Electronic Supplementary Material (ESI) for Chemical Communications.

Simultaneous synthesis of thioesters and iron-sulfur clusters in water: two universal components of energy metabolism

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1. General Methods

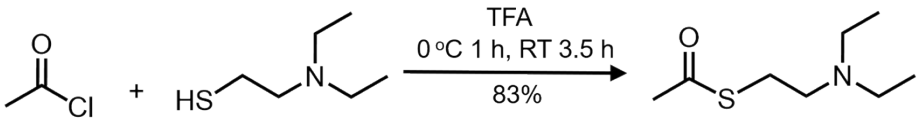
1.1 General considerations. All experiments were carried out in 18.2 MΩ water using a MilliQ (Merck Millipore, Billerica, MA, USA) purification system. All reagents were purchased from Sigma Aldrich (Tokyo, Japan) or TCI (Tokyo Chemical Industry, Japan) and used without further purification.

1.2 Reaction conditions in water. All solutions were degassed with 99.999% N_2 for 2h and transferred into a Coy glovebox, charged with 5% H_2 and 95% N_2 gas mix, at least one day prior to the reaction, resulting in an equilibration with the atmospheric O_2 concentration of <25 ppm. After preparation of fresh 0.1 M $FeCl_2$, $FeCl_3$, 0.2 M Et_3NS or CoM, and 0.5 M potassium thioacetate (98%, Sigma-Aldrich) or thioacetic acid (>95%, TCI) stock solutions in DI water, all reactants were diluted to 5 mM in 100 mM acetic acid buffer (pH 5.5) or 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, pH 7.5) buffer (final buffer concentration 92 mM or 97 mM respectively in case of a metal free reaction). 10ml vials were heated inside a dry water bath to 70 ± 1°C and to avoid water evaporation, sealed with Teflon coated butyl rubber stoppers (AS ONE, Japan). Initial experiments were conducted with 2-mercaptoethane-1-sulfonate at room temperature, but the yields did not exceed 4% in the absence of $FeCl_3$, and with $FeCl_3$ the yield was 7% after 5 days. Heating a 100mM pH 5.5 acetate buffer with Et_3NS at 70°C did not yield any detectable thioester, indicating that acetate in solution did not lead to acetylation of the thiol.

Prior to analysis, the samples were passed through 13mm, 0.22µm Millex-GV PVDF syringe filters (Merck) and diluted 1:5 with MQ water. Details on experimental sampling and quantification are given below.

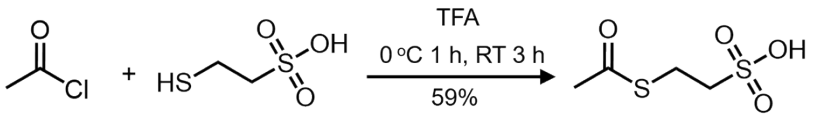
2. Synthesis of thioester standards.

2.1. Standard synthesis of S-(2-(diethylamino)ethyl) ethanethioate ($AcEt_3NS$)



The chloride salt of 2-(diethylamino)ethane-1-thiol (2.5 mmol, >96%, Tokyo Chemical Industry, TCI) was dissolved in 10 ml TFA (99%, Aldrich) at 0°C and 1.1 equivalents of acetyl-chloride (2.75 mmol, 98%, Sigma-Aldrich) were added dropwise to the solution under stirring. After 1h, the reaction temperature was raised to room temperature (25°C) and after stirring for 3.5 h, the reaction mixture was quenched with 0.1 ml methanol, and concentrated under reduced pressure to obtain $AcEt_3NS$ as colorless oil. The crude product was used as a standard without further purification. The yield of $AcEt_3NS$ was calculated from ^{1}H-NMR spectra (**Figure S1**) using 3-(Trimethylsilyl)-1-propanesulfonic acid-d_6 sodium salt (98 atom % D, Isotec) as an internal standard. From this calibration, a 83% yield of the $AcEt_3NS$ was determined. ^{1}H NMR (400 MHz, D_2O): δ 3.19–3.33 (m, 8H, CH_2), 2.41 (s, 3H, CH_3), 1.31 (t, 6H, J = 7.4 Hz, CH_3). ^{13}C NMR (100 MHz, D_2O): δ 200.08 (C=O), 50.75 (CH_2), 48.11 (CH_2), 30.21 (SCH_2), 23.12 (CH_3, Ac), 8.52 (CH_3). ESI-HRMS: m/z $[M + H]^+$ calculated for C8H17NOS: 176.110 m/z; found: 176.114 m/z.

2.2. Standard synthesis of 2-(acetylthio)ethane-1-sulfonic acid (AcCoM)



The sodium salt of 2-mercaptoethane-1-sulfonic acid (2.5 mmol, >96%, TCI) was dissolved in 10 ml 99% TFA at 0°C. Acetyl-chloride (2.75 mmol) was added dropwise to the solution and stirred for 1 h. The reaction was subsequently warmed to room temperature and after stirring for 3h, the reaction mixture was quenched with 0.1 ml methanol, and concentrated under reduced pressure to obtain AcCoM as colourless salt. The concentrated product was used as standard without further purification. The yield was calculated via integration from ^{1}H-NMR spectra (**Figure S3**) using 3-(Trimethylsilyl)-1-propanesulfonic acid-d_6 sodium salt (98 atom % D, Isotec) as an internal standard. With this calibration, a 59% yield of AcCoM was calculated. ^{1}H NMR (400 MHz, D_2O): δ 3.11-3.15 (m, 2H, J = 2.0, 6.0, 11.0 Hz, CH_2), 3.19-3.23 (m, 2H, J = 2.0, 6.0, 11.0 Hz, CH_2), 2.38 (s, 3H, CH_3). ^{13}C NMR (100 MHz, D_2O): δ 200.88 (C=O), 50.63 (CH_2), 30.29 (SCH_2), 23.96 (CH_3, Ac). ESI-HRMS: m/z $[M + H]^+$ calculated for C4H8O4S2: 182.988 m/z; found: 182.984 m/z.

3. Nuclear Magnetic Resonance Spectroscopy

3.1. Identification and Quantification via Nuclear Magnetic Resonance (NMR). ^{1}H and ^{13}C NMR spectra were recorded with a Bruker AVANCE III 400 MHz spectrometer and a 5mm ^{1}H/^{13}C dual probe (400 MHz for ^{1}H, 100 MHz for ^{13}C). Carbon chemical shifts are reported in parts per million (ppm) values on the δ scale, and were typically referenced to trifluoracetic acid and ^{1}H NMR spectra were referenced to the d6-DSS signal (δ = 0.00 ppm). All NMR spectra were recorded at room temperature and were analyzed using Topspin 3.6.1 (Bruker) and MestReNova (MestreLab Research, Santiago de Compostela, Spain).

To determine the concentration of the thioesters synthesized via acetyl chloride, four independent samples were prepared for AcCoM and three samples of $AcEt_3NS$ and dissolved in D_2O with the addition of a known amount of 3-(Trimethylsilyl)-1-propanesulfonic acid-d_6 sodium salt (98 atom % D, Isotec) as an internal standard. A 90° pulse sequence with a calibrated T_1 was used for the integration of the singlet originating from the methyl-residue of the acetyl-group. The total amount of thioester in the synthetic samples were 58.83% (standard deviation 2.65%, n=4) for the AcCoM salt and 83.25% (standard deviation 0.54%, n=3) for the $AcEt_3NS$ oil.

Figure S1 depicts a ^{1}H-NMR spectrum of the crude oil of $AcEt_3NS$ and **Figure S2** the corresponding ^{13}C-NMR spectrum. The ^{1}H-NMR spectrum of the crude salt of AcCoM is shown in **Figure S3** and the ^{13}C-NMR in **Figure S4**.

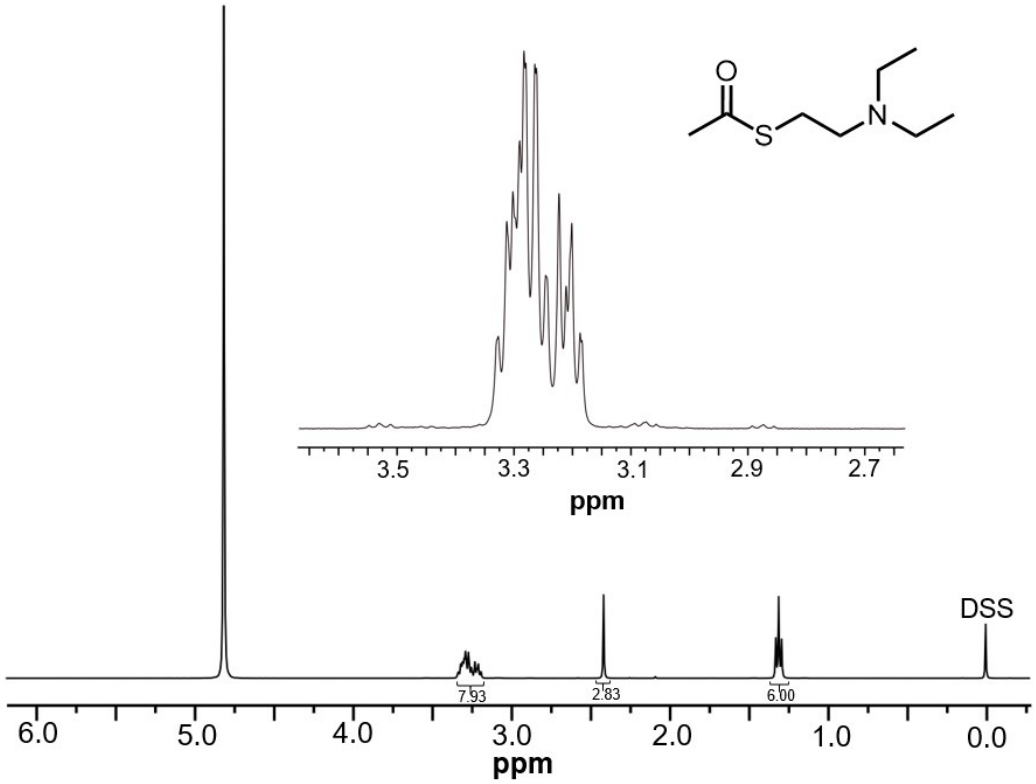


Figure S1 ^{1}H-NMR of $AcEt_3NS$ in D_2O and 3-(Trimethylsilyl)-1-propanesulfonic acid-d_6 as an internal reference. The integrated areas of the peaks are indicated with brackets and are given in reference to the ethyl amine methyl groups.

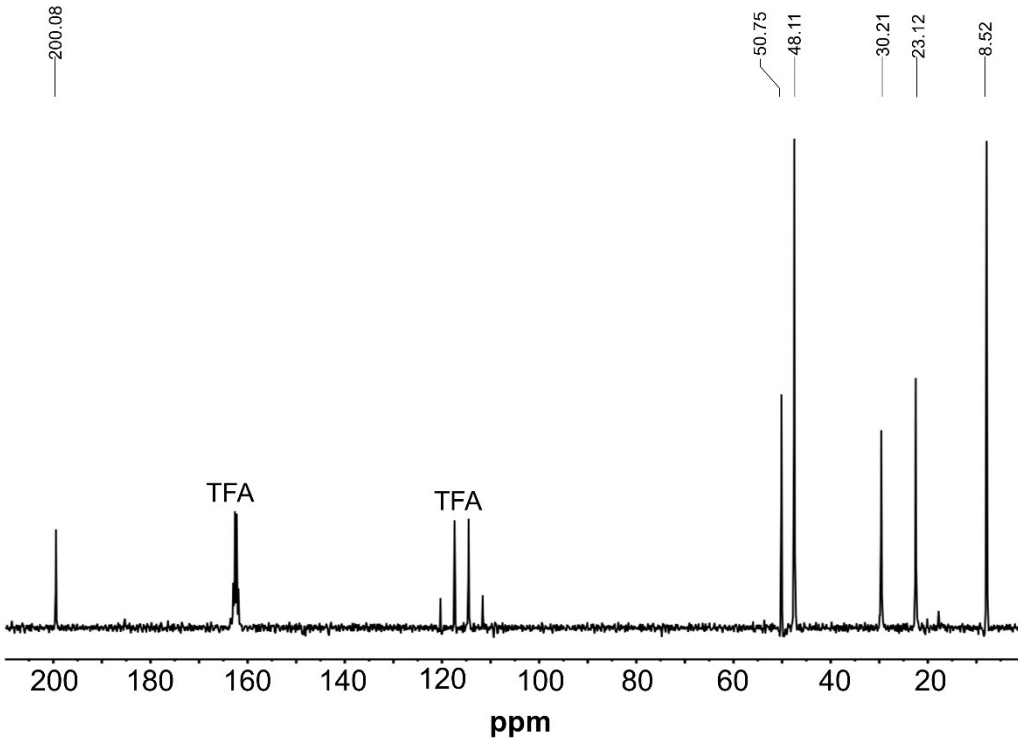


Figure S2 ^{13}C-NMR of $AcEt_3NS$ in D_2O and in the presence of trifluoracetic acid (TFA).

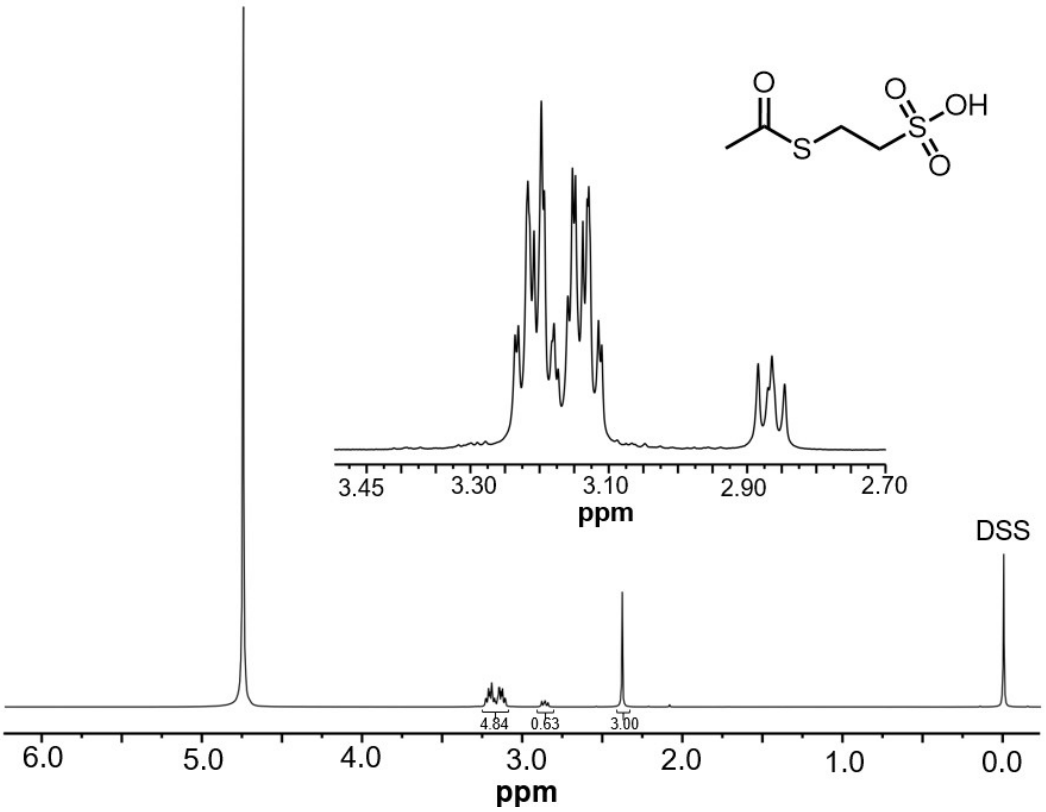


Figure S3 ^{1}H-NMR of AcCoM in D_2O with 3-(Trimethylsilyl)-1-propanesulfonic acid-d_6 as an internal reference. The integrated areas of the peaks are indicated with brackets and are given in reference to the acetyl methyl group.

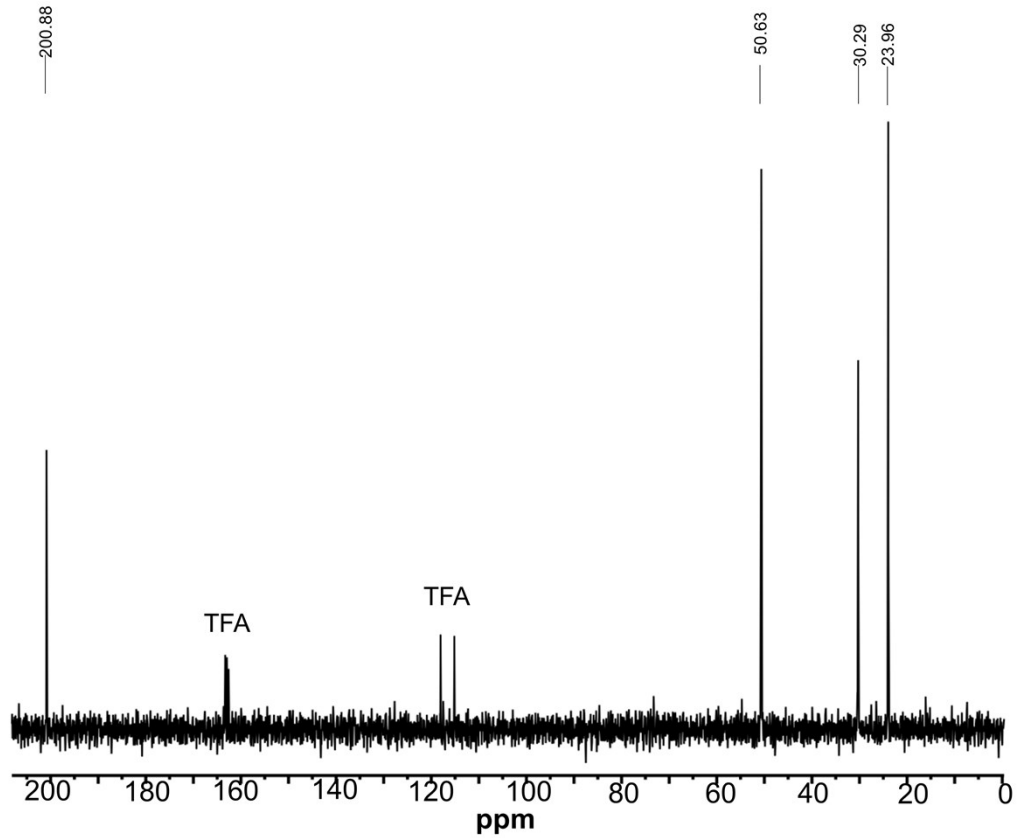


Figure S4 ^{13}C-NMR of the AcCoM reference compound in D_2O and in the presence of trifluoracetic acid.

4. Ultra-Performance-Liquid-Chromatography

Quantification via Ultra-Performance-Liquid-Chromatography (UPLC). The thioester yields were determined through chromatographic separation by an Acquity UPLC H-Class BIO (Waters) equipped with an autosampler, quaternary solvent manager and a photodiode array detector (PDA eλ). For the AcCoM thioester, a similar chromatography condition as previously reported was chosen[1], employing an isocratic gradient of 5% methanol and 95% 5 mM tetrabutylammonium hydrogen sulfate (>98%, TCI) in water at a flow rate of 0.35 ml min^{-1}. The $AcEt_3NS$ thioester concentration was determined using the gradient given in **Table S1**, using 0.1% formic acid in water as solvent A and 100% acetonitrile as solvent B. Both chromatography conditions employed an Acquity UPLC BEH C18 column with 50 mm length. Representative chromatograms for both thioester standards and thioesters produced in aqueous solution are given in **Figure S5** for AcCoM and **Figure S6** for $AcEt_3NS$.

Table S1. Chromatography profile for $AcEt_3NS$

Time	Solvent A	Solvent B
0.00 min	100%	0%
4.00 min	100%	0%
8.00 min	60%	40%
9.00 min	15%	85%
9.30 min	0%	100%
9.48 min	0%	100%
10.00min	100%	0%

For the time course measurements conducted within a time interval of 10h, a maximum of 6 samples were taken from the reaction vials at one time and injected into the UPLC to minimize the production of thioesters at room temperature and the influence of oxygen. Therefore, measurement intervals of 1h were chosen to analyze 3 different reaction conditions, prepared in duplicates, within the given time frame to allow consistent experimental conditions throughout the measurement series.

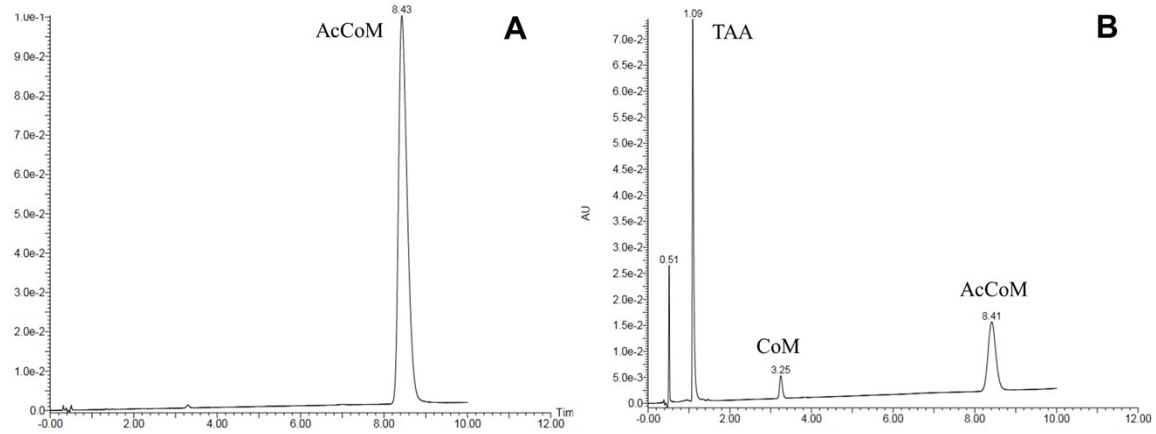


Figure S5 UPLC chromatograms of the synthetic AcCoM standard (8.43min) at 230nm (**A**) and a reaction of thioacetic acid with CoM at 70°C (**B**).

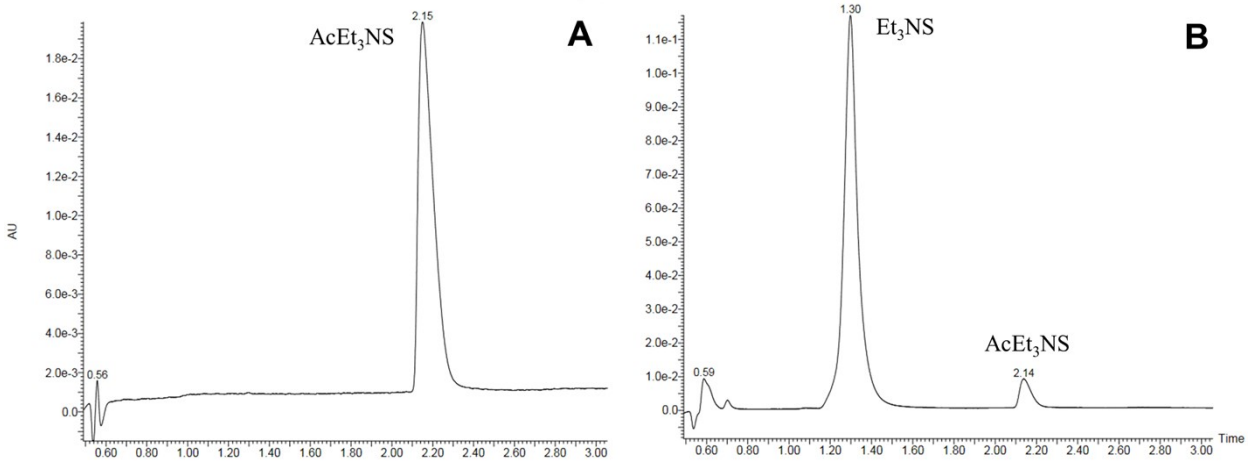


Figure S6 UPLC chromatograms at a 229nm wavelength of the $AcEt_3NS$ reference compound at 2.15min (**A**) and a reaction at 70°C between Et_3NS and thioacetic acid (**B**).

The thioesters were quantified using their characteristic absorbance at 228nm for $AcEt_3NS$ and 229nm for AcCoM and the thioester yields in aqueous solution were calculated from standard curves, in which each concentration of the dilution series was prepared in triplicates (**Figure S7**).

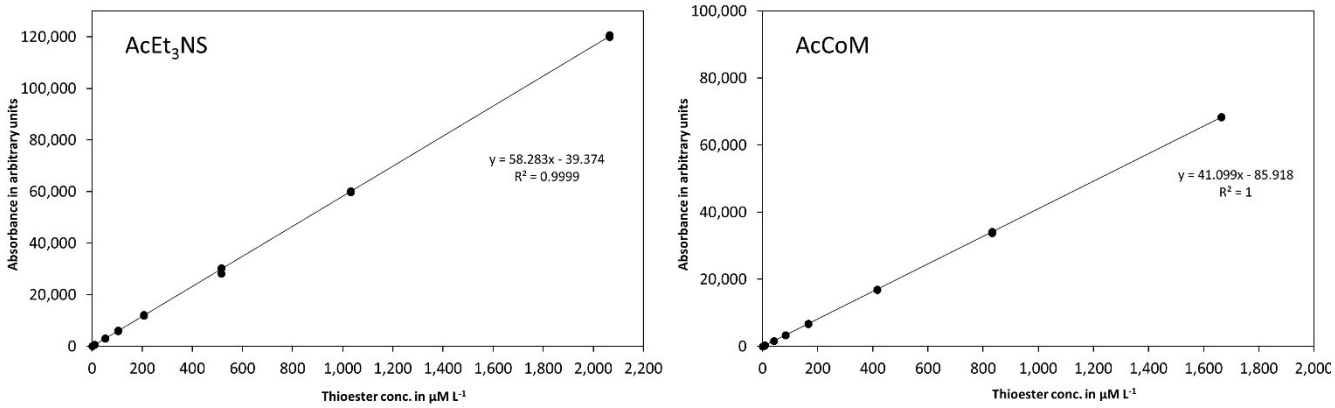


Figure S7 Calibration curves of $AcEt_3NS$ and AcCoM generated from a dilution series of their synthetic standards. Linear regressions were calculated using the LINEST function of Microsoft Excel and the linear functions and the corresponding coefficients of determination of the least square fit are displayed.

5. Liquid chromatography mass spectrometry.

5. Time of flight mass spectrometry (TOF-MS). High-resolution mass data analyses were performed on a Xevo G2-XS Q-TOF-MS platform (Waters Corporation, Manchester, UK) operated in electrospray ionization mode, combined with an Ultra-Performance Liquid Chromatography (UPLC) system (Acquity H-class; Waters Corporation, MA, USA). Liquid chromatography was carried out on an UPLC BEH C18 column (1.7 µm, 2.1 mm x 100 mm, Waters) using chromatography conditions as described for $AcEt_3NS$ (See Section **4.**, Ultra-Performance-Liquid-Chromatography). The measured mass was calibrated during the sample measurement against Leu-enkephalin as a lock mass.

Mass spectra of the reference compound AcCoM and a reaction of CoM and thioacetic acid are shown in **Figure S8**. These spectra were measured in negative ion mode with the following settings: Capillary voltage 2.5kV, Sampling cone 90.0, source temperature 100°C, desolvation temperature 300°C, cone gas flow 100 L h^{-1}. Selecting the parent ion of 182.9 m/z for a MS-MS measurement at a collision energy of 21 eV, the following major mass fragments were obtained: 74.992 m/z, 80.967 m/z and 140.972 m/z (**Figure S8**). The 95% confidence band for the calibration of the mass range from 50 to 1200 m/z with sodium formate was found to be 1.0 ppm and the residual 0.2 ppm (0.1mDa). Expected mass: 182.988 m/z, mass found in the total ion chromatogram: 182.984 m/z.

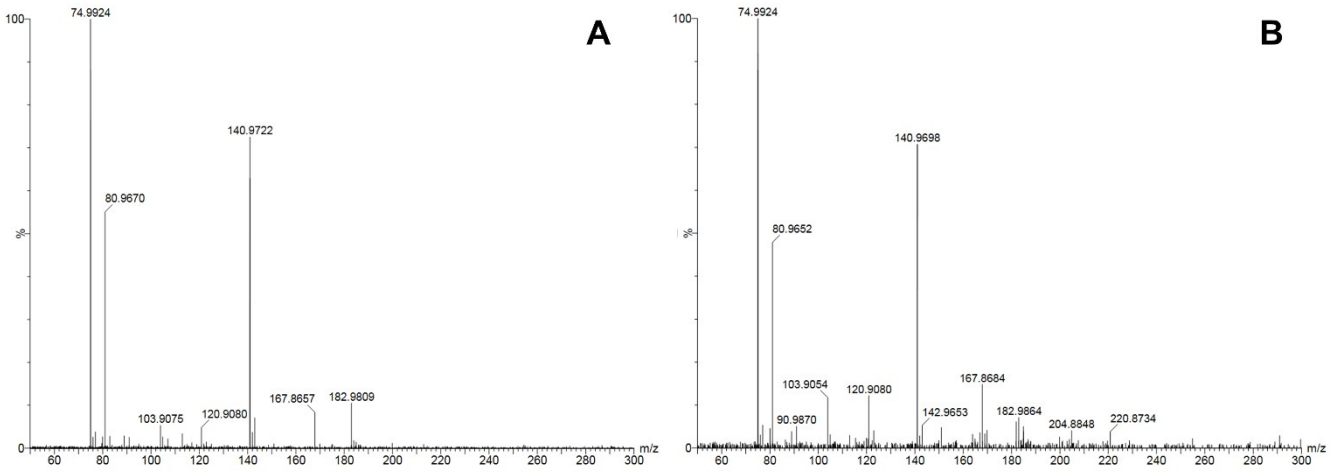


Figure S8 MS-MS spectrum of the AcCoM reference compound (**A**) and CoM and thioacetic acid reacted at 70°C for 4 days (**B**).

The high-resolution mass spectra for $AcEt_3NS$ were measured in positive ion mode with the following settings: Capillary voltage 3.0kV, Sampling cone 10.0, source temperature 100°C, desolvation temperature 250°C, cone gas flow 100 L h^{-1}. With a collision energy of 15eV, the following major fragments were observed selecting the expected mass of $AcEt_3NS$ for MS-MS analysis: 74.099 m/z, 103.025 m/z and 134.103 m/z (**Figure S9**). The 95% confidence band for this measurement was

determined to be 0.4 ppm and the residual mass 0.3 ppm (0.2 mDa). The expected mass was 176.110 m/z, and 176.114 m/z was measured.

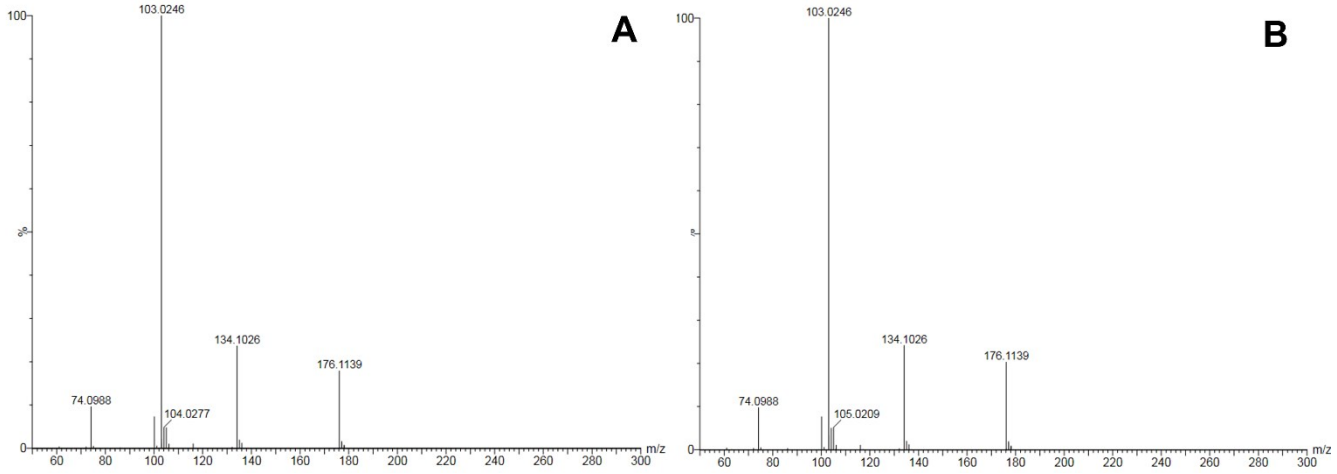


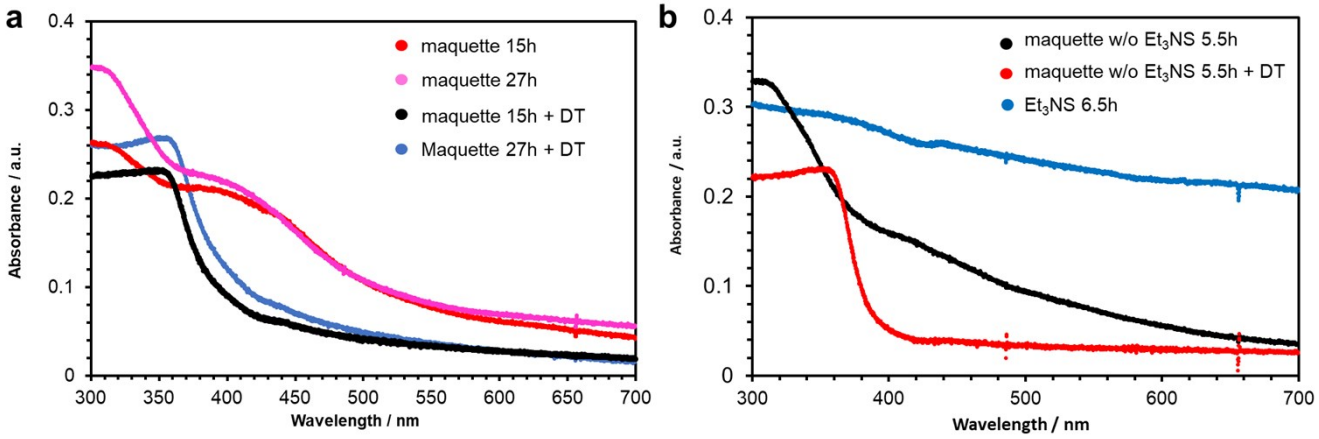
Figure S9 MS-MS spectrum of the synthetic standard of $AcEt_3NS$ (**A**) and Et_3NS and thioacetic acid reacted at 70°C for 3h (**B**).

6. UV-VIS-spectrophotometry

6. UV-VIS-spectrophotometry of [FeS] clusters. Based on an earlier study of minimum binding sequences of [4Fe4S] clusters[2], the C-terminal amidated peptide (91%, GenScript, Japan) with the sequence DLCEGGCIACGACGGD was used as a model iron-sulfur cluster binding peptide. For the FeS cluster reconstitution, Et_3NS, $FeCl_3$ and potassium thioacetate with final concentrations of 300 µM were added to 2.2 mg (~750 µM) of the above peptide and heated to 70°C in a 100 mM HEPES buffer at pH 7.5. UV-Vis spectra were acquired in a quartz cuvette under anaerobic conditions with an AvaSpec-ULS4096CL-EVO (Avantes) spectrophotometer and measurements were acquired at different time points, with and without the addition of Et_3NS or the maquette (**Figure S10**). For the reduction of the FeS cluster, sodium hydrosulfite (>82%, Sigma-Aldrich) was added to achieve a final concentration of 5 mM hydrosulfite (DT).

Whereas previous work with this peptide established the incorporation of a [4Fe4S] species after reconstitution with excess iron and sulfide,[2] our experiments contained the peptide in excess, perhaps leading to the formation of a mixture of cluster species, although future work is needed to investigate this.

Figure S10 UV-Vis spectra of 300 µM $FeCl_3$ and TAA reacted in the presence of a Ferredoxin maquette and 300 µM Et_3NS (**a**) and in the absence of Et_3NS or the maquette (**b**). The spectra in (**a**) were taken after 15h with (blue) and without (red) the addition of hydrosulfite (DT) and similarly after 27h in a separate reaction with (blue) and without DT (magenta). UV-Vis spectra without the addition of 300µM Et_3NS are displayed in (**b**) after 5.5h (black) and after adding 5mM DT (red). A measurement of the reaction of 300 µM $FeCl_3$, TAA and Et_3NS is displayed in blue.



7. Additional reaction rate measurements

Reaction yields of AcCoM at an extended timescale. The buffer pH and concentration, reactant concentrations and temperature match those give in **Section 1.2**. The yields of AcCoM are depicted in percent yield relative to the initial amount of CoM (5 mM) in **Figure S11**. The experiments were conducted in duplicates and the average yields of two experiments are connected to a trend line.

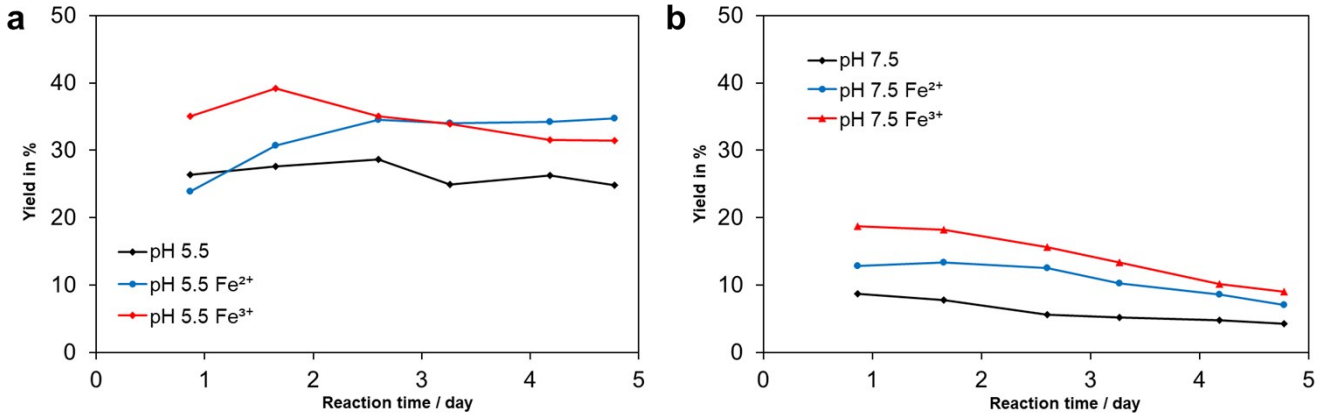


Figure S11 Time course measurement of the AcCoM thioester from thioacetate (5mM) expressed in percent yield respective to the amount of CoM (5 mM) at pH 5.5 (**a**) and pH 7.5 (**b**). The red trace corresponds to a reaction in which $FeCl_3$ was added, blue $FeCl_2$ and black contained only CoM and TAA.

Reactions presented in the main text (Figure 1 and Figure 2) and in this section were conducted as duplicates. Table S2 depicts the ranges of thioester yields that were measured via UPLC in regards to the maximum yield obtained by using either CoM or Et_3NS and with and without the addition of $FeCl_2$ or $FeCl_3$ at 70°C.

Table S2. Highest obtained thioester yields in percent relative to the initial thiol concentration and the reaction time after which these yields were measured in hours.

Experiment	No metal addition	Fe^{2+}	Fe^{3+}
AcCoM pH 5.5	28.64 ± 2.22 % (62h)	34.71 ± 0.25 % (115h)	39.23 ± 2.61 % (40h)
AcCoM pH 7.5	8.69 ± 0.69 % (21h)	13.36 ± 0.15 % (21h)	18.74 ± 1.08 % (40h)
$AcEt_3NS$ pH 5.5	20.85 ± 0.05 % (1h)	29.02 ± 0.79 % (5h)	48.37 ± 1.35 % (1h)
$AcEt_3NS$ pH 7.5	6.29 ± 0.44% (1h)	5.49 ± 0.04 % (1h)	24.93 ± 0.81 % (1h)

Reaction rates at lower thioacetic acid concentrations

Following the sample preparation for the thioester synthesis at 70°C, additional reactions were run with a thioacetic acid (96% Sigma-Aldrich) concentration of 2.3 mM and 5 mM of Et_3NS, CoM and with and without 5 mM $FeCl_2$ or $FeCl_3$. The results for the reaction rate measurements are given in **Figure S12**.

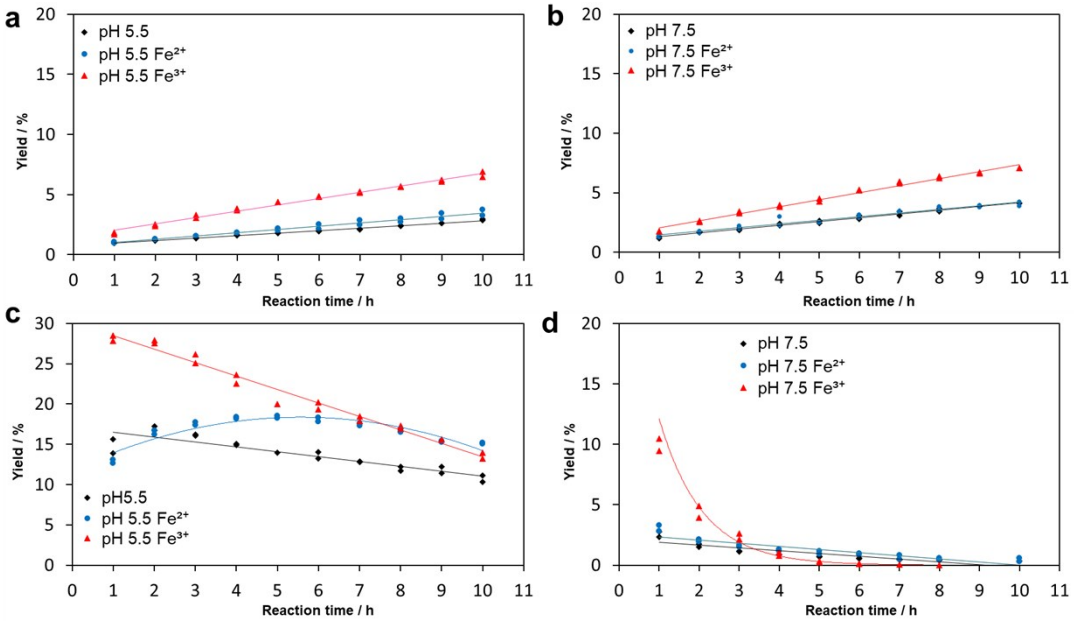


Figure S12 Time course measurement of the yields of AcCoM (**a** and **b**) and $AcEt_3NS$ (**c** and **d**) with an initial thiol concentration of either 2.3 mM (pH 5.5) or 5 mM (pH 7.5). The reactions without the addition of an iron chloride are depicted as black traces. Addition of $FeCl_2$ as blue, and addition of $FeCl_3$ as red. Reactions in the main text (Figure 1 a, b and Figure 2 a, b) contained 5mM thiol, and for comparison for the data series containing Et_3N, reactions at a 2.3 mM thiol concentration are given here. Data points from duplicate experiments are shown.

8. References

[1] Z. Apostolides, N. M. J. Vermeulen, D. J. J. Potgieter, High-performance liquid chromatography of some coenzyme M (2-mercaptoethanesulphonic acid) derivatives by ion pairing on reversed-phase columns. *Journal of Chromatography A*, **1982,** 246, 304–307.

[2] B. R. Gibney, S. E. Mulholland, F. Rabanal, P. L. Dutton, *PNAS* **1996**, *93*, 15041–15046.