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# 3-Hydroxylation of the polycyclic tetramate macrolactam in the biosynthesis of antifungal HSAF from *Lysobacter enzymogenes* C3†‡

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## Materials and Methods

*Bacterial strains, plasmids, and general DNA manipulations. Escherichia coli* strain DH5α was used as the host for general DNA propagations. *E. coli* BL21 (DE3) was used as the heterologous host for the expression of enzymes. *L. enzymogenes* strain C3 and other bacterial strains were grown in Luria-Bertani (LB) medium. All oligonucleotide primers for PCR were synthesized by Integrated DNA Technologies (IDT, Coralville, IA). Plasmid preparation and DNA gel extraction were carried out with Qiagen kits (Valencia, CA), and all other DNA manipulations were carried out according to standard methods.

*HPLC analysis of metabolites from strain C3 and mutants*. Strain C3 and individual mutant strains were cultivated on LB agar plates (supplemented with 15 g maltose for HSAFs production) at 30 ºC for 4

d. The culture was diced and extracted with EtOAc-MeOH-AcOH (80 : 15 : 5, v/v/v) at room temperature overnight. The organic solution was collected through filtration and the remaining agar residue was extracted exhaustively with the same solvent until the filtrate was colorless. The combined filtrate, upon evaporation, yielded a crude brown syrupy extract. To analyze the metabolites, the extract was dissolved in methanol and analyzed by high-pressure liquid chromatography (HPLC). The HPLC system was 1260 infinity, from Agilent Technologies with a column of Agilent ZORBAX SB-C18 (4.6×250 mm, 5 µm). The mobile phases were water (phase A) and acetonitrile (phase B) containing 0.005 M TFA, with a gradient of 5% to 40% B in A in the first 10 min, increased to 60% at 15 min, 60% B from 15 to 20 min, to 100% at 22 min, 100% B from 22 to 24 min, back to 5% B at 26 min, and 5% B from 26 to 30 min. The flow rate was 1 ml/min. The peaks were detected at 318 nm on a UV-visible detector.

*Isolation of HSAF and 3-deOH-HSAF and MS and NMR analysis.* To isolate HSAF and 3-deOH-HSAF, the crude extract from 3 liters of solid culture (LB agar plates) of strain C3 was partitioned between water and EtOAc (1: 1) until the EtOAc layer was colorless. The combined organic layer was concentrated under vacuo to obtain EtOAc extract (2 g). The extract was subjected to MPLC (RP-18, 30 g), with gradient aq. MeOH (60%, 80%, and 100%, respectively, 600 ml each) to obtain three fractions Fr.a – Fr.c. Fr.b was concentrated and further purified by using a preparative HPLC column (Agilent ZORBAX Eclipse XDB-C18, 9.0×250 mm, 5 µm), with the same solvent systems described above. The peaks with retention times of 18.7 and 21.1 min were collected and dried to obtain HSAF (20 mg) and 3-deOH-HSAF (9.3 mg), respectively. To analyze the chemical structure of 3-deOH-HSAF, HR-Q-TOF-MS data was acquired by using Agilent Q-TOF 6520 mass spectrometer. NMR spectra ($^{1}H$ and $^{13}C$ NMR and HSQC) were recorded on a Bruker DRX-500 spectrometer, at 600/150 MHz, respectively, in DMSO-$d_6$, in ppm relative to Me4Si.

*Construction of plasmids and expression of SD and FNR in E. coli.* The sterol desaturase (SD) gene was amplified by PCR using a subclone, pGEM-3ZF-3.0Kb, as template and Taq DNA polymerase as the enzyme. The forward primer was 5′- CGC GGA TCC AAC GGA TTA TCA GAG CAC-3′ (*Bam*HI underlined) and the reverse primer was 5′- TTT AAG CTT TCA GGG CGG CGC GCG GTC-3′ (*Hin*dIII underlined). The 1062 bp PCR fragment was cloned into pGEM-3ZF and its correctness was confirmed by DNA sequencing. SD was then transferred into pET28a (Novagen) for expression in *E. coli* BL21(DE3). The expected molecular weight of SD was ~41 kDa (41394 Da). To co-express the ferredoxin reductase (FNR) gene, the FNR was amplified by PCR using *L. enzymogenes* strain C3 genomic DNA as template and Taq DNA polymerase as the enzyme. The forward primer was 5′-ATA TCC ATG GCC CAG TTG CGC ACC GAA CGC-3′ (*Nco*I underlined) and the reverse primer was 5′-GGA AGA TCT CTT CTC GAC GAA GGC CCG-3′ (*Bgl*II underlined). The 768 bp PCR fragment was cloned into pANT841 and its correctness was confirmed by DNA sequencing. FNR was transferred into pQE-60 (Qiagen) and transformed into *E. coli* SG13009 that contains a pREP4 plasmid for FNR expression. The expected molecular weight of FNR is ~30 kDa (30333 Da). After confirming FNR expression, the construct was then transferred into *E. coli* BL21(DE3) that already contained the SD expression construct. To express the proteins, a single colony was inoculated into LB containing 25 µg/ml kanamycin and 100 µg/ml ampicillin, and the culture was grown at 37 ºC, shaken at 250 rpm. When the optical density ($A_{600}$) of the culture reached 0.4 – 0.6, the protein expression was induced with 1 mM IPTG. The cells were allowed to further grow for 15 h at a lower temperature (16 ºC). Finally, the cells were harvested by centrifugation at 4 ºC and resuspended in 20 mM PBS, pH 7.5, containing 500 mM NaCl, followed by sonication and centrifugation. The presence of SD and FNR was monitored by analyzing the cell extracts by SDS-PAGE.

*Precursor feeding experiments*. One microliter of 3-deOH-HSAF stock solution (2 mM, in DMSO) was added to 100 μl of freshly prepared cells (pET28a+pQE60, pET28a/SD, or pET28a/SD+pQE60/FNR), which had grown in the induction medium at 16 ºC, 250 rpm, for 15 h. The cultures were allowed to grow at 30 ºC for another 3 h and quenched by addition of 100 μl methanol. After centrifugation at 12,000 g for 5 min, a fraction of the supernatant (80 μl) of each of the samples was injected into the HPLC system for analysis.

*In vitro activity assay*. The *E. coli* cells containing empty vectors or the expression constructs were cultured and collected in the same way as described above. After sonication, the supernatant of the cells, in 20 mM PBS, pH 7.5, containing 500 mM NaCl, was used as the enzyme source for *in vitro* activity assay. The reaction mixture contains the enzymes (100 μl, 20 μg/ml of SD, 5 μg/ml of FNR), 3-deOH-HSAF (1 μl, 2 mM, in DMSO) and NADPH (1 μl, 100 mM). All reactions were performed at 30 ºC for 3 h and then quenched by addition of 100 μl methanol. After centrifugation at 12,000 g for 5 min, a fraction of the supernatant (80 μl) of each of the reactions was injected into the HPLC system for analysis. Reactions with boiled enzymes were also conducted in parallel.

Fig. S1 (Li et al.)

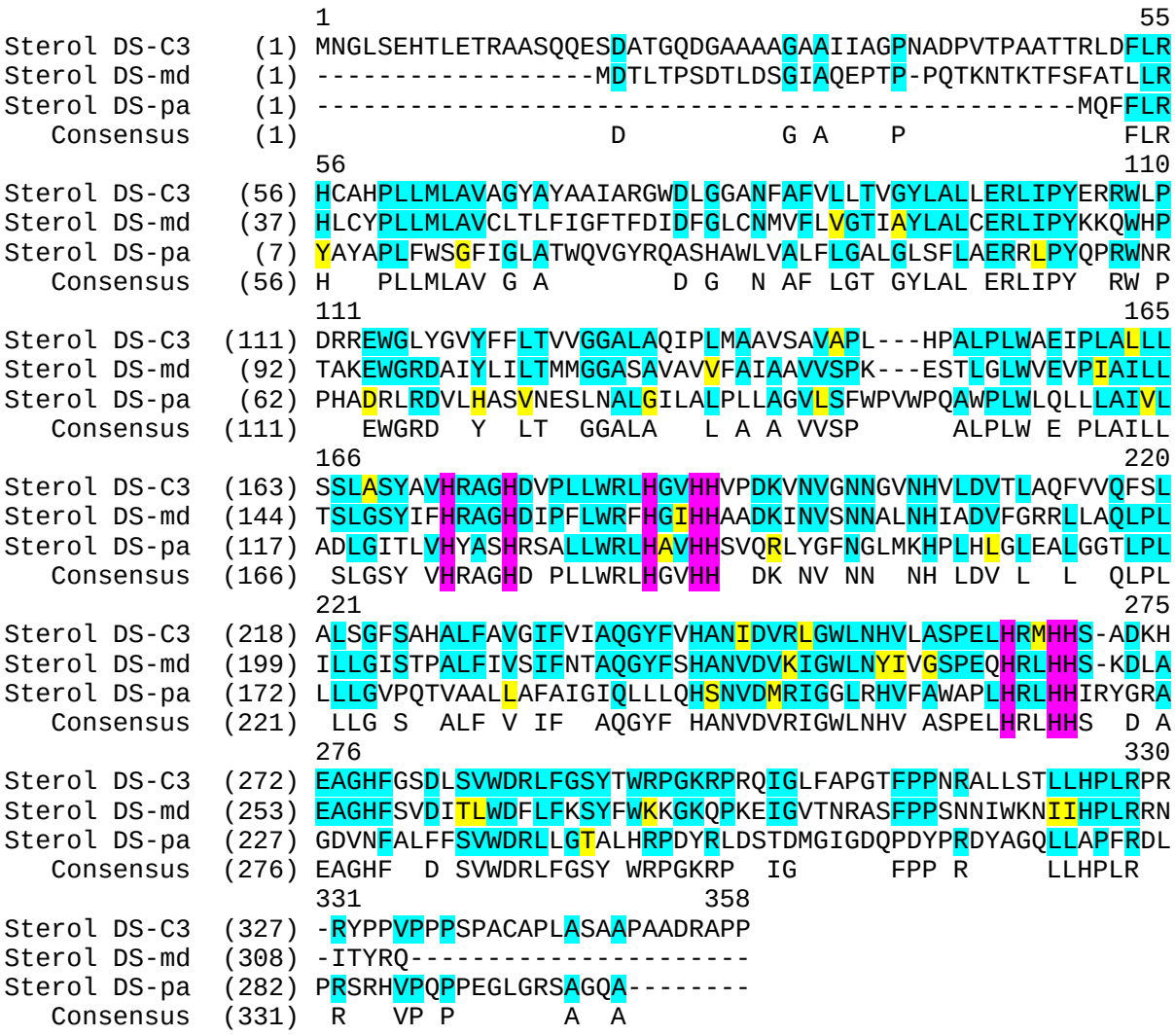
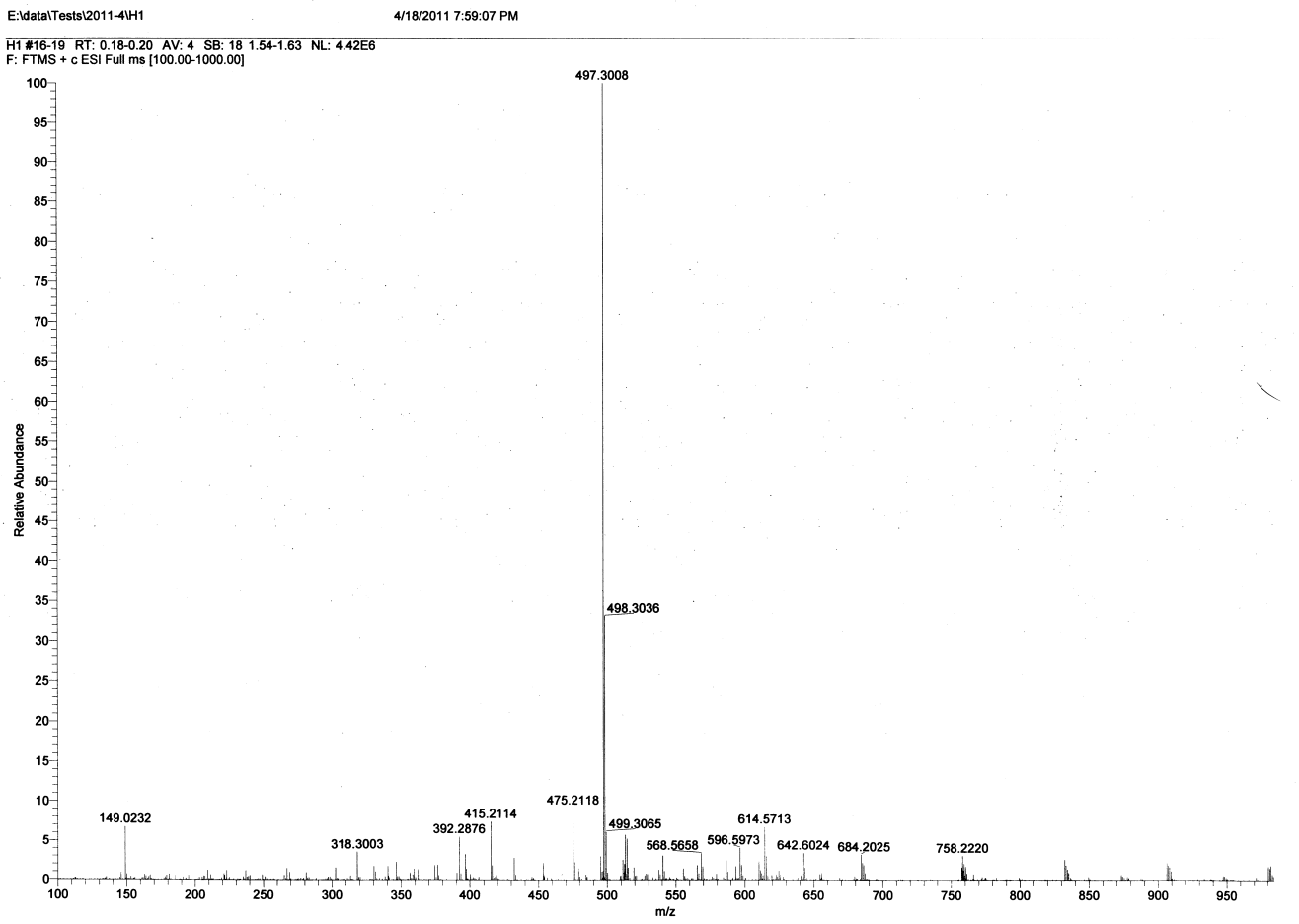



**Fig. S1.** Sequence comparison of three putative sterol desaturases. DS-C3, from *Lysobacter enzymogenes* C3 (accession number ABL86392); DS-md, from *Microbulbifer degradans* 2-40 (accession number ZP_00316392); DS-pa, from *Pseudomonas aeruginosa* UCBPP-PA14 (accession number ZP_00138305). Note that DS-C3 and DS-md have an *N*-terminal putative transmembrane region. The three highly conserved histidine rich motifs ($HX_3H$, $HX_2HH$, and $HX_2HH$) are highlighted with purple color to indicate the hallmarks of the sterol desaturases, alkane hydroxylases, and xylene monooxygenases.[1]

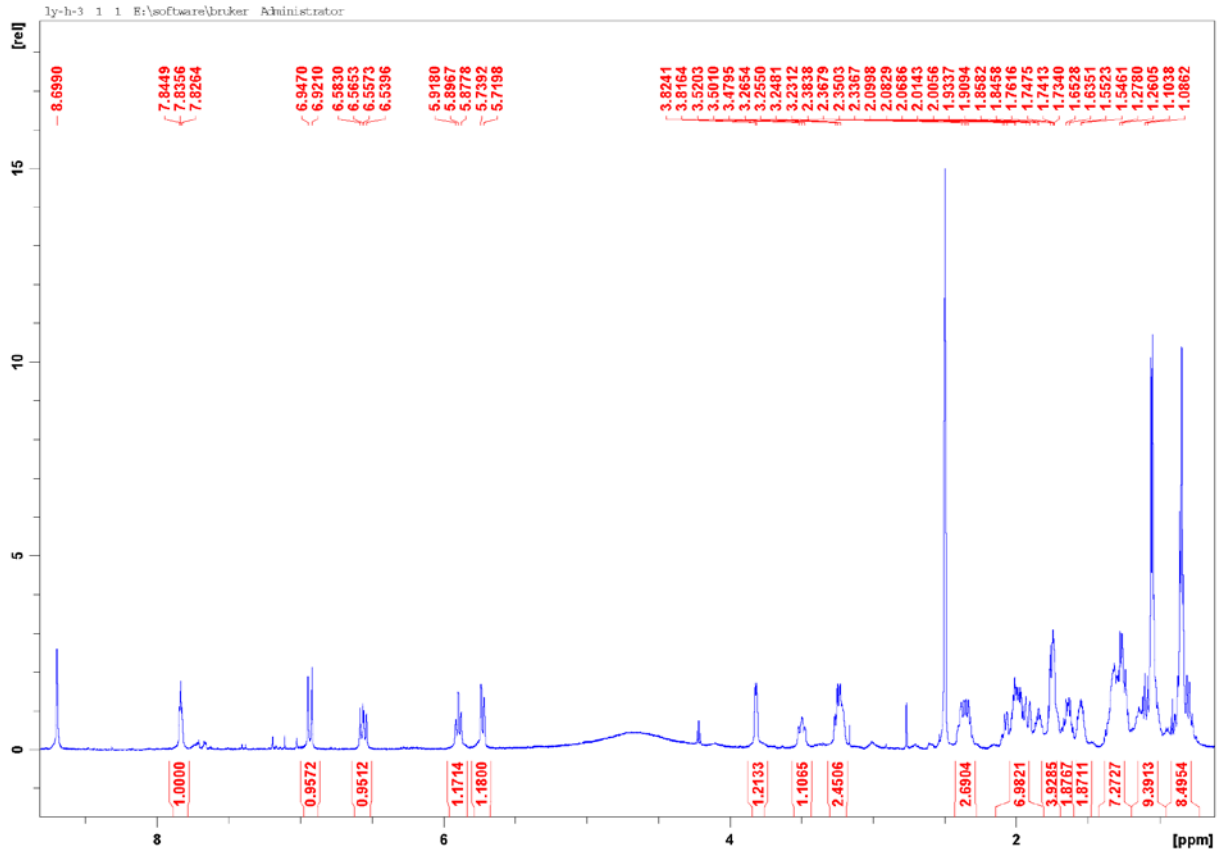
## Spectra of 3-deOH-HSAF

The spectroscopic data include HR-Q-TOF-MS, $^{1}$H-NMR spectrum, $^{13}$C-NMR spectra, and HSQC correlations for 3-deOH-HSAF.

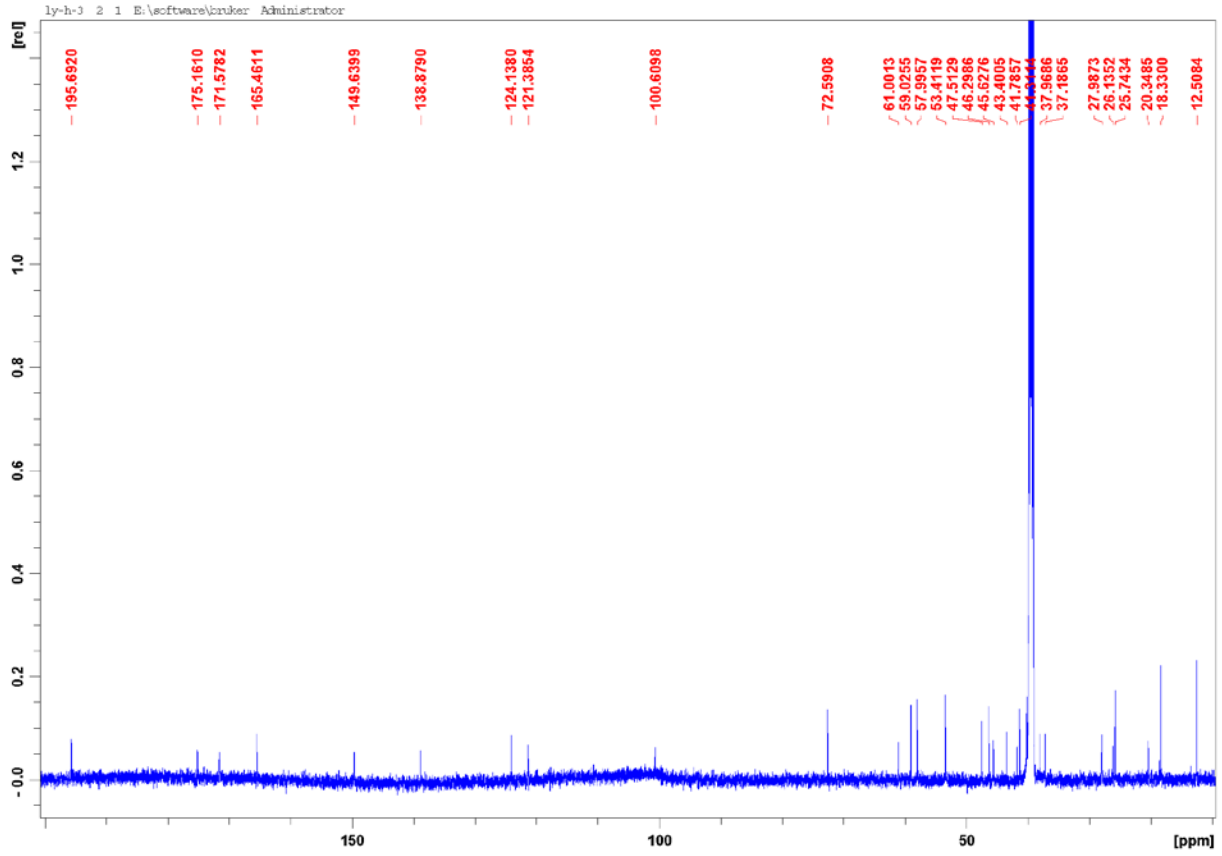
The HR-Q-TOF mass spectrum of 3-deOH-HSAF



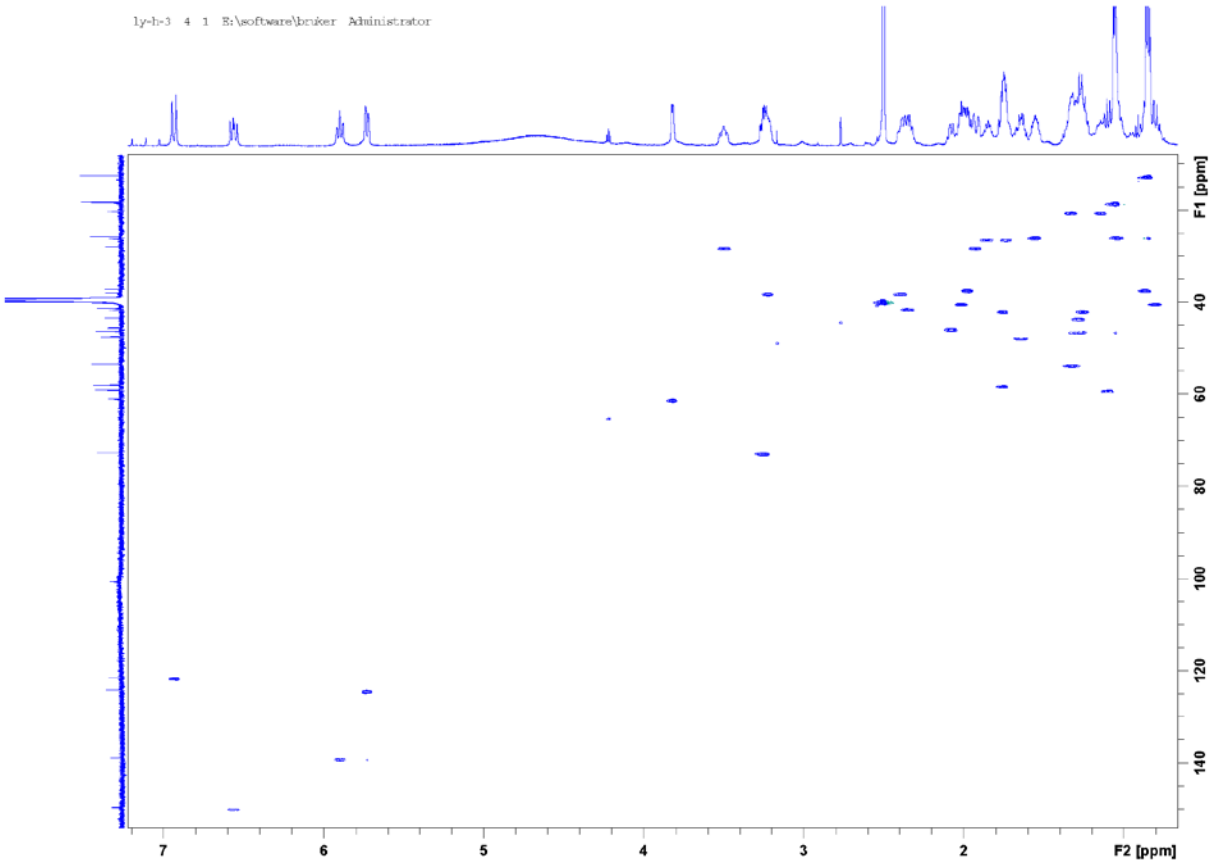
The $^{1}$H-NMR spectrum of 3-deOH-HSAF in DMSO-$d_6$



The $^{13}$C-NMR spectrum of 3-deOH-HSAF in DMSO-$d_6$



The HSQC spectrum of 3-deOH-HSAF in DMSO-$d_6$




Reference

1. J. Shanklin, E. Whittle and B. G. Fox, *Biochemistry-Us*, 1994, **33**, 12787-12794.