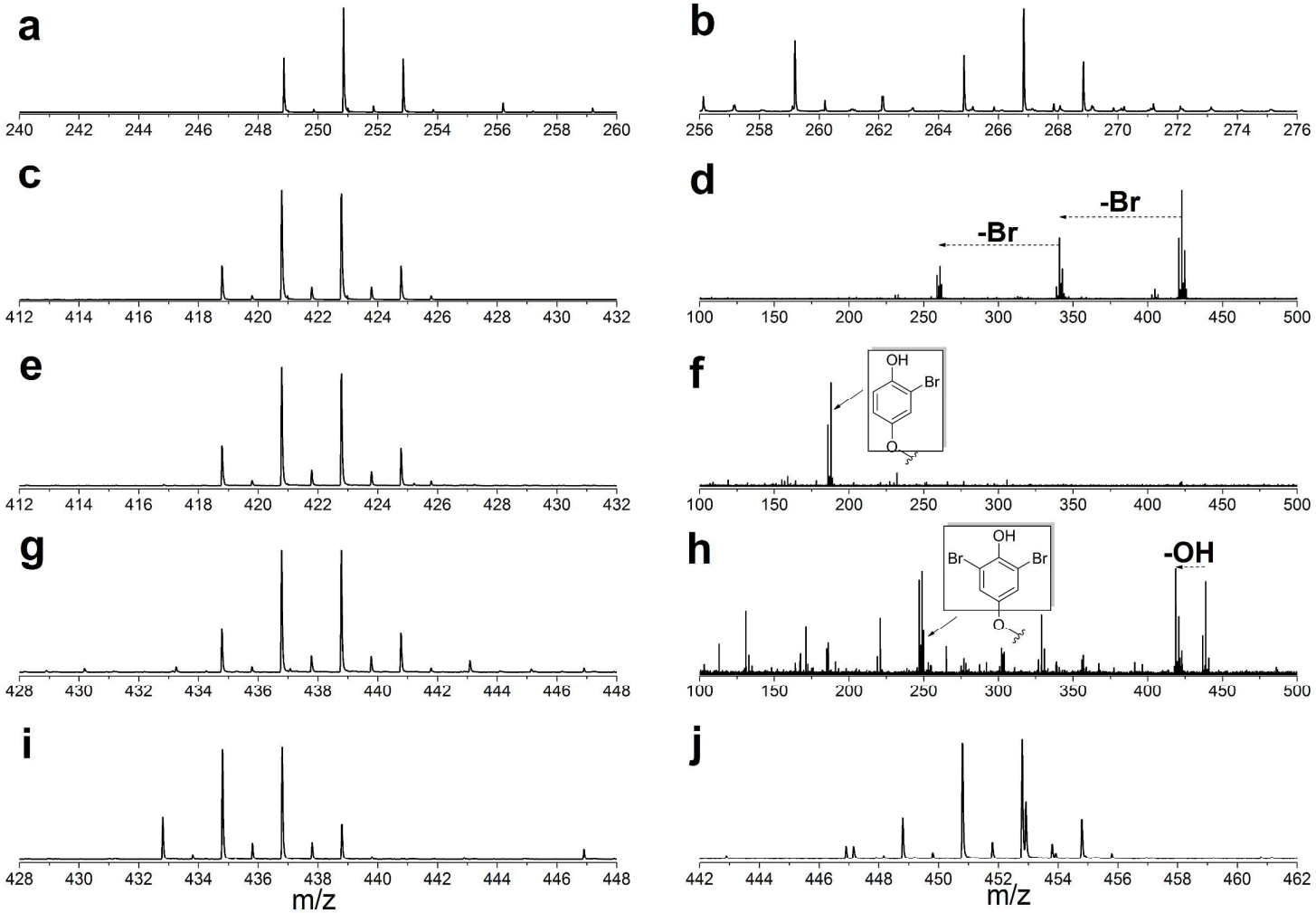
Supplementary Figure 40: 2D-NOESY spectrum for **14**.



Supplementary Figure 41: MS spectra obtained after TMS derivatization and GC-MS analysis for **13** (top) and **14** (below). Preparatively isolated molecules were dissolved in DMSO and an excess of N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) (Sigma-Aldrich: 33027) was added. The reaction was diluted with MeCN and directly analyzed by GC-MS without further purification. The cluster of molecular ions shown above correspond to two TMS adducts for the molecular formula $C_{12}H_5Br_3O_4$. Note that three TMS adducts (theoretical m/z position on the plot denoted by dashed line) were not observed.



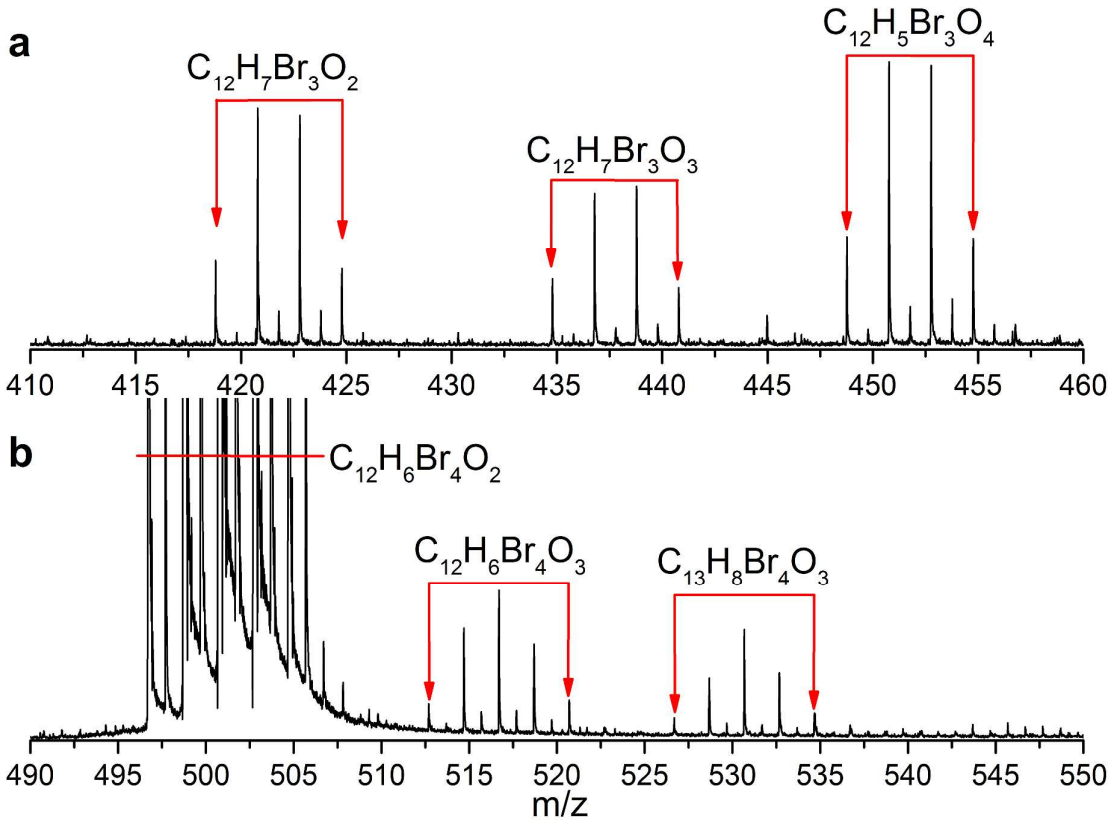
Supplementary Figure 42: MS2 spectra corresponding to the species denoted by ■ in **Figure 4b**. The two major MS2 ions can be annotated as dibromophenol product ion, and a trihydroxylated monobromobenzene product ion. A likely deduced structure of the di-OH-BDE that corresponds to these two product ions is shown with the product ions boxed.



Supplementary Figure 43: LC-MS/MS analysis for an extract of *E. coli* culture coexpressing Bmp5, Bmp7 and *m3553* grown in the presence of exogenous **1**. MS1 plots showing the characteristic isotopic distribution, which along with retention time relate to the presence of (**a**) **2** and (**b**) **5** in the culture extract.

(**Supplementary Figure 43** continued)

In addition to **5**, the production of **4** could also be observed. (**c**) MS1 and (**d**) MS2 plots showing the presence of a tribrominated biphenyl molecule pertaining to the chemical formula $C_{12}H_7Br_3O_2$. (**e**) MS1 and (**f**) MS2 plots showing the presence of a tribrominated OH-BDE pertaining to the chemical formula $C_{12}H_7Br_3O_2$. Based on their MS1 spectra (panels **c** and **e)**, biphenyls and OH-BDEs are indistinguishable as they are isomeric. However, they can be distinguished on the basis of their MS2 spectra. Biphenyls show successive loss of bromine atoms (panel **d**), while OH-BDEs demonstrate scission of the ether linkage to generate, as shown in panel **e**, a characteristic monobromobenzenediol MS2 ion (boxed).[1] (**g**) MS1 and (**h**) MS2 plots showing the presence of a tribrominated di-OH-BDE pertaining to the chemical formula $C_{12}H_7Br_3O_3$. This species corresponds to ■ as shown in **Figure 4**. Assignment as a di-OH-BDE is supported by the MS2 spectra (panel **h**) that shows loss of hydroxyl, as well as a characteristic dibromobenzenediol MS2 ion (boxed). MS1 plots showing the presence of (**i**) mono- and (**j**) di-hydroxylated dibenzo-*p*-dioxins that respectively correspond to species denoted by ● and ♦ as shown in the main text. Note the characteristic loss of two protons between panels **g** and **i** that is analogous to the *in vitro* observation described in **Figure 4**.



Supplementary Figure 44: Metabolomic analysis of *Dysidea* spp. sponge extract reveals the concomitant presence of polybrominated molecules likely derived from bromophenols and bromocatechols. The sponge extract, generated as described in **Supplementary Figure 12** was analyzed by LC-MS/MS using elution gradient II. Numerous polybrominated phenolic molecules could be identified, guided in part by the characteristic isotopic distribution of polybrominated species. Six of these species, along with their molecular formulae as determined by high resolution mass spectrometry are shown above. (**a**) Three tribrominated molecules that differ in the number of oxygen atoms and thus likely originate via differential homo- and hetero- coupling of bromophenols and bromocatechols elute together. Molecular formula $C_{12}H_7Br_3O_2$ corresponds to tribrominated OH-BDE species that have never before been described from *Dysidea*, but has instead been isolated from marine algae,[5] and shown to be produced by bromophenol-bromophenol coupling by marine bacteria.[1] Molecular formula $C_{12}H_7Br_3O_3$ corresponds to a di-OH-BDE generated by bromophenol-bromocatechol coupling, and corresponds to the species denoted by ▪ **Figure 4**. Note that this species has also never been described from marine sponges before. Molecular formula $C_{12}H_5Br_3O_4$ corresponds to the species denoted by ♦ **Figure 3**, and has never been isolated from sponges before. As shown in **Figure 3**, this species likely originates via bromocatechol-bromocatechol coupling. (**b**) Three tetrabrominated molecules that differ in the number of oxygen atoms, and by methylation of di-OH-BDEs. Molecular formula $C_{12}H_6Br_4O_2$ corresponds to an OH-BDE. Judging by the very high amounts of this molecule present within the sponge, prior literature[6,7-8] leads to a postulate of its identity as 2'OH-BDE-68, a molecule shown to be generated by bromophenol-bromophenol coupling.[1] Note that the MS1 spectrum of this molecule has been truncated for illustrative purposes to allow for demonstration of spectra related to other species concomitantly eluting in the LC-MS/MS run. Molecular formula $C_{12}H_6Br_4O_3$ corresponds to a di-OH-BDE, likely generated via bromophenol-bromocatechol coupling. di-OH-BDEs pertaining to this formula have been described from *Dysidea* sponges previously.[7,9] Molecular formula $C_{13}H_8Br_4O_3$ corresponds to

a di-OH-BDE in which one of the hydroxyl oxygen atoms has been methylated. Again, there is literature precedence for the presence of methylated di-OH-BDEs corresponding to this molecular formula from *Dysidea* spp. sponges.[6,9]

Thus, marine invertebrates such as *Dysidea* spp. sponges concomitantly harbor polybrominated molecules that are derived from both bromophenol and bromocatechol initiators. The complexity of the sponge extract, and the non-equitable amounts of different polybrominated molecules present makes comprehensive structural elucidation of all different species an analytical challenge. This has likely masked the description of several low abundance species in literature.

Supplementary references

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