Supplementary information

for

Catalytic Degradation of *N*-Acyl Homoserine Lactone Using Copper Complex of TACN Derivative: Implications for Quorum Sensing Interference

Denisa Skurková[1,2] & Hanna Zhukouskaya[1,3], Andrii Mahun[1], Michaela Buziková[4], Lívia Kanizsová[1], Miroslav Vetrík[1], Jan Kotek[4], Martin Hrubý[1]*, Tomáš Tobrman[2]*

[1] Institute of Macromolecular Chemistry, Czech Academy of Sciences, Heyrovského náměstí 2, 162 06 Prague 6, Czech Republic

[2] Department of Organic Chemistry, Faculty of Chemistry and Technology, University of Chemistry and Technology, Prague, Technická 5, 166 28 Prague 6, Czech Republic

[3] Department of Physical and Macromolecular Chemistry, Faculty of Science, Charles University, Hlavova 8, Prague, 128 43, Czech Republic

[4] Department of Inorganic Chemistry, Charles University, Hlavova 8, 128 40 Prague 2, Czech Republic

* Correspondence: mhruby@centrum.cz (M.H.); Tel.: +420-296-809-130; tomas.tobrman@vscht.cz (T.T.); Tel: +420 220 444 245

Contents

1. Conditions of measurements

1.1. Experimental conditions of NMR measurements

^{1}H NMR experiments. The ^{1}H NMR spectra were recorded at 25 °C on a Bruker Avance III 600 (14.1 T) spectrometer operating at $v(^{1}H)$ of 600.13 MHz. Samples were prepared in deuterated solvents. The spectra were acquired using a 90^{o} pulse (width = 18 ⌾s) with a recycle delay of 10 s and 64 scans. The chemical shifts were calibrated relative to TMS using hexamethyldisiloxane (HMDSO, 0.05 ppm from TMS in ^{1}H spectra) as an external standard. In the case of *in-situ* NMR kinetics measurements "zgdelay" pulse sequence was used employing a 90^{o} pulse (width = 18 ⌾s) with a recycle delay of 10 s, and 8 scans.[S1]

^{13}C NMR experiments. The ^{13}C NMR spectra were recorded at 25 °C on a Bruker Avance III 600 (14.1 T) spectrometer operating at $v(^{13}C)$ of 150.90 MHz. Samples were prepared in deuterated solvents. The spectra were acquired using a 90^{o} pulse (width = 14 ⌾s) with a recycle delay of 10 s and 6000 scans. The inverse-gated decoupling was used for removal of ^{1}H-^{13}C heteronuclear interactions. The chemical shifts were calibrated relative to TMS using hexamethyldisiloxane (HMDSO, 2.0 ppm from TMS in ^{13}C spectra) as an external standard. The ^{13}C -APT and DEPT spectra were acquired under standard conditions using 90° pulses (pulse width = 18 μs) and optimized delays for polarization transfer (typically based on a J_{CH} value of 145 Hz). A recycle delay of 2 s and 128 scans were used.

^{1}H-^{13}C HSQC experiments. The 2D ^{1}H-^{13}C multiplicity edited HSQC NMR experiments were recorded at 25 °C on a Bruker Avance III 600 (14.1 T) spectrometer. The spectral window of 16 ppm in the direct dimension (F2) and 240 ppm in the indirect dimension (F1) was used. The number of transients in the indirect dimension was 256, each with 160 scans and 10 s recycle delay. In the acquired spectra the phase of the 2D NMR signals from -CH_2 groups is opposite to the phase of the signals yielded by -CH_3 and -CH groups (denoted by a different color of the respective signals)

All the NMR spectra obtained by the above-described experiments were processed and analyzed using Bruker TopSpin 4.5.0 software.

1.2. Experimental conditions of GC/MS and MALDI-TOF spectrometry.

ESI-MS mass spectrometry measurements for establishing the molecular mass of compounds were determined on an LCQ Fleet mass analyser with electrospray ionisation (Thermo Fisher Scientific; Waltham, MA, USA).

MALDI-TOF mass spectrometry was performed on an ultrafleXtreme TOF–TOF instrument (Bruker Daltonics, Germany) equipped with a 2000 Hz smartbeam-II laser (355 nm), operated in positive-ion reflectron mode with panoramic pulsed ion extraction. Deposition of the samples onto the target plate was done by the dried droplet method. A volume of 0.5–1 μL of the mixture was deposited on the target plate. External calibration was used.

Acetonitrile (Honeywell, Germany) was used as a solvent both for the sample (ADC or MAHL; concentration of the sample was 10 mg mL^{-1}), and 2,5-dihydroxy benzoic acid (20 mg mL^{-1}) as a matrix. The mixing volume ratio sample:matrix was 1:5.

MilliQ purity water (Milli-Q® IQ 7000; Merck KGaA, Darmstadt, Germany) was used as a solvent for preparation of mixture of the sample (hydrolytic products of NAHL) with 2,5-dihydroxybenzoic acid (Sigma–Aldrich, 98%, 20 mg mL^{-1}) as a matrix and sodium chloride (NaCl; Sigma–Aldrich, 10 mg mL^{-1}) as an ionizing agent. The mixing volume ratio sample:matrix:ionizing agent was 4:20:1.

To distinguish the peak of matrix from the phenylacetic acid peak derivatization was used. The 2nd hydrolysis product sample was weighed into a glass vial and left to evaporate. Two-fold volume of *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA, LiChropur™, ≥99.0 %, Sigma Aldrich, Switzerland) was added into vial. The sample was derivatized at ambient temperature for 30 min and then subjected to MALDI TOF analysis.

GC/MS – Gas chromatography coupled with mass spectrometry was used for compound analysis. Samples were dissolved in acetonitrile (Honeywell, Germany) and derivatized with *N,O*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA, ≥99.0%, Sigma-Aldrich). Analysis was performed using a Clarus 680 GC coupled with a Clarus SQ 8 T MS (PerkinElmer, USA) and a DB-35MS capillary column (30 m × 0.25 mm, 0.25 μm film). Helium (1 mL/min) was used as the carrier gas. Injection volume was 0.5 μL, with splitless mode for 0.25 min, then split flow at 50 mL/min. The oven was programmed from 200 °C (1 min) to 340 °C at 10 °C/min, held for 5 min. The interface and EI source were set at 300 °C and 190 °C, respectively. Mass spectra (m/z 15–620) were identified using the NIST library and Spectrus (ACD/Labs).

2. UHPLC kinetic studies of MAHL and NAHL

2.1. UHPLC kinetic studies of MAHL and Cu(II)-*i*Pr$_2$-TACN complex catalysis

All compounds were first measured as standards in UHPLC using mobile phase A (H_2O/ACN/TFA; 95/5/0.1) to establish retention times and spectral characteristics, as you can see in **Figure S18**. These included the starting material model **(B)** Model *N*-acyl homoserine lactone (MAHL), which undergoes hydrolysis in the presence of the **(A)** Cu(II)-*i*Pr$_2$-TACN complex catalyst, and the **(C)** Azo Dye Chromophore (ADC), expected hydrolysis products and precursor used in MAHL synthesis. The observed retention times (t_r) and absorbance maximum (λ_{max}) were as follows: **(A)** Cu(II)-*i*Pr$_2$-TACN complex catalyst eluted between t_r=1.4–2.3 min. (overlapping with the solvent peak), (**B)** MAHL showed a t_r=6.2 min. with a λ_{max}=524 nm, and **(C)** ADC eluted at t_r=6.7 min., also with a λ_{max}=524 nm.

To ensure that hydrolysis was promoted specifically by the Cu(II)-*i*Pr$_2$-TACN complex and not merely by the presence of water, acidic or basic conditions, or heating, we performed a stability test of MAHL in TRIS/MES buffers at pH 5.0, 6.5, and 7.4, each subjected to heating at 50 °C. As shown in **Figure S19**, at **(A)** pH 5.0, no hydrolysis products were observed, likely due to the mildly acidic environment maintaining an equilibrium that favours the closed form

of the lactone ring. At **(B)** pH 6.5, a minor degradation product appeared after 48 hours at a t_r= 5.7 min., corresponding to the 1st hydrolysis product, an opened form of lactone ring. Finally, at **(C)** pH 7.4, both the 1st t_r= 5.7 min and 2nd t_r= 5.2 min hydrolysis products were detected after 48 hours. These results demonstrate the high stability of MAHL under buffered conditions over the course of two days.

The shift in t_r of the 2nd hydrolysis product, ADC, from 6.7 min to 5.2 min, along with the hypsochromic (blue) shift in the absorption maximum from λ_max = 524 nm to 514 nm, may reflect protonation of the para-dimethylamino group on the benzene ring of ADC under buffered conditions. This shift is primarily attributed to protonation of the aromatic dimethylamino group.

2.2. Qualitative comparison of MAHL and NAHL Cu(II)-*i*Pr$_2$-TACN complex catalysed hydrolysis with PGA (Penicilin G-acylase) enzymatic degradation

To ensure that hydrolysis was promoted specifically by the Cu(II)-*i*Pr$_2$-TACN complex and not merely by the presence of water, or heating conditions, we performed a stability test in same conditions as experiment (Tris buffer at 7.4, subjected to heating at 50 °C) but without the presence of of Cu(II)-*i*Pr$_2$-TACN complex. As shown in **Figure S22.**, at **(A)** pH 7.4, a minor degradation product appeared after 24 hours at a t_r= 4.2 min., corresponding to the 1st hydrolysis product, an opened form of lactone ring. These results demonstrate the high stability of NAHL under buffered conditions over the course of two days. Observance of new signals emerging in presence of Cu(II)-*i*Pr$_2$-TACN, **Figure S22** at **(B)** suggested degradation of NAHL. As signals of the 1st and 2nd hydrolysis product peaks gradually merged after 24 hours as t_r=3.6-4.2 min, it was not possible to analyse them separately by ESI-MS to establish structure of degradation products.

For the 1st hydrolysis product, standard NAHL (1 mg) was dissolved in 500 µL of 2 mM NaOH and heated at 50 °C for 30 min to 1 h. The reaction mixture was then analysed by UHPLC and MALDI-TOF to monitor the formation of the 1st hydrolysis product. For the 2nd hydrolysis product, 50 µL of 10 mM HCl was added to 250 µL of the solution obtained from the 1st hydrolysis step, or HCl was titrated until the appearance of the 2nd hydrolysis product was observed by UHPLC. The mixture was subsequently heated at 50 °C for an additional 30 min to 1 h (or longer if necessary). Reaction progress and product formation were monitored by UHPLC and MALDI-TOF. To distinguish the phenylacetic acid peak (2nd hydrolysis product) from the matrix signal in MALDI-TOF analysis, derivatization was performed. The reaction mixture was evaporated to dryness in a glass vial, followed by the addition of a two-fold volume of *N,O*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA, LiChropur™, ≥99.0%, Sigma-Aldrich, Switzerland). Derivatization was carried out at ambient temperature for 30 minutes prior to MALDI-TOF measurement.

We decided to use the experimentally determined retention time of NAHL (4.7 min) for all further calculations, even when the peak was sometimes shifted by 0.1-0.2 min (resulting

in the retention time of 4.6-4.8 min). It was done for better representation of experimental results and did not influence the calculations of kinetic parameters. This shift could be once again resolving from either TRIS/MES buffer solutions or Cu(II)-*i*Pr_2-TACN complex or, as mentioned before.

3. NMR studies of hydrolytic products of MAHL, and SAHL

For 1st hydrolysis product standard SAHL (10 mg) was dissolved in 1 mL of 2 mM NaOH prepared in deuterated water (D_2O). If a more concentrated NaOH stock was used for dilution, D_2O was employed as the diluent. The solution was heated at 50 °C for 30 min. The formation of the 1st hydrolysis product was monitored by ^1H NMR spectroscopy (Figure S26 (b)). For the 2nd hydrolysis product an aliquot (500 μL) of the solution from the 1st hydrolysis was transferred to an NMR tube, and 100 μL of 10 mM deuterated hydrochloric acid (DCl in D_2O) was added. The mixture was heated at 50 °C for an additional 30 min. No significant changes were observed (Figure S26 (c)) apart from a slight change in chemical shift of the signals due to the change of pH. The spectrum of neat benzyl alcohol was used for comparison and its identification in the degradation mixture. The signal 4'' (at 4.65 ppm) was used as a marker of this compound. Since this signal was not found in the degradation mixture (Figure S26 (c)) it might be concluded that the second step of hydrolysis didn't occur. A spectrum of the degradation products was recorded at 25 °C (Figure S27 (a)) and compared with that after addition of 10 μL of benzyl alcohol (Figure S27 (b)) to confirm its absence in the degradation mixture.

4. Figures and Schemes

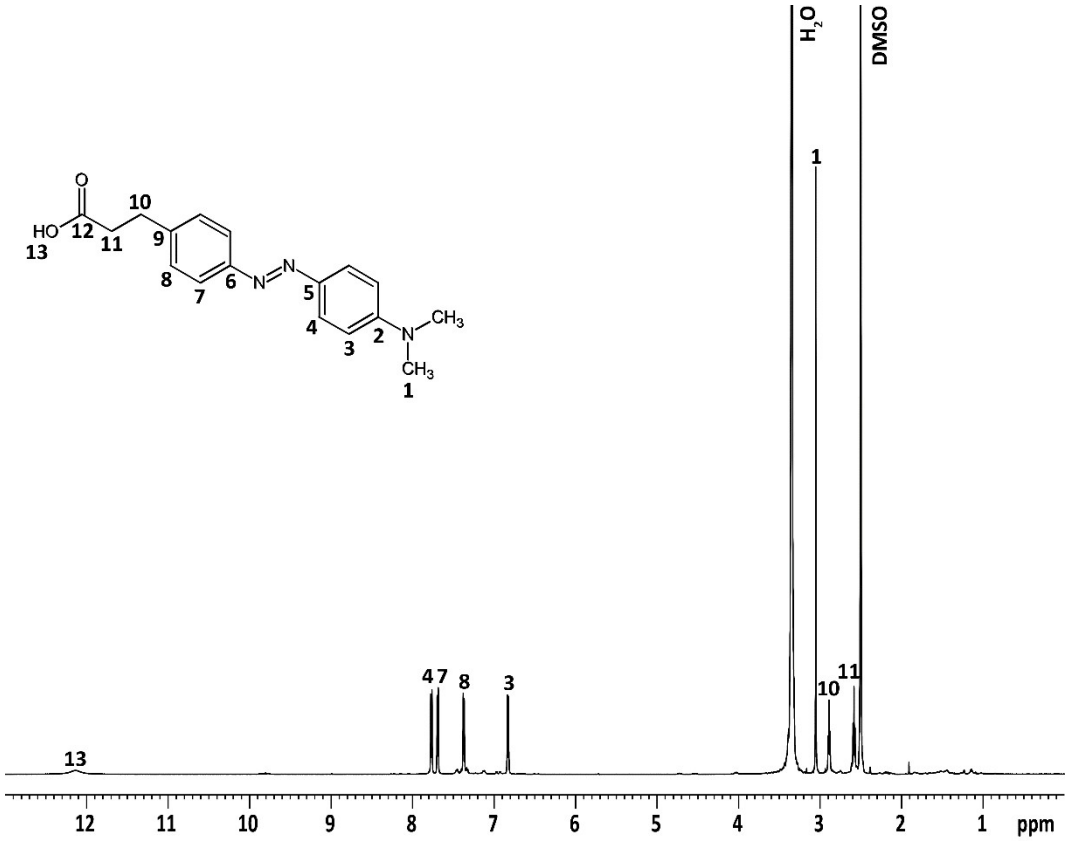


Figure S1. ^{1}H NMR spectrum of azo dye chromophore (ADC) in DMSO-d_6.

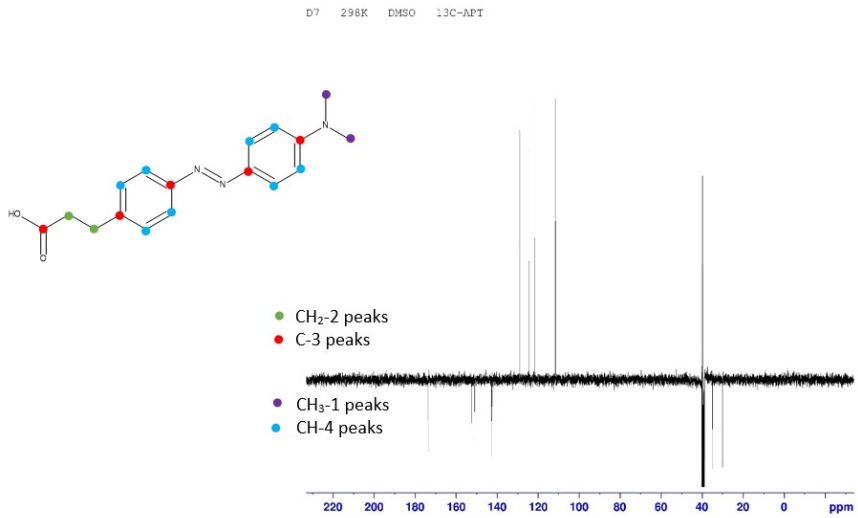


Figure S2. ^{13}C-APT-NMR spectrum of azo dye chromophore (ADC) in DMSO-d_6.

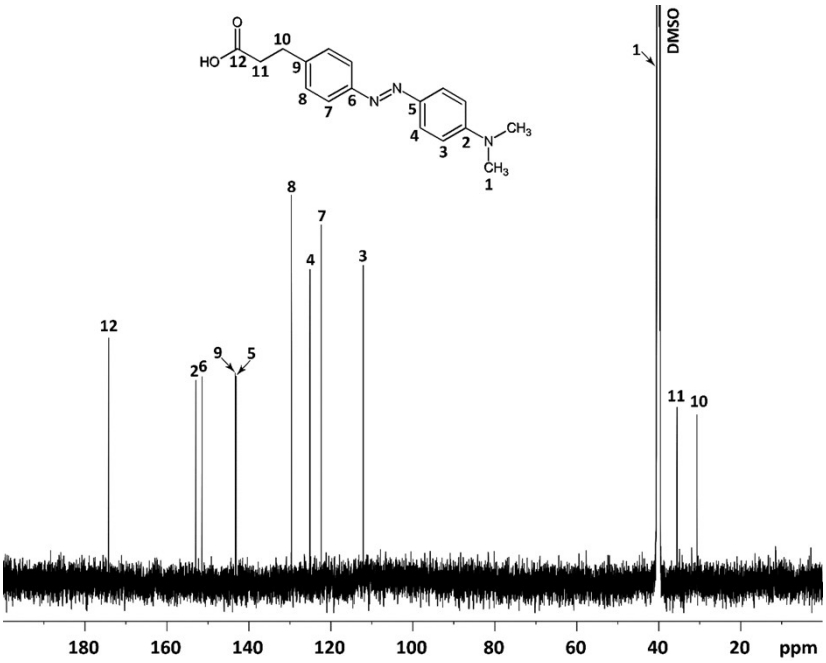


Figure S3. ^{13}C NMR spectrum of azo dye chromophore in DMSO-d_6.

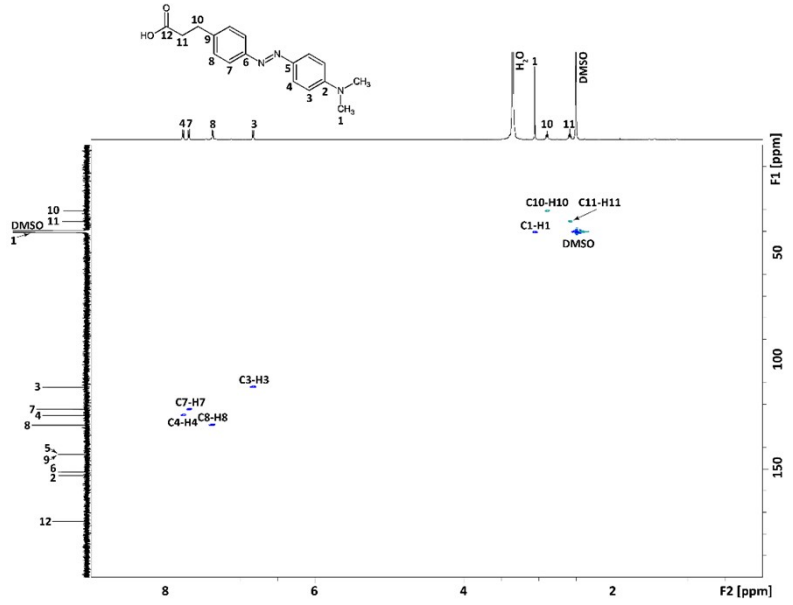


Figure S4. ^{1}H -^{13}C HSQC NMR spectrum of azo dye chromophore in DMSO-d_6. Blue cross peaks indicate CH and CH_3 groups and turquoise cross peaks indicate CH_2 groups.

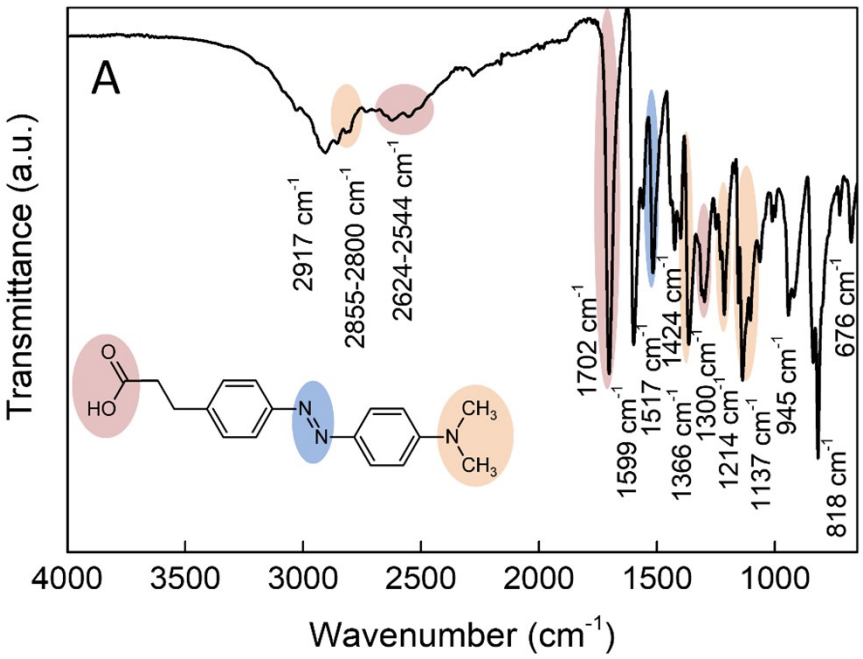


Figure S5. ATR-FTIR spectrum of ADC.

ADC: ν(N-H) = 3301 cm^{-1}, ν(-CH_2-) = 2925 cm^{-1}, ν ($N(CH_3)_2$) = 2855–2800 cm^{-1}, ν(lactone C=O) = 1777 cm^{-1}, ν(amide C=O) = 1644 cm^{-1}, δ(aromatic C-H) = 1600 cm^{-1}, ν(amide C-N) = 1547 cm^{-1}, ν(N=N) = 1516 cm^{-1}, ν(aromatic ring) = 1444 cm^{-1}, ν($N(CH_3)_2$) = 1361 cm^{-1}, ν(amine C-N) = 1223–1140 cm^{-1}, δ(aromatic C-H) = 1012 and 820 cm^{-1}; δ(N-H) = 772 cm^{-1}

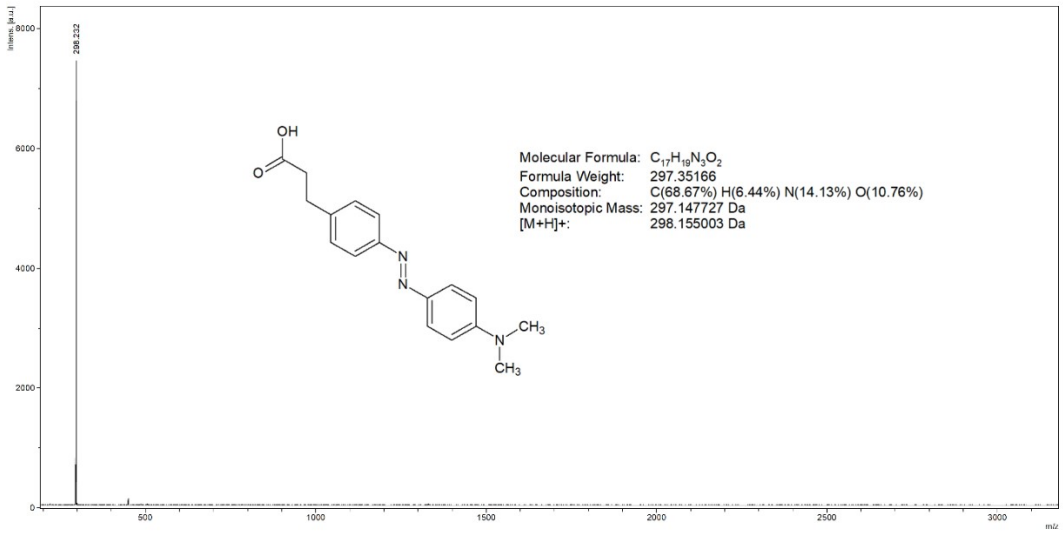


Figure S6. MALDI-TOF spectrum of ADC.

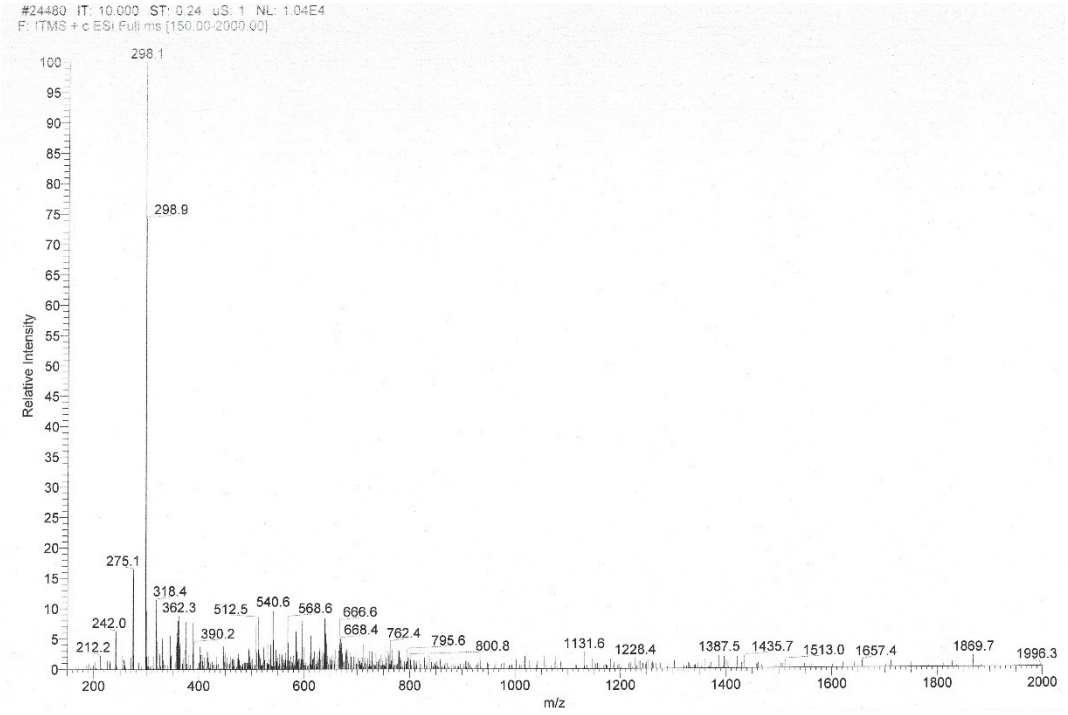
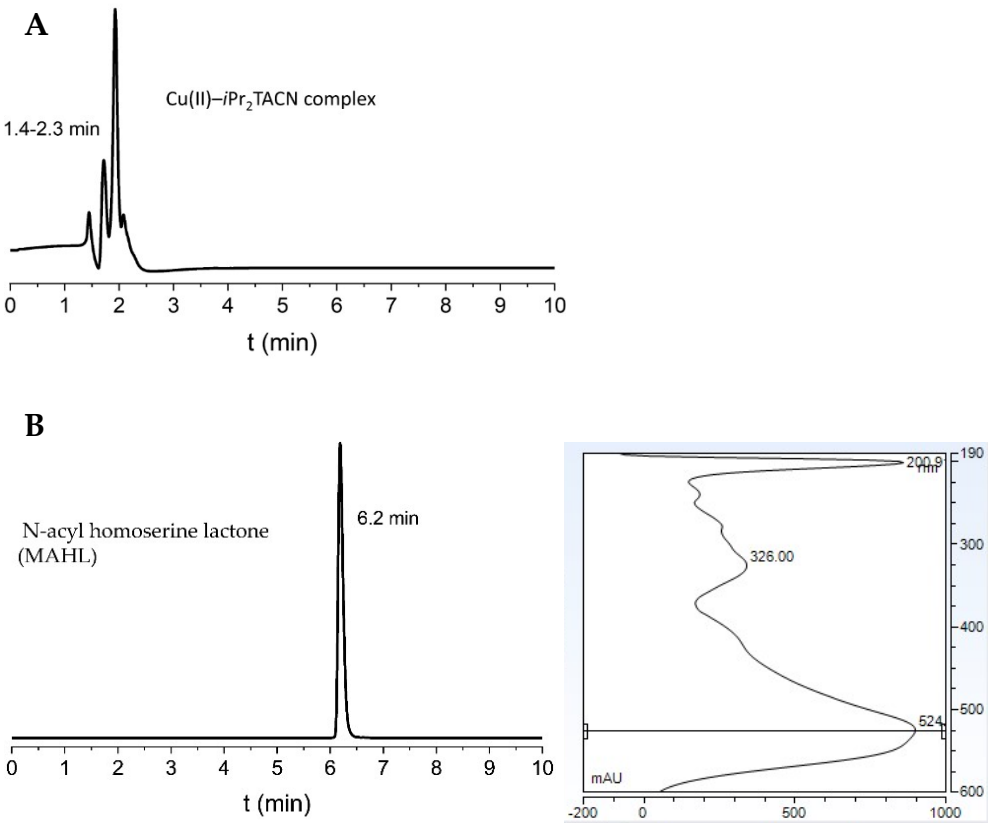


Figure S7. MS spectrum of ADC.



C

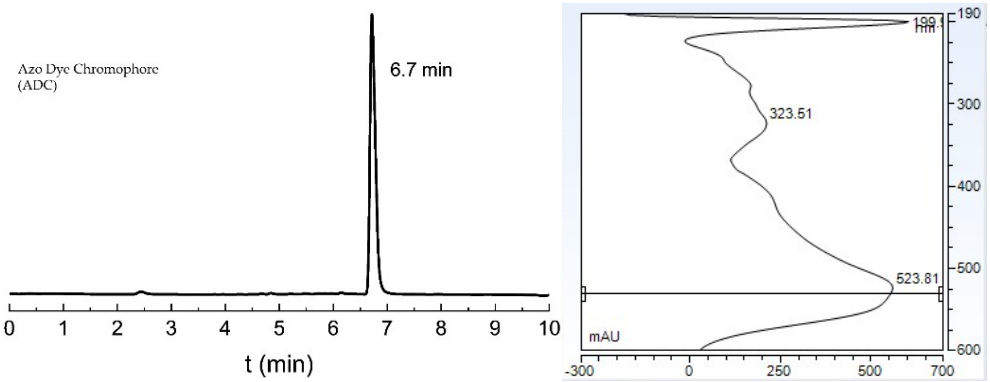


Figure S8. UHPLC curve of standards: (A) Cu(II)-*i*Pr_2-TACN complex, (B) MAHL and (C) ADC in UHPLC phase A (H_2O/ACN/TFA; 95/5/0.1).

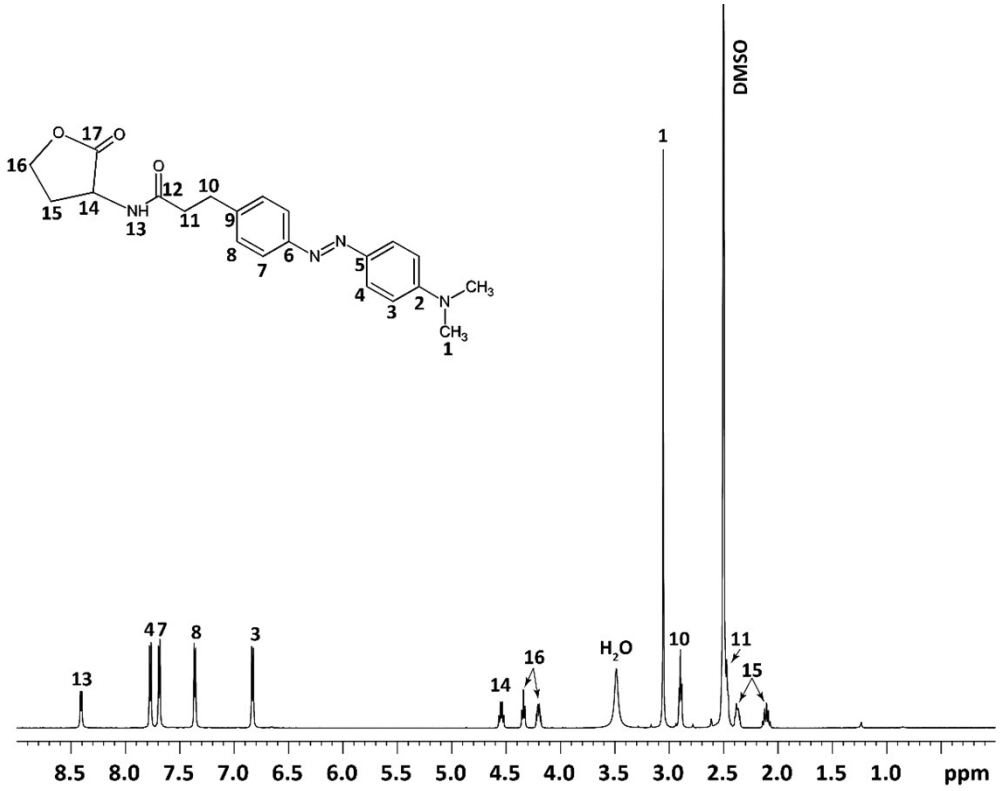


Figure S9. ^{1}H NMR spectrum of Model *N*-acyl homoserine lactone (MAHL) in DMSO-d_6.

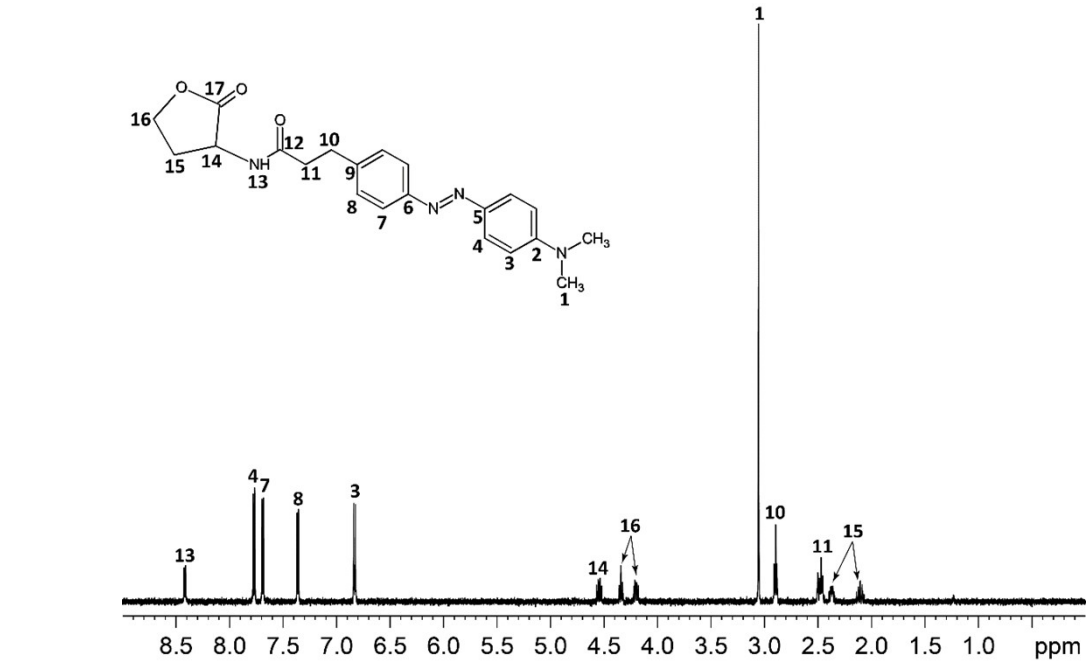


Figure S10. 1D ^{1}H-PFG NMR spectrum of Model *N*-acyl homoserine lactone (MAHL) in DMSO-d_6 (signals from water and DMSO are removed using the features of the PFG NMR experiment).

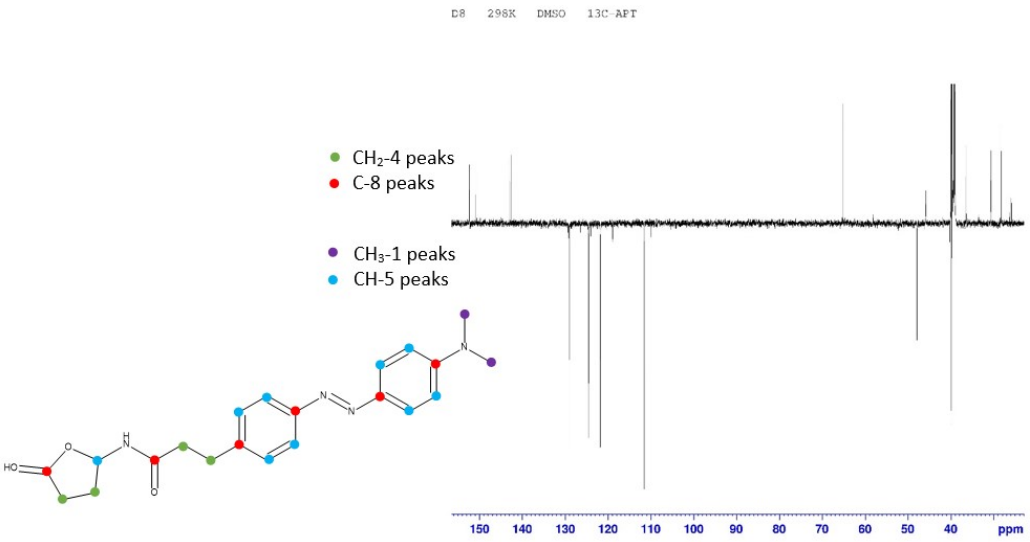


Figure S11. ^{13}C-APT-NMR spectrum of Model *N*-acyl homoserine lactone (MAHL) in DMSO-d_6.

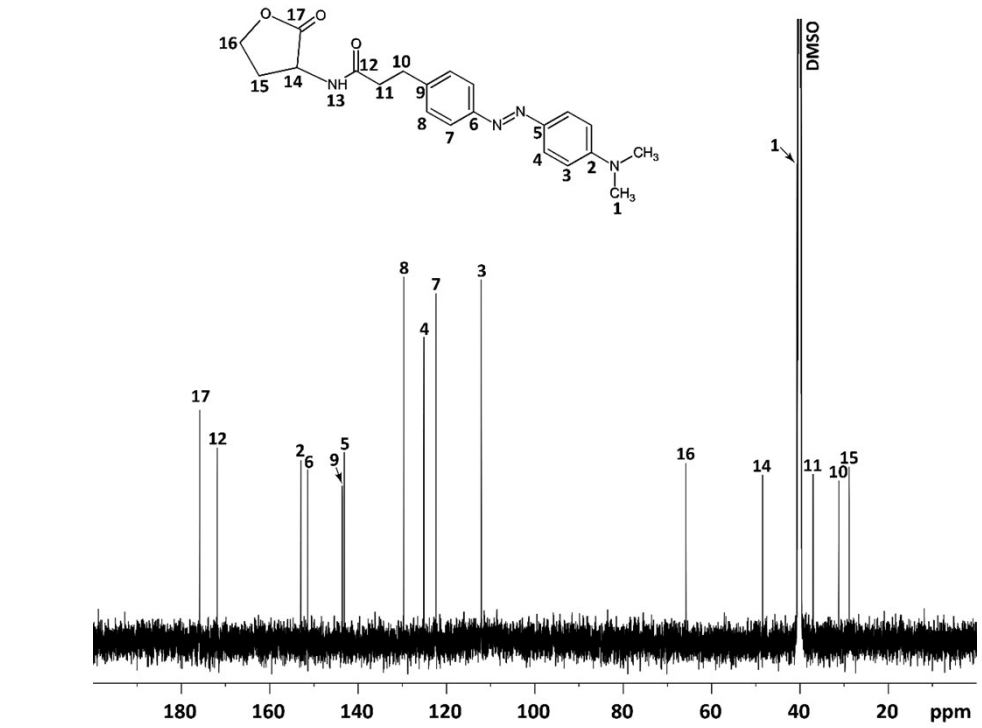


Figure S12. ^{13}C NMR spectrum of Model *N*-acyl homoserine lactone (MAHL) in DMSO-d_6.

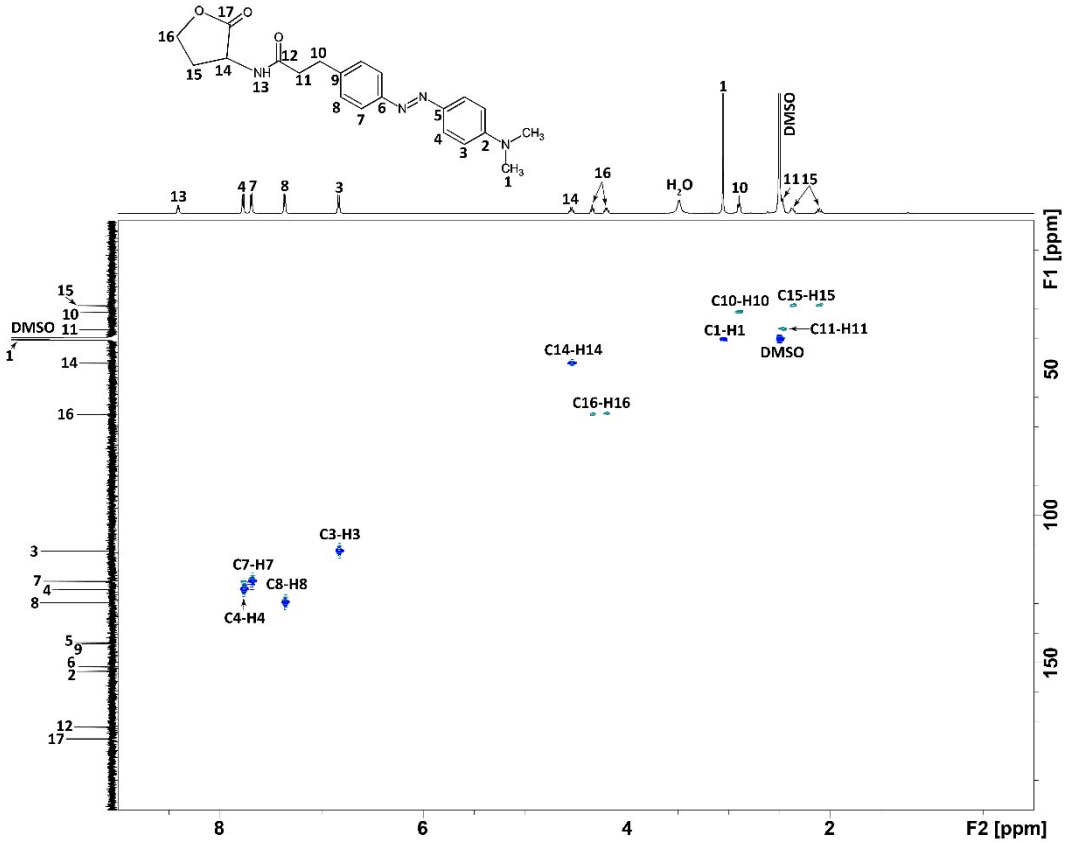


Figure S13. ^{1}H -^{13}C HSQC NMR spectrum of Model *N*-acyl homoserine lactone (MAHL) in DMSO-d_6. Blue cross peaks indicate CH and CH_3 groups and turquoise cross peaks indicate CH_2 groups.

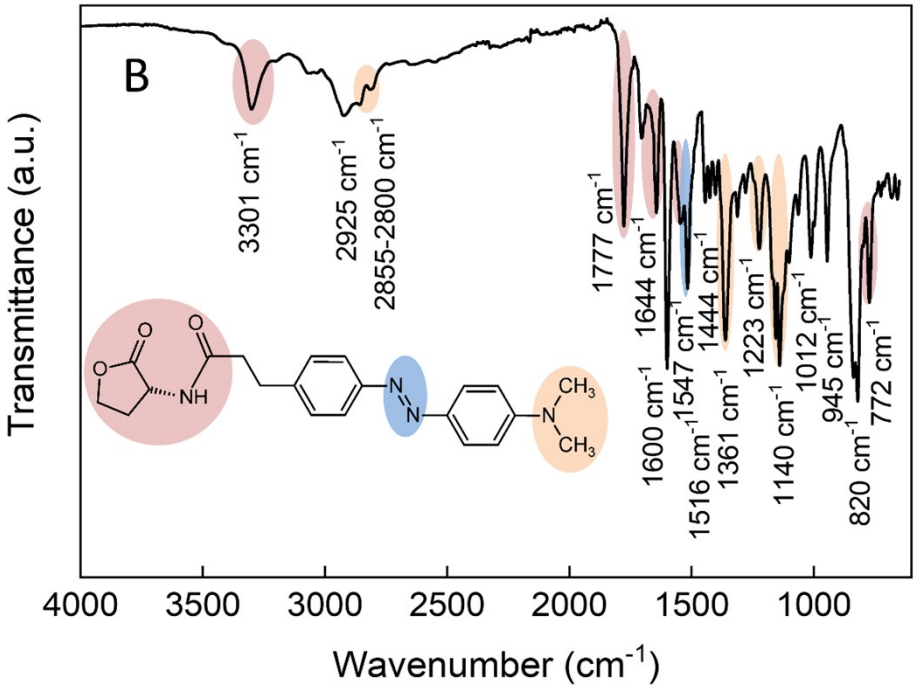


Figure S14. ATR-FTIR spectrum of MAHL.

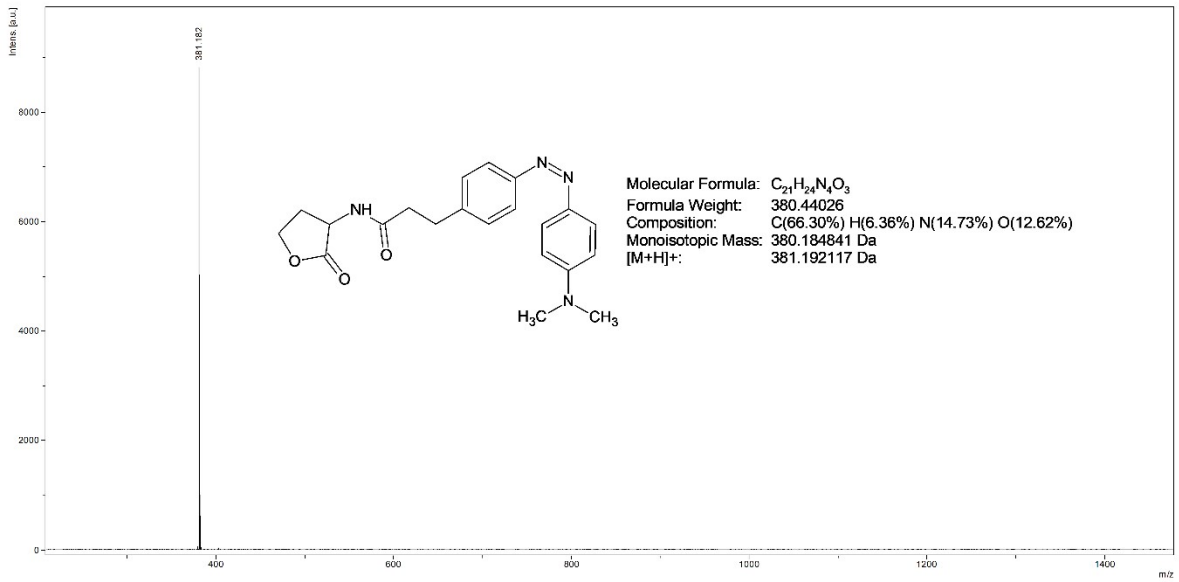


Figure S15. MALDI-TOF spectrum of MAHL.

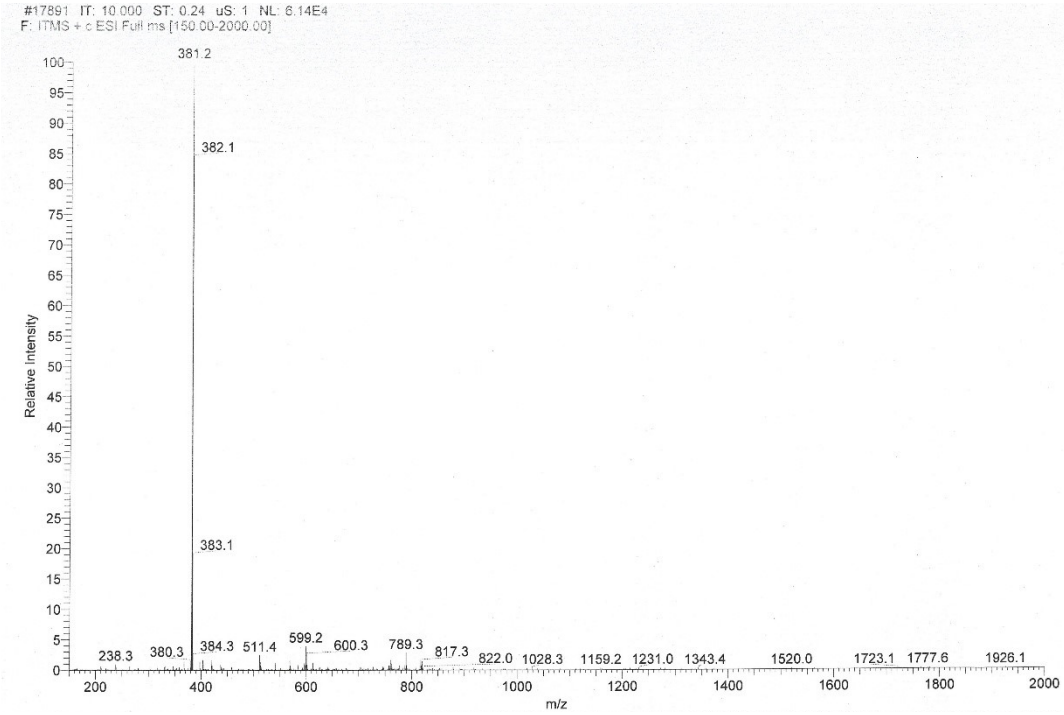


Figure S16. MS spectrum of MAHL.

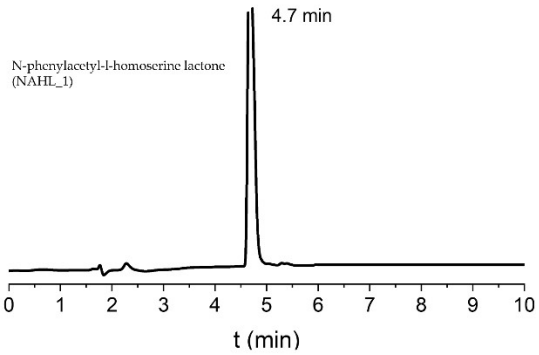


Figure S17. UHPLC curve of standard for NAHL in UHPLC mobile phase A (H_2O/ACN/TFA; 95/5/0.1).

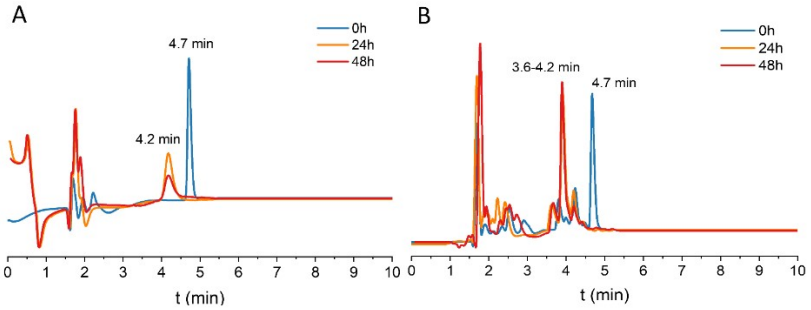


Figure S18. UHPLC curves of NAHL (A) stability in buffer solution without catalyst: hydrolysis at 50 °C in 0.15M Tris buffer solution and (B) decomposition by Cu(II)-*i*Pr_2-TACN complex at constant temperature 50°C and pH 7.4.

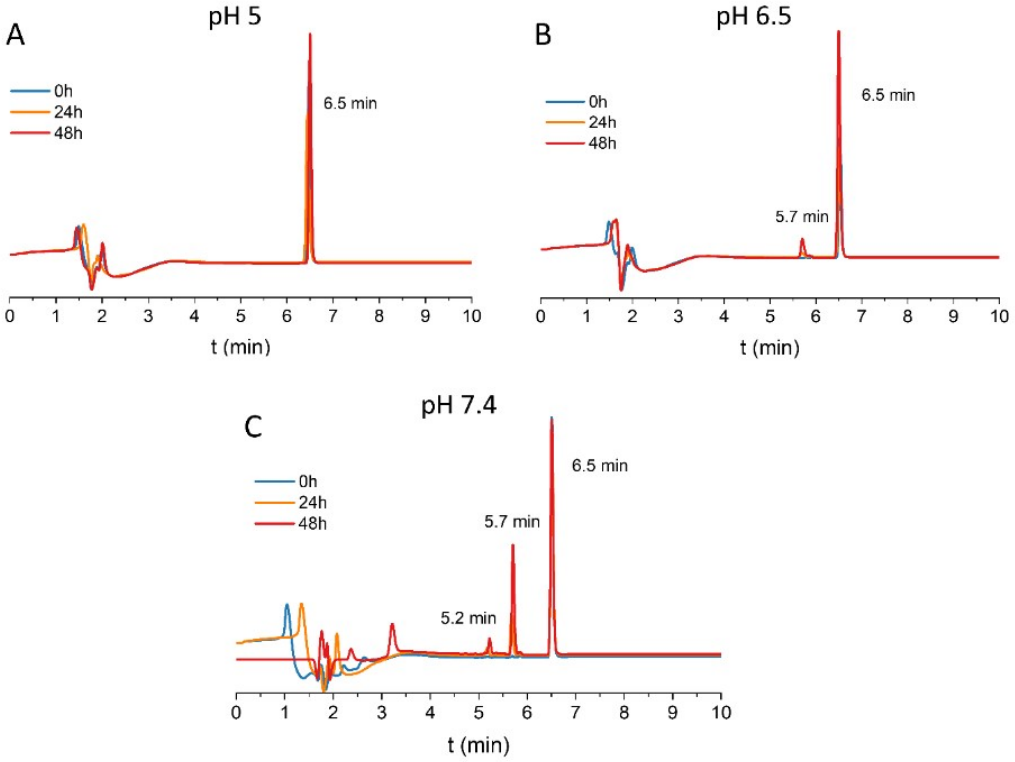


Figure S19. UHPLC curves of MAHL stability in buffer solutions without catalyst at 50 °C in 0.15M MES (A-B) and TRIS (C) buffer solutions.

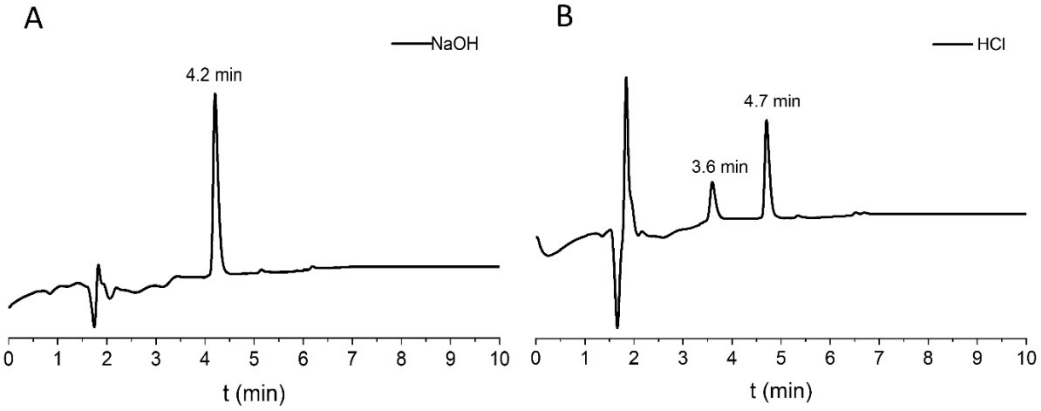


Figure S20. **U**HPLC curves NAHL hydrolysis standards after reaction in aggressive (A) basic and (B) acidic conditions. The experiments were performed in aqueous solutions.

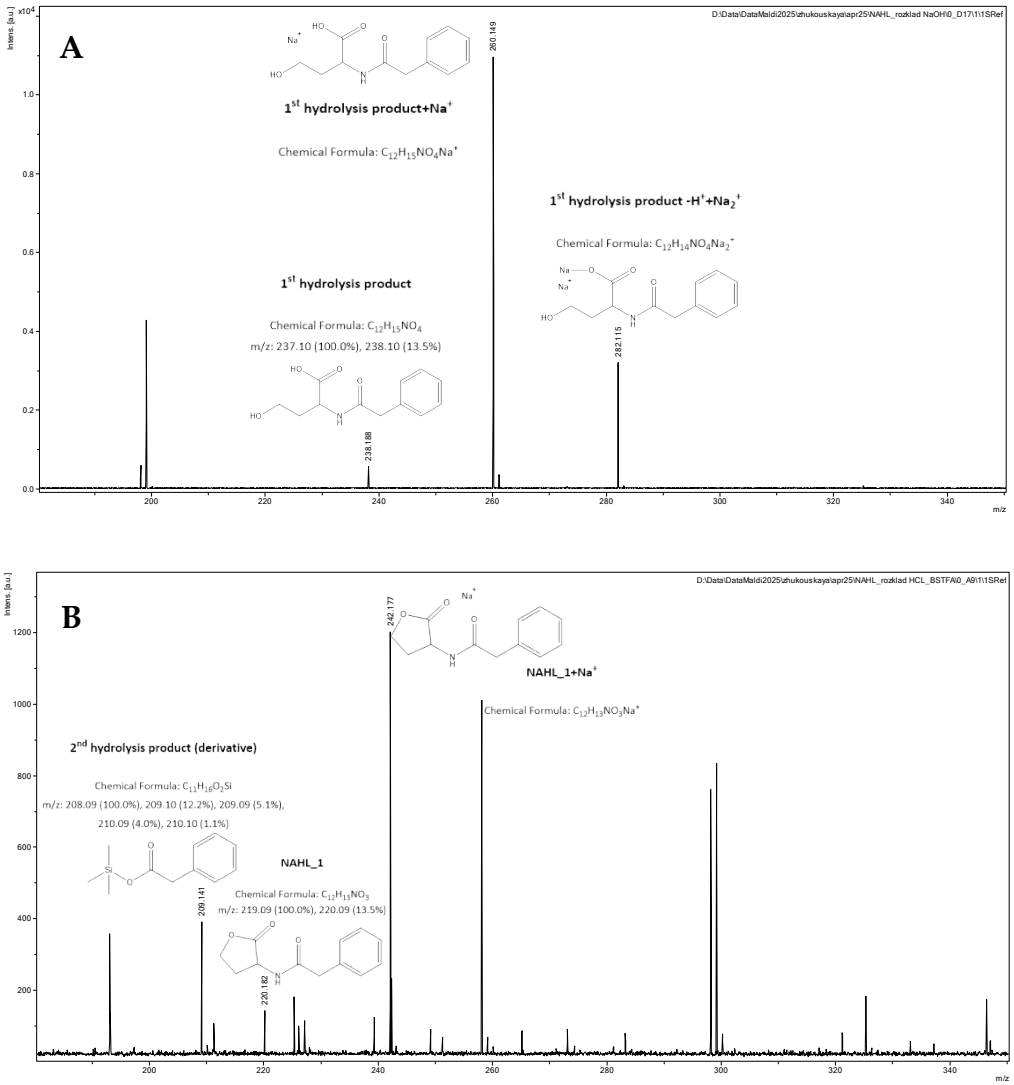


Figure S21. MALDI-TOF analysis of standards in aggressive (A) basic (1st hydrolysis product, t_r = 4.2 min) and (B) acidic conditions (NAHL, t_r = 4.7 min and 2nd hydrolysis product, t_r = 3.9 min)

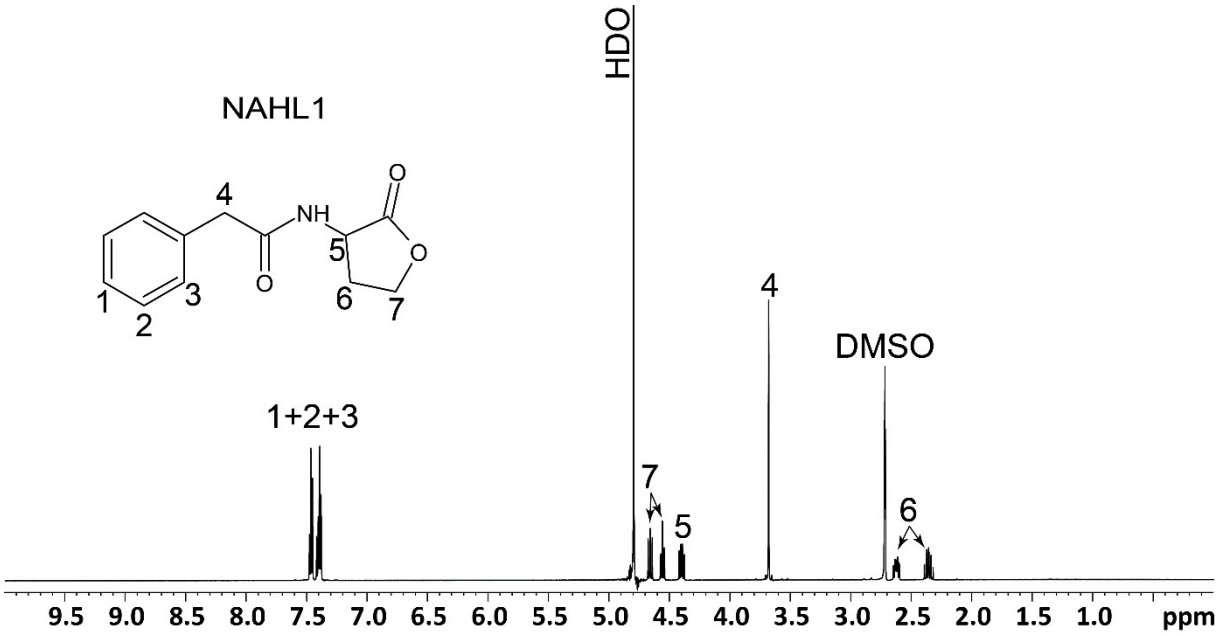


Figure S22. ^{1}H NMR spectrum of NAHL in DMSO-d_6-D_2O (25:75 v/v) mixture.

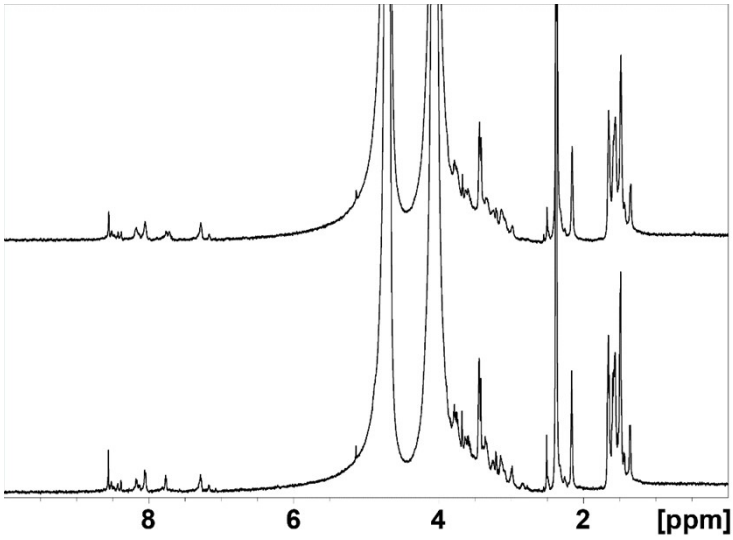


Figure S23. Degradation of MAHL in the presence of Cu(II)–*i*Pr2-TACN followed by ^{1}H NMR

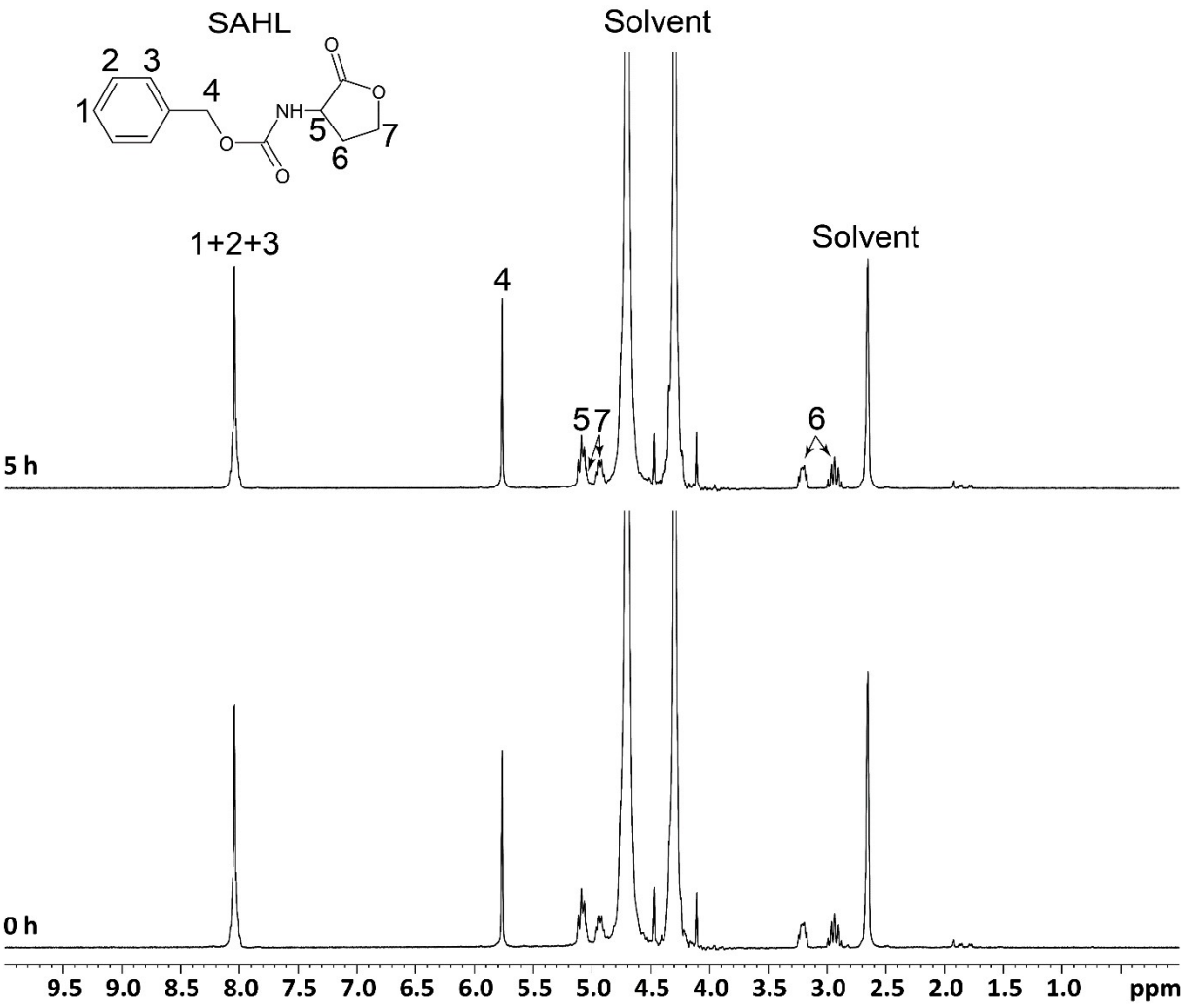


Figure S24. Stability test of SAHL in TRIS buffer (D_2O and CD_3CN solvents were used) at 50 °C.

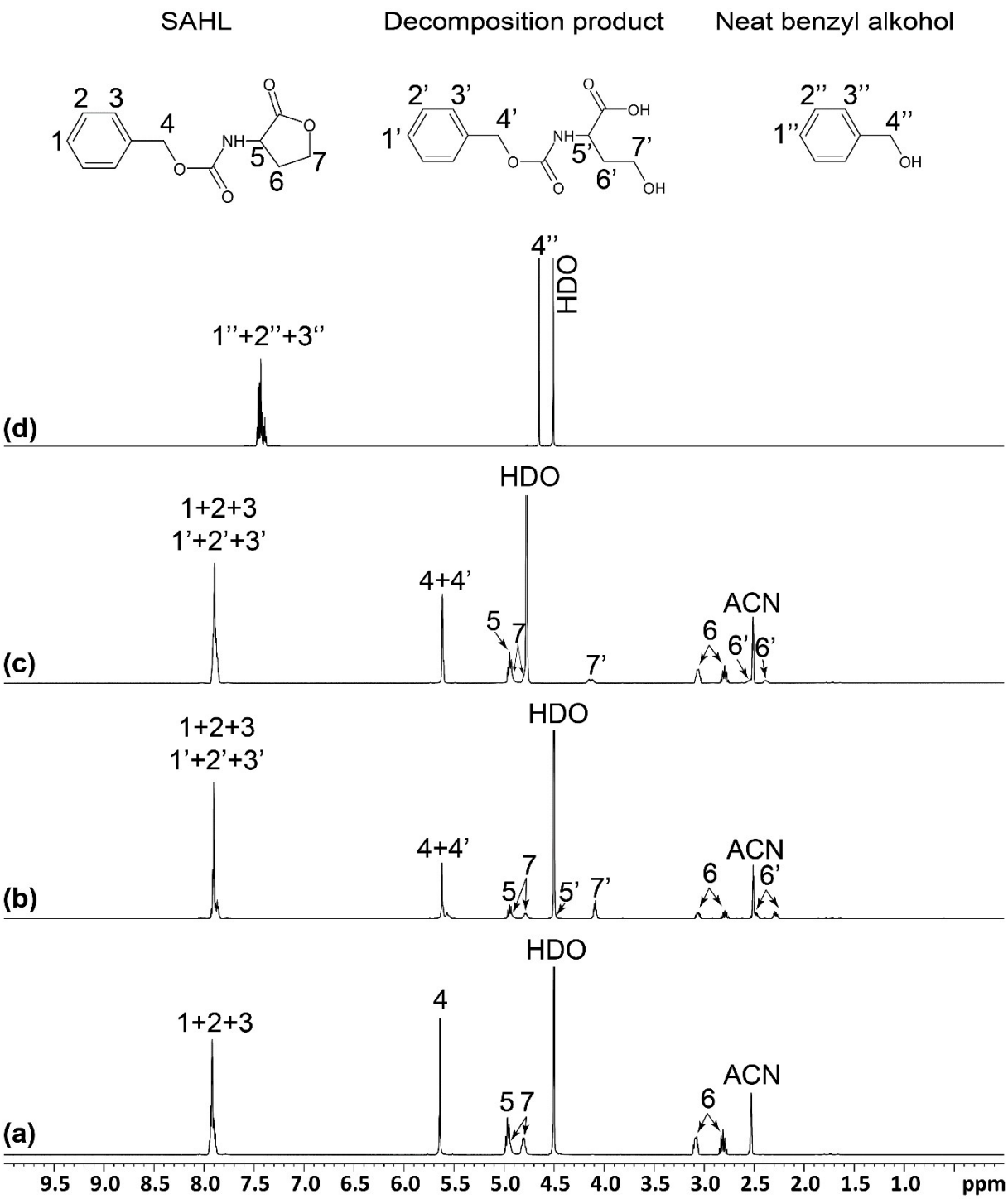


Figure S25. ^{1}H NMR spectra of SAHL standard (in D_2O-CD_3CN) (a), and its degradation products in basic (b) and acidic (c) medium, and ^{1}H NMR spectrum of neat benzyl alcohol (d) recorded at 50 ^{o}C. Peak assignment has been done based on the literature data.[S2]

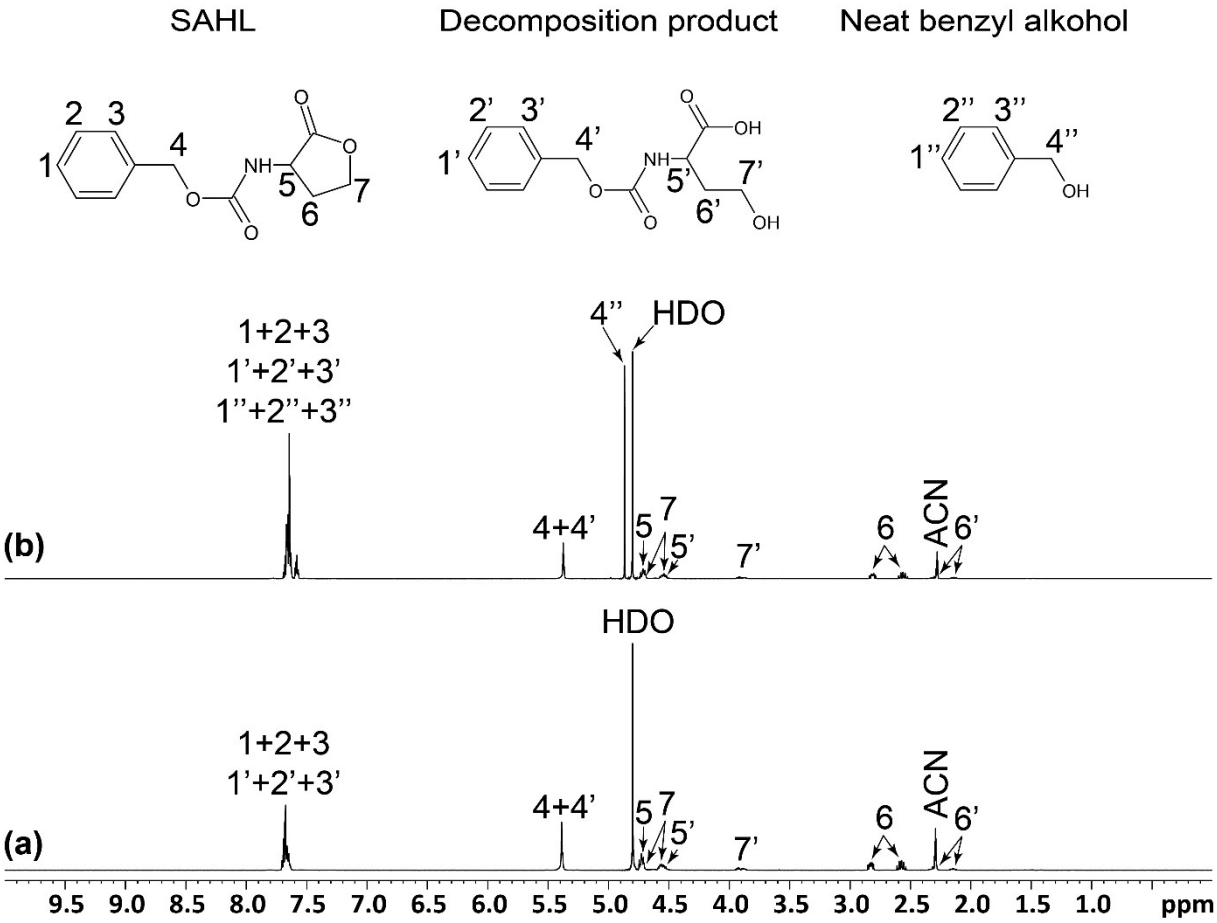
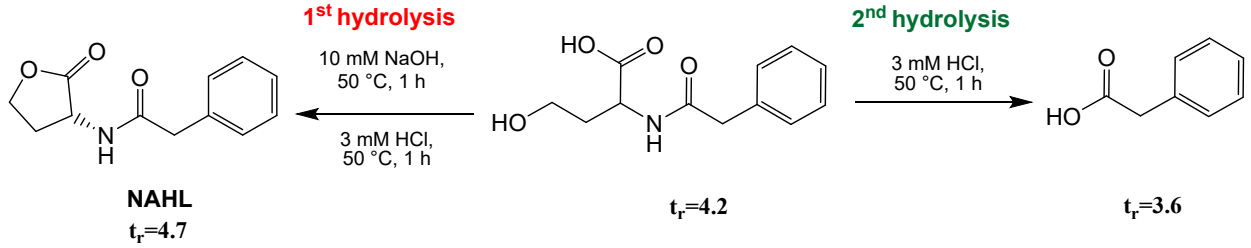
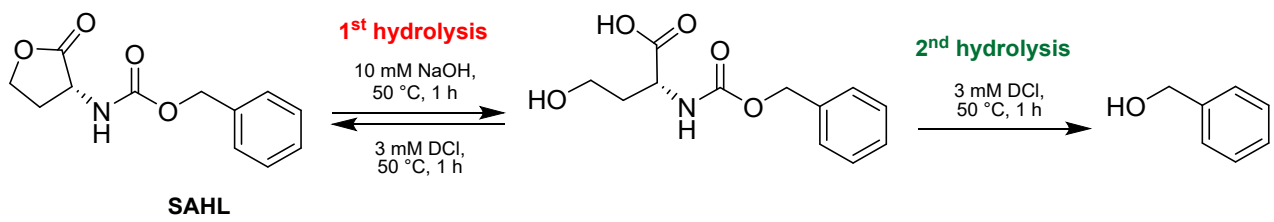


Figure S26. ^{1}H NMR spectrum (in D_2O-CD_3CN) of the SAHL degradation products (a) and the spectrum after addition of 10 µL of benzyl alcohol (b) recorded at 25 °C.



Scheme S1. NAHL hydrolysis standards preparation



Scheme S2. SAHL hydrolysis standards preparation

References

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S2. E. W. Ziegler, A. B. Brown, N. Nesnas and A. G. Palmer, *Eur. J. Org. Chem.*, **2019**, 2850.