

Supplementary Information

**Exploring a *Streptomyces* wax synthase using Acyl-SNACs as donor substrates**

Federica Casolari<sup>1</sup>, Saad Alrshadi<sup>1,2</sup>, Reuben Carr<sup>3</sup> and Hai Deng<sup>1</sup>,

1 Department of Chemistry, University of Aberdeen, Aberdeen AB24 3UE, United Kingdom;

2 College of Science and Arts in Gurayat, Jouf University, King Khaled Road, Saudi Arabia

3 Ingenza Ltd Scotland, United Kingdom

Corresponding author: [h.deng@abdn.ac.uk](mailto:h.deng@abdn.ac.uk)

Table of Contents

1. Experimental.....	2
1.1. General Experimental Procedures.....	2
1.2. Instrumentation.....	2
2. Cloning and overexpression of WS-SCo in <i>E. coli</i> .....	3
3. Purification of His <sub>6</sub> -WS-SCo .....	3
4. Enzymatic reactions for wax esters production.....	4
5. Enzymatic studies .....	6
5.1. Standard kinetic profiling and calculations.....	6
5.2. Spectroscopic analysis of wax ester synthase activity .....	6
6. Site directed mutagenesis .....	6
7. Spectroscopic and spectrometric analysis of key products and substrates.....	7
References .....	21

## 1. Experimental

### 1.1. General Experimental Procedures

Chemicals and reagents were purchased from Sigma Aldrich (Dorset, UK), Fisher Scientific (Loughborough, UK), Merck (Dorset, UK), Goss Scientific (Crewe, UK), and BIO-RAD (Watford, UK) and did not require any additional purification. Sigma Aldrich (Dorset, UK) and Invitrogen (Thermo Scientific Inc, Dorset, UK) provided all buffer and media components. GenScript Biotech (UK) Limited supplied the synthetic genes used in this study.

### 1.2. Instrumentation

Laminar Flow Cabinets (Bioaire® Safemate 1.5, EuroClone®, Italy) were employed for bacterial work for the maintenance of sterile working condition. All media and glassware for bacterial cultivation underwent autoclave sterilization prior use for 20 minutes at 121°C in Priorclave Tactrol 2 (BioCote, UK).

High-Resolution Electrospray Ionisation Mass Spectrometry (HR-ESIMS) was utilised to confirm presence of enzymatic reaction products. All measurements were taken on a Bruker MaXis II Q-ToF system coupled to an Agilent 1290 Infinity UPLC equipped with a quaternary pump (G4204A), autosampler (G4226A) and diode array detector (G4212A). All samples were separated on Kinetex® 2.6 µm EVO C18 (100Å, 100 x 2.1 mm) column with a solvent gradient of water (0.1% LC-MS grade formic acid) and acetonitrile (0.1% LC-MS grade formic acid) starting at 5% ACN to 100% ACN over 15 minutes. HR-ESIMS analysis was run with the following parameters: ion capillary voltage 4500 V, ion capillary temperature 250 °C, N<sub>2</sub> flow rate 9.0 L/min, nebulizer spray pressure 4.0 bar and mass range of 100-2000 m/z for positive ionisation mode and 100-1000 m/z for negative ionisation mode. Instrument was calibrated prior each run and this applied to each sample data set. All obtained data were analysed with Bruker Compass DataAnalysis 5.1 (Bruker, Germany).

Proton nuclear magnetic resonance (<sup>1</sup>H NMR) spectra and carbon nuclear magnetic resonance (<sup>13</sup>C NMR) were recorded on Bruker AVANCE III HD 400 MHz (Ascend™ 9.4 Tesla) or Bruker AVANCE III HD 600 MHz (Ascend™ 14.1 Tesla) with Prodigy TCI™ cryoprobe. Chemical shifts for protons are reported in parts per million scale (δ scale) downfield from tetramethylsilane (TMS) and are referenced to residual protium in the NMR solvents, CD<sub>3</sub>OD δ3.31 and CDCl<sub>3</sub> δ7.26 (Goss Scientific).

All enzymatic studies were performed with EZ Read 400 Microplate Reader (Biochrom Ltd, UK).

## 2. Cloning and overexpression of WS-SCo in *E. coli*

The synthetic pET28(+)-WS-SCo plasmid was transformed into BL21/pGro7 by heat shock and recovered in 700  $\mu$ L of Luria-Bertani medium (37 °C for 90 minutes). Single colonies from each transformation were grown overnight in LB medium (5 mL) containing the corresponding antibiotic (for DH10B Kanamycin 50  $\mu$ g/mL; and for BL21 (pGro7), Kanamycin and Chloramphenicol 50  $\mu$ g/mL).

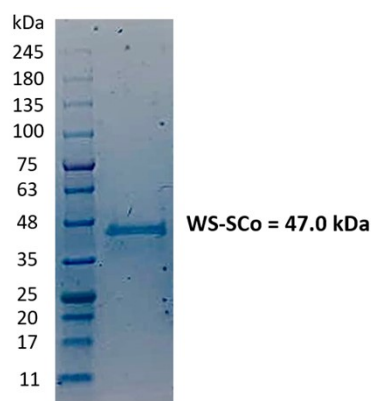
The overnight culture was transferred to 300 mL of previously autoclaved Auto Induction medium (Formedium™, UK) supplemented with kanamycin (50  $\mu$ g/mL), chloramphenicol (50  $\mu$ g/mL), L-arabinose (0.5 mg/mL). Cultures were allowed to grow to OD<sub>600</sub> of 0.6 and subsequently chilled on ice for 15 minutes. Cultures were then incubated overnight at 16°C at 220 rpm. Cell pellets were harvested by centrifugation 4 °C in Heraeus Multifuge 3 S-R bucket (Thermo Fisher Scientific).

## 3. Purification of His<sub>6</sub>-WS-SCo

The cell pellets were resuspended in ice-cold Binding Buffer (Imidazole, 10 mM in Phosphate-buffered saline Buffer) and further lysed by Ultrasonic Homogenizer JY92-IIN.

After centrifugation, the supernatant of cell free extracts was purified via immobilised Nickel-ion affinity chromatography on an NGC™ Medium Pressure Liquid Chromatography System Quest™ 10 (BIO-RAD) equipped with NGC™ Fraction Collector connected to Bio-Scale™ Mini Nuvia™ IMAC Cartridge. The column was pre-equilibrated with 5 CV of MilliQ water previously sterile filtered with 0.45  $\mu$ m filters and Binding Buffer (Imidazole, 10 mM in PBS Buffer) until the UV<sub>280</sub> absorption was stabilised. The cell lysates were loaded on the column at 4 mL/min of flow rate. The column was then washed by a gradient of Elution Buffer (Potassium Phosphate Buffer, 50 mM, pH 8.0; Imidazole, 500 mM; NaCl 300 mM) from 5 to 40% over 10 minutes to elute proteins for competitive binding. Each fraction was collected with a total volume of 5 mL and fractions containing the protein of interest by UV<sub>280</sub> visualisation were subjected to SDS-PAGE analysis for further confirmation. The desired elution fractions were combined and concentrated using a Centrifugal Filter Unit (Millipore). Final protein concentration was quantified with Colibri Microvolume spectrometer (Titertek Berthold) by using the corresponding molecular weight and extinction coefficient before being stored in equal amounts of protein preservation buffer (protein: buffer ratio 1:1, Tris-HCl, 10 mM; NaCl, 50 mM; DTT, 1 mM; 20% v/v glycerol) at -80°C.

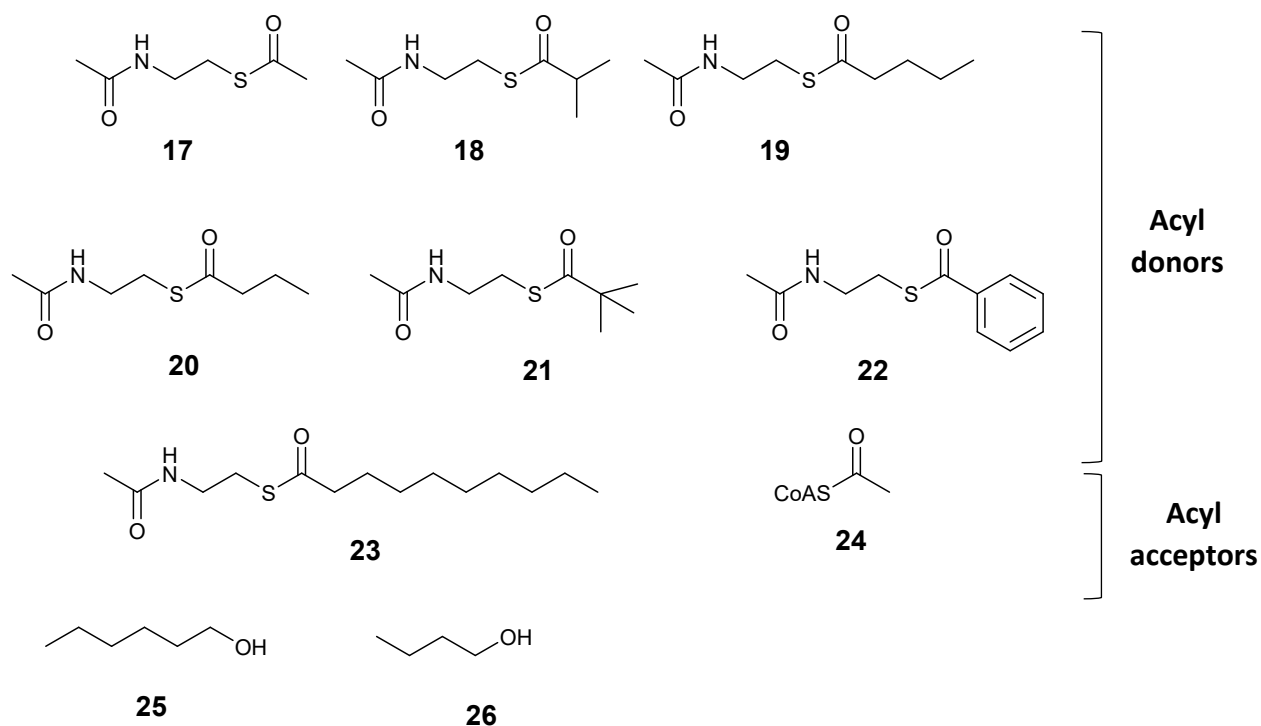
The purity and size of the desired protein was confirmed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis (**Figure S2**).



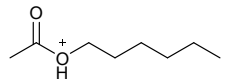
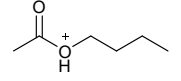
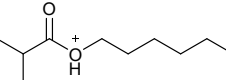
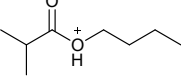
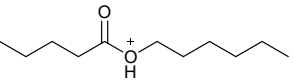
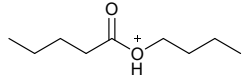
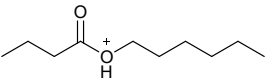
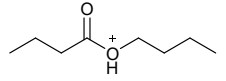
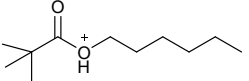
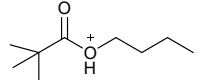
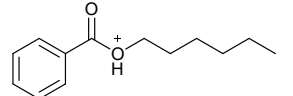
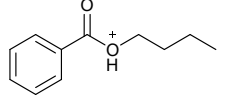
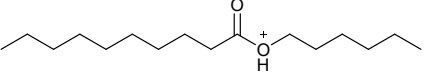
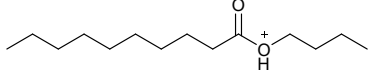
**Figure S1.** SDS-PAGE electrophoresis gel of WS-SCo highlighting the final size of the protein (47.0 kDa).

#### 4. Enzymatic reactions for wax esters production

Wax esters formation was monitored with LC-MS analysis. Enzymatic reactions contained DTNB in 0.5 mM Tris-HCl pH Buffer, 2.5 mM of alcohol (hexanol **25** and butanol **26**), 10  $\mu$ M of enzyme and 1 mM acyl-donor species (acyl-CoAs or acyl-SNACs, Figure S2). All reactions were incubated at 37  $^{\circ}$ C for 30 minutes and then injected (10  $\mu$ L) in Bruker MaXis II Q-ToF system coupled to an Agilent 1290 Infinity UPLC using the method described above (**1.2. Instrumentation**).



**Figure S2.** List of substrates for WS-SCo used in this study.

<p><b>A</b></p> 	<p>Hexyl acetate [M+H]<sup>+</sup>=145.1223 Mass error = 3.1 ppm</p>	<p><b>B</b></p> 	<p>Butyl acetate [M+H]<sup>+</sup>= 117.0911 Mass error = 4.2 ppm</p>
	<p>Hexyl isobutyrate [M+H]<sup>+</sup>=173.1536 Mass error = 2.6 ppm</p>		<p>Butyl isobutyrate [M+H]<sup>+</sup>= 145.1224 Mass error = 3.4 ppm</p>
	<p>Hexyl valerate [M+H]<sup>+</sup>=187.1693 Mass error = 4.9 ppm</p>		<p>Butyl valerate [M+H]<sup>+</sup>= 159.1380 Mass error = 3.9 ppm</p>
	<p>Hexyl butyrate [M+H]<sup>+</sup>=173.1536 Mass error = 4.7 ppm</p>		<p>Butyl butyrate [M+H]<sup>+</sup>= 145.1224 Mass error 4.8 ppm</p>
	<p>Hexyl <i>tert</i>-butyrate [M+H]<sup>+</sup>=187.1693 Mass error = 3.9 ppm</p>		<p>Butyl <i>tert</i>-butyrate [M+H]<sup>+</sup>= 159.1380 Mass error 2.2 ppm</p>
	<p>Hexyl benzoate [M+H]<sup>+</sup>=207.1380 Mass error = 1.8 ppm</p>		<p>Butyl benzoate [M+H]<sup>+</sup>= 179.1067 Mass error = 1.5 ppm</p>
	<p>Hexyl decanoate [M+H]<sup>+</sup>=257.2475 Mass error = 3.3 ppm</p>		<p>Butyl decanoate [M+H]<sup>+</sup>= 229.2163 Mass error = 1.2 ppm</p>

**Figure S3.** Obtained hexyl (A) and butyl (B). Enzymatic reactions were run in the presence of WS-SCo (10  $\mu$ M), acyl-SNACs (1 mM) and alcohols (2.5 mM, hexanol and butanol). Decanol reactions did not yield any detectable esters.

## 5. Enzymatic studies

### 5.1. Standard kinetic profiling and calculations

Michaelis-Menten kinetics were measured using non-linear regression. All kinetic rates were calibrated with the background noises and calculated using the TNB<sup>2-</sup>'s extinction coefficient (14.15 mM<sup>-1</sup> cm<sup>-1</sup>) for wax ester synthases kinetic calculations.

### 5.2. Spectroscopic analysis of wax ester synthase activity

DTNB assay (5,5'-dithiobis(2-nitrobenzoic acid), Ellman's assay) is used to quantify free sulfhydryl groups and indirectly measures the wax ester synthase activity of WS-SCo. The assay was performed as described by Maglangit et al. at 37 °C using a EZ Read 400 Microplate Reader (Biochrom Ltd, UK)(1). The reaction (200 µL) contained DTNB in 0.5 mM Tris-HCl Buffer, 2.5 mM of alcohol (hexanol and butanol), 10 µM of enzyme and acyl-SNACs (acetyl-, isobutyryl-, valeryl-, butyryl-, *tert*-butyl-, phenyl-, decanoyl-) in increasing concentrations (25, 50, 100, 500 µM, 1 mM). The reaction will monitor the release of the thiol by-product by measuring an increase in absorbance at 412 nm(2).

**Table S1.** Kinetic constants measuring enzymatic activity of WS-SCo towards acyl-SNACs and alcohols.

Acyl-SNACs	$K_m$ (µM ± STD Dev)		$k_{cat}$ (min <sup>-1</sup> ± STD Dev)		$k_{cat}/K_m$ (mM·min <sup>-1</sup> ± STD Dev)	
	Hexanol	Butanol	Hexanol	Butanol	Hexanol	Butanol
Acetyl-SNAC	28.261 ± 0.104	296.796 ± 0.400	0.133 ± 0.020	0.157 ± 0.133	4.897 ± 1.093	2.513 ± 2.943
Isobutyryl-SNAC	5.229 ± 0.003	28.795 ± 0.024	0.037 ± 0.015	0.084 ± 0.028	7.475 ± 1.291	3.900 ± 2.321
Valeryl-SNAC	28.636 ± 0.011	30.347 ± 0.013	0.549 ± 0.062	0.101 ± 0.021	20.369 ± 5.939	3.494 ± 0.741
Butyryl-SNAC	23.651 ± 0.007	19.640 ± 0.011	0.653 ± 0.081	0.090 ± 0.012	28.485 ± 5.565	5.401 ± 2.648
<i>tert</i> -butyl-SNAC	25.374 ± 0.007	22.135 ± 0.001	0.575 ± 0.025	0.132 ± 0.054	23.333 ± 5.072	6.019 ± 2.654
Phenyl-SNAC	27.376 ± 0.001	22.201 ± 0.006	0.419 ± 0.039	0.137 ± 0.016	15.298 ± 1.152	6.284 ± 0.872
Decanoyl-SNAC	15.206 ± 0.001	30.208 ± 0.009	0.617 ± 0.012	0.115 ± 0.003	40.548 ± 0.343	3.984 ± 1.111

## 6. Site directed mutagenesis

To generate a L25F variant of WS-SCo, the whole plasmid PCR was performed using two corresponding pairs of overlapping primers (**6.1. Primers**) and pET-28(+)-WS-SCo as DNA template. The mutated construct was treated with Dnpi restriction enzyme to eliminate the parental strands. The sequence of the isolated mutated plasmid was confirmed by DNA sequencing. All designed primers used for mutation studies are listed in Table S3 (6.1. Primers). The plasmid containing WS-SCo-L25F was transferred into BL21 (pGro7) competent cells for producing a Leu25Phe variant.

## 6.1. Primers

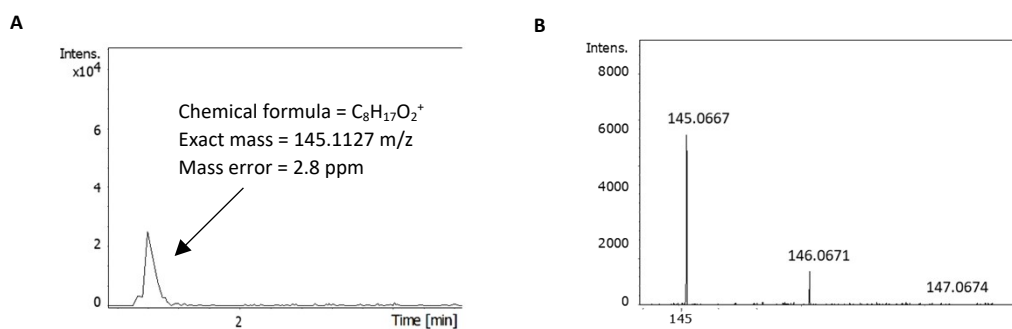
**Table S2.** PCR primers used in this study.

Construct	Primer sequence (5' → 3')
WSSCo-L25F-F	cat <u>ttt</u> ggggccttaggggttttcgaagc
WSSCo-L25F-R	ca <u>aaa</u> tgcatcggatgttctgcagattc

## 7. Spectroscopic and spectrometric analysis of key products and substrates

All ester products were synthesised in-house as follows: hexyl acetate **1** was obtained by reacting acyl-CoA **24** (1 mM) and hexanol **25** (2.5 mM) and incubating the reaction at 37 °C for 30 minutes before confirming its production with HR-ESIMS.

All acyl-SNACs (**17-23**) were also synthesised in-house with a previously reported method(1). <sup>1</sup>H, <sup>13</sup>C NMR and HR-ESIMS analysis of yielded compounds are also shown below.



**Figure S4.** Extracted Ion Chromatogram (A) and MS data (B) of hexyl acetate **1** yielded from WS-SCo with acetyl-CoA **24** and hexanol **25**.

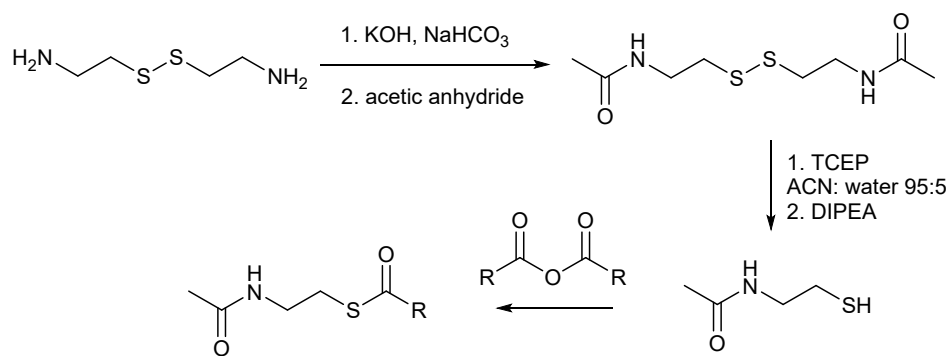


Figure S5. One-pot synthesis of acyl-SNAC substrates.

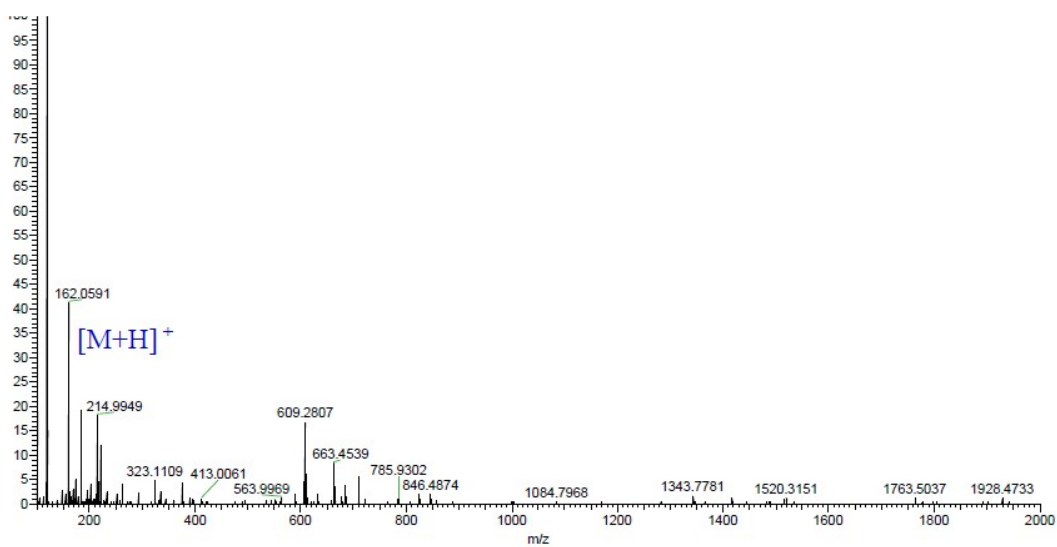
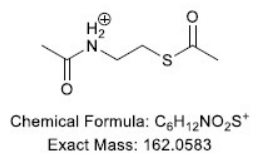


Figure S6. HR-ESIMS of acetyl-SNAC 17.



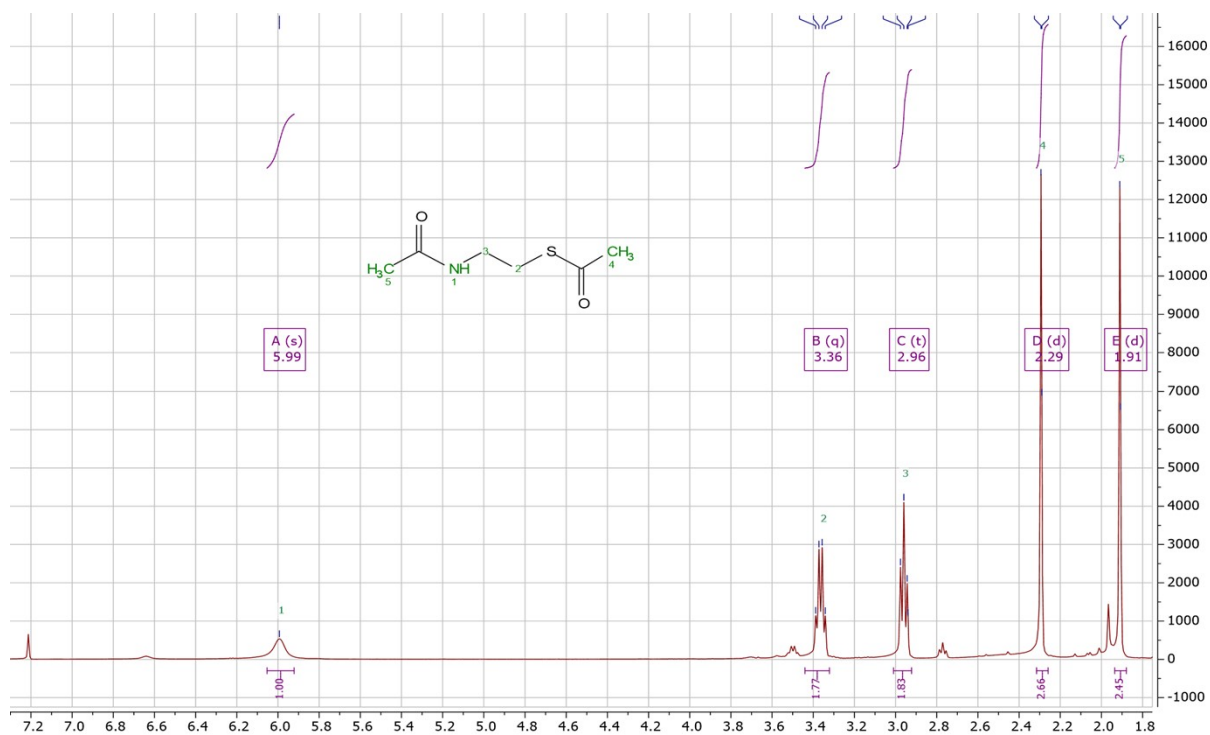


Figure S7. <sup>1</sup>H-NMR of acetyl-SNAC 17 (600MHz, CDCl<sub>3</sub>, Me<sub>4</sub>Si).

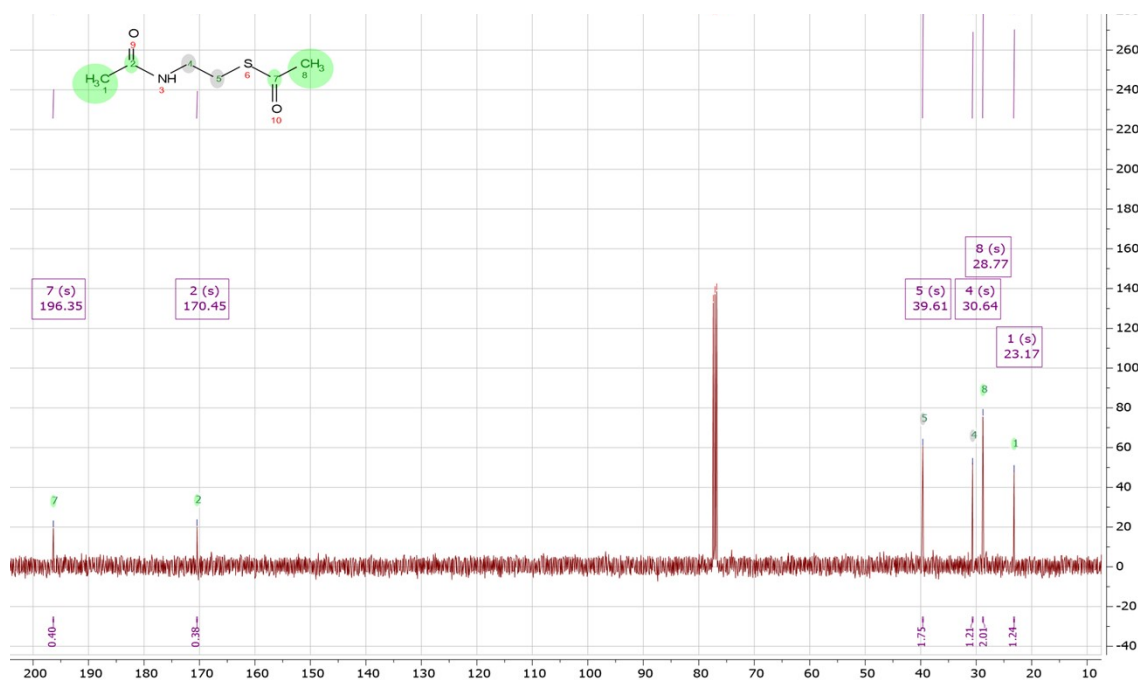


Figure S8. <sup>13</sup>C-NMR of acetyl-SNAC 17 (600MHz, CDCl<sub>3</sub>, Me<sub>4</sub>Si).

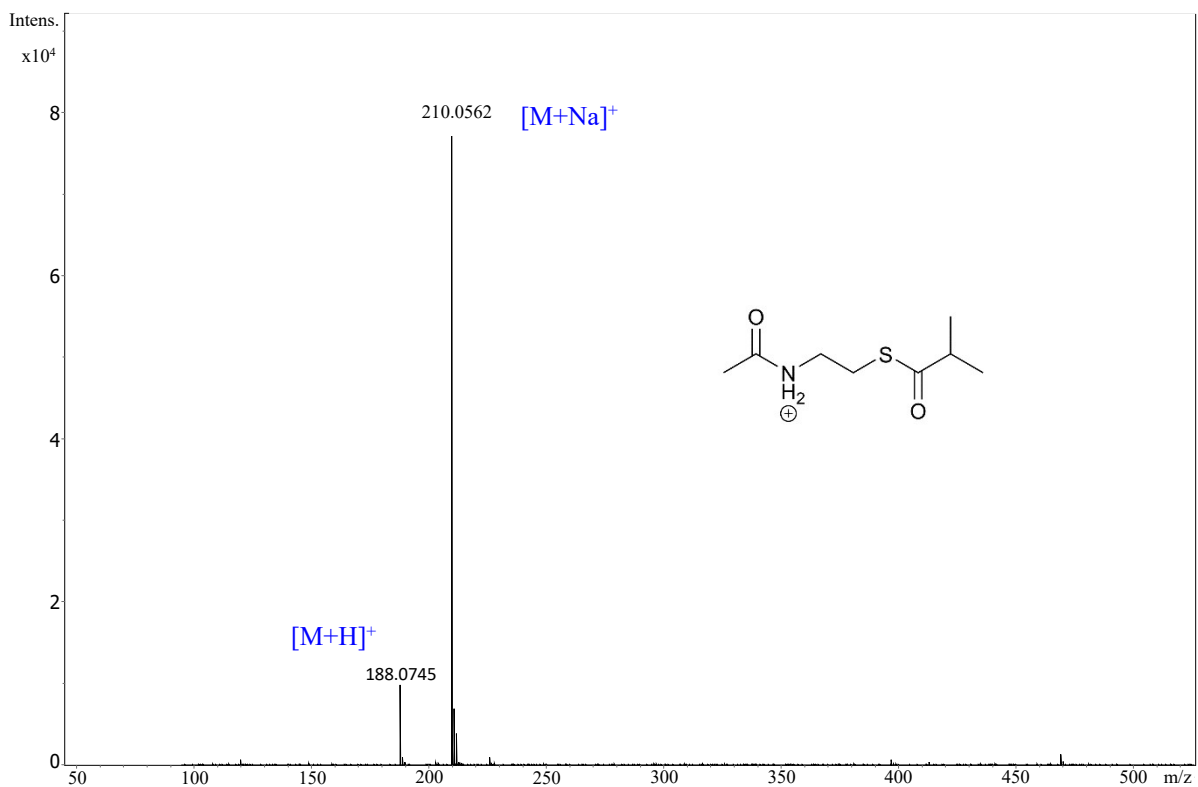


Figure S9. HR-ESIMS of isobutyryl-SNAC 18.

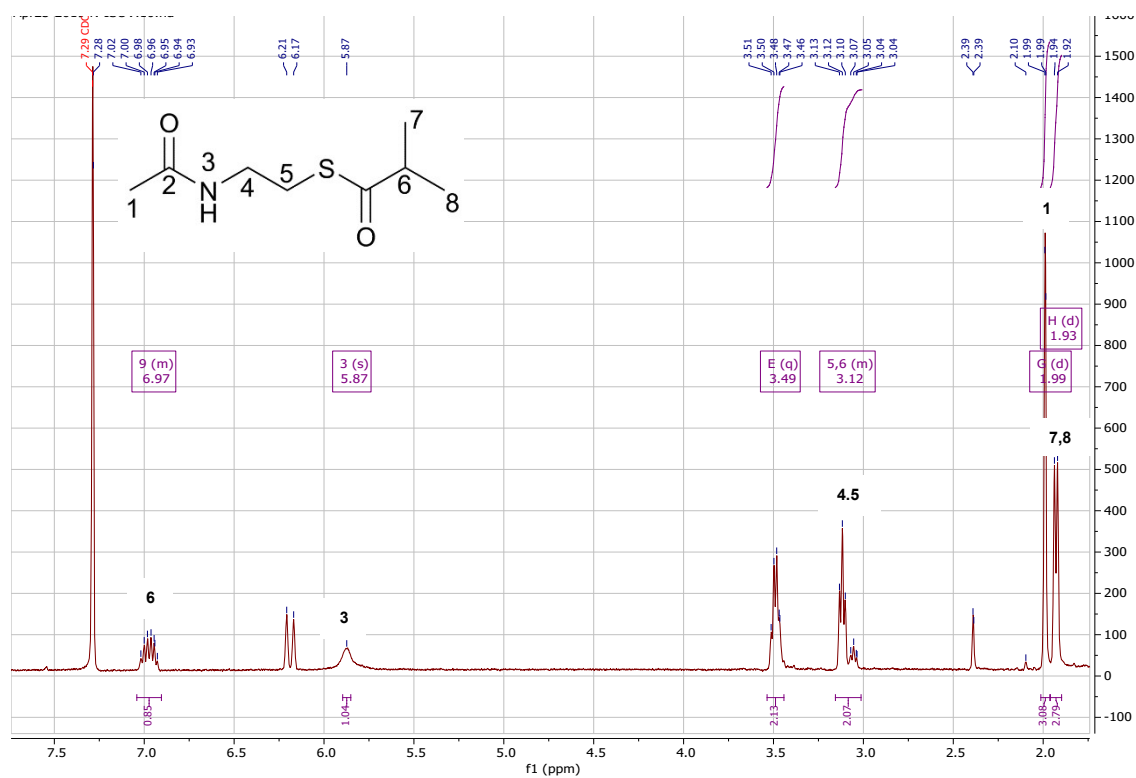


Figure S10. <sup>1</sup>H-NMR of isobutyryl-SNAC 18 (600MHz, CDCl<sub>3</sub>, Me<sub>4</sub>Si).

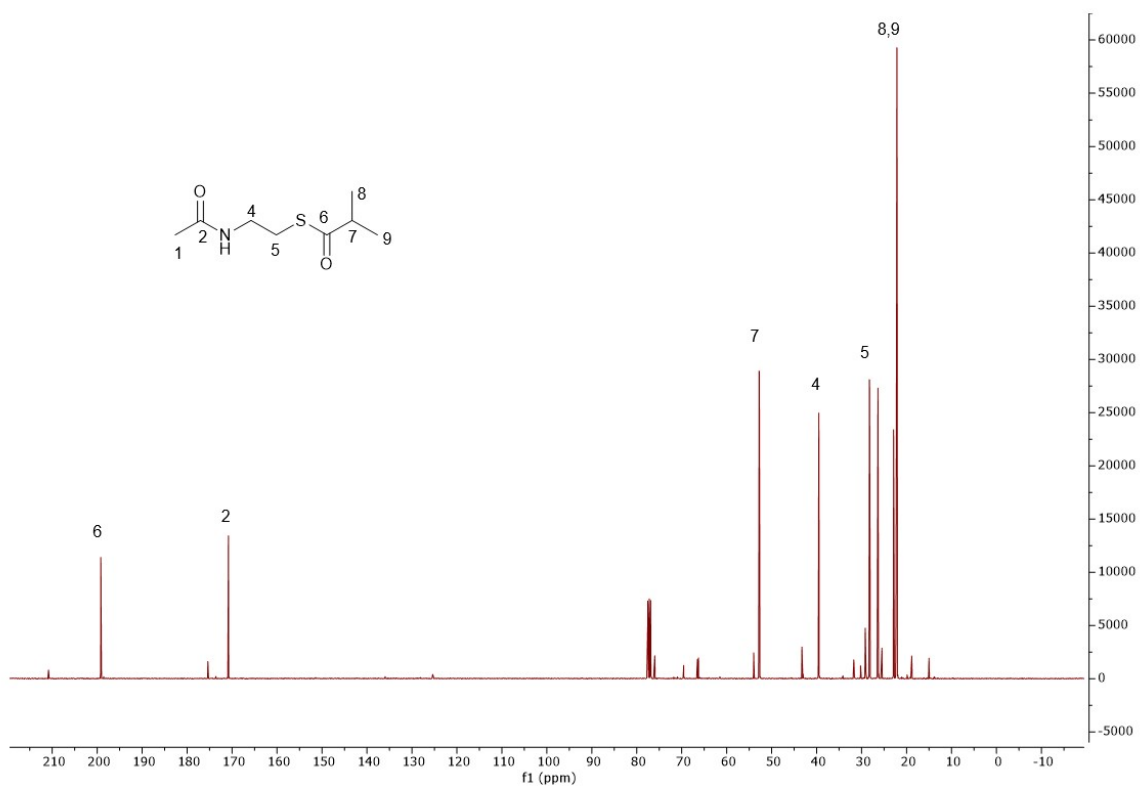


Figure S11.  $^{13}\text{C}$ -NMR of isobutyryl-SNAC **18** (600MHz,  $\text{CDCl}_3$ ,  $\text{Me}_4\text{Si}$ ).

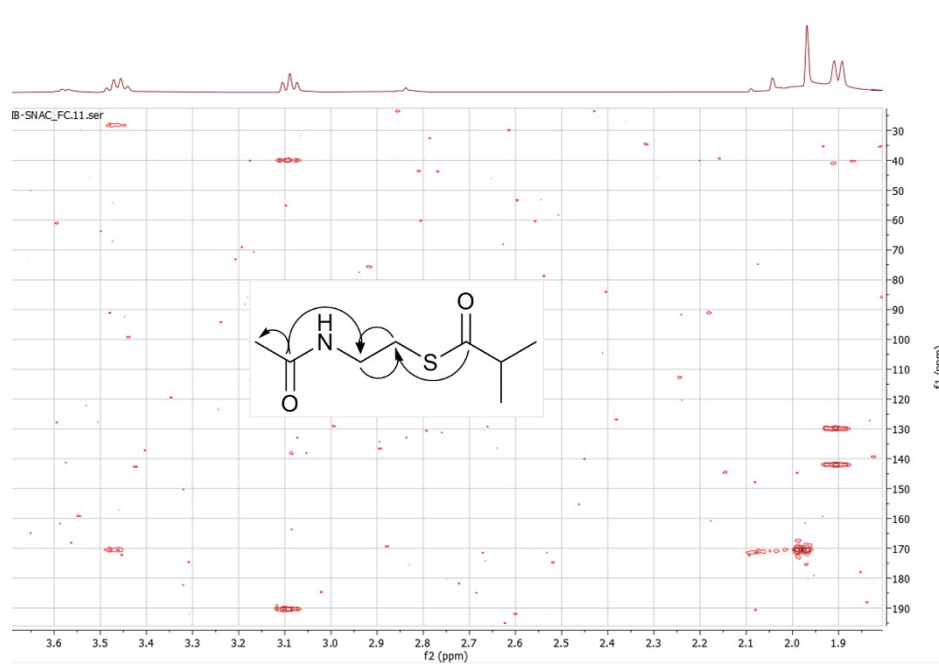
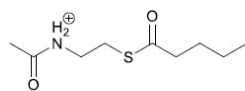


Figure S12. HMBC spectrum and correlations of isobutyryl-SNAC **18** (600MHz,  $\text{CDCl}_3$ ,  $\text{Me}_4\text{Si}$ ).



Chemical Formula:  $C_9H_{18}NO_2S^+$   
Exact Mass: 204.1053

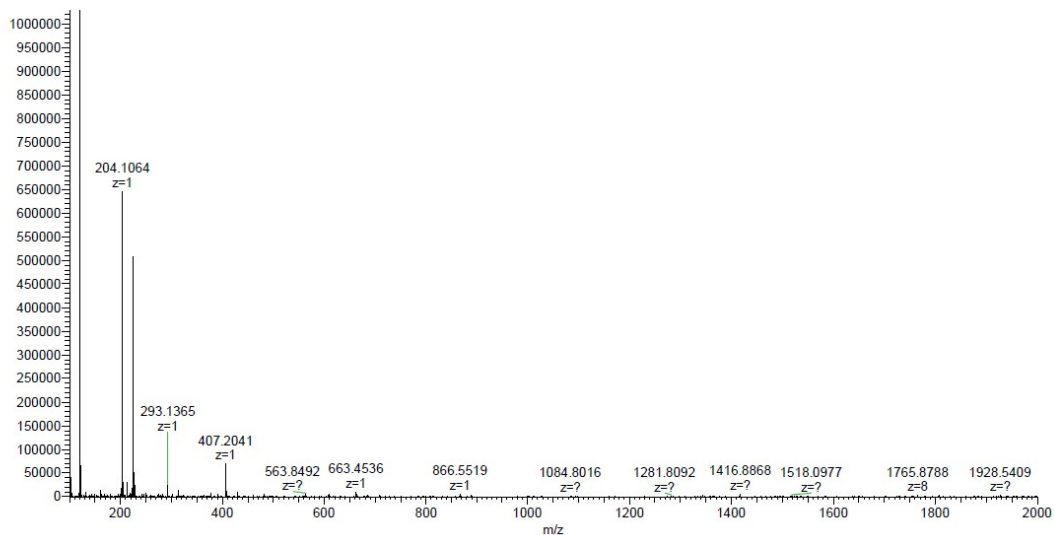


Figure S13. HR-ESIMS of valeryl-SNAC 19.

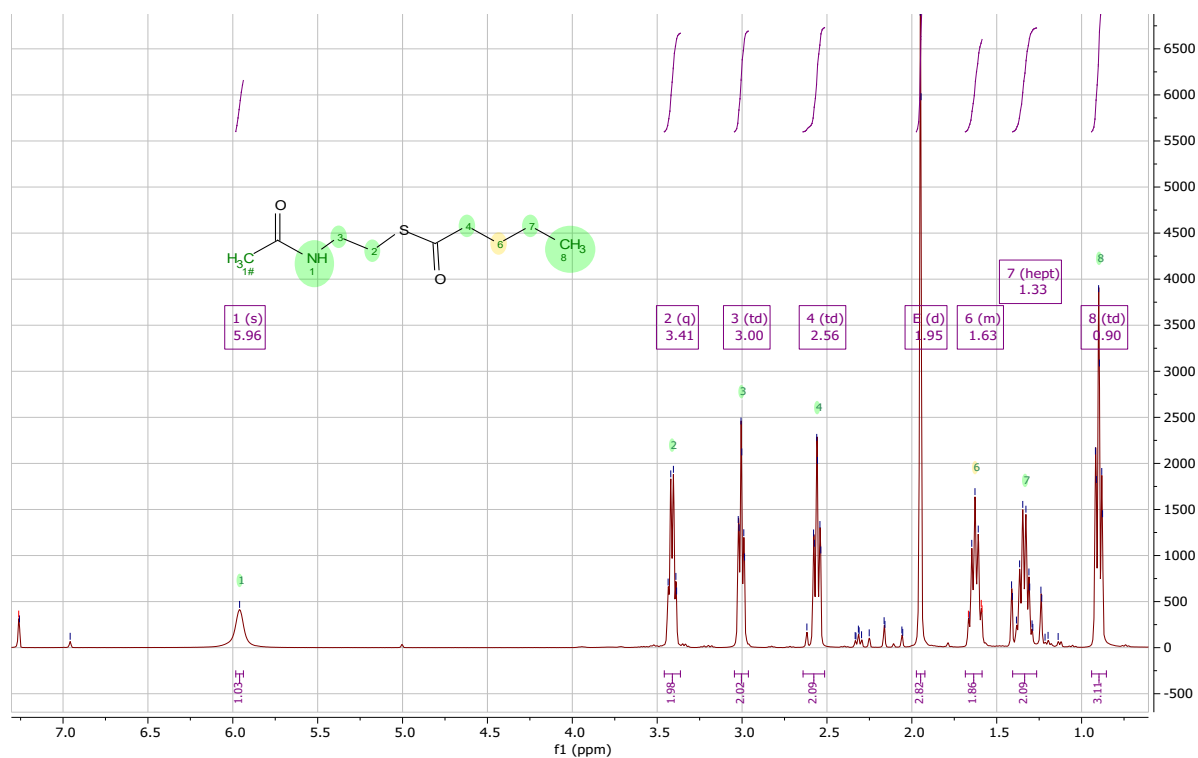


Figure S14.  $^1H$ -NMR of valeryl-SNAC 19 (600MHz,  $CDCl_3$ ,  $Me_4Si$ ).

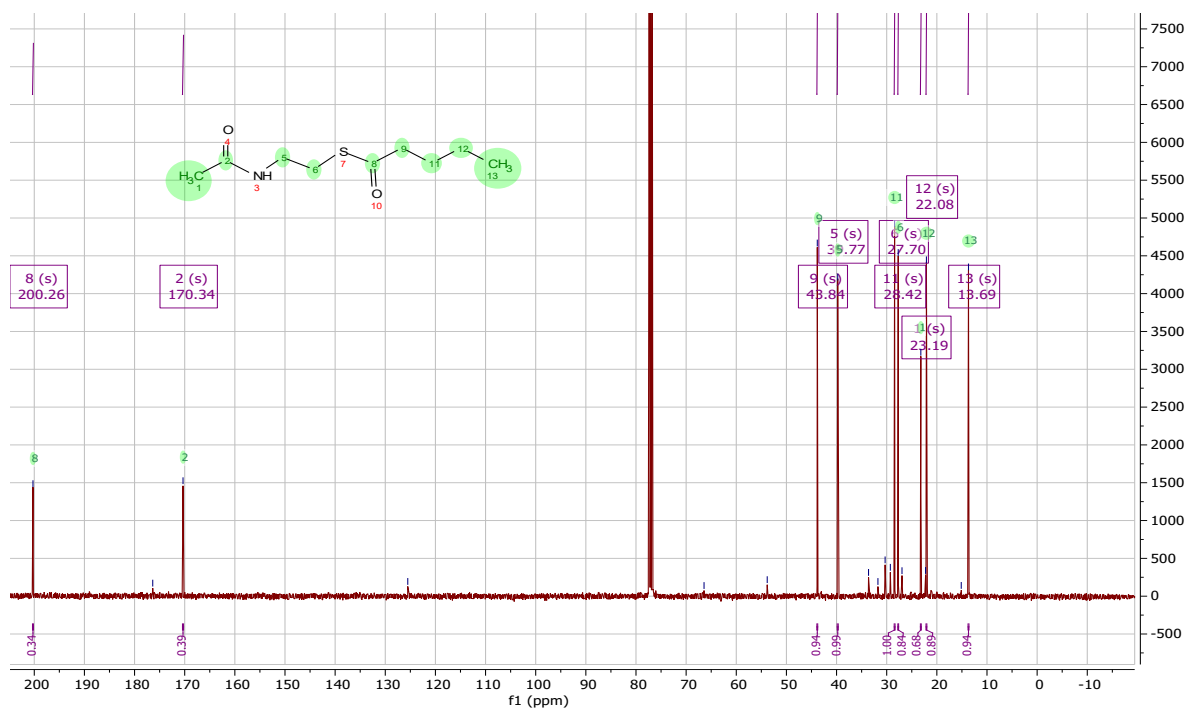


Figure S15. <sup>13</sup>C-NMR of valeryl-SNAC 19 (600MHz, CDCl<sub>3</sub>, Me<sub>4</sub>Si).

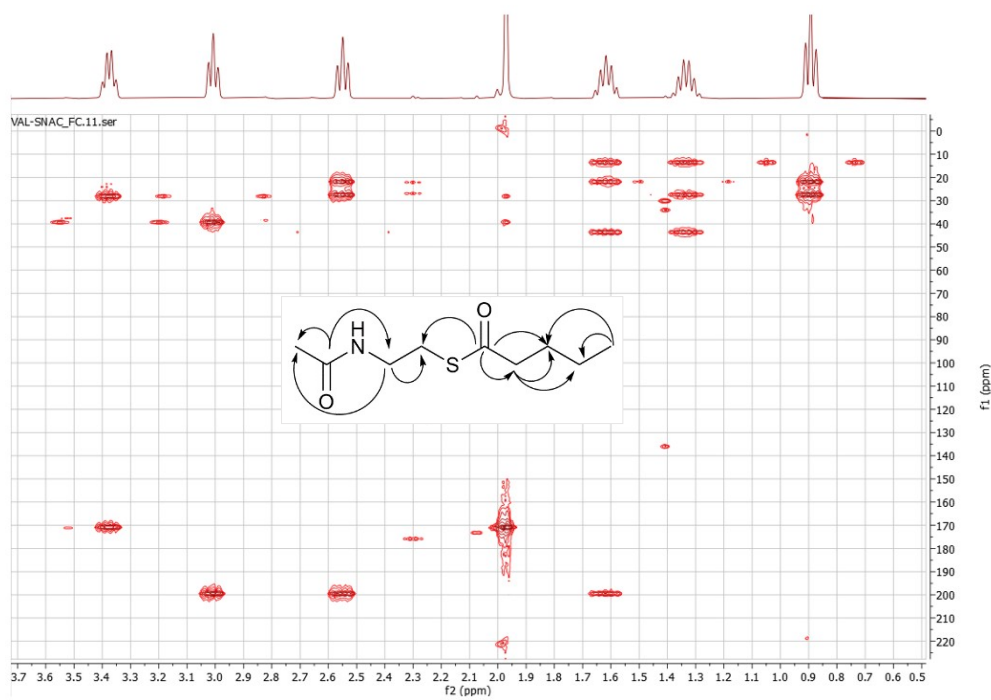
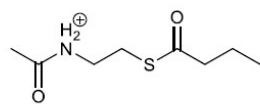


Figure S16. HMBC spectrum and correlations of valeryl-SNAC 19 (600MHz, CDCl<sub>3</sub>, Me<sub>4</sub>Si).



Chemical Formula:  $C_8H_{16}NO_2S^+$   
Exact Mass: 190.0896

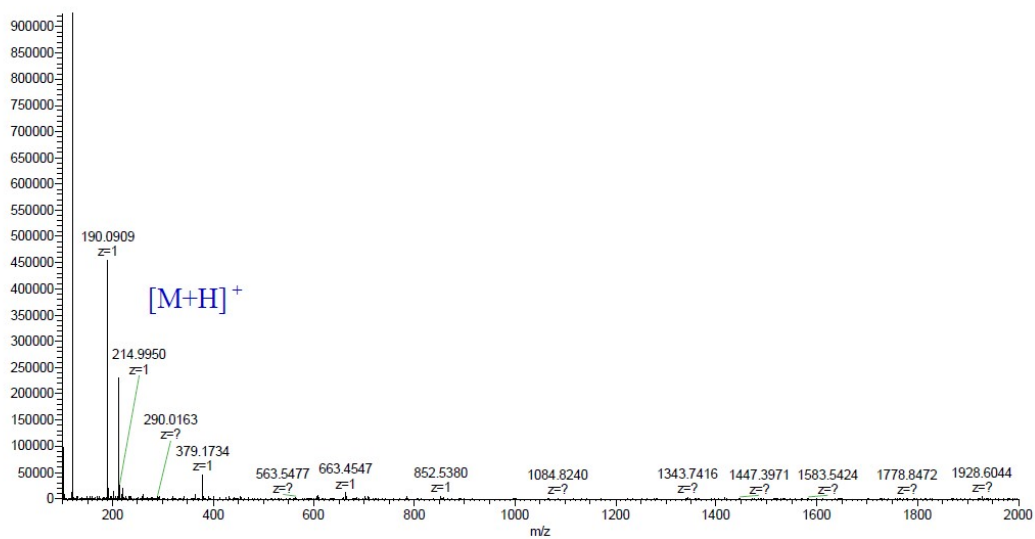


Figure S17. HR-ESIMS of butyryl-SNAC 20.

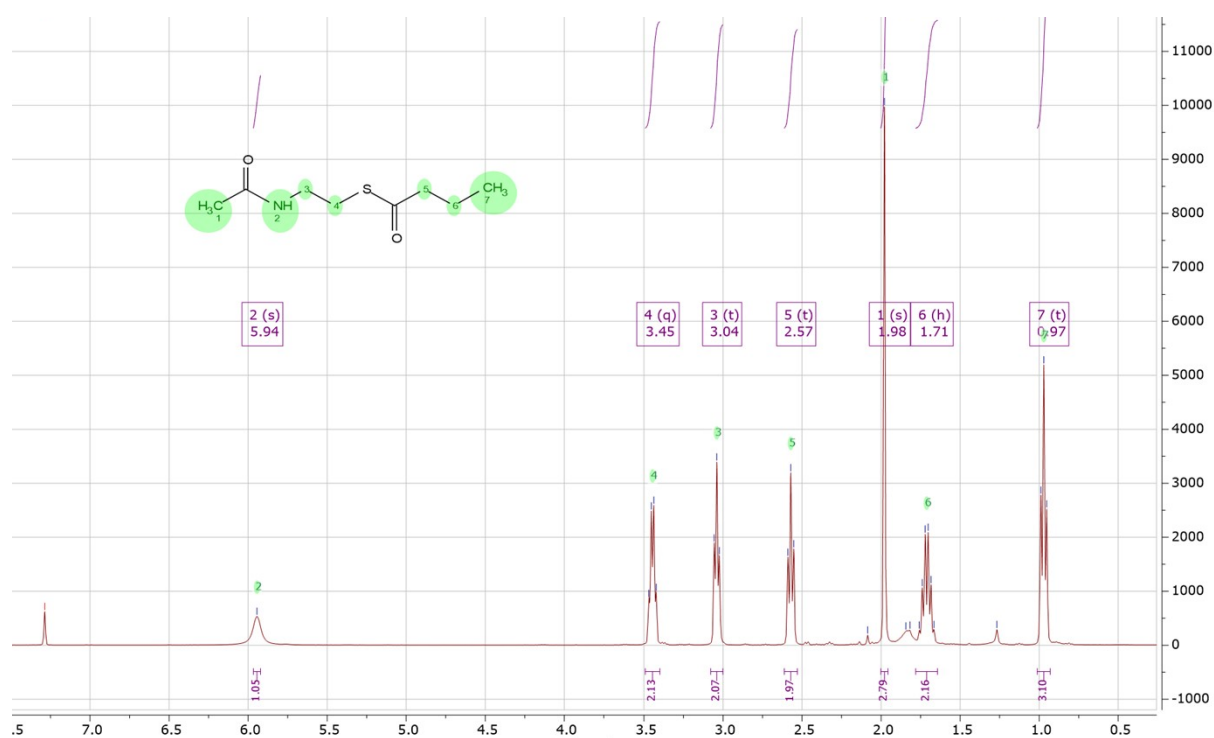


Figure S18.  $^1H$ -NMR of butyryl-SNAC 20 (600MHz,  $CDCl_3$ ,  $Me_4Si$ ).

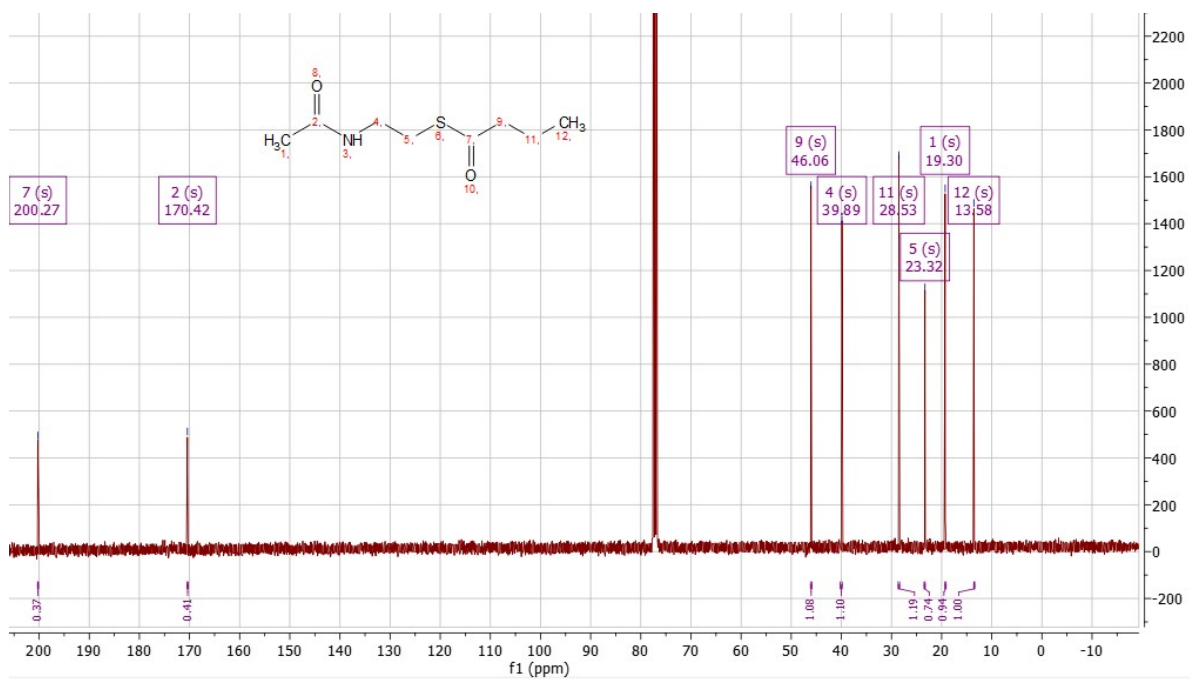


Figure S19.  $^{13}\text{C}$ -NMR of butyryl-SNAC **20** (600MHz,  $\text{CDCl}_3$ ,  $\text{Me}_4\text{Si}$ ).

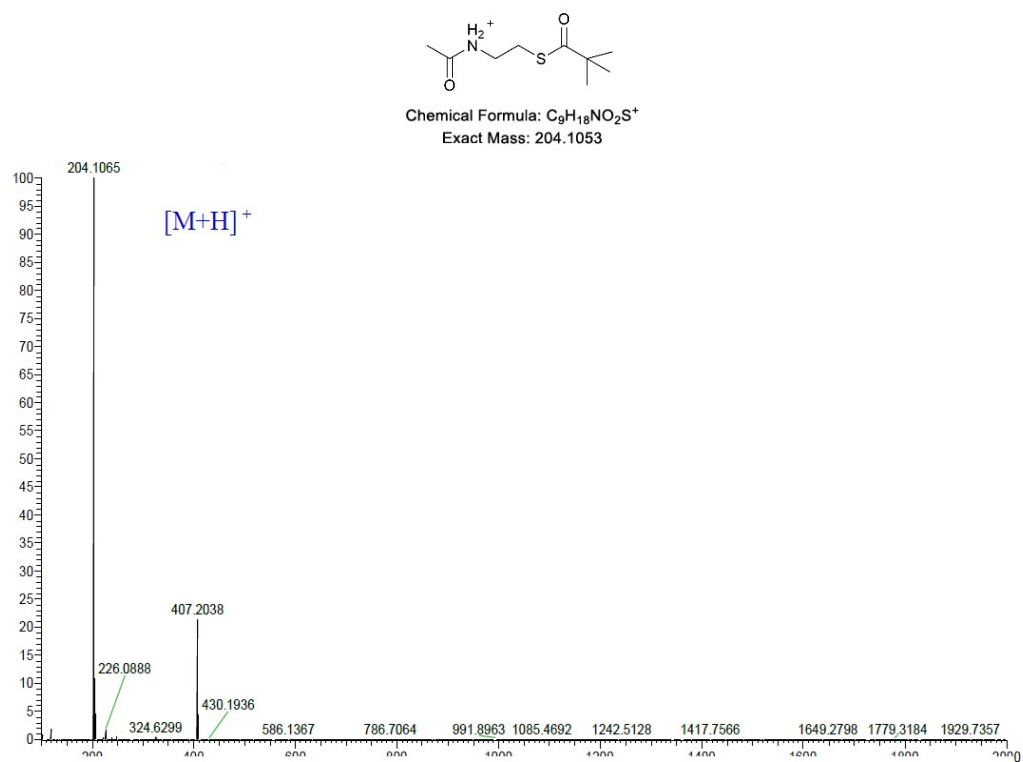


Figure S20. HR-ESIMS of *tert*-butyl-SNAC **21**.

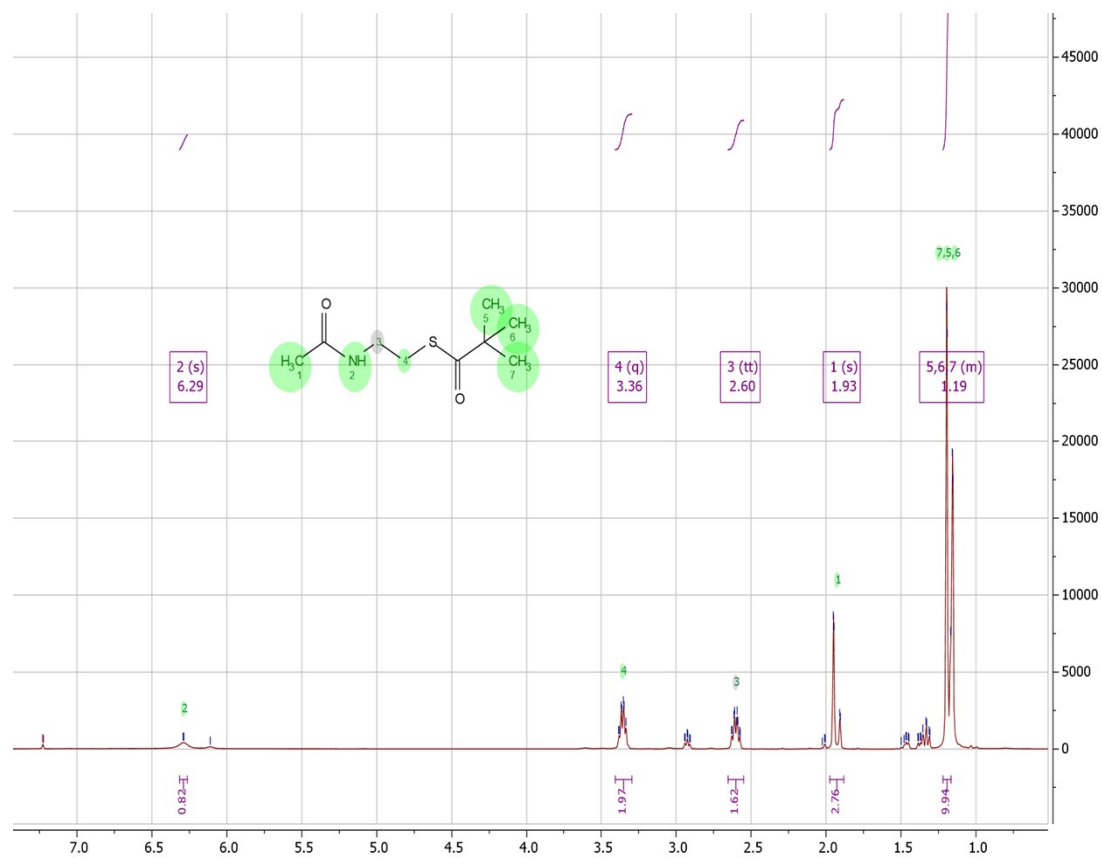


Figure S21.  $^1\text{H-NMR}$  of *tert*-butyl-SNAC **21** (600MHz,  $\text{CDCl}_3$ ,  $\text{Me}_4\text{Si}$ ).

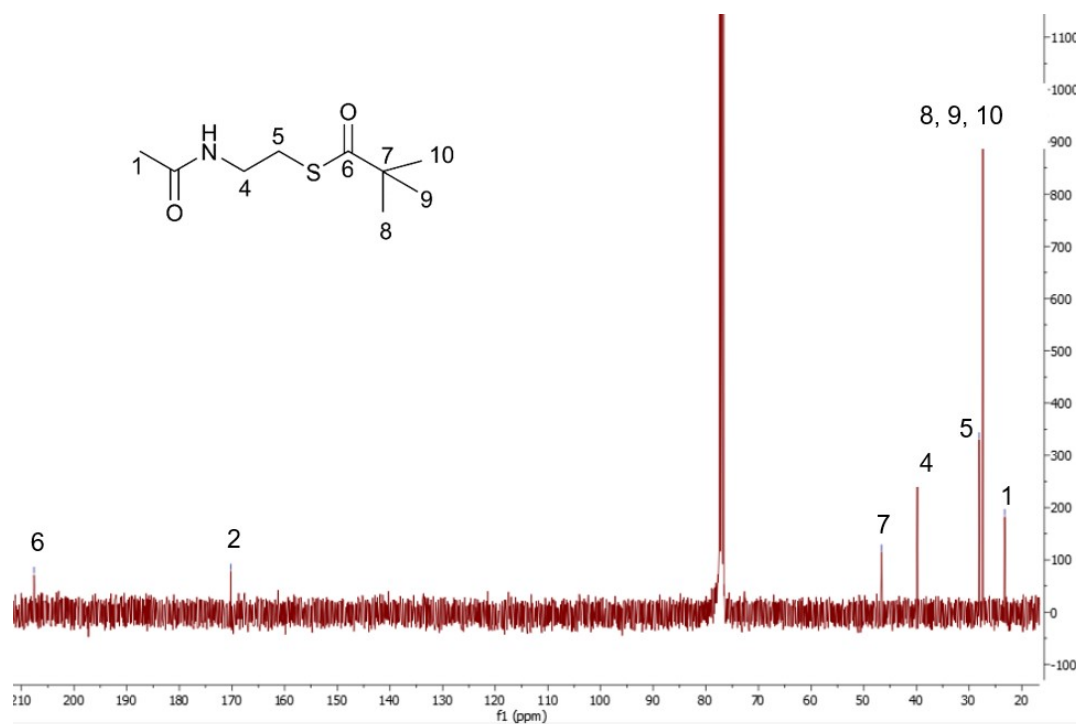
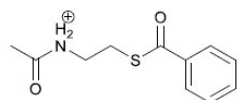


Figure S22.  $^{13}\text{C-NMR}$  of *tert*-butyl-SNAC **21** (600MHz,  $\text{CDCl}_3$ ,  $\text{Me}_4\text{Si}$ ).





Chemical Formula: C<sub>11</sub>H<sub>14</sub>NO<sub>2</sub>S<sup>+</sup>  
Exact Mass: 224.0740

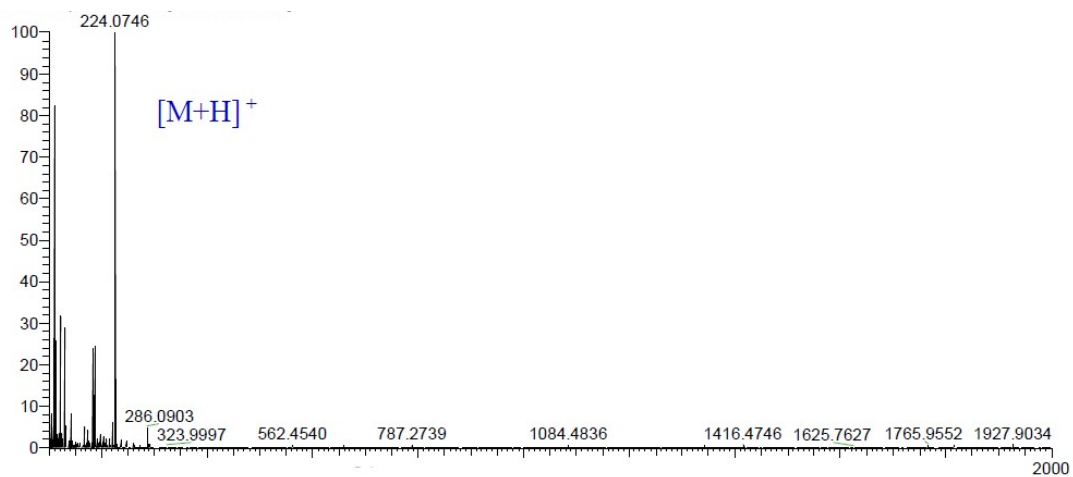


Figure S23. HR-ESIMS of phenyl-SNAC **22**.

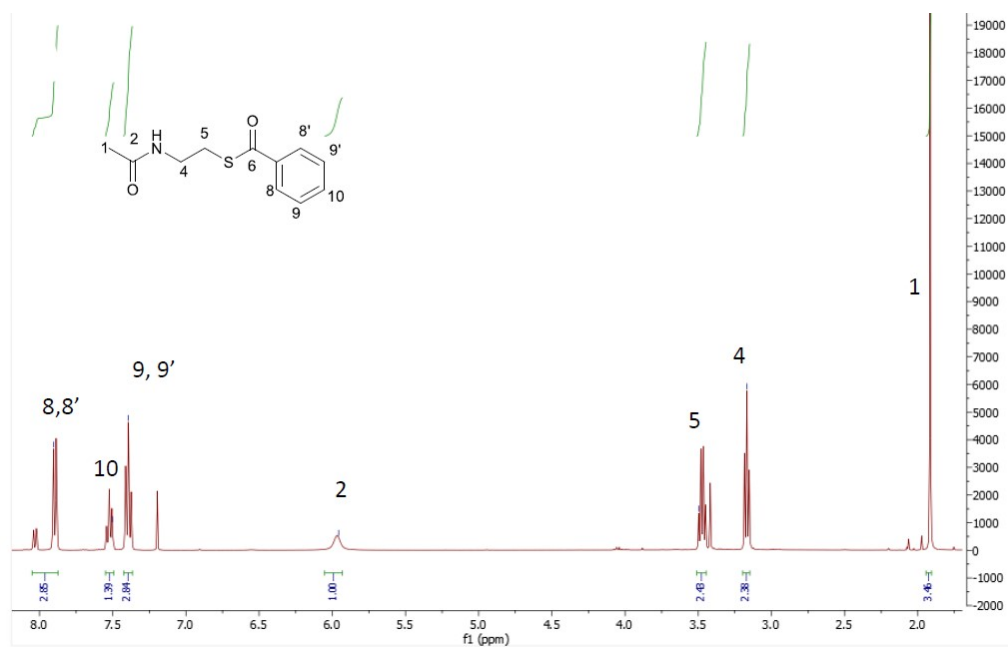


Figure S24. <sup>1</sup>H-NMR of phenyl-SNAC **22** (600MHz, CDCl<sub>3</sub>, Me<sub>4</sub>Si).

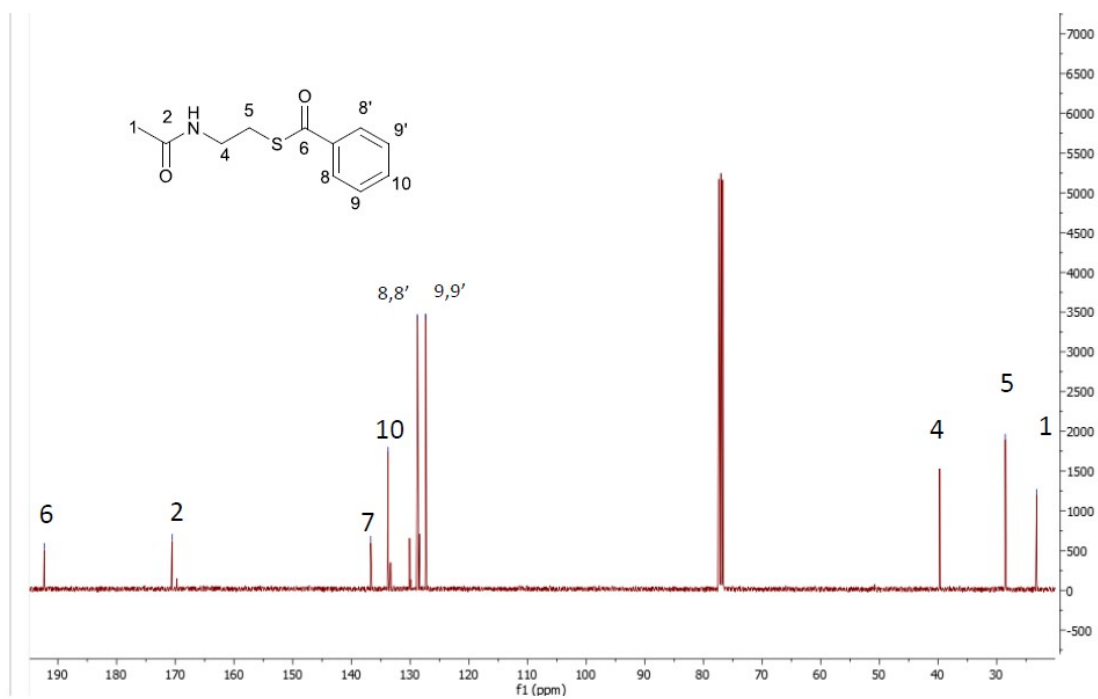
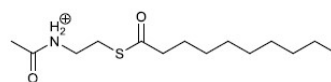


Figure S25.  $^{13}\text{C}$ -NMR of phenyl-SNAC **22** (600MHz,  $\text{CDCl}_3$ ,  $\text{Me}_4\text{Si}$ ).



Figure S26. HMBC spectrum and correlations of phenyl-SNAC **22** (600MHz,  $\text{CDCl}_3$ ,  $\text{Me}_4\text{Si}$ ).



Chemical Formula:  $C_{14}H_{28}NO_2S^+$   
 Exact Mass: 274.1835

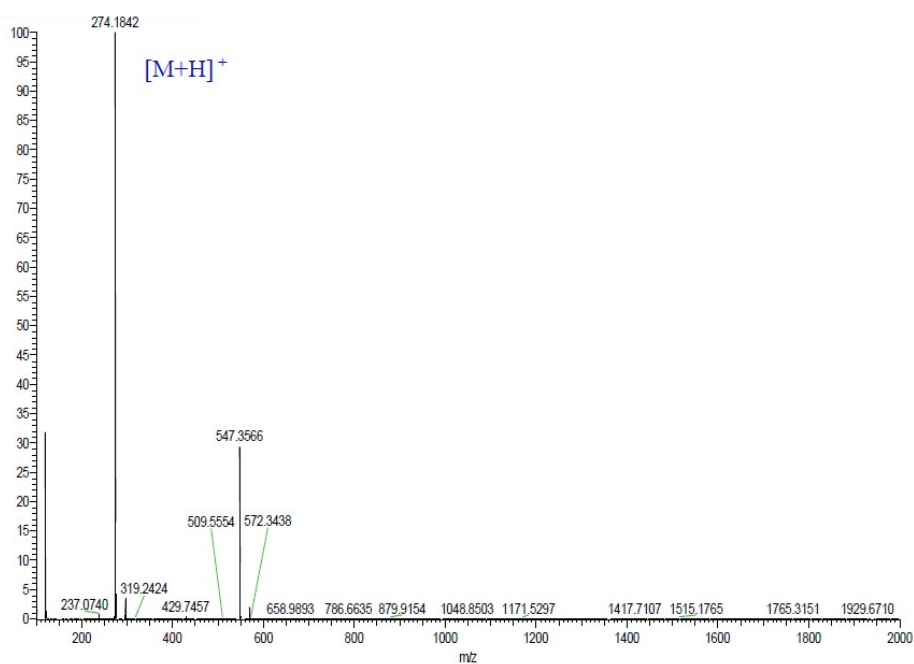


Figure S27. HR-ESIMS of decanoyl-SNAC 23.

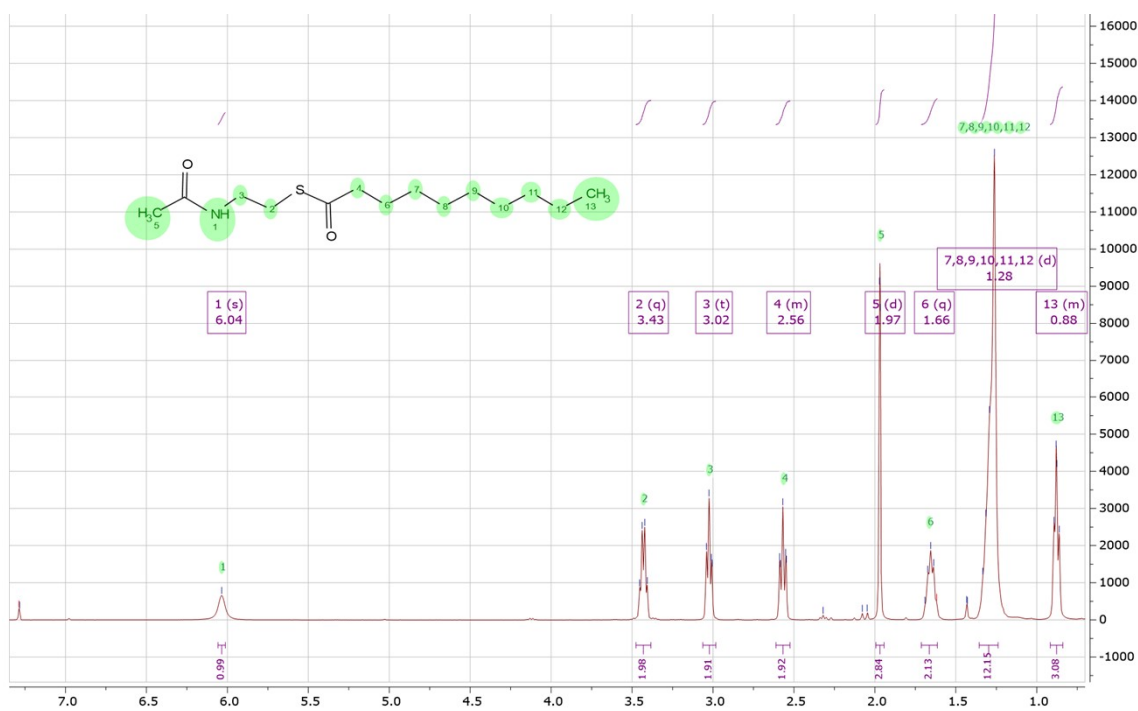
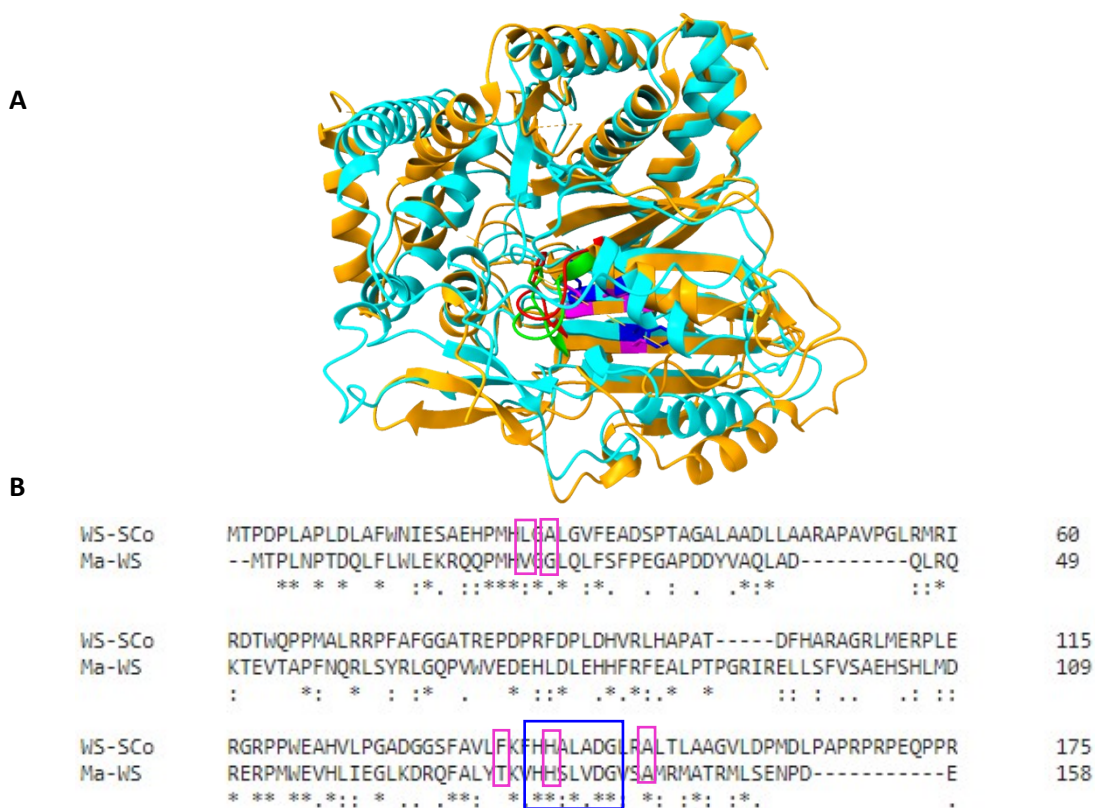


Figure S28.  $^1H$ -NMR of decanoyl-SNAC 23 (600MHz,  $CDCl_3$ ,  $Me_4Si$ ).



**Figure S29.**  $^{13}\text{C}$ -NMR of decanoyl-SNAC **23** (600MHz,  $\text{CDCl}_3$ ,  $\text{Me}_4\text{Si}$ ).



**Figure S30. A.** Overlap of 3D structures for WS-SCo (AlphaFold prediction) and *Ma*-WS (UniProt accession number A1TX06). **B.** Alignment of protein sequence between WS-SCo and *Ma*-WS with highlighted key residues (magenta) and binding pocket (blue).

## References

1. Maglangit F, Alrashdi S, Renault J, Trembleau L, Victoria C, Tong MH, et al. Characterization of the promiscuous: N-Acyl CoA transferase, LgoC, in legonoxamine biosynthesis. *Org Biomol Chem*. 2020 Mar 28;18(12):2219–22.
2. Ellman GL. Tissue Sulfhydryl Groups. *Arch Biochem Biophys*. 1959;82:70–7.