Supporting information

Thiazolidine chemistry revisited: A fast, efficient and stable click-type reaction at physiological pH

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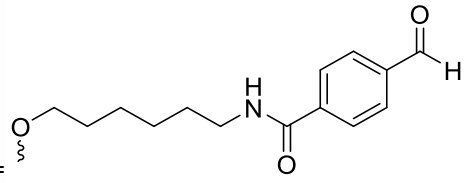
Contents:

1. Materials

All reagents were of the highest purity available from Sigma-Aldrich (Sweden) and were used without further purification. Sodium deuteroxide (NaOD, 30wt% solution in D_2O) was purchased from Acros Organics (Sweden). (2RS,4R)-2-ethylthiazolidine-4-carboxylic acid (**3** in Figure 1) was synthetized following a previously reported procedure.[1] Peptide elastin sequence (CVGVAPG) and peptide TAT sequence (CYGRKKRRQRRR) were purchased from PepMic (China). The siRNA (Sense strand- 5'-M-[rG][rC][rC][rA][rG][rC][rA][rA][rA][rG][rA][rA][rU][rC][rA][rC][rA][rU][rG][dT][dT]-3') with benzaldehyde modification and complementary unmodified antisense strands were obtained from ChemGenes corporation (USA).

M = 

2. NMR spectroscopy experiments

General. All spectra were recorded on a JEOL JNM-ECP Series FT NMR spectrometer at a magnetic field strength 9.4 T, operating at 400 MHz for ^{1}H-NMR and 100 MHz for ^{13}C-NMR. Compounds were dissolved in 0.1 M deuterated phosphate buffered saline (dPBS). pD (pH + 0.4) of the resulting solutions was adjusted by adding NaOD or HCl 0.1 M in D_2O. The residual solvent peak at 4.79 ppm was used as internal reference for ^{1}H-NMR spectra. Chemical shifts were expressed in ppm.

Thiazolidine formation at different pDs. L-Cysteine (10 mM) and propionaldehyde (160 mM) were dissolved in dPBS. After pD adjustment (pD = 3, 5, 7.4 or 9), the solutions were mixed, and the pD of the reaction mixture was adjusted again. Thiazolidine formation was too fast to be followed at a suitable concentration for NMR because cysteine resonances disappeared before the first ^{1}H-NMR spectrum could be recorded.

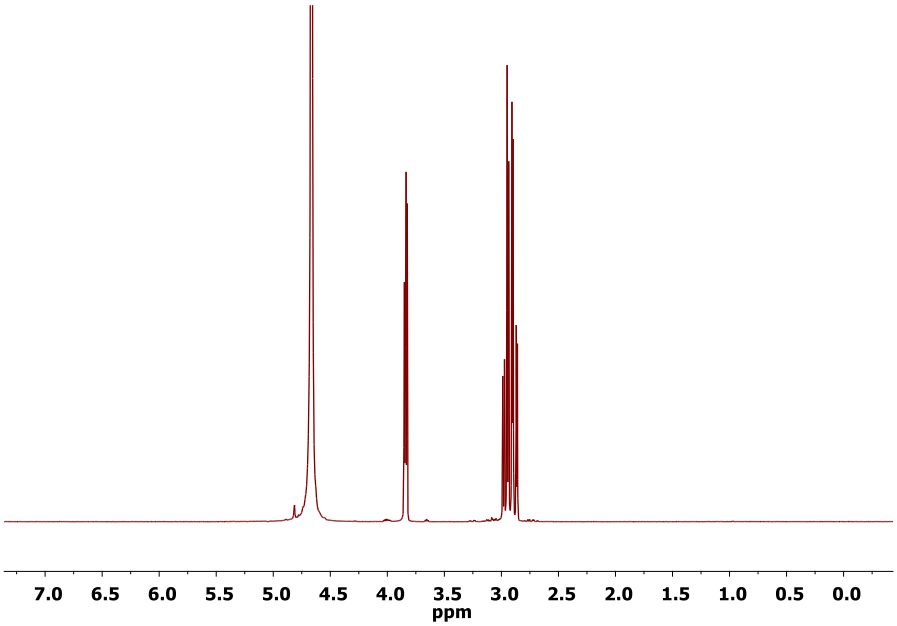


Figure S1. The ^{1}H-NMR spectrum of L-cysteine at pD = 5.

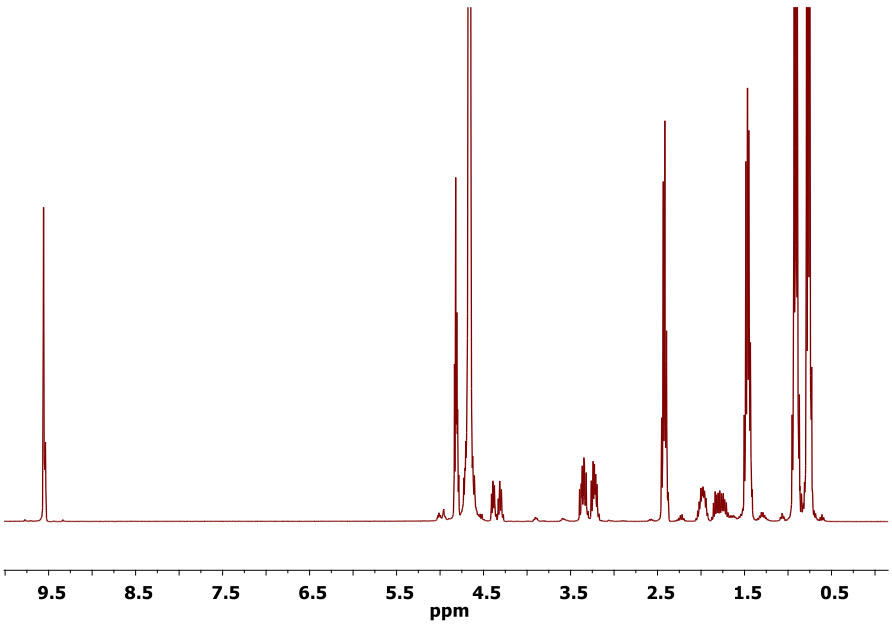


Figure S2. The ^{1}H-NMR spectrum of the reaction mixture between L-cysteine and 16 equivalents of propionaldehyde at pD = 5 after 5 minutes.

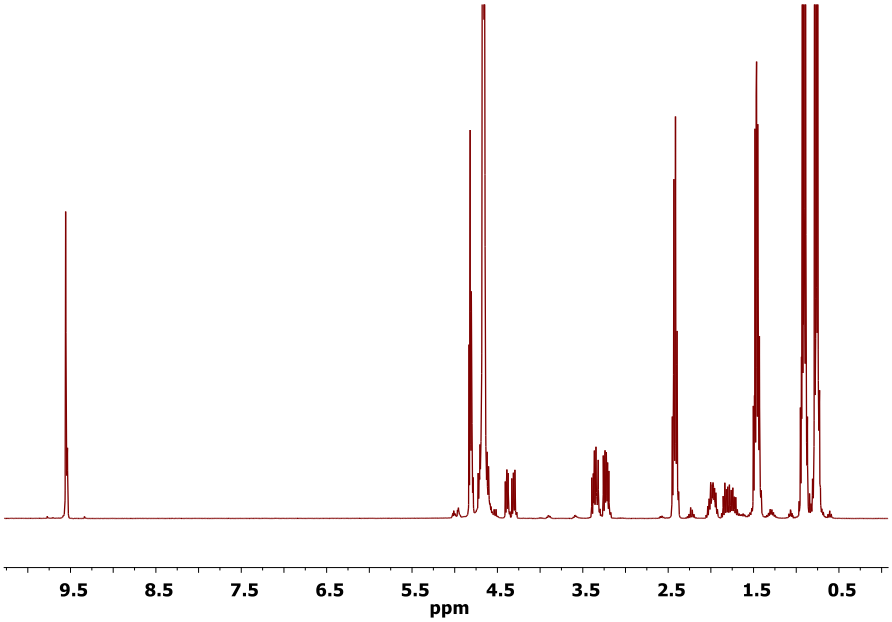


Figure S3. The ^{1}H-NMR spectrum of the reaction mixture between L-cysteine and 16 equivalents of propionaldehyde at pD = 5 after 120 minutes.

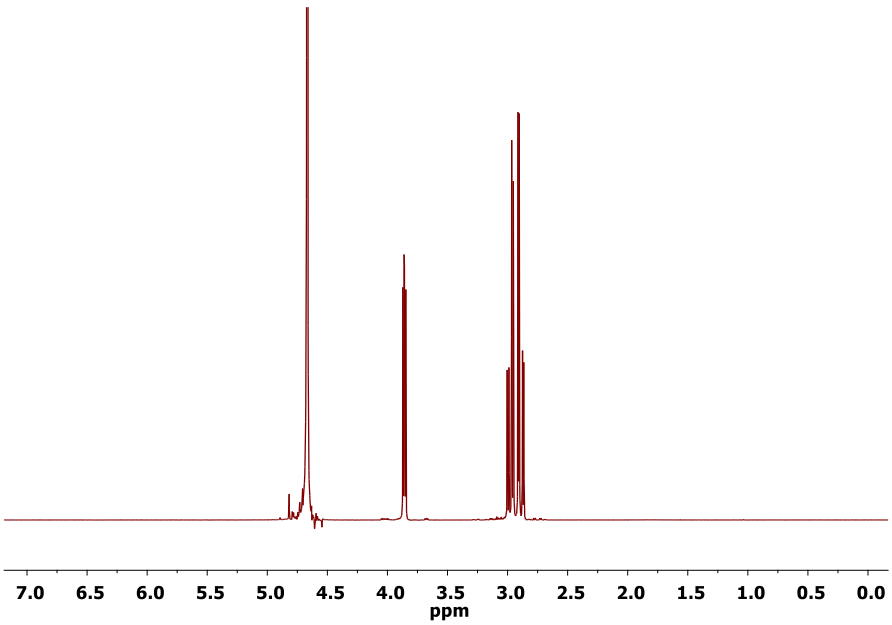


Figure S4. The ^{1}H-NMR spectrum of L-cysteine at pD = 7.4.

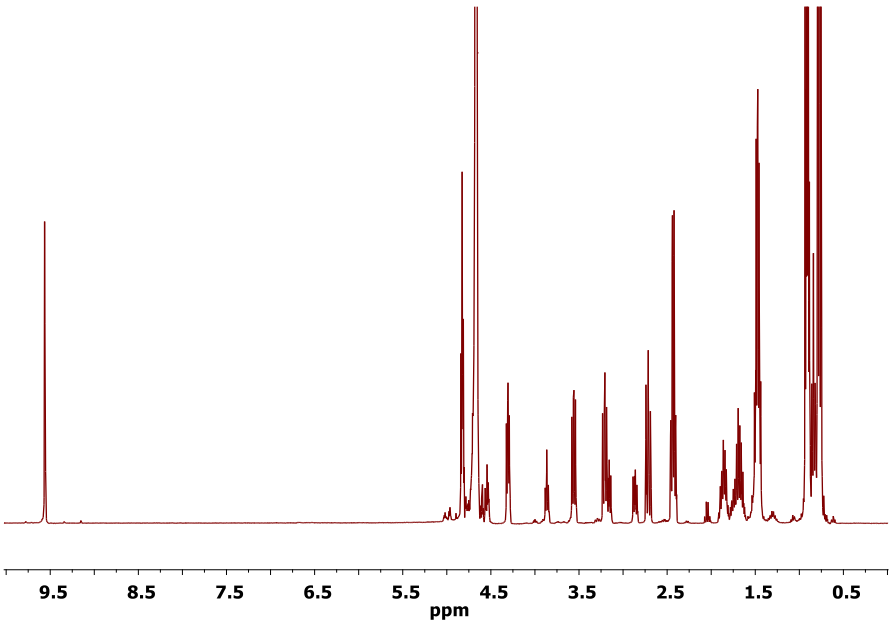


Figure S5. The ^{1}H-NMR spectrum of the reaction mixture between L-cysteine and 16 equivalents of propionaldehyde at pD = 7.4 after 5 minutes.

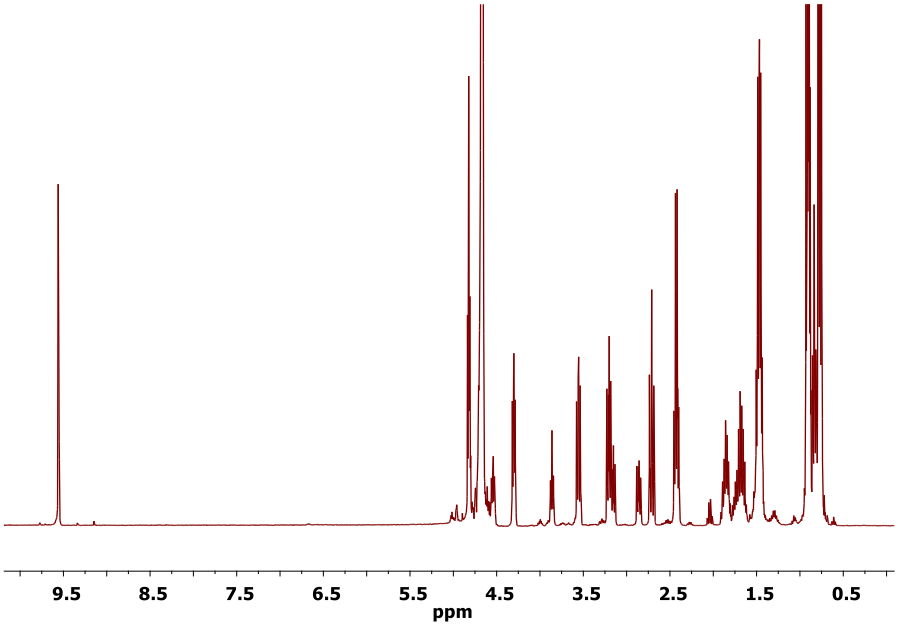


Figure S6. The ^{1}H-NMR spectrum of the reaction mixture between L-cysteine and 16 equivalents of propionaldehyde at pD = 7.4 after 120 minutes.

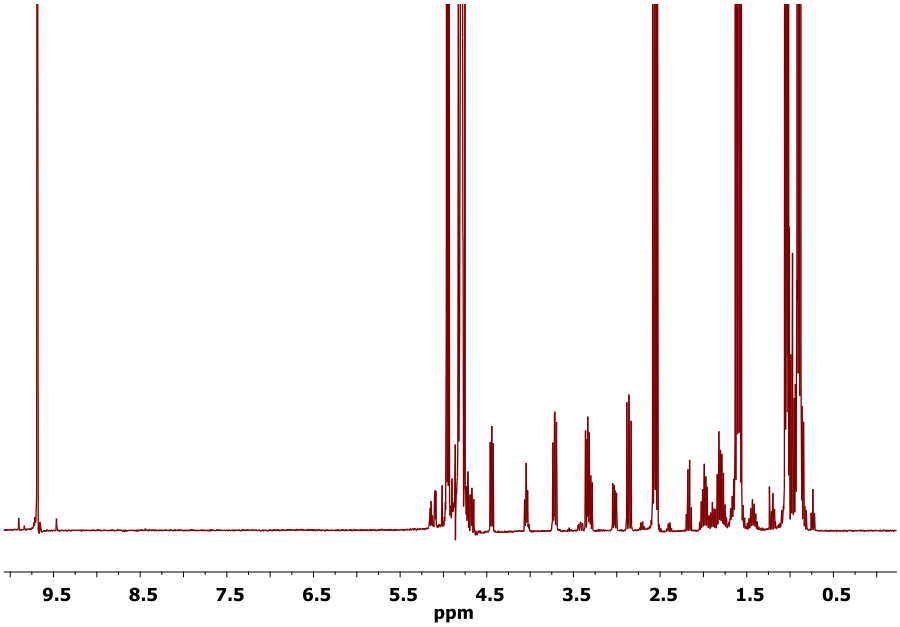


Figure S7. The ^{1}H-NMR spectrum of the reaction mixture between L-cysteine and 16 equivalents of propionaldehyde at pD = 9 after 5 minutes.

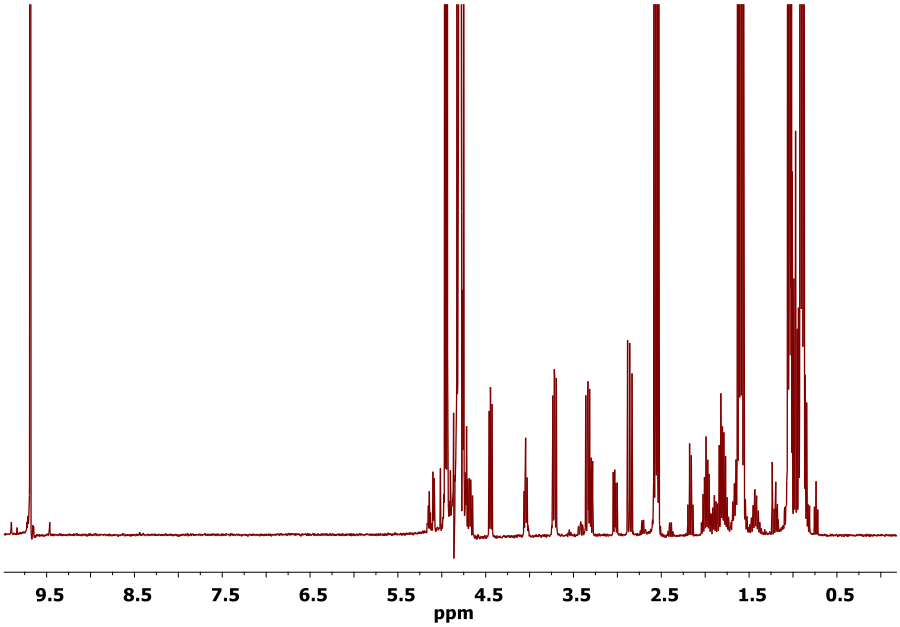


Figure S8. The ^{1}H-NMR spectrum of the reaction mixture between L-cysteine and 16 equivalents of propionaldehyde at pD = 9 after 120 minutes.

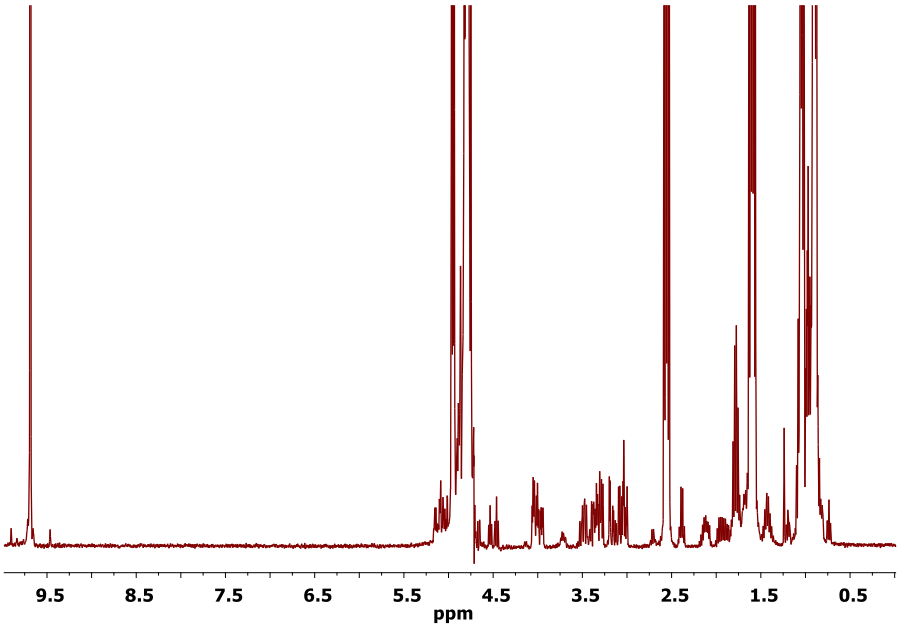


Figure S9. The ^{1}H-NMR spectrum of the reaction mixture between L-cysteine and 16 equivalents of propionaldehyde at pD = 3 after 5 minutes.

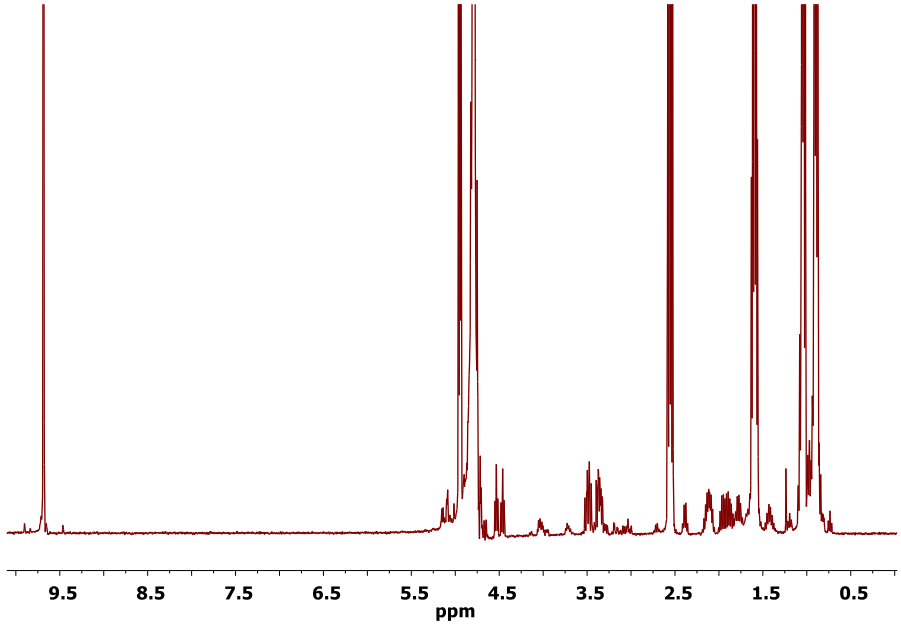


Figure S10. The ^{1}H-NMR spectrum of the reaction mixture between L-cysteine and 16 equivalents of propionaldehyde at pD = 3 after 30 minutes.

Interconversion of thiazolidine species upon pD regulation. (2RS,4R)-2-ethylthiazolidine-4-carboxylic acid (**3** in Figure S7) was dissolved in a dPBS at acidic condition (pD = 5). The resulting solution was neutralized (pD = 7.4) and again acidified (pD = 5) by adding NaOD (0.1 M) or HCl (0.1 M) in D_2O. ^{1}H-NMR spectrum was recorded after adjusting the pDs. The inter-conversion of the two products upon pD adjustment indicates that both the structures have a thiazolidine ring. Further characterization of the thiazolidine ring was carried out using 2D-NMR experiments indicated below.

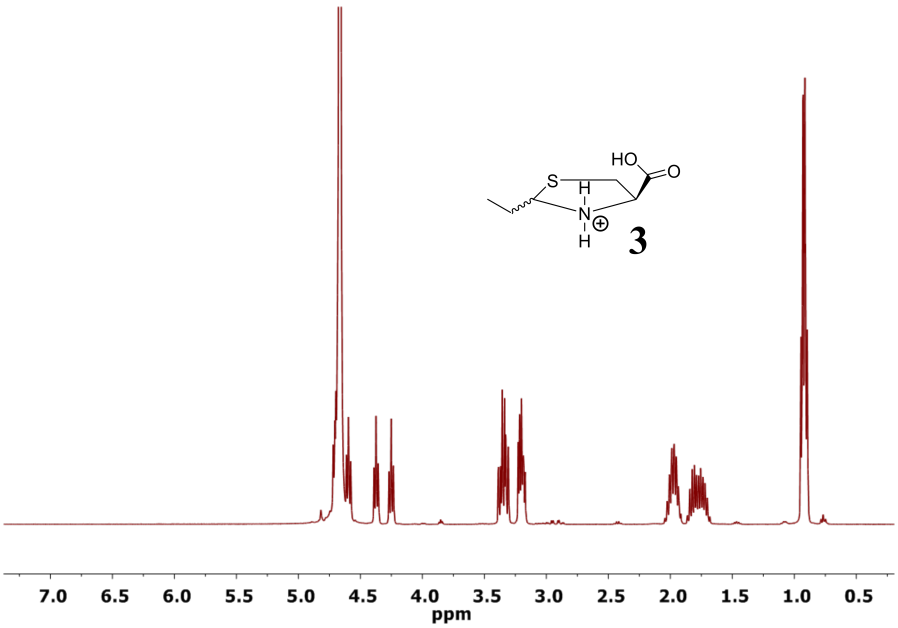


Figure S11. The ^{1}H-NMR spectrum of **3** at pD = 5.

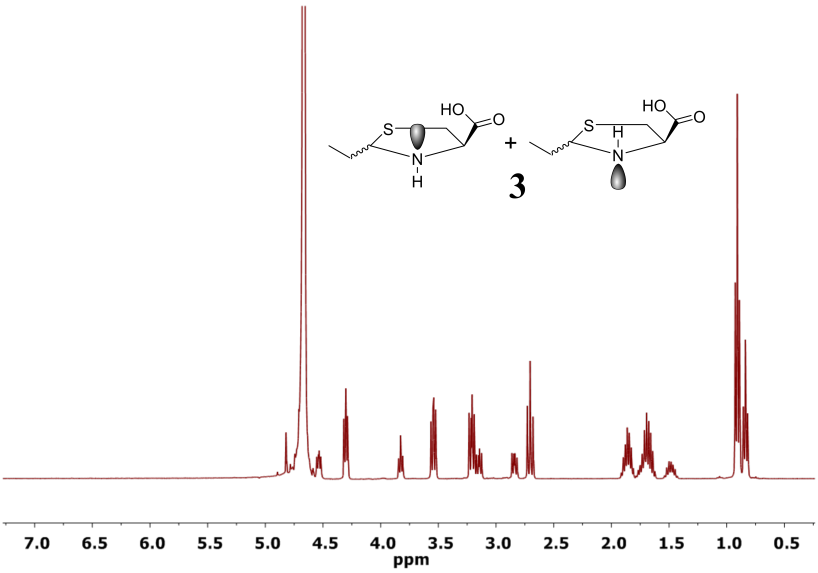


Figure S12. The ^{1}H-NMR spectrum of **3** after pD adjustment from pD = 5 to pD = 7.4.

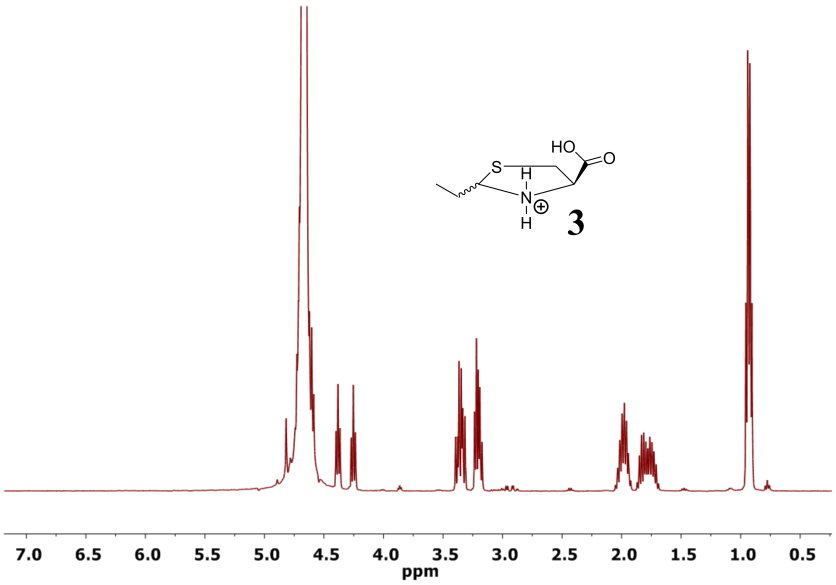


Figure S13. The ^{1}H-NMR spectrum of **3** after two separate pD adjustments, namely from pD = 5 to pD = 7.4 followed by acidification of the product from pD = 7.4 to pD = 5.

Spectroscopy characterization of thiazolidine at pD 5 and pD 7.4. The thiazolidine compound **3** was dissolved in a dPBS solution and the pD was adjusted to 5 for NMR characterization. The ^{1}H-NMR spectrum of **3** showed the typical resonance splitting of diastereomeric isomers (**Figure S14**). ^{1}H-NMR resonances were assigned by performing the 2D-COSY experiment. The ^{13}C-NMR spectrum of **3** showed two sets of the signal corresponding to the diastereomeric mixture with a clear splitting of the vicinal carbons at the diastereomeric centres (C_2, C_4 and C_7 in Figure S10d). The ^{13}C-NMR resonance for **3** was assigned with the help of the 2D-HSQC experiments. The HMBC spectrum showed the expected pattern for a thiazolidine product. Crossover peaks between C_5 with H_2, C_4 with H_2, as well as C_2 with H_5 and H_4, confirmed the cyclic nature of the thiazolidine structure. The same characterization method was employed at pD 7.4 (**Figure S15-18**). However, we could not assign the ^{1}H and ^{13}C signals as there was a mixture of diastereomers.

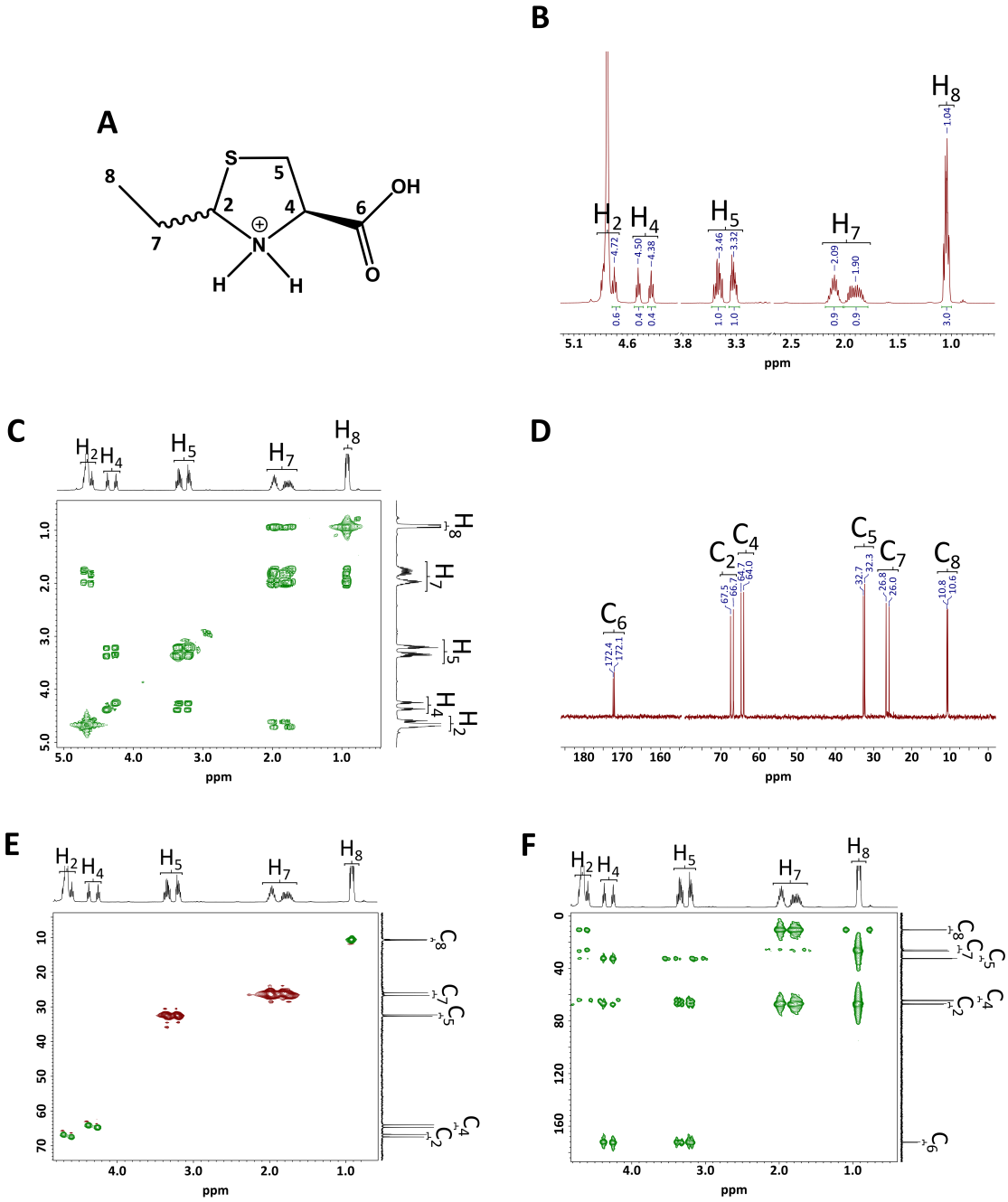


Figure S14. a) Chemical structure of **3** followed by its b) ^{1}H-NMR spectrum, c) COSY spectrum, d) ^{13}C-NMR spectrum, e) HSQC spectrum and f) HMBC spectrum at pD = 5.

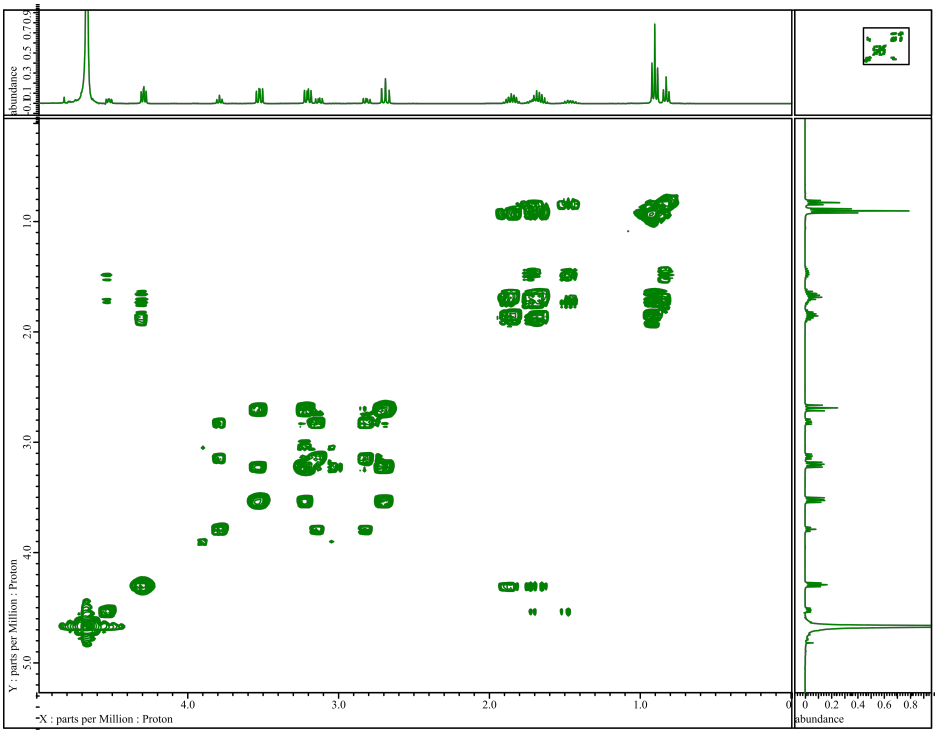


Figure S15. COSY spectrum of **3** at pD = 7.4.

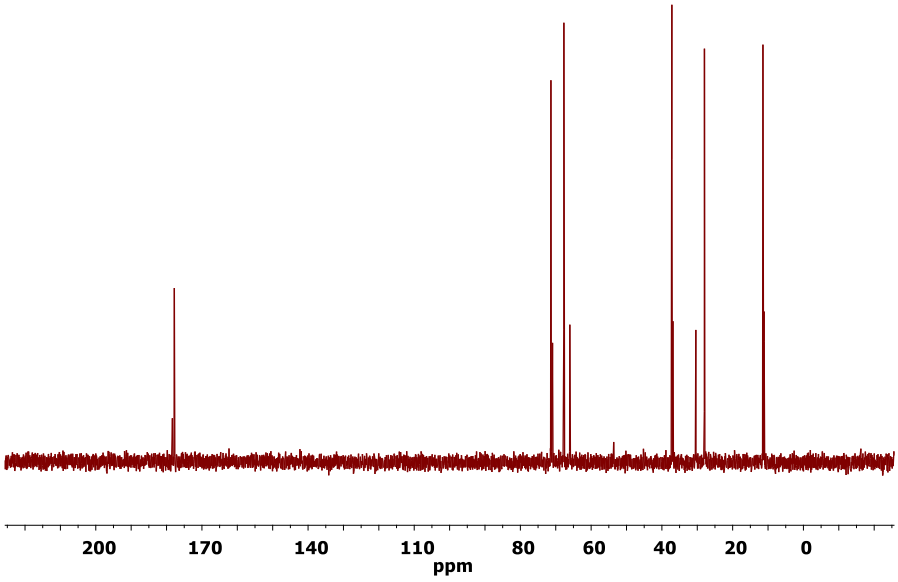


Figure S16. The ^{13}C-NMR spectrum of **3** at pD = 7.4.

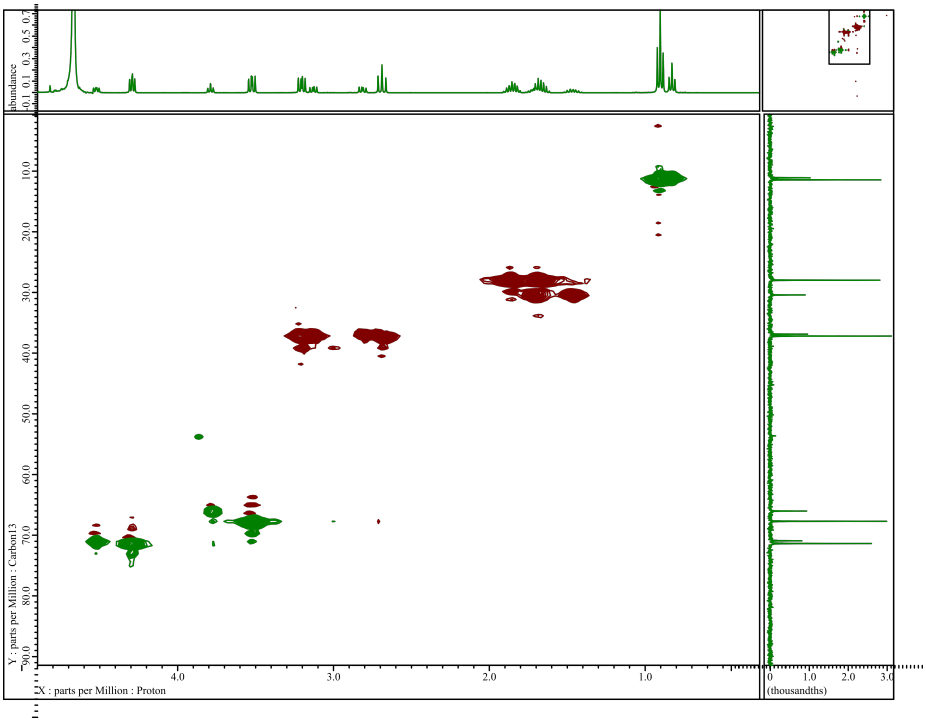


Figure S17. HSQC spectrum of **3** at pD = 7.4.

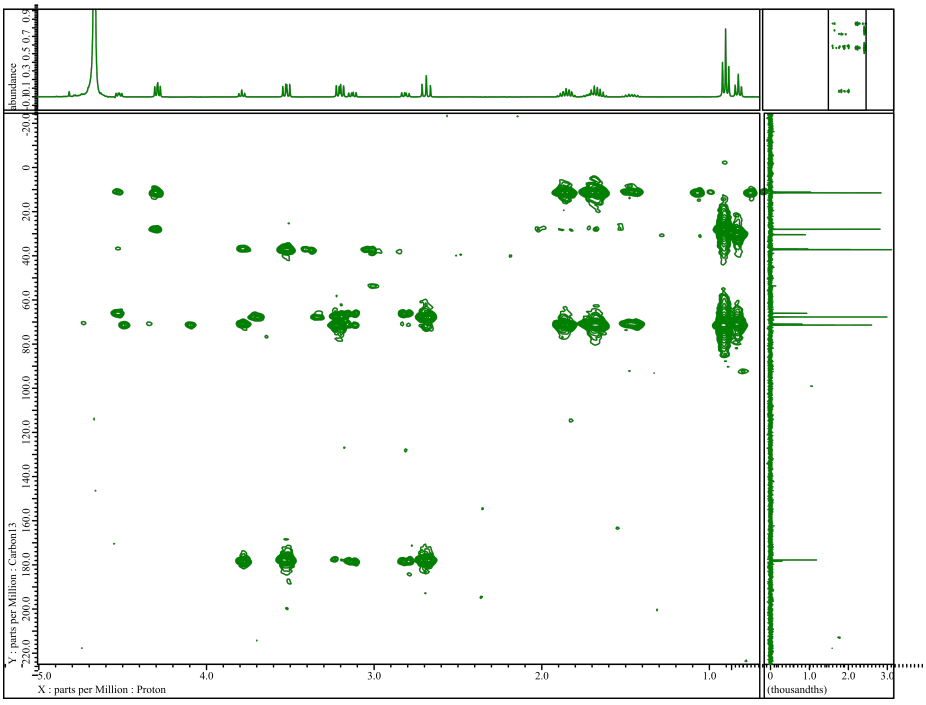


Figure S18. HMBC spectrum of **3** at pD = 7.4.

Stability of thiazolidine at different pDs. The compound **3** was dissolved in dPBS solution and the pD was adjusted to 3, 5 ,7.4 and 9 by adding NaOD (0.1 M) or HCl (0.1M) in D_2O. Thereafter, the ^{1}H-NMR spectra were recorded at different time points (**Figure S19**). The

thiazolidine ring showed excellent stability at all studied conditions, as there was no visible sign of product degradation after 7 days of the experiment.

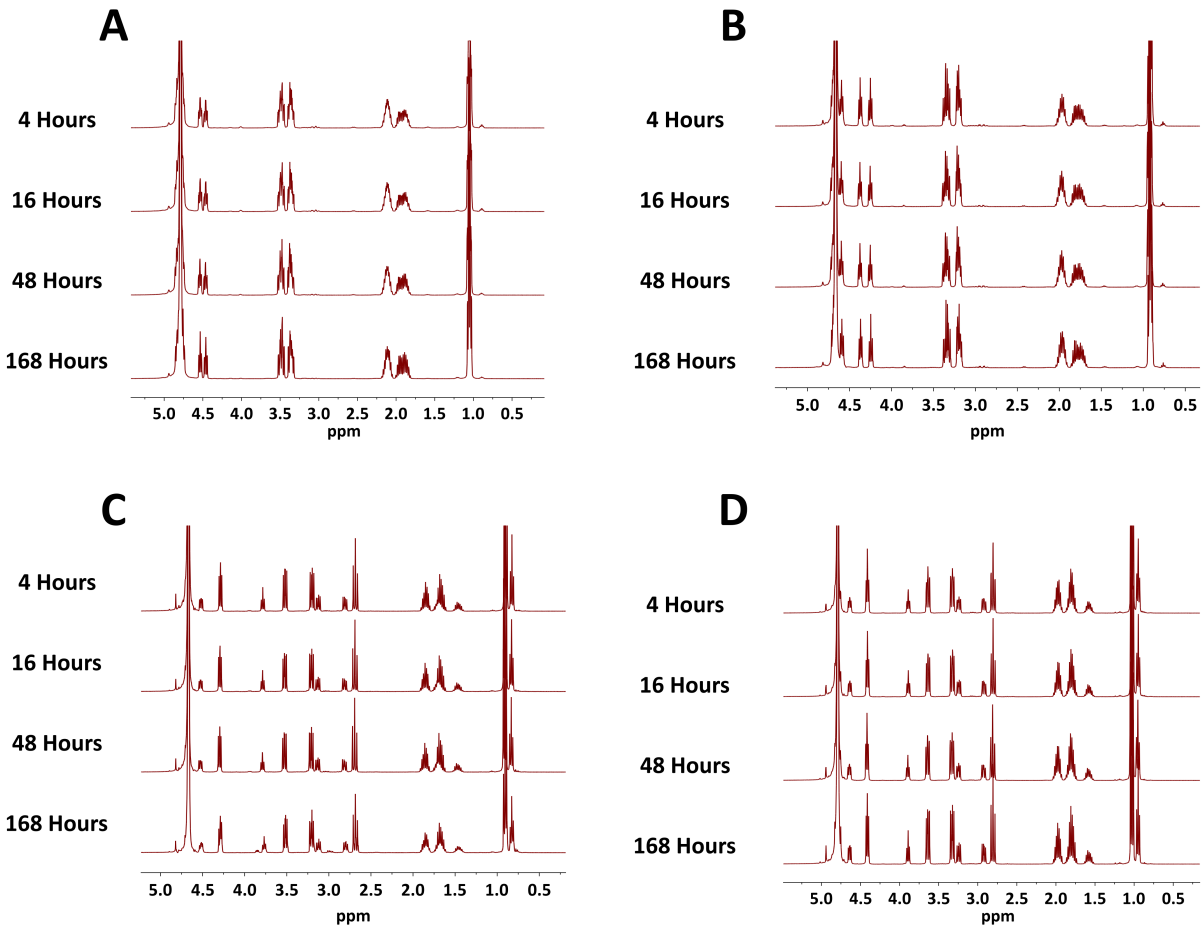


Figure S19. The ^{1}H-NMR spectra of **3** at a) pD = 3, b) pD = 5, c) pD = 7.4 and d) pD = 9 at different time points from 4 hours till 7 days. These experiments confirmed the stability of thiazolidine product after 7 days.

Thiazolidine formation with different aldehyde substrates. L-Cysteine (5 mM) and aldehyde (80 mM) were dissolved in dPBS. After pD adjustment (pD = 7.4), the solutions were mixed and the pD of the reaction mixture was adjusted again. Thiazolidine formation was too fast to be followed with butyraldehyde and trimethylacetaldehyde. Aromatic aldehydes were also studied. For aromatic aldehydes 10% d-DMSO was added to improve their solubility in aqueous media and dithiothreitol (5mM) was added to prevent disulfide formation. Pseudo-first order rate constant was calculated for benzaldehyde and 4-hydroxybenzaldehyde. 4-Nitrobenzaldehyde was not soluble at the studied conditions.

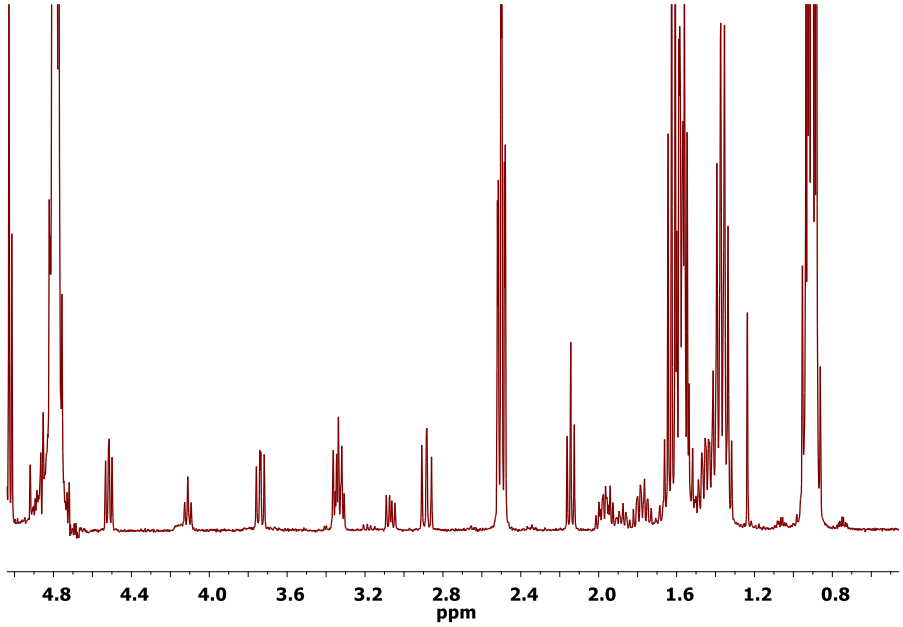


Figure S20. The ^{1}H-NMR spectrum of the reaction mixture between L-cysteine and 16 equivalents of butyraldehyde at pD = 7.4 after 5 minutes.

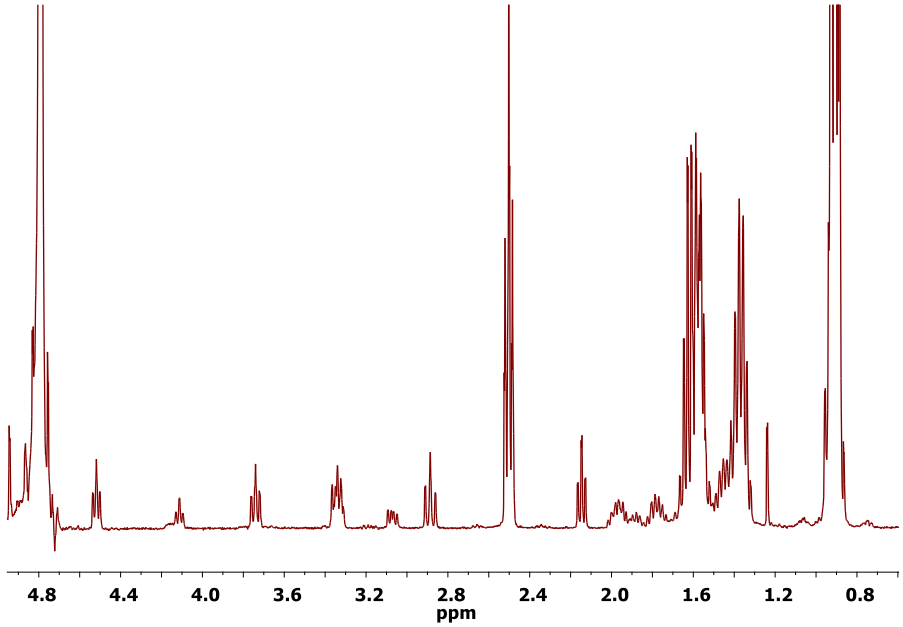


Figure S21. The ^{1}H-NMR spectrum of the reaction mixture between L-cysteine and 16 equivalents of butyraldehyde at pD = 7.4 after 20 minutes.

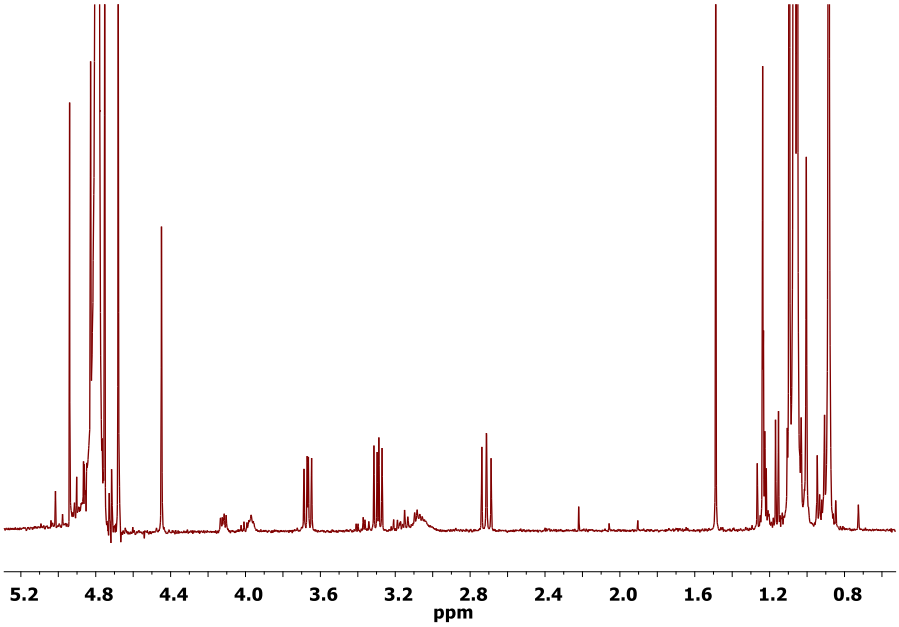


Figure S22. The ^{1}H-NMR spectrum of the reaction mixture between L-cysteine and 16 equivalents of trimethylacetaldehyde at pD = 7.4 after 5 minutes.

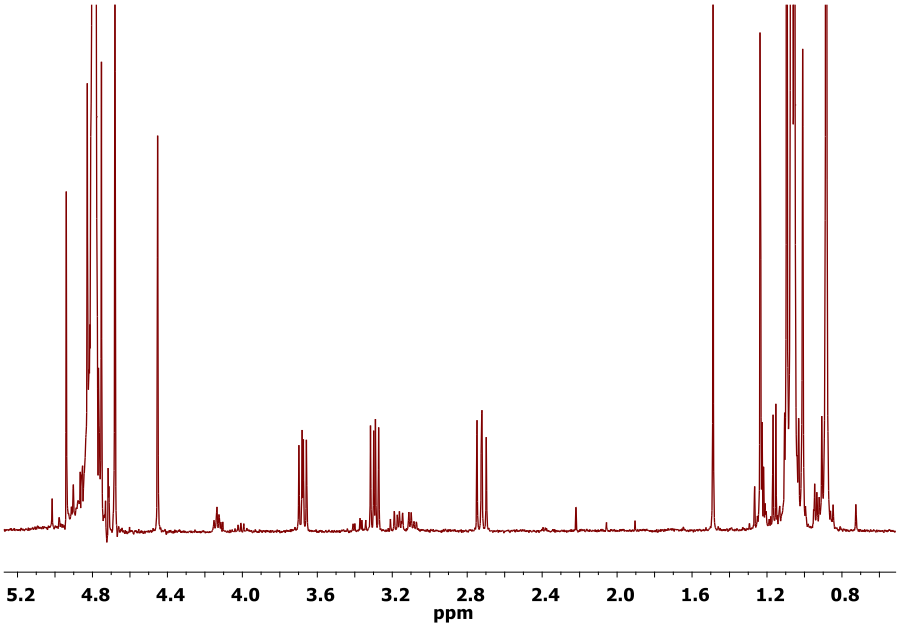


Figure S23. The ^{1}H-NMR spectrum of the reaction mixture between L-cysteine and 16 equivalents of trimethylacetaldehyde at pD = 7.4 after 20 minutes.

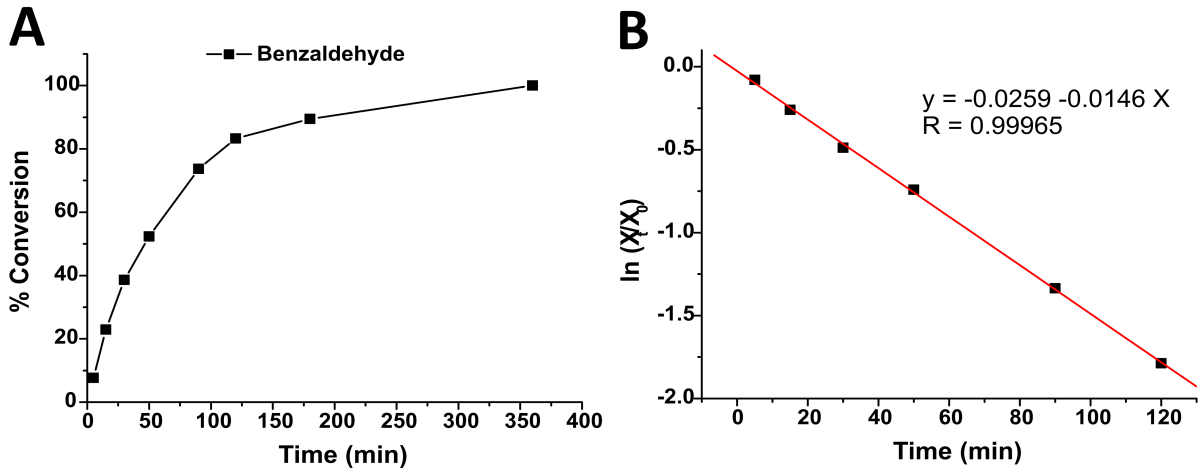


Figure S24. a) Representative plots of time-dependent conversion of thiazolidine using benzaldehyde at pD 7.4. b) Representative linear least-squares fits for thiazolidine formation using benzaldehyde at pD 7.4.

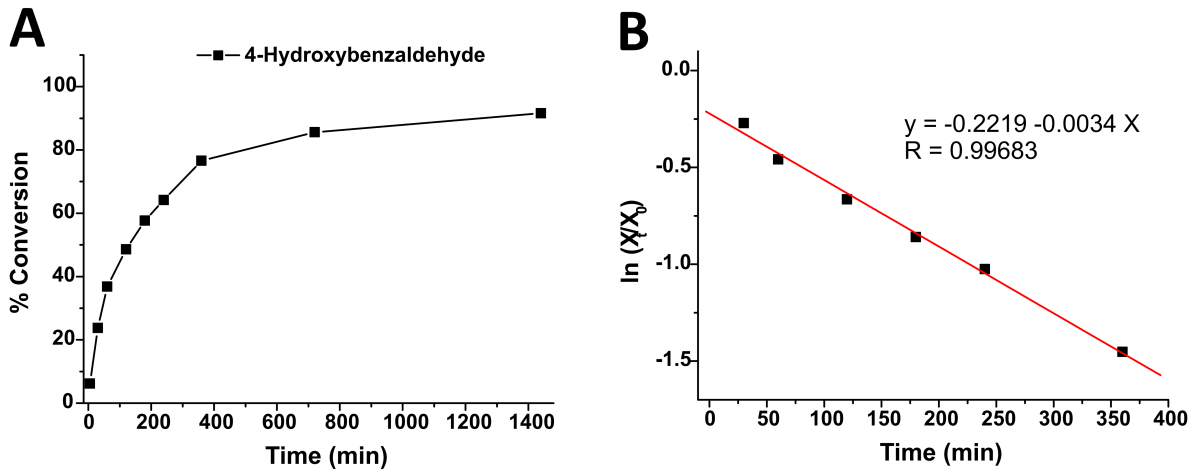


Figure S25. a) Representative plots of time-dependent conversion of thiazolidine using 4-hydroxybenzaldehyde at pD 7.4. b) Representative linear least-squares fits for thiazolidine formation using 4-hydroxybenzaldehyde at pD 7.4.

3. HPLC experiments

General conditions. The progress of the reaction was monitored by reverse phase HPLC (Waters XBridge, C18, 3 μm, 4.6×50 mm) at 214 nm for small molecule and 260 nm for RNA, if not specified otherwise. A linear gradient of 0–40% acetonitrile containing 0.1% TFA in water (pH 7.4) for 15 minutes with a flow rate of 1 mL/min at 25°C was employed.

Conjugation of elastin mimetic peptide to small molecule aldehydes. In general, with both the aliphatic and aromatic aldehyde substrates, reactions were performed in PBS (pH 7.4). The N-terminal cysteine modified elastin peptide (CVGVAPG) was used as a model to study the conjugation with small molecule aldehyde molecules. Propionaldehyde/4-nitrobenzaldehyde and peptide stock solutions were prepared in PBS (pH 7.4). For experiments containing DTT, 2 mM DTT solution was added to the peptide stock solution to avoid possible disulfide formation before performing the conjugation reaction. The reaction was performed with a different molar ratio of aldehyde (ranging from 0.5 to 5 equivalents of propionaldehyde and from 1 to 25 equivalents of 4-nitrobenzaldehyde) with 2 mM final concentration of peptide.

The free peptide was readily distinguishable from the conjugation product with propionaldehyde using the HPLC conditions mentioned before. Peptide chromatogram was observed at a retention time of 6.3 minutes, while peptide conjugate was observed at a retention time of 7.8 minutes. The conjugation reaction of the peptide (2 mM final concentration) with 2.5 equivalents of propionaldehyde was very fast and efficient (**Figure S27**) as the starting material completely disappeared in less than 30 minutes with nearly 75% conversion after 1 minute (approximate time employed in mixing the solutions and injecting the sample into the column). The rate of the reaction could be further improved using 5 equivalents of propionaldehyde, where 85% conversion was obtained after 1 minute of reaction (**Figure S28**).

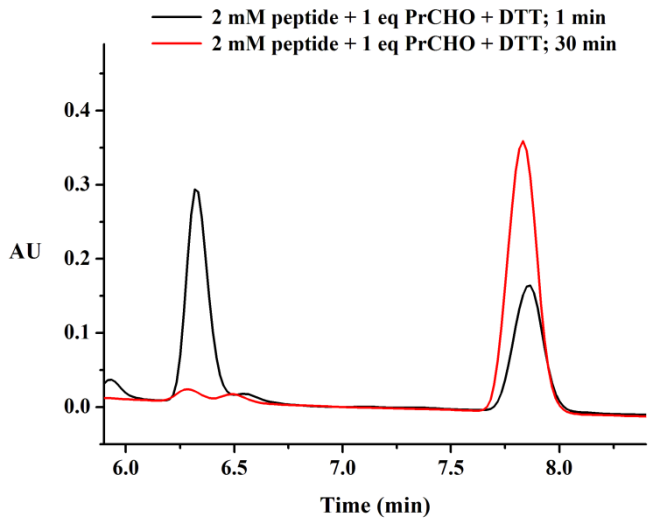


Figure S26. HPLC chromatogram showing the conjugation reaction between N-terminal cysteine peptide and 1 equivalent of propionaldehyde in the presence of DTT. The conjugation was complete in less than 30 minutes and showed 44% conversion when it was analysed after mixing (approx. 1 minute).

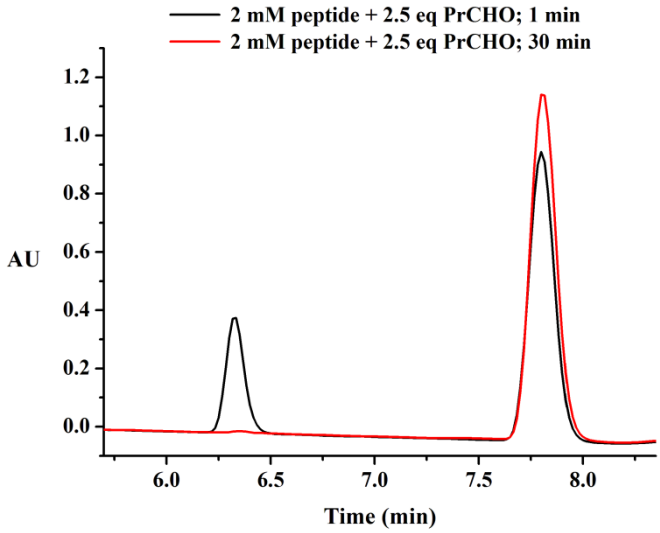


Figure S27. HPLC chromatogram showing the conjugation reaction between N-terminal cysteine peptide (2 mM) and 2.5 equivalents of propionaldehyde. The conjugation was complete in less than 30 minutes and showed 75% conversion when it was analysed just after mixing (approx. 1 minute).

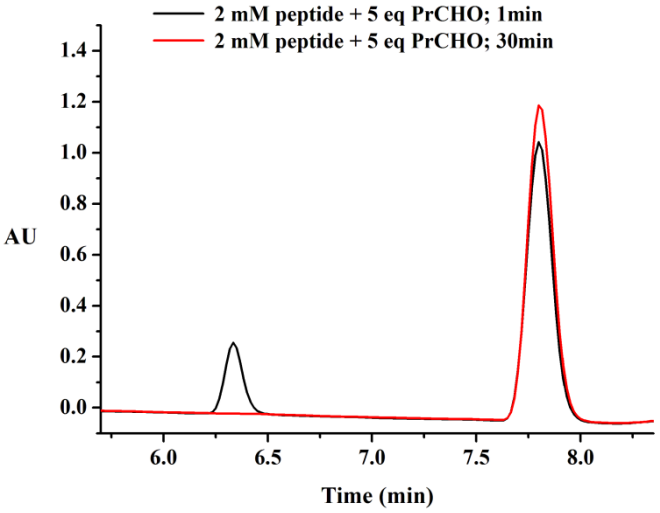


Figure S28. HPLC chromatogram showing the conjugation reaction between N-terminal cysteine peptide and 5 equivalents of propionaldehyde. The conjugation was complete in less than 30 minutes and showed 85% conversion when it was analysed just after mixing (approx. 1 minute).

The conjugation reaction was further repeated using 4-nitrobenzaldehyde as a model substrate. This reaction was also effective but showed slower reaction rate. The conjugation rate could be significantly improved by increasing the substrate concentration (**Figure S29, Figure S30**).

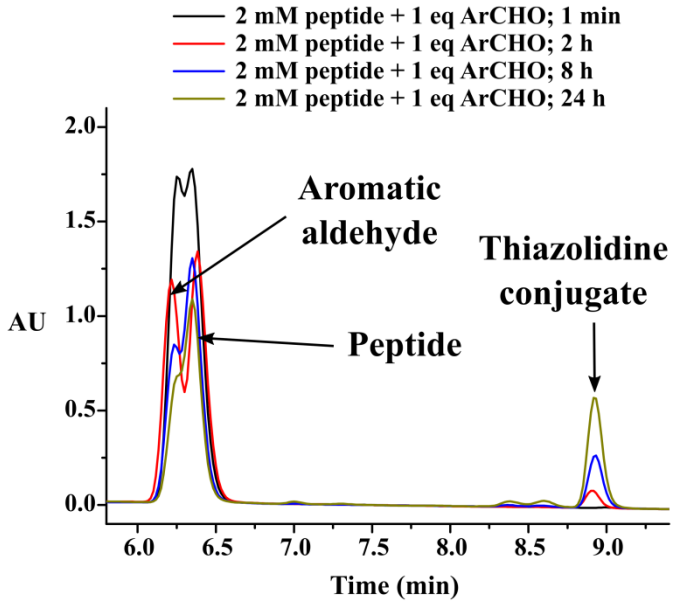


Figure S29. HPLC chromatogram showing the conjugation reaction between N-terminal cysteine containing peptide (final peptide concentration of 2 mM) and 1 equivalents of 4-nitrobenzaldehyde. The conjugation was inefficient because only 27% conversion was obtained after 24 h and 12% after 8 hours.

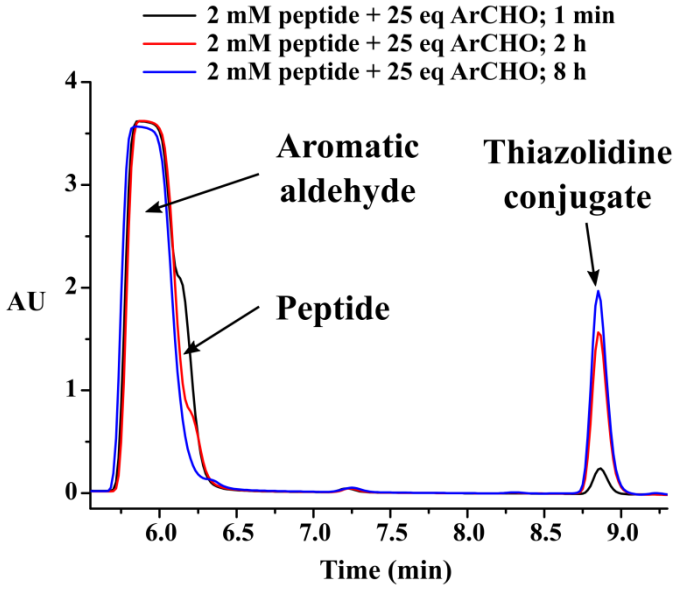


Figure S30. HPLC chromatogram showing the conjugation reaction between N-terminal cysteine containing peptide (2 mM final peptide concentration) and 25 equivalents of 4-nitrobenzaldehyde. The conjugation was complete in less than 8 hours with 77% conversion observed in less than 2 hours.

Conjugation of the peptide to aldehyde functionalised siRNA. In general, conjugation reaction between N-terminal cysteine containing TAT peptide (CYGRKKRRQRRR) and 5'-aldehyde modified siRNA was performed in PBS (pH 7.4). A 20 µL of siRNA (50 µM stock

solution in PBS, pH 7.4) was diluted with 70 µL of PBS (pH 7.4). To this solution, 10 µL of the peptide stock solution (10 mM peptide and 5 mM DTT in PBS, pH 7.4) was added. The progress of the reaction was monitored by reverse phase HPLC at 260 nm with a gradient as mentioned above.

4. References

(1) Nagasawa, H. T.; Goon, D. J.; Muldoon, W. P.; Zera, R. T. *J. Med. Chem.* **1984,** *27*, 591.