An optimized protocol for the synthesis of *N*-2-hydroxybenzyl-cysteine peptide crypto-thioesters.

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Supporting Information

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1- General information

All reagents and solvents were used without further purification. Fmoc-Rink MBHA polystyrene resin, Fmoc-Rink linker, aminomethyl NovaGel resin and Oxyma were purchased from Merck Biosciences (Nottingham, UK). Protected amino acids were purchased from Gyros Protein Technology (Uppsala, Sweden). Aminomethyl TentaGel R resin was purchased from Rapp polymers (Tuebingen, Germany). HCTU and HATU were purchased from IRIS Biotech GmbH (Marktredwitz, Germany). DIEA was purchased from Carlo ERBA (Val-de-Reuil, France). Rink amide ChemMatrix resin was purchased from Biotage (Uppsala, Sweden). Spheritide resin was a gift from CEM (Orsay, France). Peptide synthesis grade DMF was obtained from VWR (Fontenay-sous-Bois, France). All other chemicals were from Sigma Aldrich (St-Quentin-Fallavier, France) and solvents from SDS-Carlo Erba (Val de Reuil, France). Ultrapure water was obtained using a Milli-Q water system from Millipore (Molsheim, France). Polypropylene syringes fitted with polypropylene frits were obtained from Torviq (Niles, MI, USA) and were equipped with PTFE stopcock bought from Biotage. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AVANCE III 600 instrument, at a constant temperature of 25°C. Chemical shifts are reported in parts per million from low to high field and referenced to tetramethylsilane (TMS). Coupling constants (*J*) are reported in hertz (Hz). Standard abbreviations indicating multiplicity were used as follows: s = singlet, d = doublet, dd =doublet of doublets, m = multiplet, b=broad signal. HPLC analyses were carried out on a Hitachi Chromaster system equipped with a 5160 pump, a 5430 diode array detector and a 5260 auto sampler and semi-preparative purifications were carried out on a Hitachi LaChromElite system equipped with a L-2130 pump, a L-2455 diode array detector and a L-2200 auto sampler. Chromolith High Resolution RP-18e (150 Å, 10 × 4.6 mm, 3 mL/min flow rate) columns were used for analysis, Nucleosil C18 (300 Å, 5µm, 250 × 10mm, 3 mL/min flow rate) and Jupiter C4 (300 Å, 5µm, 250 × 10mm, 3 mL/min flow rate) for purification. Solvents A and B are 0.1 % TFA in H_2O and 0.1 % TFA in MeCN, respectively.) Each gradient was followed by a washing step to elute any compound not eluted during the gradient (up to 95% B/A over 0.5 min, then isocratic 95% B/A over 0.5 min for Chromolith). LC-ESI-MS analyses were carried out on an Agilent 1260 Infinity HPLC system, coupled with a 6120 mass spectrometer, and fitted with an Aeris Widepore XB-C18 2 (3.6 µm, 150 × 2.1 mm, 0.5 mL/min flow rate, 60°C) column. The reported *m*/*z* values correspond to the monoisotopic ions if not specified otherwise. Solvents A' and B' were 0.1 % formic acid in H_2O and 0.1 % formic acid in MeCN, respectively. Gradient: 3% B'/A' for 1 min, then 3 to 50% B'/A' over 15 min. Low resolution MS of pure compounds were obtained using this system. High-resolution ESI-MS analyses were performed on a maXisTM ultra-high-resolution Q-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany), using the positive mode. Relative rates of Hnb-containing compounds were determined by HPLC peak integration at λ = 320 nm which is a wavelength of maximum absorption (λmax) for the Hnb group.

2-General procedures for manual and automatic SPPS

Protocol PS1 – peptide elongation: Manual couplings were performed on polypropylene syringes fitted with polypropylene frits using rotation stirring. Fmoc-based solid phase peptide syntheses (SPPS) were carried out on a Prelude synthesizer from Protein technologies. The side-chain protecting groups used were Arg(Pbf), Asp(O*t*Bu), Cys(Acm), Cys(Trt), Cys(S*t*Bu), Glu(O*t*Bu), His(Trt), Ser(*t*Bu), Thr(*t*Bu), Trp(Boc) and Tyr(*t*Bu). Syntheses were performed on a 0.025 mmol-per-reactor scale. Protected amino acids (0.25mmol, 10 equiv.) were coupled using HCTU (98 mg, 0.238 mmol, 9.5 equiv.) and *i*Pr_2NEt (87 µL, 0.5 mmol, 20 equiv.) in NMP (3 mL) for 30 min. Coupling of Fmoc-Ser(*t*Bu)-OH *N*-Hnb-cysteine secondary amine were performed for 18 h. For recommended coupling protocols of the different C-terminal amino acids of peptide segments, see the experimental part of the article. Capping of eventual unreacted amine groups was achieved by treatment with acetic anhydride (143 µL, 1.51 mmol, 60 equiv.), *i*Pr_2NEt (68 µL, 0.39 mmol, 15.5 equiv.) and HOBt (6 mg, 0.044 mmol, 1.8 equiv.) in NMP (3 mL) for 7 min (4 x 7 min in the case of *N*-Hnb-cysteine secondary amine). Fmoc group was deprotected by three successive treatments with 20% piperidine in NMP (3 mL) for 3 min. As stated in the experimental section of the article, variable amount (5-90 %) of *O*-acylation of the Hnb phenol are observed after each coupling. This ester is cleaved within seconds upon piperidine treatment during Fmoc deprotection. As a consequence, a final piperidine treatment (standard 3 x 3 min, 20% in DMF or NMP) is thus necessary even if the N-terminal residue is not Fmoc-protected.

Protocol PS2 – **Cleavage**: The crude peptide was deprotected and cleaved from the resin through a treatment with TFA/H_2O/*i*Pr_3SiH/phenol, 88:5:2:5 for 2 h, and the peptide was precipitated by dilution into an ice-cold diethyl ether/petroleum ether 1:1 mixture, recovered by centrifugation and washed twice with diethyl ether.

Protocol PS3 – **Small peptides cleavage** (less than 4 residues): they were deprotected and cleaved from the resin through a treatment with TFA/H_2O/*i*Pr_3SiH, 93:5:2 for 30 min following by concentration under vacuum.

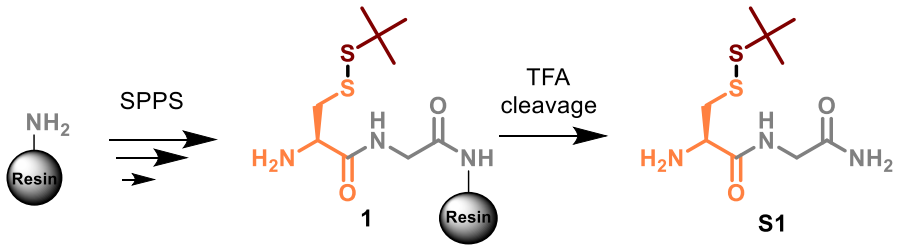
Protocol PS4 – **Procedure for selective Hnb ester cleavage to allow UV titration of Fmoc deprotection**: As a consequence of the formation of variable amount (5-90 %) of *O*-acylated Hnb during each coupling, this ester being cleaved upon piperidine treatment during Fmoc deprotection, standard UV titration of the fluorenylmethyl-piperidine adduct after Fmoc deprotection is useless unless using a prior treatment for selective ester cleavage before piperidine treatment.

Ester cleavage mixture was prepared as follows: 1.25 g (1.80 mmol) of $NH_2OH{\cdot}HCl$ and 0.918 g (1.35 mmol) of imidazole were suspended in 5 mL of NMP, the mixture was sonicated until complete dissolution. This solution can be stored for few months at -20°C. 5 volumes of this this solution is diluted with 1 volume of DCM prior to utilization, and the resin is treated for 3x20 min for quantitative ester cleavage.

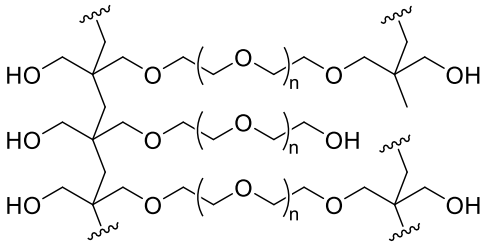
The fluorenylmethyl-piperidine adduct is quantified by UV spectroscopy at λ = 301 nm (ε =7800 mol^{-1} cm^{-1}) in order to evaluate the Fmoc SPPS elongation yield of crypto-thioester peptides.

3-Optimization of the reductive amination and coupling of first amino acid

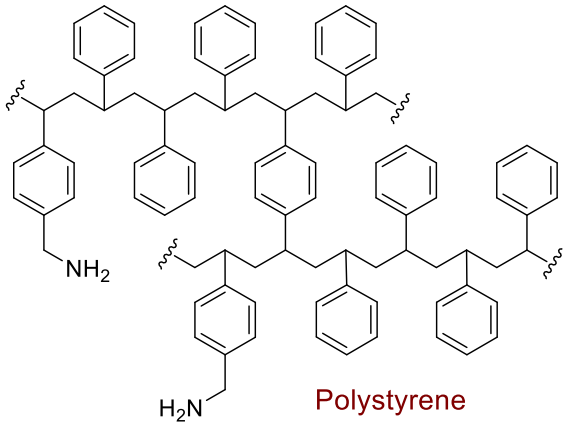
3-1- Synthesis of cysteinyl peptide resins 1a-e



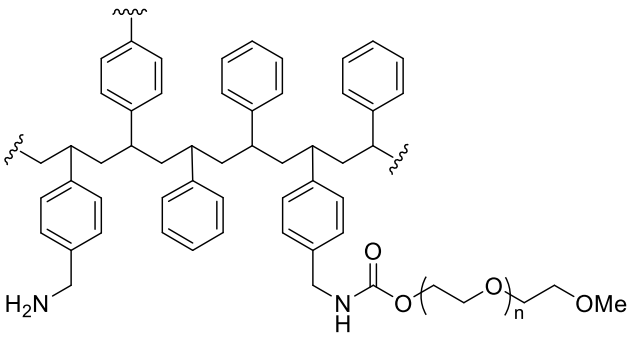
1a-Resin = Rink ChemMatrix
1b-Resin = Rink Polystyrene
1c-Resin = Rink Tentagel
1d-Resin = Rink Novagel
1e-Resin = Rink Spheritide



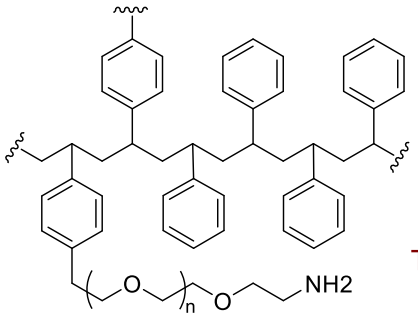
ChemMatrix



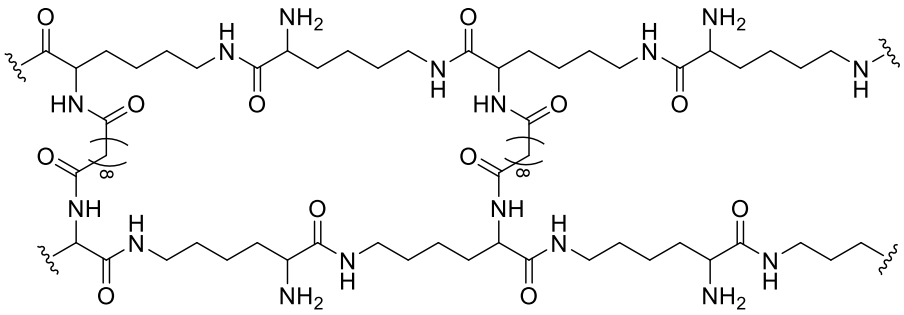
Polystyrene



Novagel



Tentagel



Spheritide

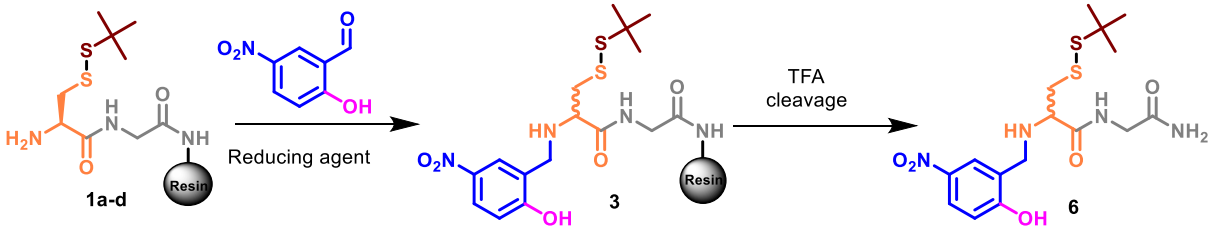
Peptidyl resins **1a-e** were obtained through manual SPPS (protocol PS1) starting from different resins. Resins loading: Rink ChemMatrix: 0.54 mmol/g, Rink-MBHA polystyrene: 0.89 mmol/g, Tentagel R: 0.164 mmol/g, Novagel: 0.77 mmol/g, Rink Spheritide: 0.15 mmol/g. In the cases of Tentagel and Novagel, Fmoc-Rink linker was coupled manually. Small amounts of peptide-resins were cleaved for analytical purpose (protocol PS3 - small peptides cleavage) to give peptide **S1**.

- H-Cys(S*t*Bu)-Gly-NH_2 (**S1**)

ESI-MS (*m/z*): $[MH]^+$ calcd. for $C_9H_{20}N_3O_2S_2$: 266.1, found: 266.1

HPLC analysis: t_R = 2.14 min (Chromolith, gradient: 1-50% B/A over 15 min)

3-2- Solid-supported reductive amination



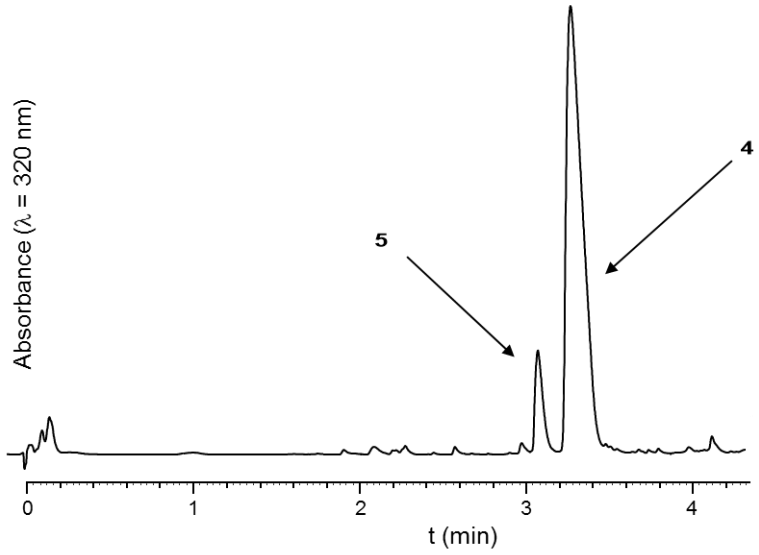
- H-(Hnb)Cys(S*t*Bu)-Gly-NH_2 (**6**)

ESI-MS (*m/z*): $[MH]^+$ calcd. for $C_{16}H_{25}N_4O_5S_2$: 417.1, found: 417.0

HPLC analysis: t_R = 6.8 min (Aeris Widepore XB-C18 2, gradient: 3-50% B'/A' over 15 min)

Protocol PS5-1: Previously reported reductive amination protocol with $NaBH_3CN$ [1] (see main text, Table 1, entry 1)

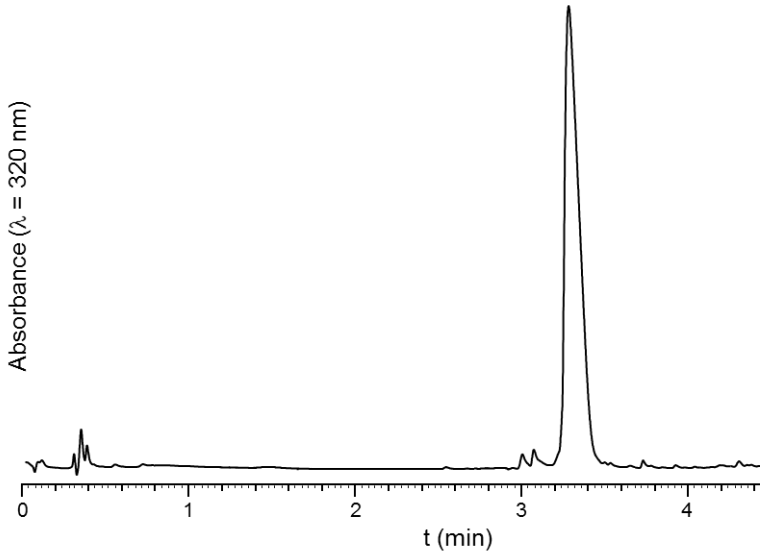
25 µmol of H-Cys(S*t*Bu)-Gly-Rink-resin (**1a-d**) was washed with 1:1 DMF/MeOH (4 x 3 mL, 30 s) then treated with 9:9:2 DMF/MeOH/AcOH mixture (3 mL, 5 min). The resin was washed with 1:1 DMF/MeOH (3 x 3 mL, 30 s) and 2-hydroxy-5-nitrobenzaldehyde (42 mg, 10 equiv.) in 2 mL 1:1 DMF/MeOH (125 mM aldehyde concentration) was then added, and the reactor was stirred for 1 h. The reactor was drained and the resin was washed with 1:1 DMF/MeOH (3 x 3 mL, 5 s). Without delay, a fresh solution of sodium cyanoborohydride (32 mg, 20 equiv.) in 3 mL 9:9:2 DMF/MeOH/AcOH (250 mM, $NaBH_3CN$ concentration) was added and the reactor stirred for 1 h. The reactor was drained and the resin was washed with 1:1 DMF/MeOH (4 × 3 mL, 30 s), NMP (3 x 3 mL, 30 s), 20% piperidine in NMP (3 x 3 mL, 3 min), NMP (3 x 3 mL, 30 s), dichloromethane (3 x 5 mL, 30 s) and NMP (2 x 3 mL, 30 s). Peptidyl resins were cleaved using protocol PS2 - small peptides cleavage.



Supplementary figure S1: HPLC trace (Chromolith, gradient: 5-50% B/A over 5 min) of **4** (resin: ChemMatrix) obtained using previously reported two-steps reductive amination with $NaBH_3CN$ protocol PS2-1 corresponding to table 1, entry 1)

Protocol PS5-2 : Optimized reductive amination protocol with $NaBH_4$ (see main text, table 6, entry 1)

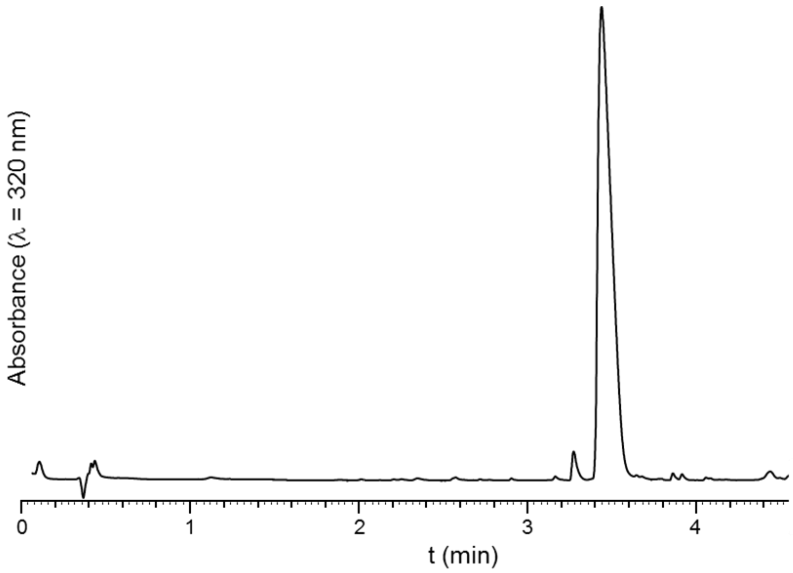
25 µmol of H-Cys(StBu)-Gly-Rink-resin (**1a-c**) was washed with 1:1 DMF/MeOH (4 x 3 mL, 30 s). 2-Hydroxy-5-nitrobenzaldehyde (42 mg, 10 equiv.) in 2 mL 44.5:44.5:1 DMF/MeOH/AcOH (125 mM aldehyde concentration) was then added, and the reactor was stirred for 5 min. The reactor was drained and the resin was washed with 1:1 DMF/MeOH (3 x 3 mL, 5 s) then DMF (3 x 3 mL, 5 s). Without delay, a fresh solution of sodium borohydride (19 mg, 20 equiv.) in 2 mL DMF (250 mM borohydride concentration) was added and the reactor was stirred for 20 min. The reactor was drained and the resin was washed with DMF (4 × 3 mL, 30 s), 20% *v*/*v* piperidine in NMP (3 × 3 mL, 3 min), NMP (3 × 3 mL, 30 s), dichloromethane (3 × 5 mL, 30 s) and NMP (3 × 3 mL, 30 s). Peptidyl resins were cleaved using protocol PS2 - small peptides cleavage.



Supplementary figure S2: HPLC trace (Chromolith, gradient: 5-50% B/A over 5 min) of **4** obtained using two-steps reductive amination with $NaBH_4$

Protocol PS5-3 : Optimized one pot reductive amination protocol with borane pyridine complex (see main text, table 5, entry 5)

25 µmol of H-Cys(S*t*Bu)-Gly-Rink-ChemMatrix **1a** was washed with 3 mL of a 1:1 DMF/MeOH mixture (4 x 3 mL, 30 s). A solution of 2-hydroxy-5-nitrobenzaldehyde (42 mg, 10 equiv.) in 1 ml 1:1 DMF/MeOH (250 mM, aldehyde concentration) was prepared, next acetic acid (20 µL, 14 equiv., 1% *v*/*v* final) was added and finally a solution of reducing agent (46.5 mg, 20 equiv.) in 1 ml DMF/MeOH (250 mM final borane concentration) was added. The mixture was added to the resin without delay, and the reactor was stirred for 20 min. The reactor was drained and the resin was washed with 1:1 DMF/MeOH (4 × 3 mL, 30 s), NMP (3 × 3 mL, 30 s), 20% piperidine in NMP (3 x 3 mL, 3 min), NMP (3 × 3 mL, 30 s), dichloromethane (3 × 5 mL, 30 s) and NMP (3 × 3 mL, 30 s).



Supplementary figure S3: HPLC trace (Chromolith, gradient: 5-50% B/A over 5 min) of **4** obtained through one-pot reductive amination with borane pyridine complex

- Other reductive amination protocols tried not detailed in the manuscript

❖ Table S1: Different other reductive amination conditions tested following literature protocols[a]

Entry	Ref.	One pot / two steps	Reducing agent	Additive (equiv.)	Desired product **4** (%)[b]	Epimer **5** (%)[b]	Imidazolidinones **10** (%)[b]	Other side products (%)[b] [*m/z*][c]
1[d]	[2]	One pot	$NaBH_3CN$	$SnCl_2$ (20)	91	6	-	3 [447.0]
2[e]	[3]	Two steps	$BH_3{\cdot}Me_2NH$	-	66	4.5	25	4.5 [534.1]
3[f]	[4]	Two steps	$BH_3{\cdot}Me_2S$	-	90	9	0.5	-
4[g]	[5]	Two steps	$NaBH_4$	$ZnCl_2$ (15)	75	8	-	16 [534.1]
5[g]	[6]	Two steps	$NaBH_4$	$MgCl_2$ (10)	95	2.5	2	1.6 [534.1]
6[h]	[7]	One pot	$NaBH(OAc)_3$	-	<2	-	-	<98 [581.9]
7[i]	[8]	Two steps	$NaBH_4$	$TiCl_4$ (4)	51	5	-	27 [534.1] 17 [409.1]
8[j]	[9]	One pot	$NaBH_3CN$	$Ti(iPrO)_4$ (2)	14	0.7	-	70.1 [353.0] 15.2 [385.1]

[a] All reactions were conducted at room temperature at a 12.5 mM peptidyl resin concentration using 20 equiv. of the reducing agent; [b] Relative rates determined by HPLC peak integration at λ= 320 nm; [c] *m/z* not attributed to putative structures except for the [M-2Da] byproducts that likely correspond to imidazolidinones **10a-d**; [d] 5 min reaction in MeOH; [e] Imination in DMF/MeOH 1:1 for 5 min, reduction in DMF/MeOH 1:1 for 5 min; [f] Imination in DMF/MeOH 1:1 for 5 min, reduction in THF for 5 min; [g] Imination in DMF for 5 min, reduction in DMF for 5 min; [h] 1 h reaction in THF/DMF 1:1; [i] Imination in THF for 5 min, reduction in THF for 5 min; [i] 5 min reaction in THF.

The different conditions described in table S1 were inspired from the following reports. Note that in some cases, reaction conditions were slightly modified and adapted to the current work.

3-3 Characterization of compounds 4 and 5.

- H-Ser-(Hnb)Cys(S*t*Bu)-Gly-NH_2 (**4**)

ESI-MS (*m/z*): $[MH]^+$ calcd. for $C_{19}H_{30}N_5O_7S_2$: 504.2, found: 504.1

HPLC analysis: t_R = 7.30 min (Aeris Widepore XB-C18 2, gradient: 3-50% B'/A' over 15 min)

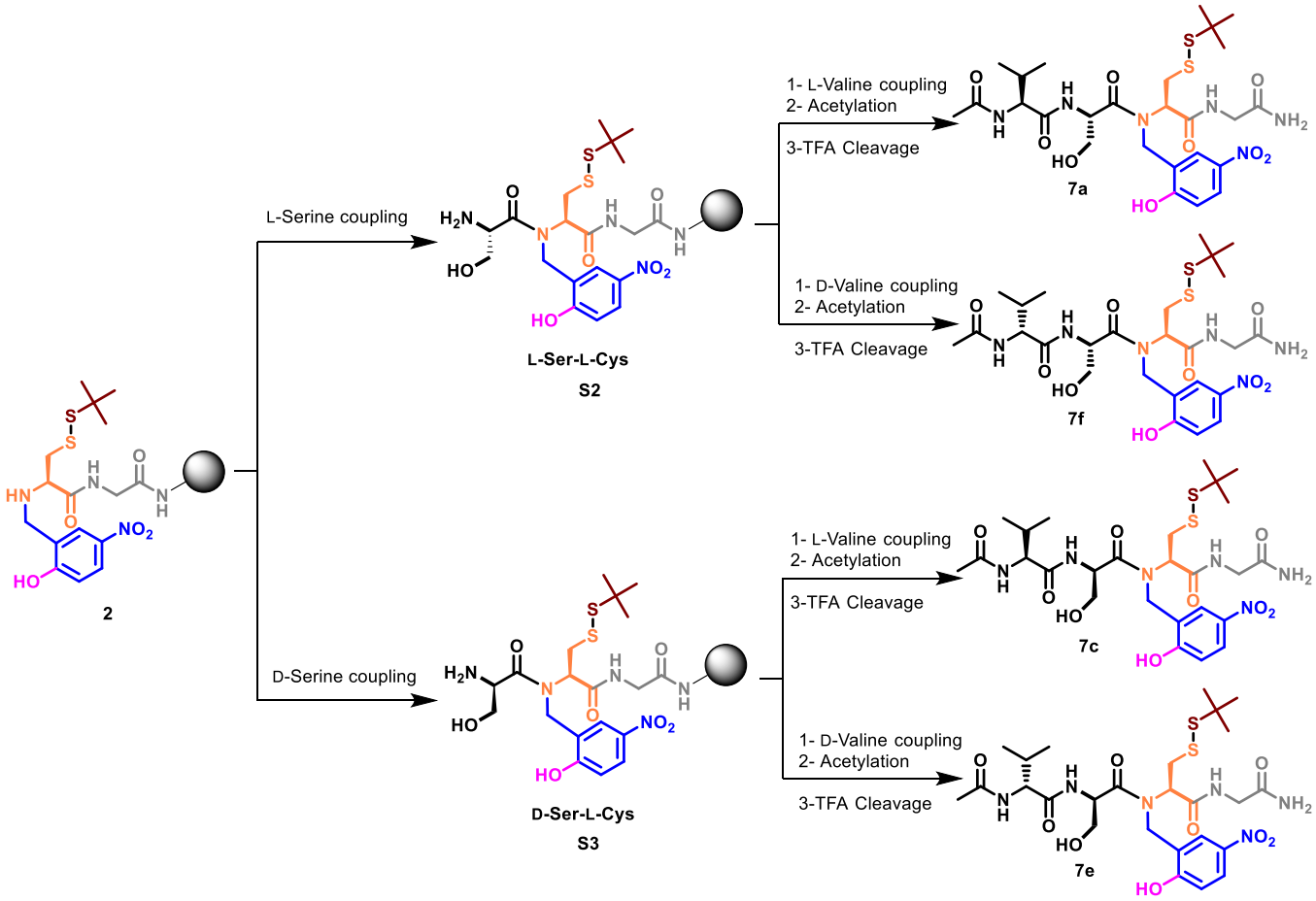
- H-Ser-(Hnb)D-Cys(S*t*Bu)-Gly-NH_2 (**5a**) and H-D-Ser-(Hnb)Cys(S*t*Bu)-Gly-NH_2 (**5b**)

ESI-MS (*m/z*): $[MH]^+$ calcd. for $C_{19}H_{30}N_5O_7S_2$: 504.2, found: 504.1

HPLC analysis: t_R = 6.5 min (Aeris Widepore XB-C18 2, gradient: 3-50% B'/A' over 15 min)

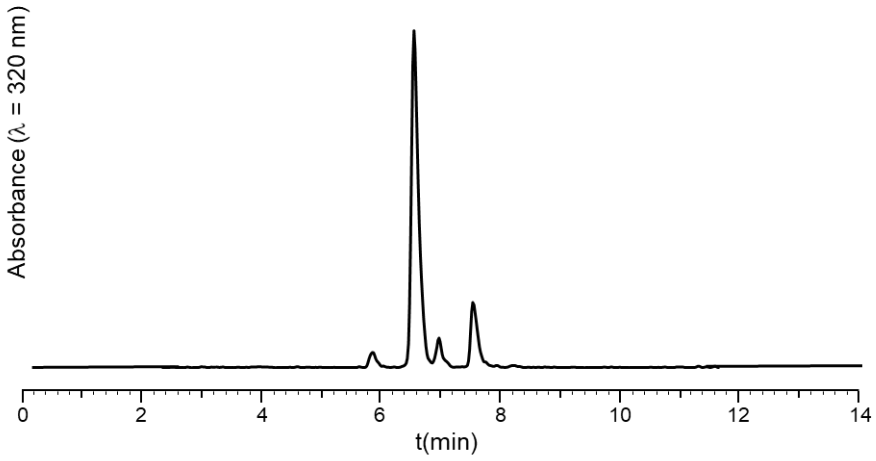
4-Synthesis of HPLC standards 7a, 7c, 7d and 7f

In the case of **7b** and **7d**, we synthesized their enantiomeric counterpart **7e** and **7f**, respectively, as the later compounds were less synthetically demanding while being equivalent in achiral HPLC (fig. 2A).



The four HPLC standards (**7a**, **7c**, **7f** and **7e**) were synthesized from **2** according to general coupling protocol PS1. *N*-acetylation was performed with 20% anhydride acetic in DCM for 20 min followed by piperidine treatment. Peptidyl resins were then cleaved following protocol PS1-small peptides cleavage.

- Ac-Val-Ser-(Hnb)Cys(S*t*Bu)-Gly-NH_2 (**7a**)

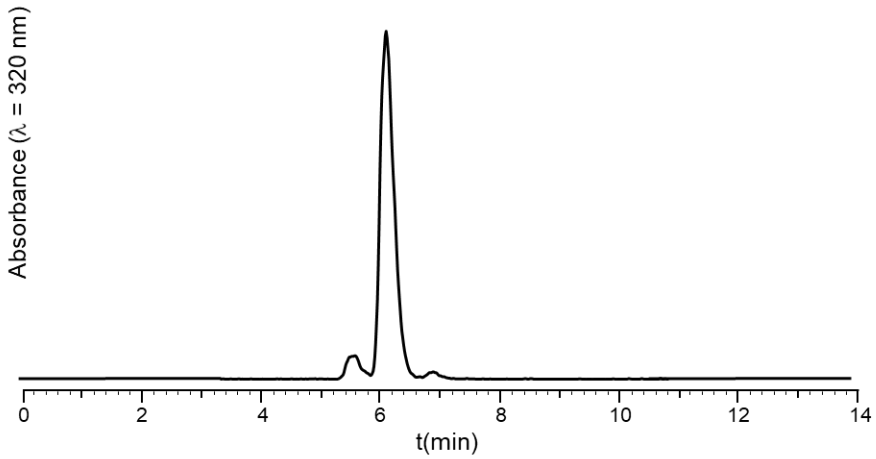


Supplementary figure S4: HPLC trace of crude **7a**

ESI-MS (*m/z*): $[MH]^+$ calcd. for $C_{26}H_{41}N_6O_9S_2$: 645.2, found: 645.2

HPLC analysis: t_R = 6.77 min (Aeris Widepore XB-C18 2, gradient: 23-33% B'/A' over 15 min)

- Ac-Val-D-Ser-(Hnb)Cys(S*t*Bu)-Gly-NH_2 (**7c**)



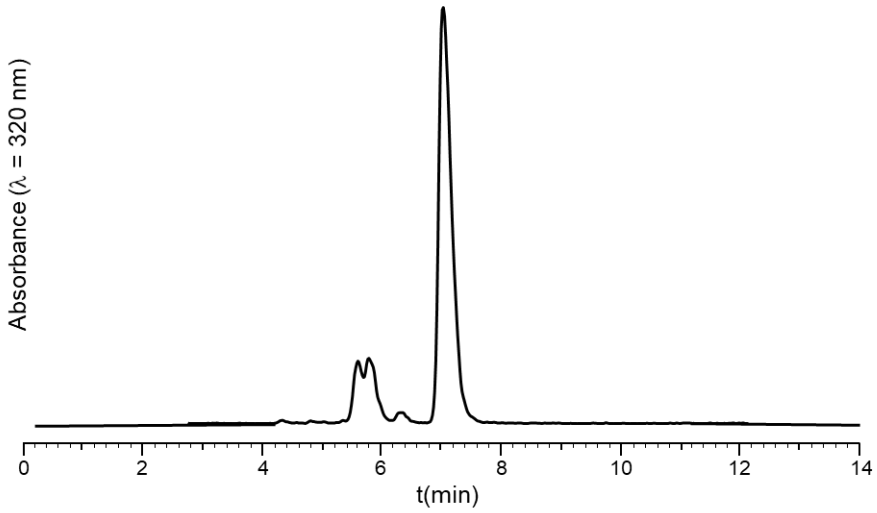
Supplementary figure S5: HPLC trace of crude **7c**

ESI-MS (*m/z*): $[MH]^+$ calcd. for $C_{26}H_{41}N_6O_9S_2$: 645.2, found: 645.2

HPLC analysis: t_R = 6.23 min (Aeris Widepore XB-C18 2, gradient: 23-33% B'/A' over 15 min)

- Ac-D-Val-D-Ser-(Hnb)Cys(S*t*Bu)-Gly-NH_2 (**7e**)

For synthesis simplicity reasons, **7e** has been synthesized to be used as an HPLC standard for its enantiomer Ac-Val-Ser-(Hnb)D-Cys(S*t*Bu)-Gly-NH_2 (**7b**).



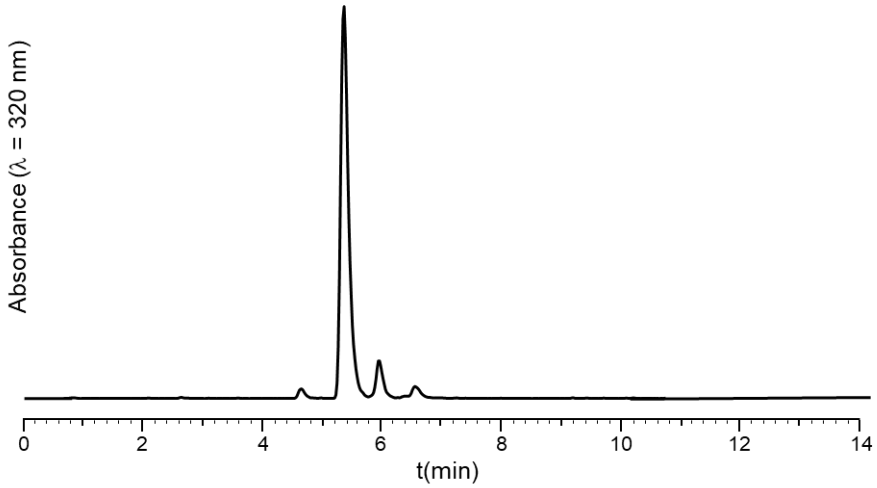
Supplementary figure S6: HPLC trace of crude **7e**

ESI-MS (*m/z*): $[MH]^+$ calcd. for $C_{26}H_{41}N_6O_9S_2$: 645.2, found: 645.1

HPLC analysis: t_R = 7.01 min (Aeris Widepore XB-C18 2, gradient: 23-33% B'/A' over 15 min)

- Ac-D-Val-Ser-(Hnb)Cys(S*t*Bu)-Gly-NH_2 (**7f**)

For synthesis simplicity reasons, **7f** has been synthesized to be used as an HPLC standard for its enantiomer Ac-Val-D-Ser-(Hnb)D-Cys(S*t*Bu)-Gly-NH_2 (**7d**).

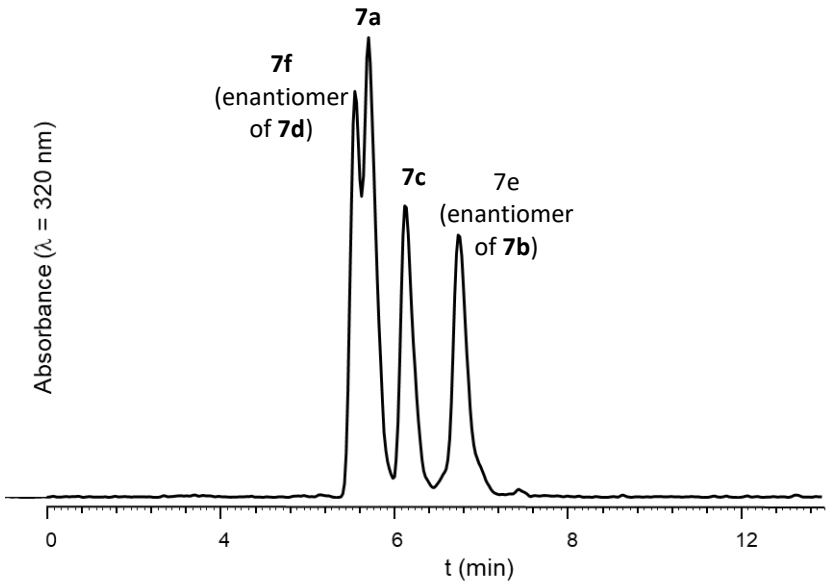


Supplementary figure S7: HPLC trace of crude **7f**

ESI-MS (*m/z*): $[MH]^+$ calcd. for $C_{26}H_{41}N_6O_9S_2$: 645.2, found: 645.2

HPLC analysis: t_R = 5.56 min (Aeris Widepore XB-C18 2, gradient: 23-33% B'/A' over 15 min)

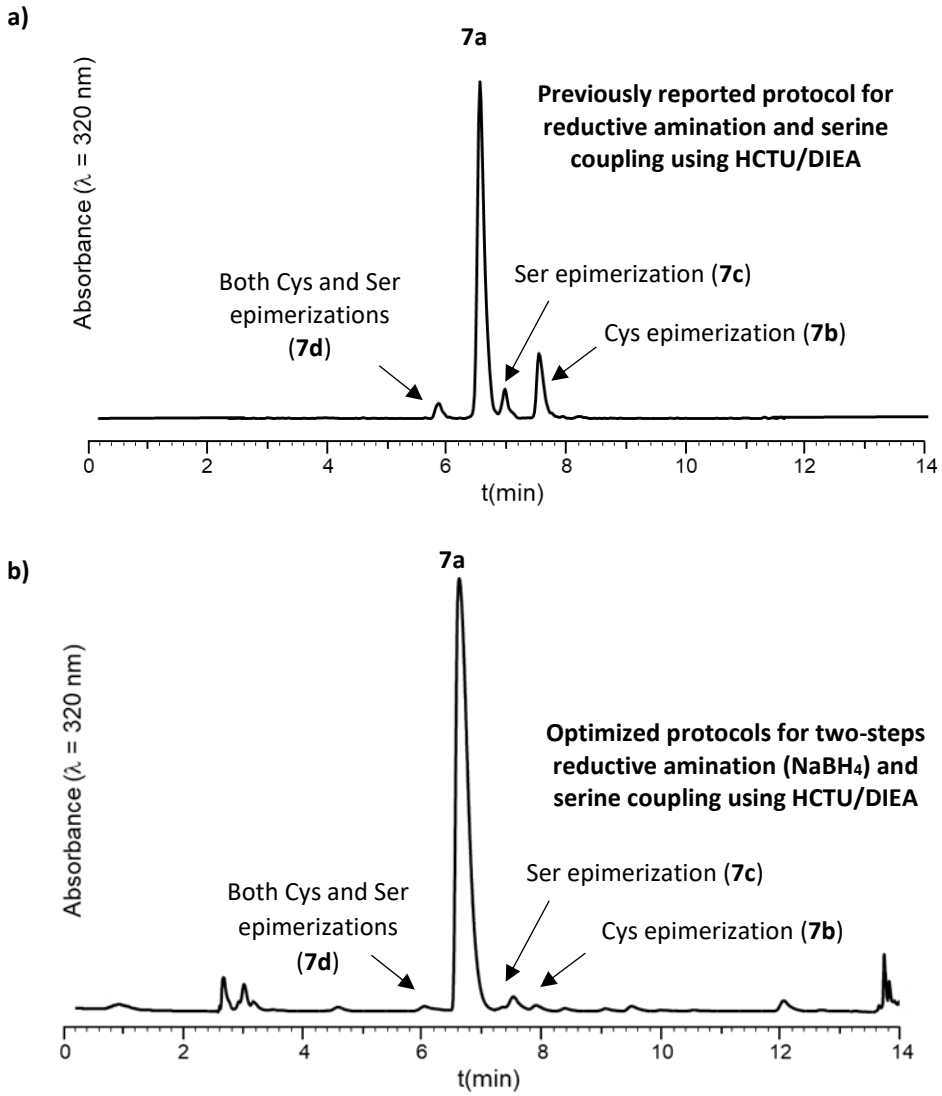
- Co-injection of the four HPLC standards **7a**, **7b**, **7e** and **7f**



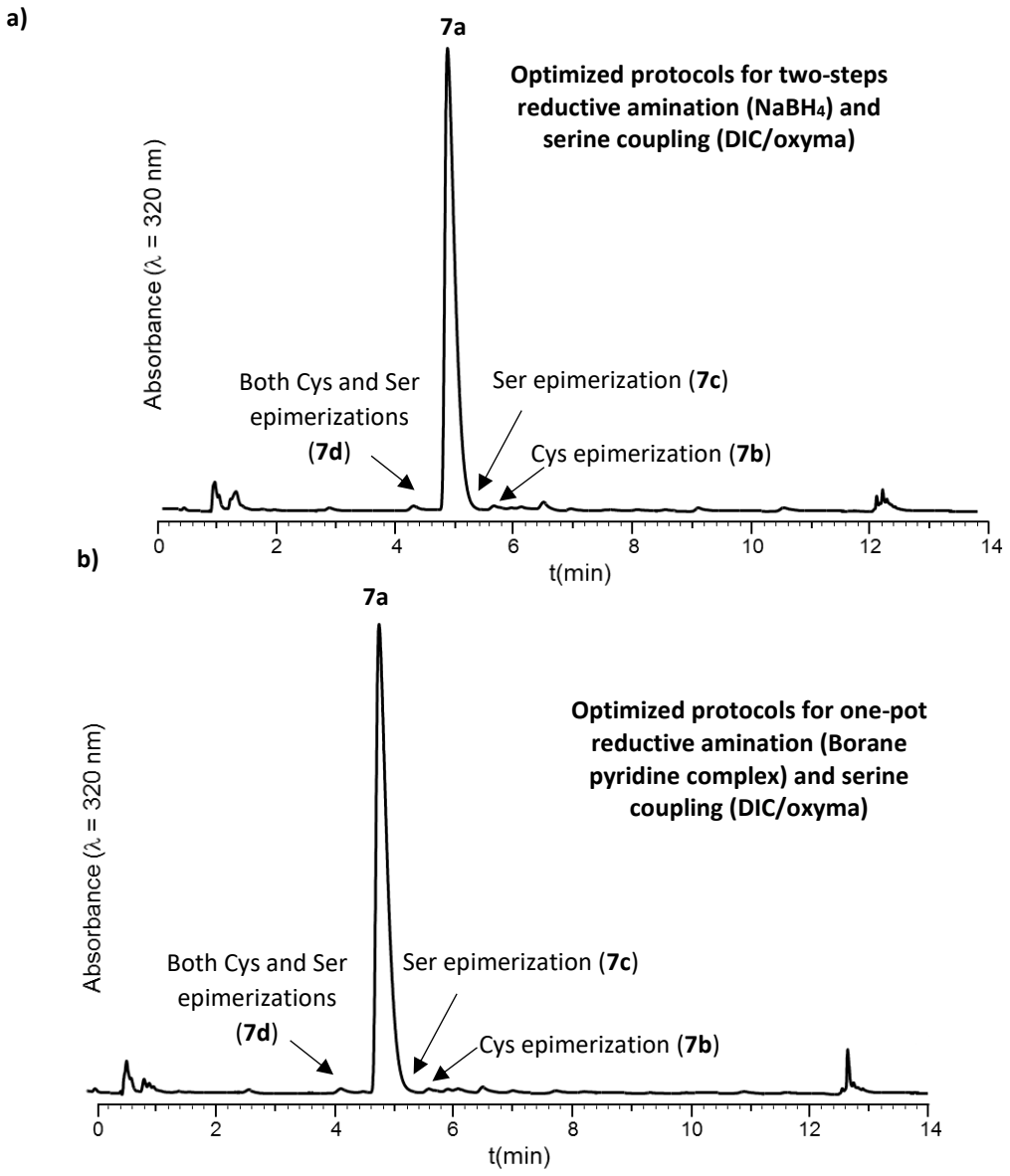
Supplementary figure S8: HPLC trace (Aeris Widepore XB-C18 2, gradient: 23-33% B'/A' over 15 min) of co-injection of the 4 HPLC standards

- Use of the HPLC standards for quantification of Ser and Cys epimerization

The mixture of the 4 HPLC standards was co-injected with the three crude mixtures obtained from the initial and optimized reductive amination protocols in order to unambiguously identify compounds arising from Cys, Ser and both Cys and Ser epimerization.



Supplementary figure S9: HPLC trace (Aeris Widepore XB- C18 2, gradient: 23-33% B'/A' over 15 min) of crude **7** obtained using previously reported reductive amination protocol (resin: ChemMatrix) (S9a) and the optimized protocol for two-steps reductive amination (NaBH4) (resin: ChemMatrix) (S9b). Serine was coupled using the HCTU/DIEA standard protocol in both cases

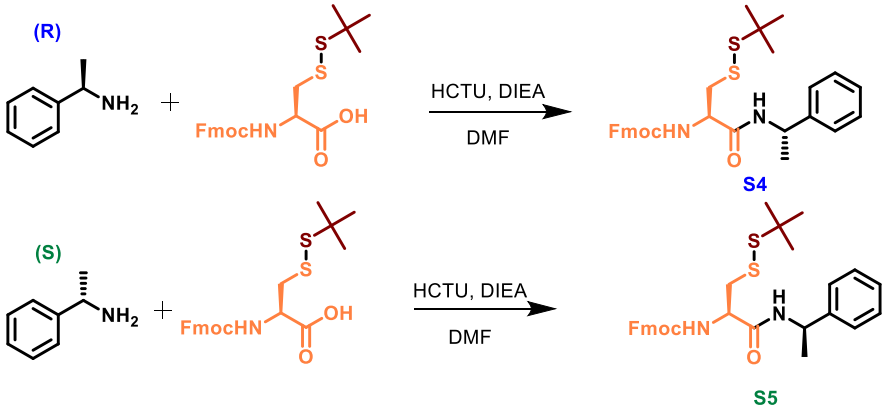


Supplementary figure S10: HPLC trace trace (Aeris Widepore XB- C18 2, gradient: 3-90 % B'/A' over 15 min) of crude **7** obtained using optimized two-steps reductive amination ($NaBH_4$) protocol (resin: ChemMatrix) (S10a) and the optimized one-pot reductive amination (borane pyridine complex) (resin: ChemMatrix) protocol (S10b). Serine was coupled using the optimized DIC/Oxyma protocol in both cases

5-Identification of the origin of the Cys epimerization

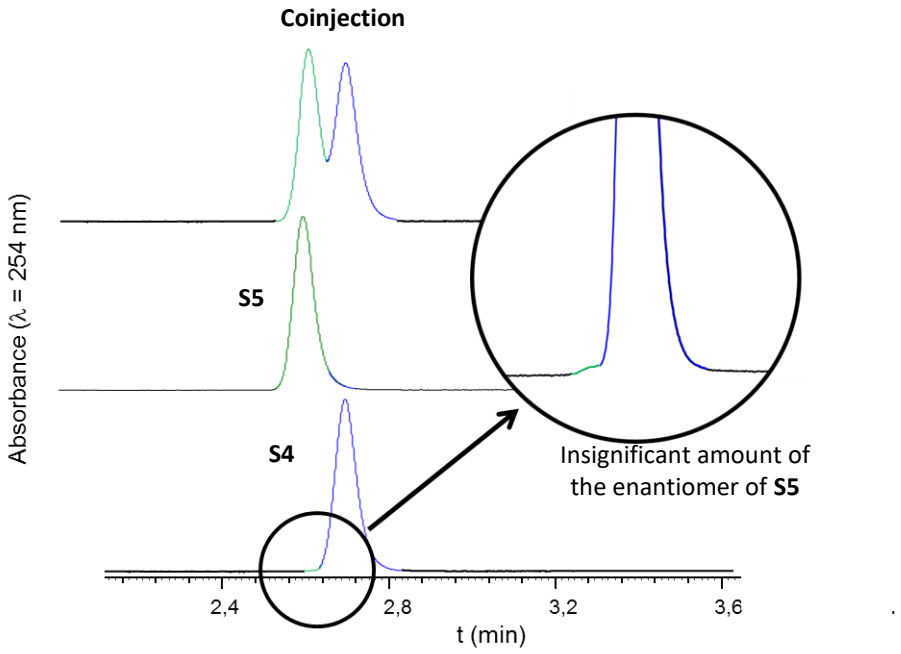
As described in the article main text, the following experiments allowed us to unambiguously conclude that Cys epimerization occurred during reductive amination.

- Verification of the enantiomeric purity of commercial Fmoc- Cys(S*t*Bu)-OH



(*R*)- and (*S*)-methylbenzylamine (89 µl, 0.7 mmol, 1 equiv.) were coupled to Fmoc-Cys(S*t*Bu)-OH (302 mg, 0.7 mmol) using HCTU (350 mg, 0.83 mmol, 1.2 equiv.) and DIEA (122 µL, 0.14 mmol, 2 equiv.) in DMF (5 ml) for 30 min giving **S4** and **S5**, respectively. No further purification was performed, crude products were directly analysed by HPLC.

S5 was used as a HPLC standard for its enantiomer H-D-Cys(St*B*u)-(*R*)-methylbenzylamine that would be present in **S4** if Fmoc-Cys(StBu)-OH was not optically pure.



Supplementary figure S11: HPLC trace of L-Cys(S*t*Bu)-(*R*)-methylbenzylamine (**S4**), L-Cys(S*t*Bu)-(*S*)-methylbenzylamine (**S5**) and co-injection of both

- H-L-Cys(S*t*Bu)-(*R*)-methylbenzylamine (**S4**)

ESI-MS (*m/z*): $[MH]^+$ calcd. for $C_{30}H_{34}N_2O_3S_2$: 535.2, found: 535.7

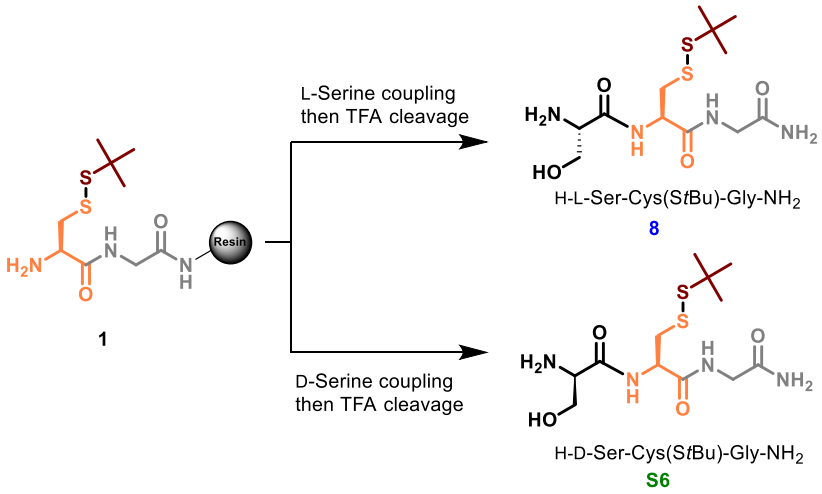
HPLC analysis: t_R = 2.73 min, (Chromolith, gradient: 10-50% B/A over 5 min)

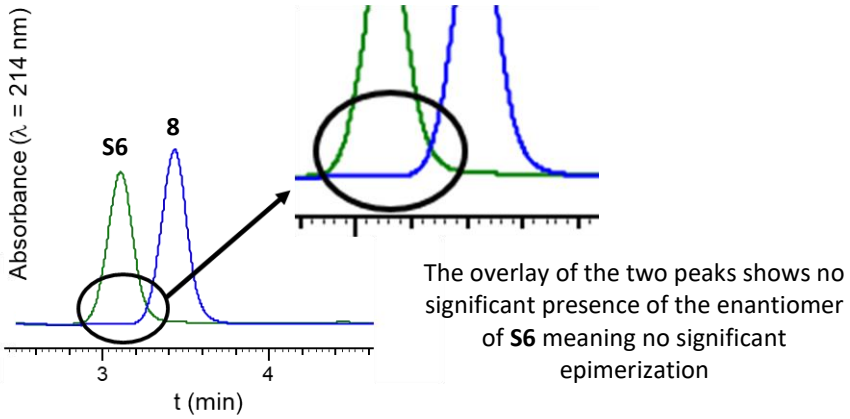
- H-L-Cys(S*t*Bu)-(*S*)-methylbenzylamine (**S5**)

ESI-MS (*m/z*): $[MH]^+$ calcd. for $C_{30}H_{34}N_2O_3S_2$: 535.2, found: 535.7

HPLC analysis: t_R = 2.63 min (Chromolith, gradient: 10-50% B/A over 5 min)

- Verification of the epimerization rate without the Hnb group





Supplementary figure 12: HPLC trace of the overlay of H-L-Ser-Cys(S*t*Bu)-Gly-NH_2 (**8**) and H-D-Ser-Cys(S*t*Bu)-Gly-NH_2 (**S6**) peaks

S6 was used as a HPLC standard for its enantiomer H-L-Ser-D-Cys(S*t*Bu)-Gly-NH_2 that would be formed if L-Cys was epimerized during peptide elongation.

- H-L-Ser-L-Cys(S*t*Bu)-Gly-NH_2 (**8**)

ESI-MS (*m/z*): $[MH]^+$ calcd. for $C_{12}H_{25}N_4O_4S_2$: 353.1, found: 353.5

HPLC analysis: t_R = 5.44 min (Chromolith, gradient: 20-30% B/A over 5 min)

- H-D-Ser-L-Cys(S*t*Bu)-Gly-NH_2 (**S6**)

ESI-MS (*m/z*): $[MH]^+$ calcd. for $C_{12}H_{25}N_4O_4S_2$: 353.1, found: 353.5

HPLC analysis: t_R = 5.32 min, (Chromolith, gradient: 20-30% B/A over 5 min)

6-Effect of temperature and concentration on Cys epimerization during reductive amination

❖ Table S2: Effect of temperature on side product **5** formation during the synthesis of **4**.[a]

Entry	Temperature (°C)	**4** (%)[b]	**5** (%)[b]
1	5	92	8
2	25	89	11
3	60	68	32

[a] Reductive amination conditions : imination with 10 equiv. aldehyde, 1 equiv. AcOH in 1:1 DMF/MeOH, 12.5 mM peptidyl resin, RT; reduction with 20 equiv. $NaBH_3CN$ in 9:9:2 DMF/MeOH/AcOH, 12.5 mM peptidyl resin [b] Relative rates determined by HPLC peak integration at λ = 320 nm.

❖ Table S3: Effect of peptidyl resin concentration on side product **5** formation during the synthesis of **4**.[a]

Entry	[Peptidyl resin] (mM)	equiv. aldehyde	equiv. $NaBH_3CN$	**4** (%)[b]	**5** (%)[b]
1	12.5	10	20	89	11
2	3	10	20	84	16
3	25	10	20	89	11
4	12.5	2	4	77	23
5	12.5	50	100	89	11
6	12.5	2	20	78	22

[a] Reductive amination conditions : imination with 10 equiv. aldehyde, 1 equiv. AcOH in 1:1 DMF/MeOH, 12.5 mM peptidyl resin, RT; reduction with 20 equiv. $NaBH_3CN$ in 9:9:2 DMF/MeOH/AcOH, 12.5 mM peptidyl resin [b] Relative rates determined by HPLC peak integration at λ= 320 nm.

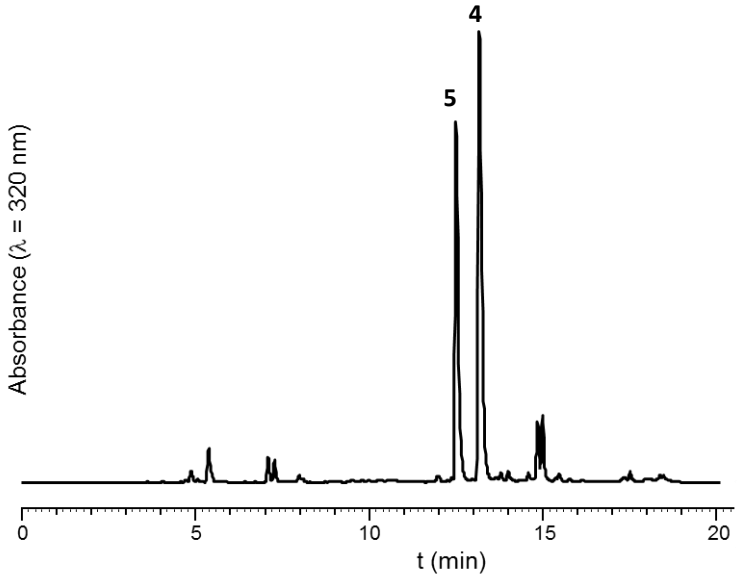
7-NMR characterisation of the [M-2] imidazolidinone byproducts 10a-d

7-1- Synthesis and isolation of products 4, 5 and 10a-d

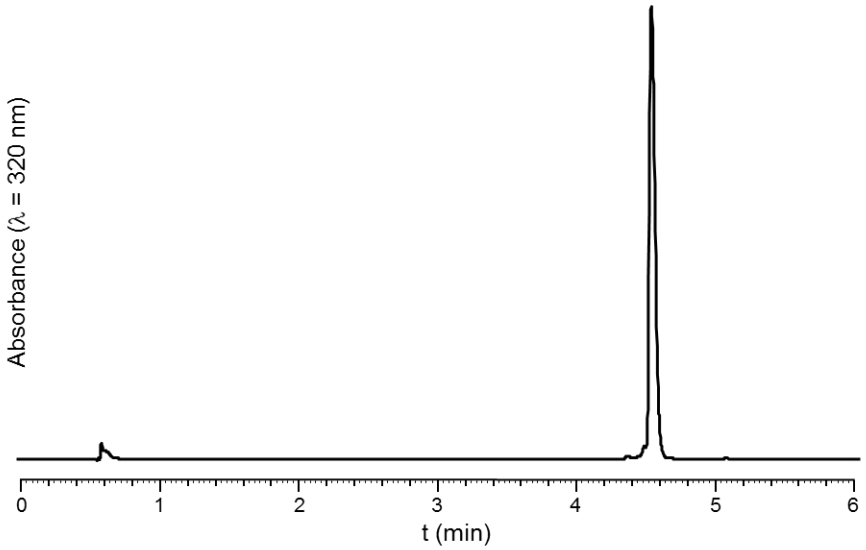
- Synthesis of **4** and **5** *via* reductive amination with $NaBH_3CN$ using a protocol designed to promote the formation of the epimerized product.

25 µmol of H-Cys(S*t*Bu)-Gly-Rink-ChemMatrix **1a** was washed with 1:1 DMF/MeOH mixture (4 x 3 mL, 30 s) then swollen in 3 mL of a 9:9:2 DMF/MeOH/AcOH mixture for 5 min. The resin was washed with 1:1 DMF/MeOH mixture (3 x 3 mL, 30 s), then 2-hydroxy-5-nitrobenzaldehyde (42mg, 10 equiv.) in 8

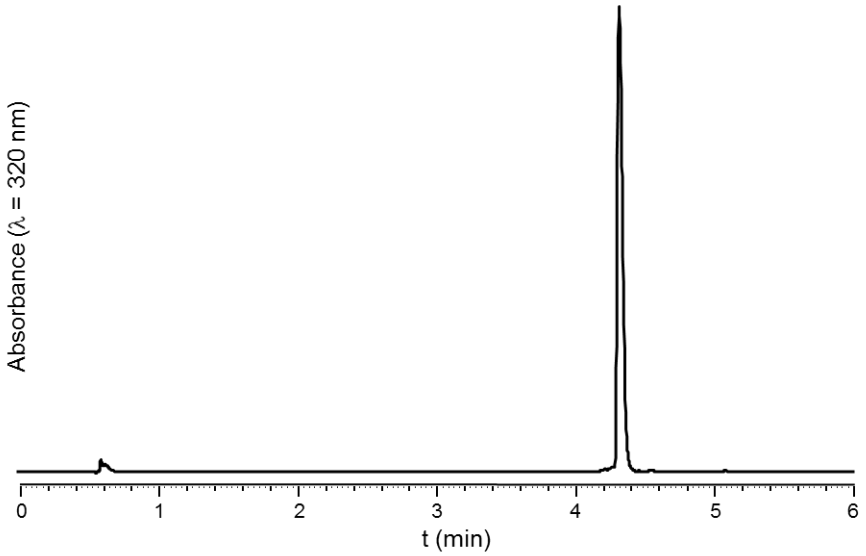
mL of 9:9:2 DMF/MeOH/AcOH was added and the reactor was stirred for 4 h at 40°C. The reactor was drained and the resin was washed with 1:1 DMF/MeOH mixture (3 x 3 mL, 5 s). A fresh solution of sodium cyanoborohydride (31.5 mg, 20 equiv.) in 3 mL of 9:9:2 DMF/MeOH/AcOH (250 mM, sodium cyano-borohydride concentration) was added and the reactor stirred for 1h. The reactor was drained and the resin was washed with 1:1 DMF/MeOH (4 × 3 mL, 30 s), NMP (3 × 3 mL, 30 s), 20% piperidine in NMP (3 × 3 mL, 3 min), NMP (3 × 3 mL, 30 s), dichloromethane (3 x 5 mL, 30 s) and NMP (2 x 3 mL, 30 s). Fmoc-Ser(*t*Bu)-OH was then coupled for 18 h followed by piperidine-mediated Fmoc deprotection, following protocol PS1. The peptidyl-resin was cleaved (protocol PS3 small peptides cleavage) to give a 42:58 mixture of **4** and **5**. The mixture was purified by semi preparative HPLC (Nucleosil C18, gradient: 15-50% B/A over 20 min) to give pure **4** and **5** for NMR characterization (see p S7 for MS characterization).



Supplementary figure S13: HPLC trace (Nucleosil C18, gradient: 5-50% B/A over 20 min) of the **4**/**5** mixture obtained using a protocol designed to promote epimerization



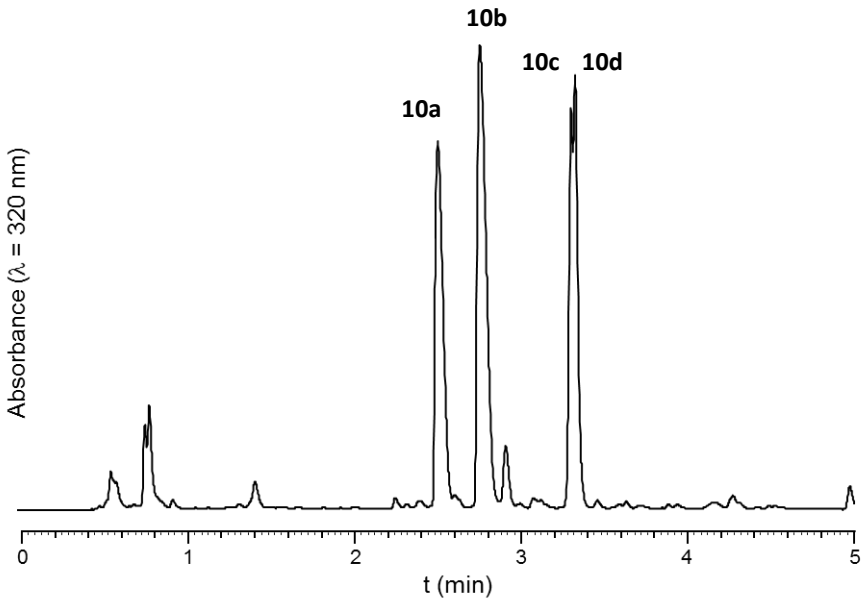
Supplementary figure S14: HPLC trace (Chromolith, gradient: 5-50% B/A over 5 min) of purified **4**



Supplementary figure S15: HPLC trace (Chromolith, gradient: 5-50% B/A over 5 min) of purified **5**

- Synthesis and purification of imidazolidinones **10a-d**

25 µmol of H-Cys(S*t*Bu)-Gly-Rink-ChemMatrix **1a** was washed with 1:1 DMF/MeOH mixture (4 x 3 mL, 30 s) then swollen in 3 mL of a 9:9:2 DMF/MeOH/AcOH mixture for 5 min. The resin was washed with 1:1 DMF/MeOH mixture (3 x 3 mL, 30 s), then 2-hydroxy-5-nitrobenzaldehyde (42 mg, 10 equiv.) in 2 ml 1:1 DMF/MeOH (125 mM aldehyde concentration) was added, and the reactor was stirred for 2 h. The reactor was drained and the resin was washed with 1:1 DMF/MeOH (4 × 3 mL, 30 s), NMP (3 × 3 mL, 30 s), 20% piperidine in NMP (3 mL, 3 min, × 3), NMP (3 × 3 mL, 30 s), dichloromethane (3 x 5 mL, 30 s) and NMP (2 x 3 mL, 30 s). Fmoc-Ser(*t*Bu)-OH was then coupled for 18 h followed by piperidine-mediated Fmoc deprotection, following general protocol PS1. The peptidyl-resin was cleaved (protocol PS3 small peptides cleavage) to give a mixture of **10a-d**. The mixture was purified by semi preparative HPLC (Nucleosil C18, gradient: 15-50% B/A over 20 min).



Supplementary figure S16: HPLC trace of imidazolidinones **10a-d**

- Imidazolidinone **10a**

ESI-HRMS (*m/z*): $[MH]^+$ calcd. for $C_{19}H_{28}N_5O_7S_2$: 502.1430, found: 502.1429

HPLC analysis: t_R = 2.47 min (Chromolith, gradient: 5-50% B/A over 5 min)

- Imidazolidinone **10b**

ESI-HRMS (*m/z*): $[MH]^+$ calcd. for $C_{19}H_{28}N_5O_7S_2$: 502.1430, found: 502.1426

HPLC analysis: t_R = 2.78 min (Chromolith, gradient: 5-50% B/A over 5 min)

- Imidazolidinone **10c**

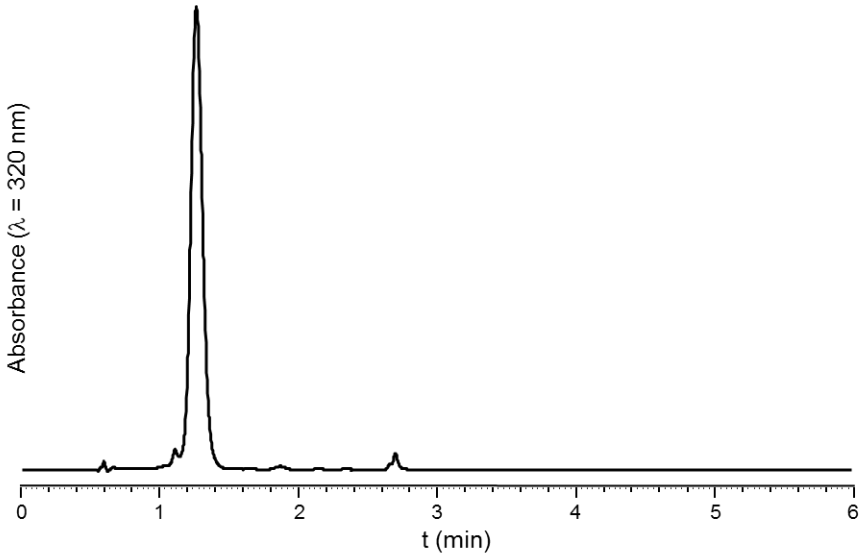
ESI-HRMS (*m/z*): $[MH]^+$ calcd. for $C_{19}H_{28}N_5O_7S_2$: 502.1430, found: 502.1428

HPLC analysis: t_R = 3.31 min (Chromolith, gradient: 5-50% B/A over 5 min)

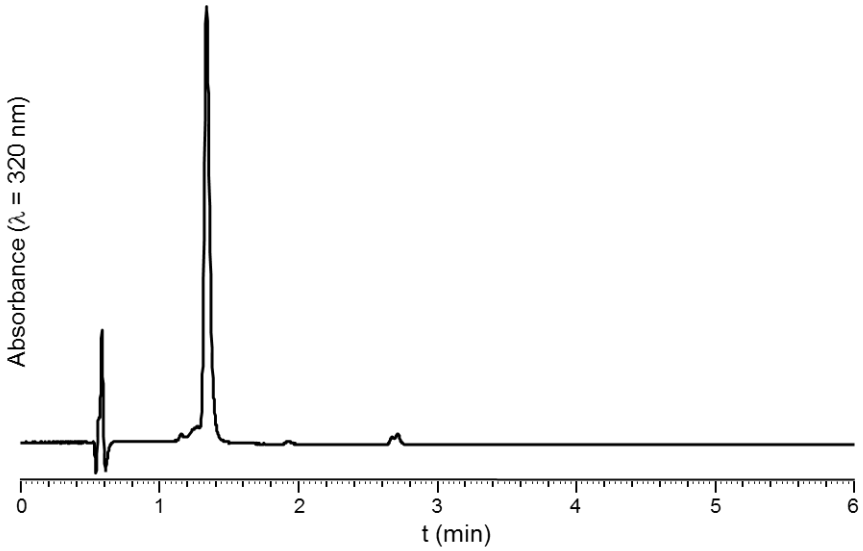
- Imidazolidinone **10d**

ESI-HRMS (*m/z*): $[MH]^+$ calcd. for $C_{19}H_{28}N_5O_7S_2$: 502.1430, found: 502.1423

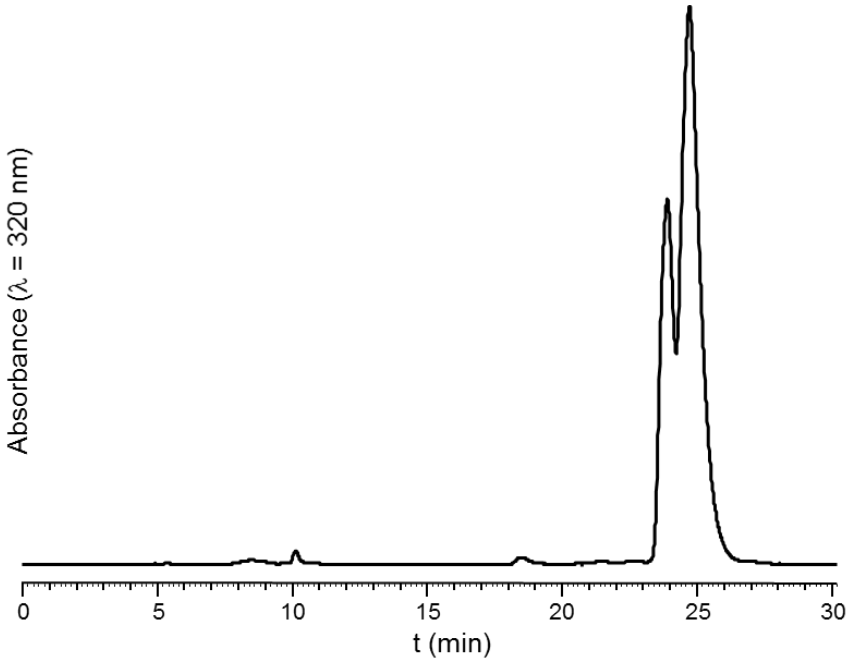
HPLC analysis: t_R = 3.33 min (Chromolith, gradient: 5-50% B/A over 5 min)



Supplementary figure S17: HPLC trace (Chromolith, gradient: 20-50% B/A over 5 min) of purified imidazolidinone **10a**

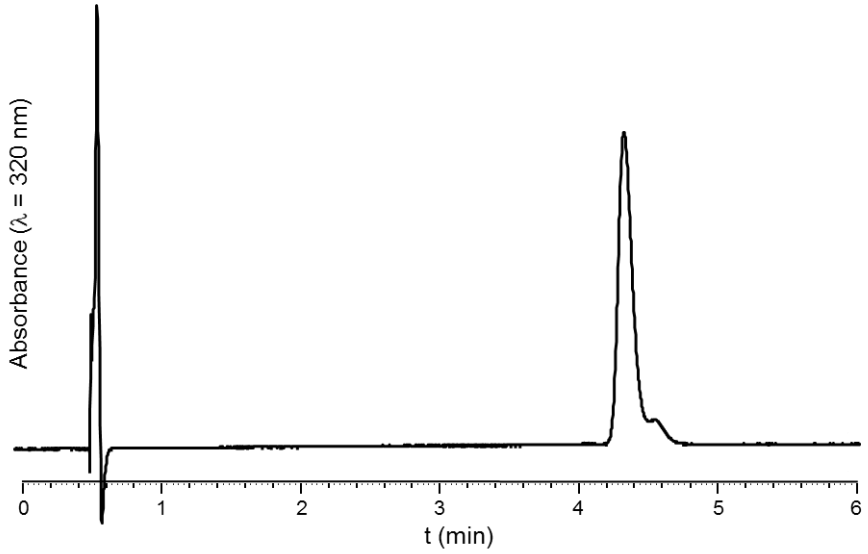


Supplementary figure S18: HPLC trace (Chromolith, gradient: 20-50% B/A over 5 min) of purified imidazolidinone **10b**

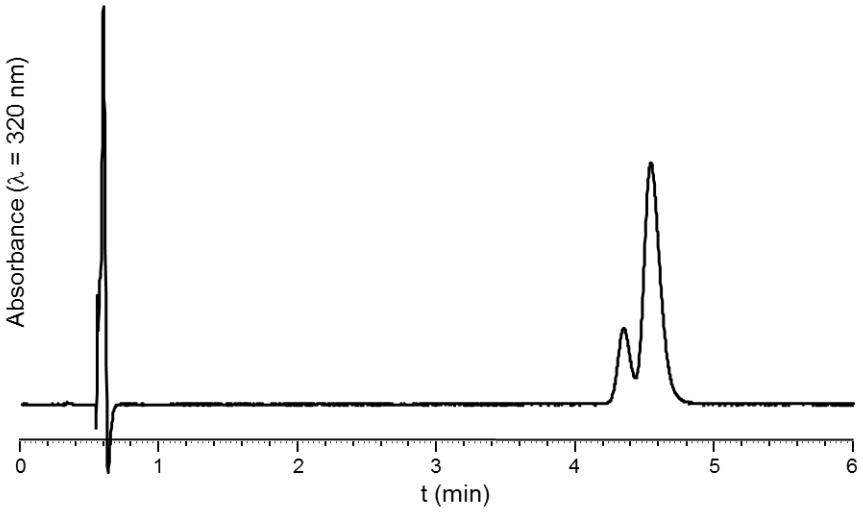


Supplementary figure S19: HPLC trace (Nucleosil C4, gradient: Isocratic 23% B/A over 20 min) of purified imidazolidinones **10c** and **10d** mixture (33:67 ratio)

Then, the 33:67 mixture of **10c** and **10d** were subjected to a second round of semi-preparative HPLC in order to try to separate them. HPLC purification: Nucleosil C4, gradient: Isocratic 23 % B/A over 20 min at 40°C. This leads to 91:9 and 19:81 **10c**/**10d** mixtures, respectively, but in very small amounts, which complicated their NMR characterization due to low signal/noise ratios. As a consequence, we mainly based the NMR characterization of **10c** and **10d** on the mixture.



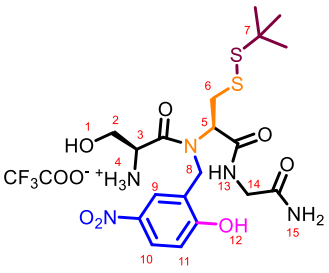
Supplementary figure S20: HPLC trace (Chromolith, gradient: Isocratic 20% B/A over 5 min) of purified imidazolidinone **10c**



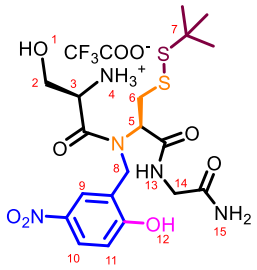
Supplementary figure S21: HPLC trace (Chromolith, gradient: Isocratic 20% B/A over 5 min) of purified imidazolidinone **10d**

7-2 NMR analysis of 4, 5 and 10a-d

- NMR analysis of **4**



trans conformer



cis conformer

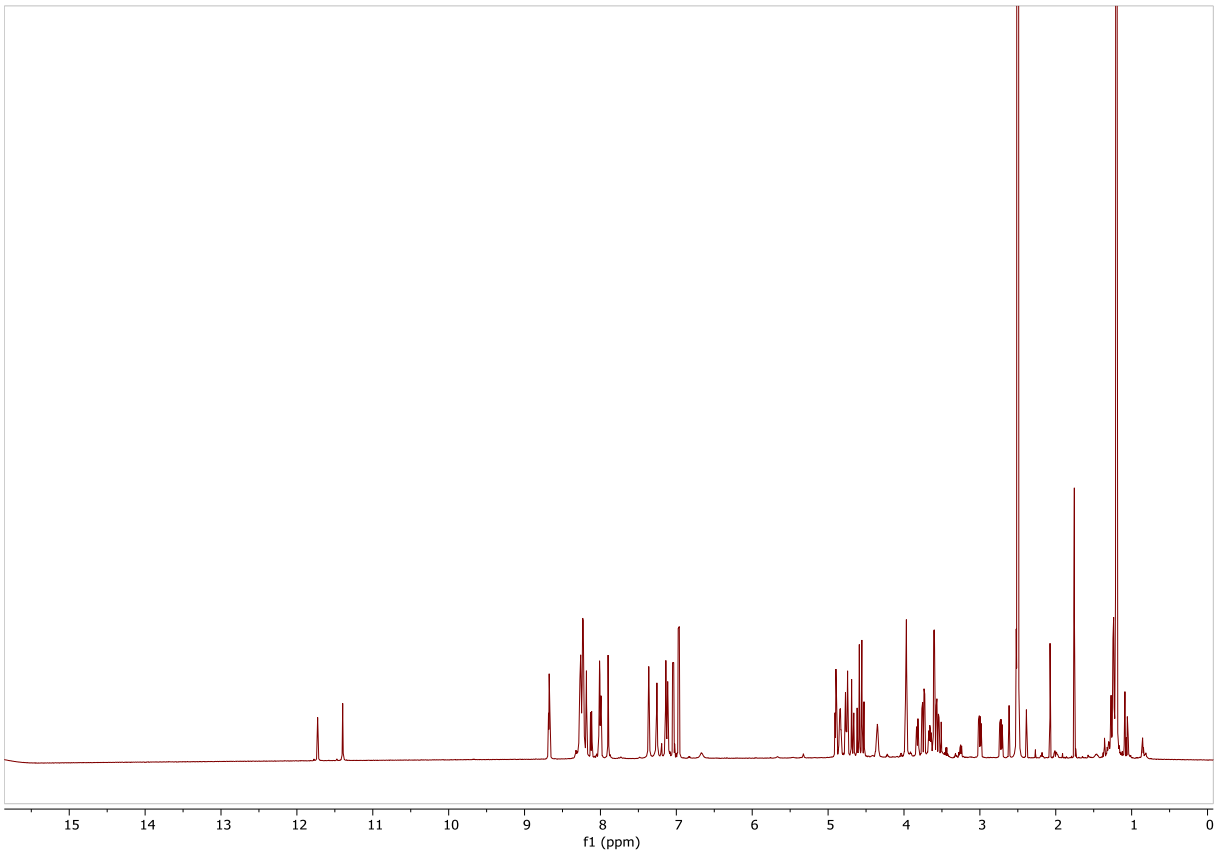
NMR analysis of pure **4** shows two sets of peaks corresponding to two amide bond rotamers (57:43 ratio), as expected from the *N*,*N*-disubstituted nature of the Ser-Cys bond. Identification of *cis* and *trans* conformers was not possible with NOESY and ROESY analysis due to the overlap between signals.

The following color code is used in the NMR description: **Major conformer, minor conformer, both.**

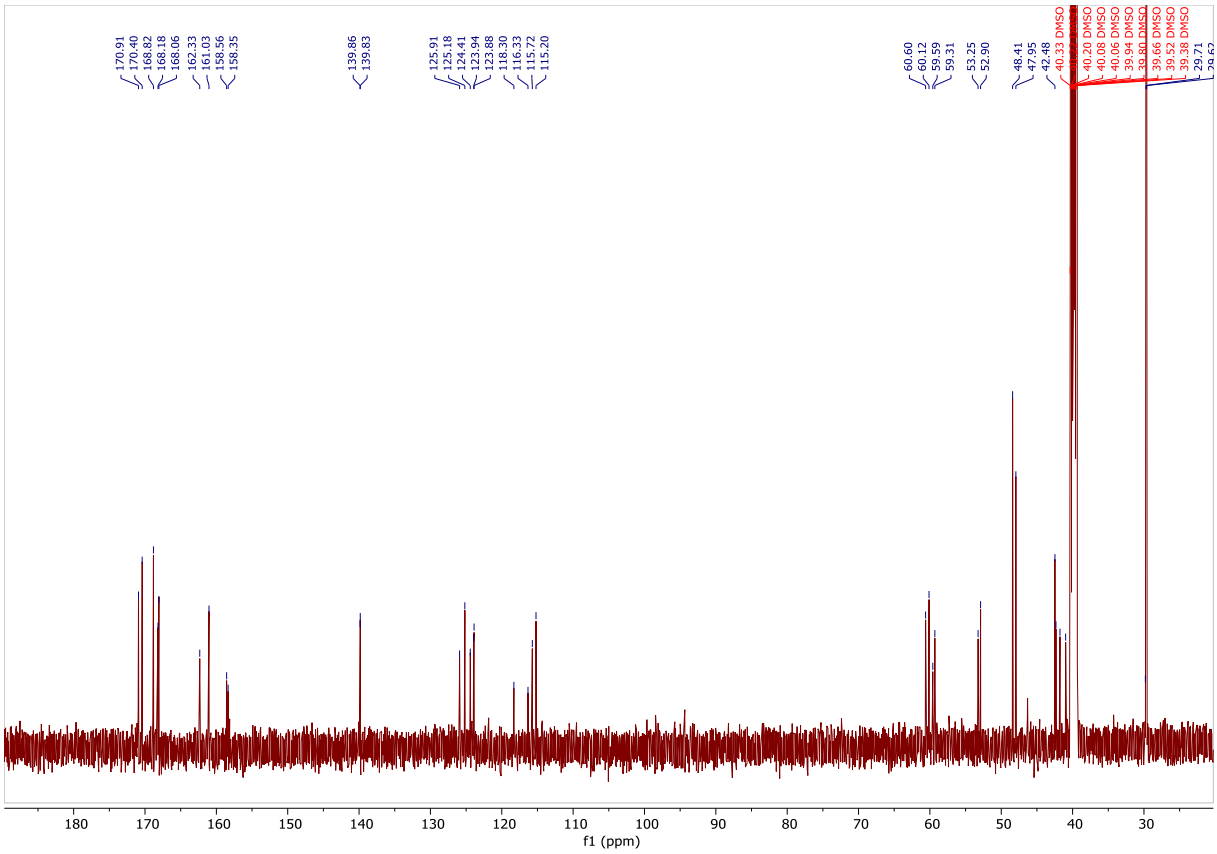
^{1}H NMR (600 MHz, DMSO-*d*6) δ 11.73 (s, 1H, H_{12}), 11.40 (s,1H , H_{12}), 8.68 (t, *J* = 5.5 Hz, 1H, H_{13}), 8.26 (d, *J* = 5.3 Hz, 3H, H_4), 8.23 (d, *J* = 5.1 Hz, 3H, H_4), 8.19 (d, *J* = 2.8 Hz, 1H, H_9), 8.12 (dd, *J* = 9, 2.8 Hz, 1H, H_{10}), 8.03 – 7.98 (m, 2x1H, H_{10} + H_{13}), 7.90 (d, *J* = 2.8 Hz, 1H, H_9), 7.36 (bs, 1H, H_{15b}), 7.26 (bs, 1H, H_{15a}), 7.14 (s, 1H, H_{15a}), 7.12 (s, 1H, H_{15a}), 7.04 (d, *J* = 9.0 Hz, 1H, H_{11}), 6.97 (d, *J* = 9.0 Hz, 1H, H_{11}), 4.90 (bs, 1H, H_5), 4.86–4.81 (m, 1H, H_3), 4.79 – 4.73 (m, 2xH, H_5, H_{8b}), 4.68 (d, *J* = 17.1 Hz, 1H H_{8a}), 4.60 (d, *J* = 17.0 Hz, 1H, H_{8b}), 4.54 (d, *J* = 17.0 Hz, 1H, H_{8a}), 4.39 – 4.32 (m, 1H, H_3), 3.97 (d, *J* = 4.7 Hz, 2H, H_2), 3.82 (dd, *J* = 11.72, 4.01, Hz, 1H, H_{2b}), 3.75 (dd, *J* = 16.9, 6.0 Hz, 1H, H_{14b}), 3.66 (dd, *J* = 11.7, 7.1 Hz, 1H, H_{2a}), 3.61–3.58 (m , 2H, H_{14}), 3.56 (dd, *J* = 16.9, 4.9 Hz, 1H H_{14a}), 3.33 (dd, *J* = 13.5, 7.6 Hz, 1H, H_{6a}), 3.26 (dd, *J* = 13.1, 7.7 Hz, 1H, H_{6a}), 3.00 (dd, *J* = 13.4, 6.7 Hz, 1H, H_{6b}), 2.72 (dd, *J* = 12.3, 6.2 Hz, 1H, H_{6b}), 1.21 (s, 9H, H_7), 1.20 (s, 9H, H_7).

^{13}C NMR (190 MHz, DMSO-*d*6) δ 170.9 C_{CO}, 170.4 C_{CO} , 168.8 2C_{CO}, 168.2 C_{CO} , 168.1 C_{CO}, 162.3 C_{OH}, 161.0 C_{OH}, 139.9 2C_{NO2}, 125.9 C_{Ar} + C_9, 125.2 C_{Ar} , 124.4 C_{10}, 123.94 C_{10}, 123.88 C_9, 115.7 C_{11}, 115.2 C_{11}, 60.6 C_2, 60.1 C_2, 59.6 C_5, 59.31 C_5, 53.25 C_5, 52.9 C_5, 48.4 C_8, 47.95 C_8, 42.5 C_{14} , 42.3 C_{14} , 41.8 C_6 , 41.0 C_6 , 29.7 C_7 , 29.6 C_7 (CMe_3 and CF_3COO^- have not been identified due to low signal-to-noise ratio)

^{15}N NMR (60 MHz, DMSO-*d*6) δ 111.9 CONH, 107.9 CONH, 105.1 $CONH_2$, 104.7 $CONH_2$, 92.4 2 x NH_3^+ (chemical shifts obtained from 2D ^{1}H-^{15}N HSQC)

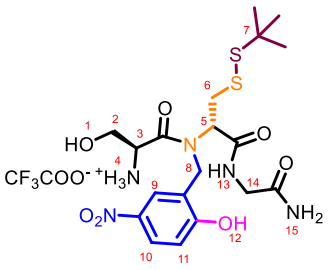


Supplementary figure S22: ^{1}H-NMR spectrum of **4**

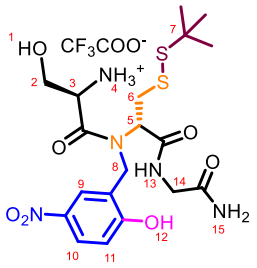


Supplementary figure S23: ^{13}C-NMR spectrum of **4**

- NMR analysis of **5**



trans conformer



cis conformer

NMR analysis of pure **5** shows two sets of peaks corresponding to two amide bond rotamers (71:29 ratio), as expected from the *N*,*N*-disubstituted nature of the Ser-Cys bond. Identification of *cis* and *trans* conformers was not possible with NOESY and ROESY analysis due to the overlap between signals.

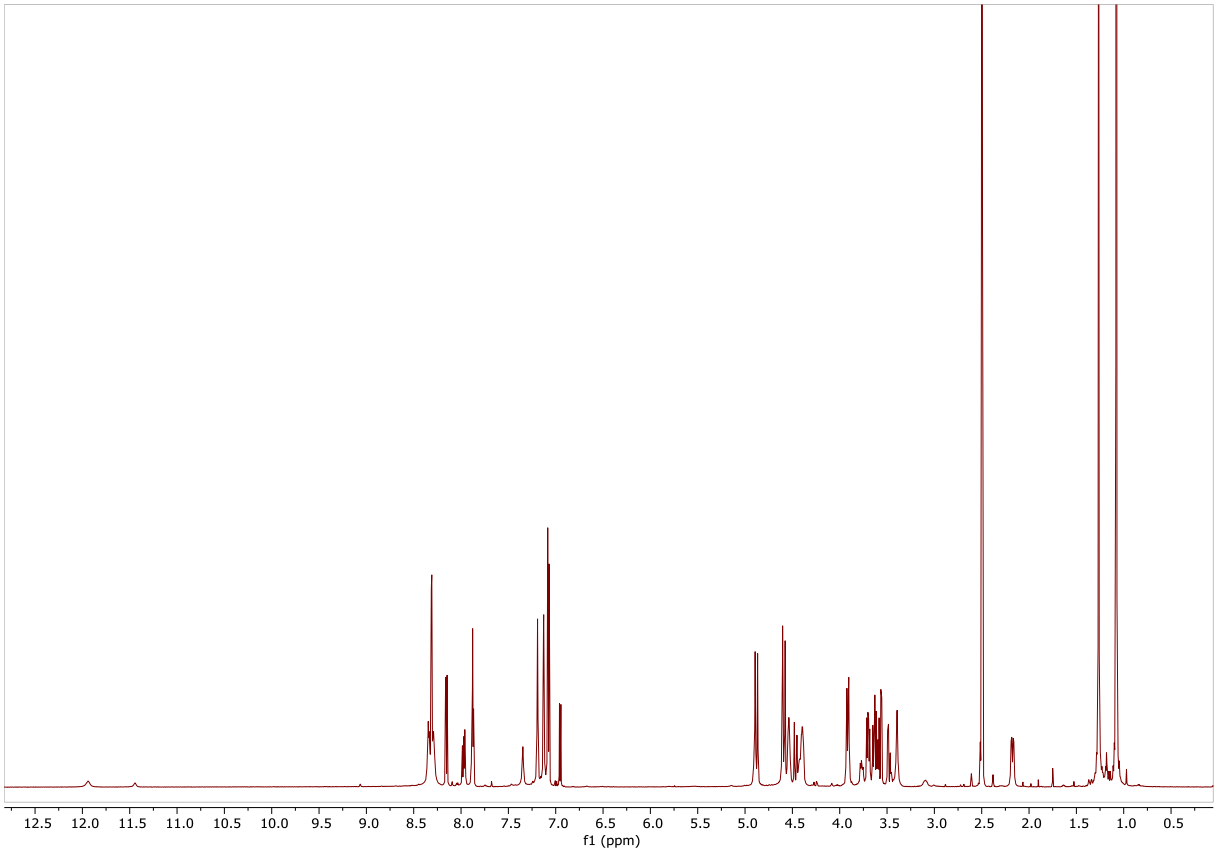
The following color code is used in the NMR description: **Major conformer, minor conformer, both.** (Assignment based on the combined analysis of 2D ^{1}H COSY, ^{1}H-^{13}C and ^{1}H-^{15}N HSQC spectra)

^{1}H NMR (600 MHz, DMSO-d_6) δ 11.94 (s, 1H, H_{12}), 11.44 (s, 1H, H_{12}), 8.37 – 8.32 (m, 3H + 1H, H_4 + H_{13}), 8.31 (d, J = 2.8 Hz, 1H, H_9), 8.30 – 8.27 (m, 3H, H_4), 8.15 (dd, J = 9.0, 2.8 Hz, 1H, H_{10}), 7.98 (dd, J = 8.8, 2.8 Hz, 1H, H_{10}), 7.96 (d, J = 2.9 Hz, 1H, H_9), 7.90–7.85 (m, 3H, H_{13}), 7.19 (bs, 2x1H, H_{15b} + H_{15b}), 7.13 (bs, 2x1H, H_{15a} + H_{15a}), 7.08 (d, J = 8.9 Hz, 1H, H_{11}), 6.95 (d, J = 8.8 Hz, 1H, H_{11}), 4.93–4.83 (m, 2x1H, H_{5b} H_{8a}), 4.59 (d, J = 15.8 Hz, 2x1H, H_{8b} + H_{8a}), 4.56–4.50 (m, 1H, H_3), 4.47 (d, J = 16.4 Hz, 1H, H_{8b}) 4.44 – 4.40 (m,

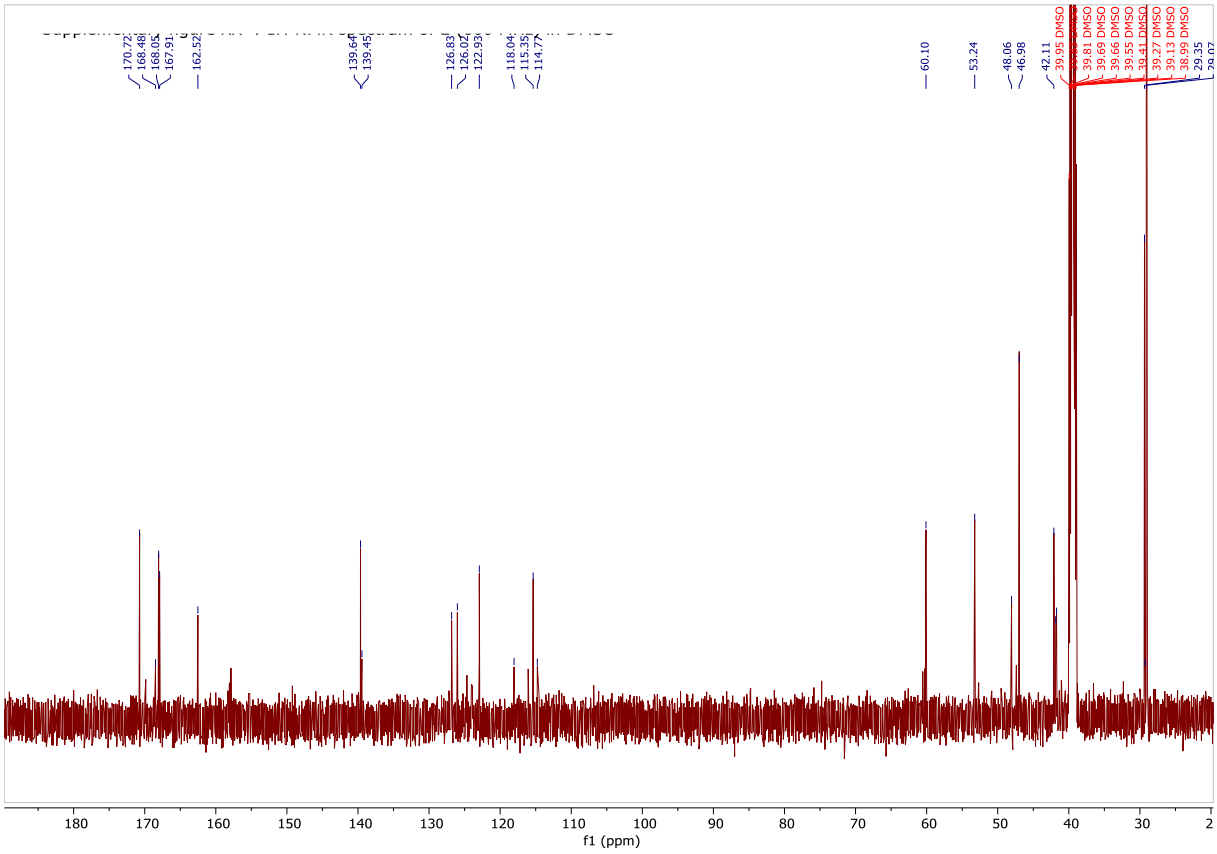
2x1H, H_3 + H_5), 3.92 (m, 2x1H, H_{2a} H_{2a}), 3.77 (dd, *J* = 11.3, 6.7 Hz, 1H H_{2b}), 3.70 (dd, *J* = 11.9, 7.0 Hz, 1H, H_{2b}), 3.66 – 3.62 (m, 2x1H, H_{6b} H_{14b}), 3.62 – 3.58 (m, 2x1H, H_{14b} H_{14b}), 3.49 (d, *J* = 5.6 Hz, 1H, H_{14a}), 3.46 (d, *J* = 5.6 Hz, 1H, H_{14a}), 3.39 (s, 1H, H_{6a}), 3.1–3.05 (m, 1H, H_{6b}), 2.18 (dd, *J* = 13.5, 4.7 Hz, 1H, H_{6a}), 1.26 (s, 9H, H_7), 1.08 (s, 9H, H_7).

^{13}C NMR (190 MHz, DMSO-d_6) δ 170.7 C_{CO}, 169.9 C_{CO}, 168.5 2C_{CO}, 168.1 C_{CO}, 167.9 C_{CO}, 162.5 2C_{OH}, 139.6 C_{NO2}, 139.4 C_{NO2}, 126.8 C_9, 126.0 C_{10}, 124.7 C_{10}, 124.0 C_9 ,123.9 C_{Ar} , 122.9 C_A, 115.3 C_{15}, 114.8 C_{15} , 60.3 C_2 , 60.13 C_2 , 60.10 C_5, 59.2 C_5 , 53.2 C_3 , 52.5 C_3 , 47.0 C_8, 42.1 2C_{14}, 41.8 C_8, 41.7 C_6, 40.9 C_6, 29.3 C_7, 29.1 C_7. (CMe_3 and CF_3COO^- have not been identified due to low signal-to-noise ratio)

^{15}N NMR (60 MHz, DMSO-*d*6) δ 111.1 CONH, 105.0 CONH, 104.9 $CONH_2$, 104.8 $CONH_2$, 91.5 NH_3^+, 92.3 NH_3^+ (chemical shifts obtained from 2D ^{1}H-^{15}N HSQC)



Supplementary figure S24: ^{1}H-NMR spectrum of **5**

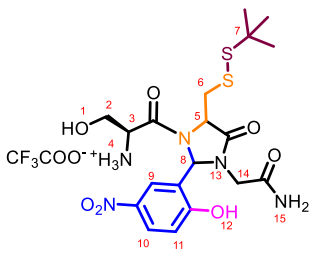


Supplementary figure 25: ^{13}C-NMR spectrum of **5**

- NMR analysis of imidazolidinones **10a-d**

Note that ^{1}H NMR spectra of pure **10a-d** were extremely complex, showing two or three different sets of peak contributing to more than 5 % in each spectrum, and probably other minor species. Based on the HPLC purity of the purified compounds, we attribute this spectral complexity to the presence of a mixture of conformers due to the two expected *cis-trans* amide rotamers, combined with different conformations of the 5-membered heterocycle. Identification of *cis* and *trans* conformers was not possible with NOESY and ROESY analysis due to the overlap between signals. For similar reasons, assignation of the relative C5/C8 stereochemistry was also not possible.

➢ NMR analysis of imidazolidinone 10a



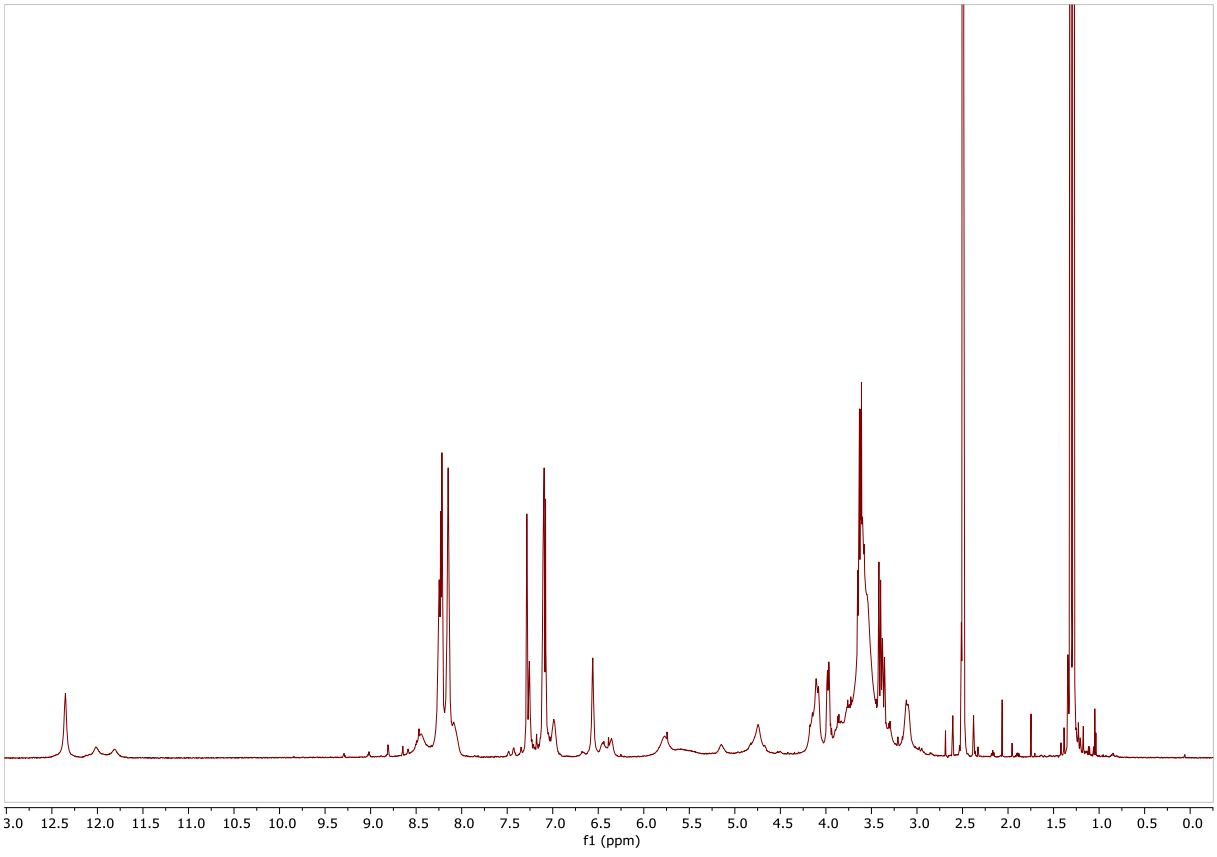
Three major conformers were observed in a 65:21:14 ratio. A red/blue/orange colour code is accordingly used to describe peaks in the following ^{1}H/^{13}C/^{15}N characterization. A black colour refers

to a signal or a multiplet not clearly attributed to only one of the conformers. Only unambiguously identified signals are reported (assignment based on the combined analysis of 2D ^{1}H COSY, ^{1}H-^{13}C and ^{1}H-^{15}N HSQC spectra).

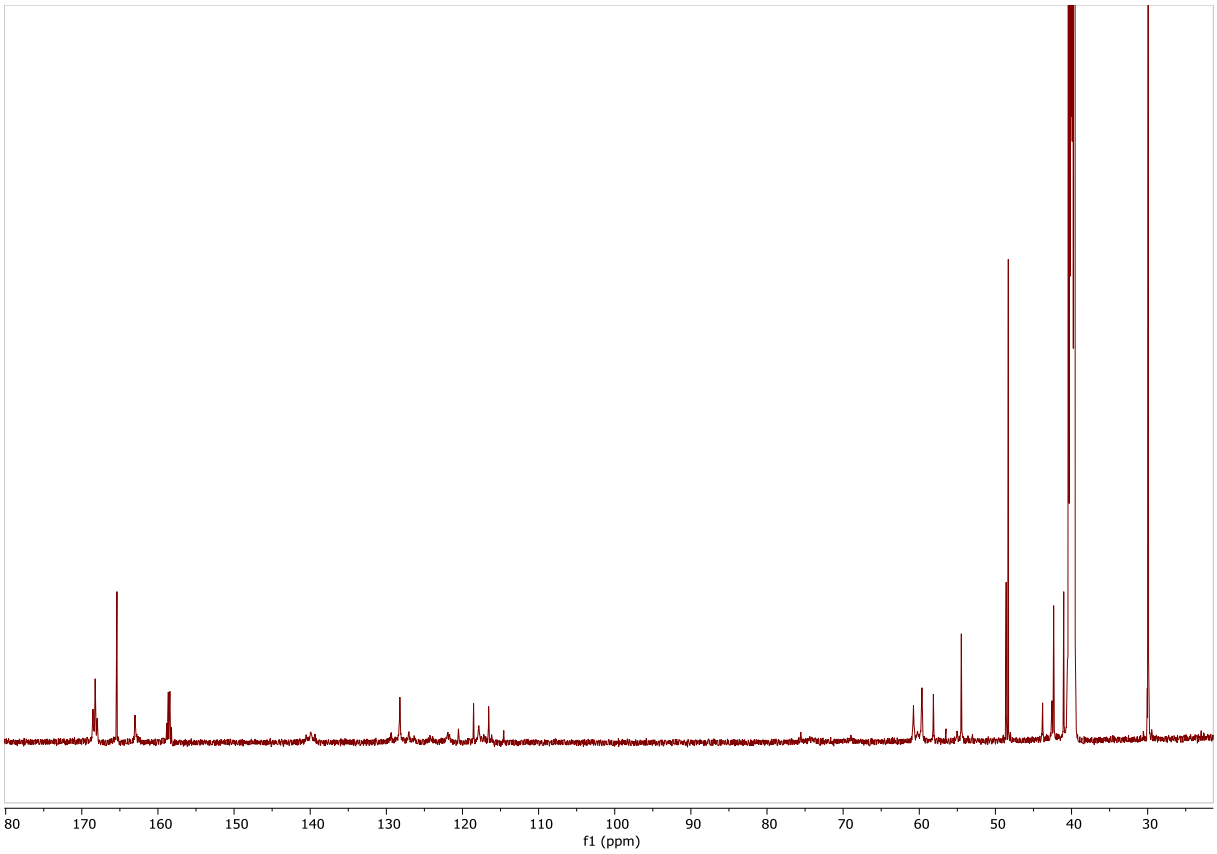
^{1}H NMR (600 MHz, DMSO-d_6) δ 12.35 (bs, 1H, H_{12}), 12.01 (bs, 1H, H_{12}), 11.81 (bs, 1H, H_{12}) 8.26 – 8.20 (m, 4H + 2x1H + 2x1H, H_4 + H_{10} + H_9), 8.16 – 8.13 (m, 3H, H_4), 7.29– 7.24 (m, 2x2H, H_{15}), 7.12 – 6.99 (m, 2x1H, H_{11}), 6.56 (s, 1H, H_8), 6.45 (bs, 1H, H_8), 6.36 (bs, 1H, H_8), 5.20 (bs, 1H, H_5), 4.75 (bs 1H, H_5), 4.15 – 4.09 (m, 1H + 2H, H_3 + H_{14}), 3.97 (d, *J* = 10.7 Hz, 2H, H_2), 3.67 – 3.56 (m, 2H + 2H + 1H, H_6 + H_2 + H_3), 3.44 – 3.36 (m, 2H, H_6), 3.11 (d, *J* = 16.0 Hz, 2H, H_{14}), 1.31 (s, 9H, H_7), 1.28 (s, 9H, H_7).

^{13}C NMR (151 MHz, DMSO-d_6) δ 168.5 $C_{CO,}$ 168.3 C_{CO}, 168.0 C_{CO} 165.4 C_{CO}, 163.0 2C_{OH}, 128.2 H_{10}, 118.5 H_{11}, 116.6 H_{11}, 76.0 C_8, 74.1 C_8, 68.9 C_8, 60.8 C_2, 59. 6 C_2, 58.1 C_5, 56.5 C_5, 55.0 C_3, 54.5 C_3, 42.6 C_{14}, 42.3 C_{14}, 41.0 C_6, 40.5 C_6, 30.0 C_7, 29.9 C_7.

^{15}N NMR (60 MHz, DMSO-*d*6) δ 105.8 $CONH_2$, 90.8 NH_3^+

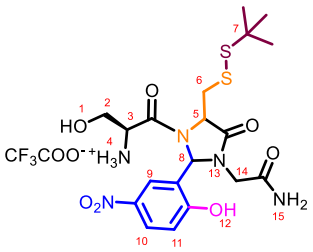


Supplementary figure S26: ^{1}H-NMR spectrum of imidazolidinone **10a**



Supplementary figure 27: ^{13}C-NMR spectrum of imidazolidinone **10a** (600 MHz) in DMSO

- NMR analysis of imidazolidinone **10b**

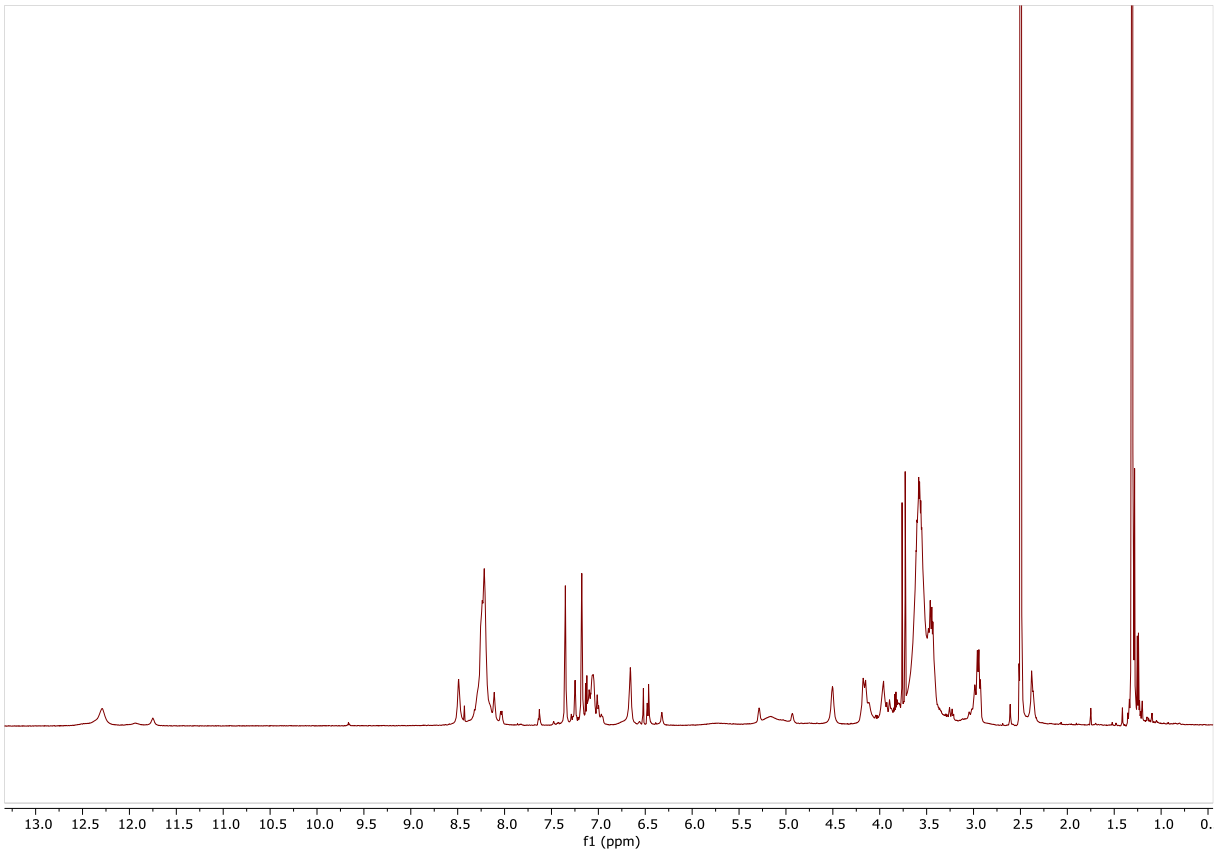


Three major conformers were observed in a 57:23:20 ratio. A red/blue/orange colour code is accordingly used to describe peaks in the following ^{1}H/^{13}C/^{15}N characterization. A black colour refers to a signal or a multiplet not clearly attributed to only one of the conformers. Only unambiguously identified signals are reported (assignment based on the combined analysis of 2D ^{1}H COSY, ^{1}H-^{13}C and ^{1}H-^{15}N HSQC spectra).

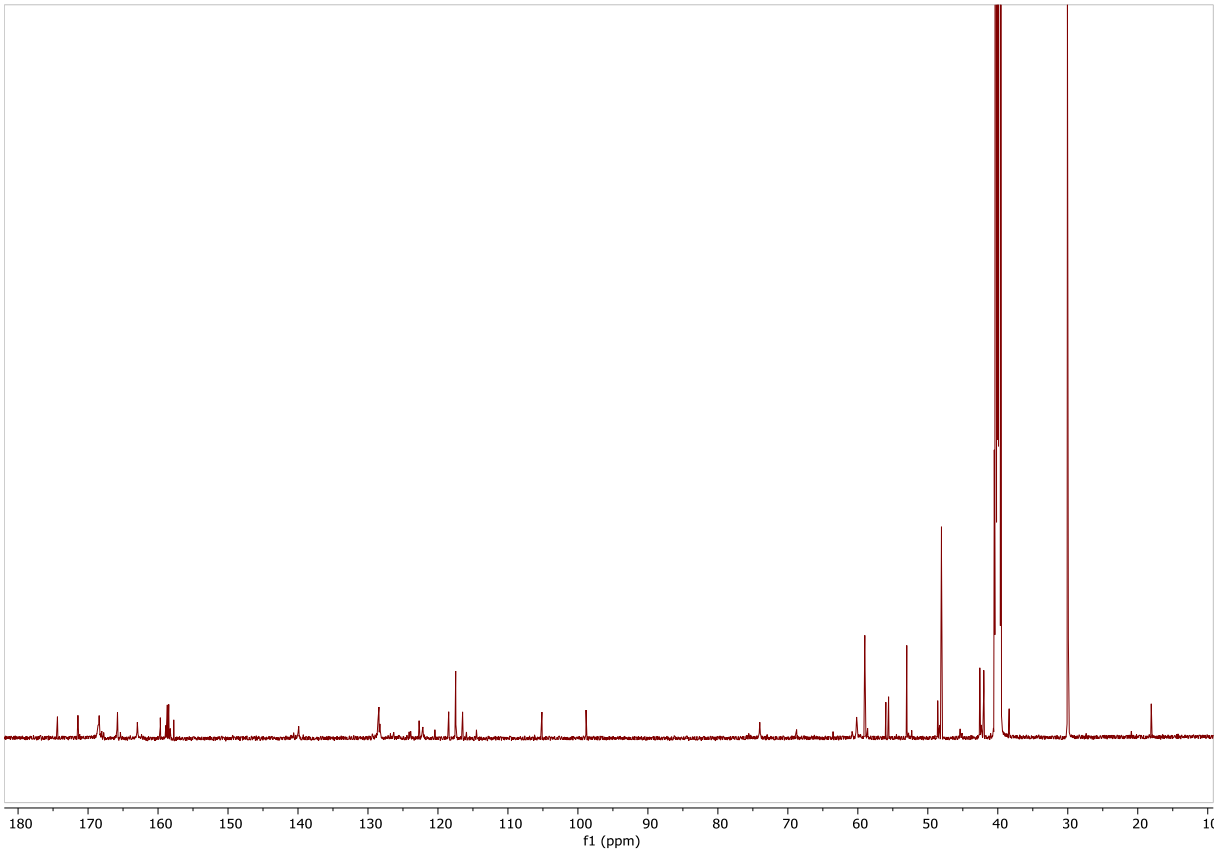
^{1}H NMR (600 MHz, DMSO-d_6) δ 12.29 (s, 1H, H_{12}), 11.75 (s, 1H, H_{12}), 8.49 (bs, 1H, H_{10}), 8.27 – 8.20 (m, 1H + 1H + 3H, H_9 + H_{10} + H_4), 8.11 (bs, 3H, H_4), 8.03 (d, J = 8.8 Hz, 1H, H_9), 7.35 (s, 1H, H_{15}), 7.18 (s, 1H, H_{15}), 7.09 – 7.03 (m, 2x1H, H_{11}), 6.66 (s, 1H, H_8), 6.56 (s, 1H, H_8), 6.32 (s, 1H, H_8), 5.29 (bs, 1H, H_5), 4.93 (bs, 1H, H_5), 4.50 (bs, 1H, H_5), 4.20 – 4.09 (m, 1H + 2H, H_3 + H_{14}), 3.96 (d, J = 10.7 Hz, 2H, H_2), 3.70 – 3.35 (m, 2H + 2H + 1H, H_6 + H_2 + H_3) , 3.01–2.91 (m, 1H, H_6), 2.40–2.34 (m, 2H, H_{14}), 1.31 (s, 9H, H_7), 1.28 (s, 9H, H_7).

^{13}C NMR (151 MHz, DMSO-d_6) δ 168.5 C_{CO}, 168.4 C_{CO}, 165.8 C_{CO}, 165.3 C_{CO}, 162.9 2C_{OH}, 128.4 H_{10}, 126.79 H_9, 126.3 H_9, 118.5 H_{11}, 117.5 H_{11}, 75.4 C_8, 74.2 C_8, 69.0 C_8, 63. 5 C_2, 60.8 C_2, 60.1 C_5, 59.0 C_5, 58.6 C_5, 53.0 C_3, 52.3 C_3, 42.6 C_{14}, 42.5 C_{14}, 42.0 C_6, 38.3 C_6, 29.9 C_7, 29.9 C_7.

^{15}N NMR (60 MHz, DMSO-d6) δ 106.1 $CONH_2$, 92.8 NH_3^+

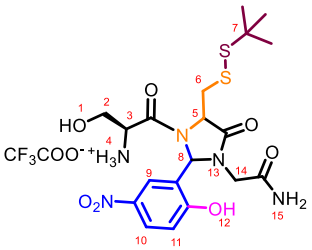


Supplementary figure S28: ^{1}H-NMR spectrum of **10b**



Supplementary figure S29: ^{13}C-NMR spectrum of **10b**

- NMR analysis of imidazolidinones **10c** and **10d** 67:33 mixture



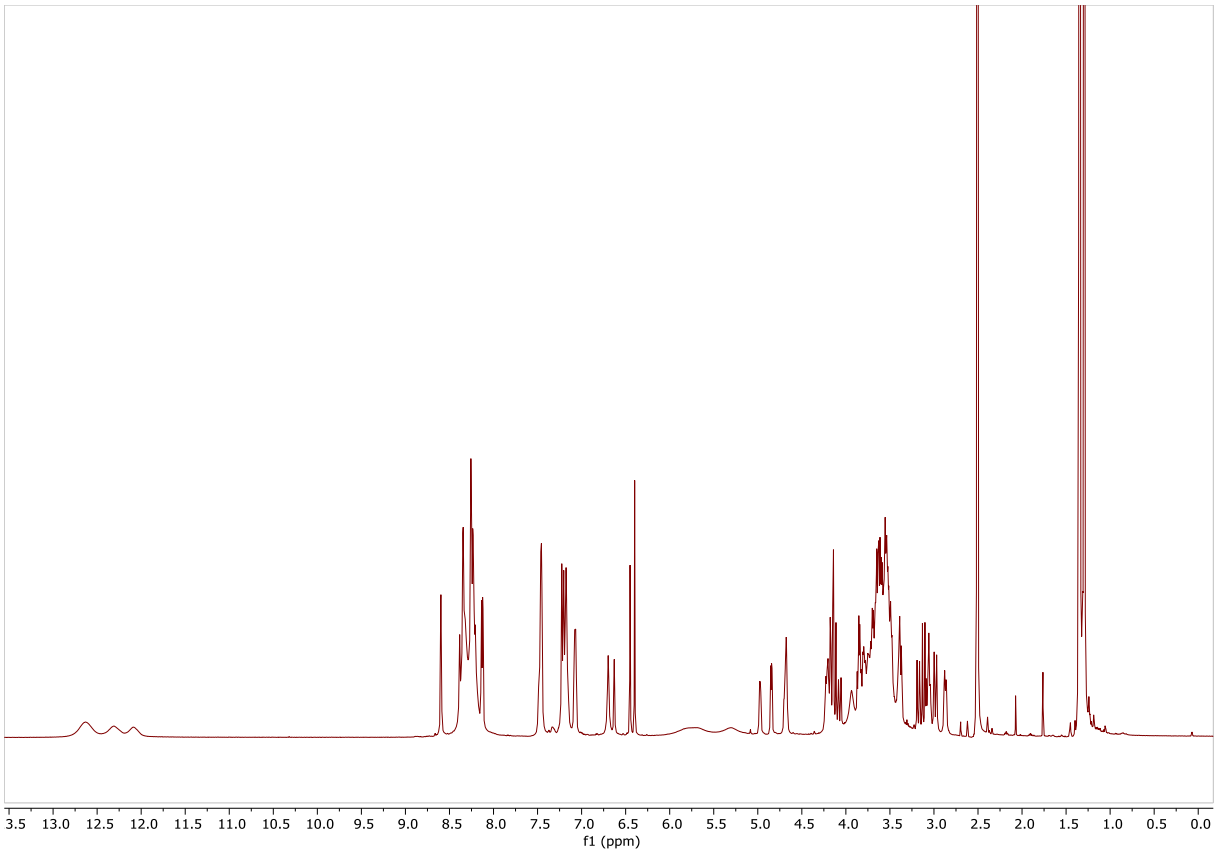
Two major conformers of both **10c** and **10d** were observed. A red/blue code colour was used to respectively describe the major and the minor conformer of **10c** in the following $^{1}H/^{13}C/^{15}N$ characterization. Similarly, a purple/green code colour was used to respectively describe the major and the minor conformer of **10d** in the following $^{1}H/^{13}C/^{15}N$ characterization. A black colour refers to a signal or a multiplet not clearly attributed to only one of the compounds **10c** and **10d**. Only unambiguously identified signals are reported (assignment based on the combined analysis of 2D ^{1}H COSY, ^{1}H-^{13}C ,^{1}H-^{15}N HSQC as well as ^{1}H of **10c** and **10d*** spectra).

* Small amounts of impure isolated **10c** and **10d** (see supplementary figures 25 and 26) were analysed as well.

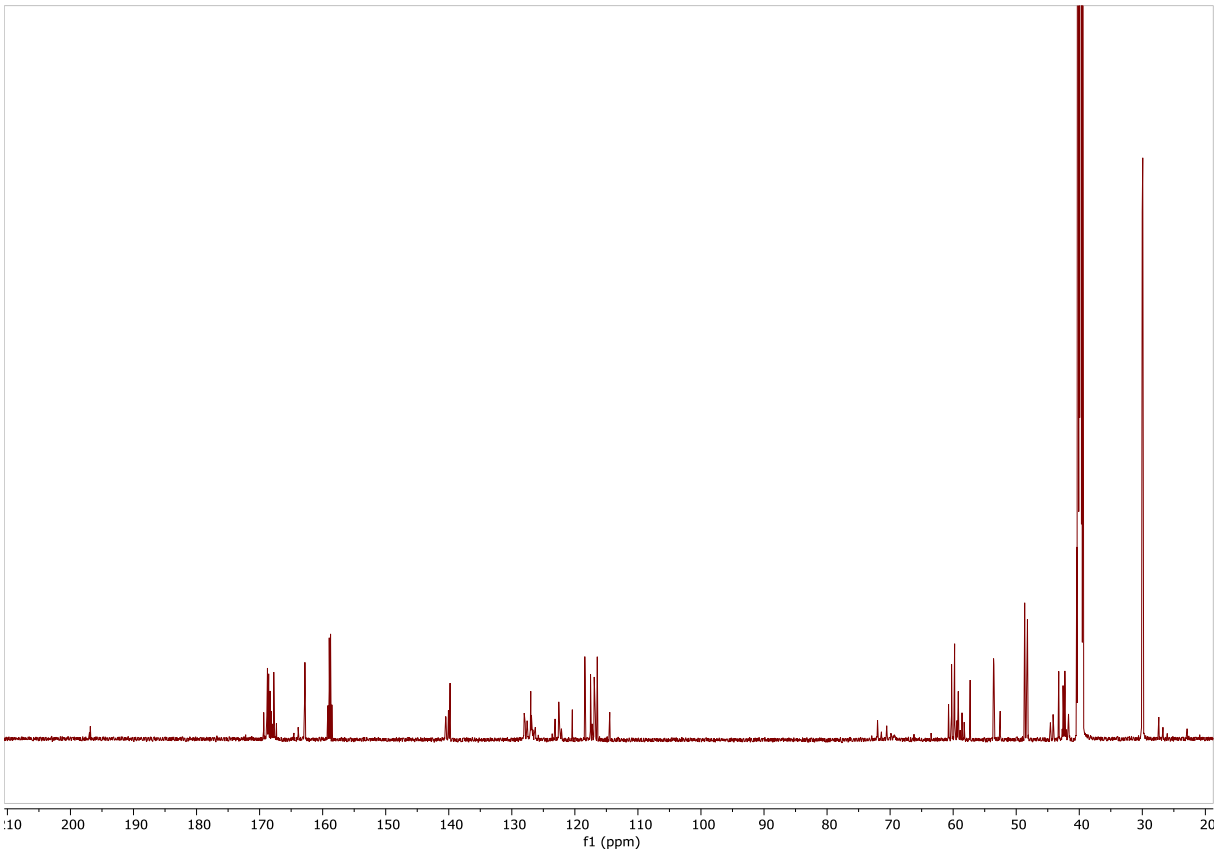
^{1}H NMR (600 MHz, DMSO-d_6) δ 12.62 (m, 2x1H $H_{12\text{-}10d}$, $H_{12\text{-}10c}$), 12.29 (s, 1H, $H_{12\text{-}10c}$), 12.07 (s, 1H $H_{12\text{-}10d}$) 6.70 (s, 1H, $H_{8\text{-}10d}$), 6.63 (s, 1H, $H_{8\text{-}10c}$), 6.45 (s, 1H, $H_{8\text{-}10d}$), 6.40 (s, 1H, $H_{8\text{-}10c}$), 4.98 (dd, J = 7.3, 3.2 Hz, 1H, $H_{5\text{-}10d}$), 4.85 (dd, J = 8.1, 3.4 Hz, 1H, $H_{5\text{-}10c}$), 4.70 – 4.64 (m, 2x1H, $H_{5\text{-}10d}$, $H_{5\text{-}10c}$)

^{13}C NMR (151 MHz, DMSO-d_6) δ 71.6 $C_{8\text{-}10d}$, 70.8 $C_{8\text{-}10c}$, 69.5 $C_{8\text{-}10d}$, 69.4 $C_{8\text{-}10c}$, 59.2 $C_{5\text{-}10c}$, 59.0 $C_{5\text{-}10d}$, 58.8 $C_{5\text{-}10d}$, 57.3 $C_{5\text{-}10c}$

^{15}N NMR (60 MHz, DMSO-d_6) δ 105.7 $CONH_2$, 104.8 $CONH_2$, 104.5 $CONH_2$, 104.4 $CONH_2$, 91.9 2xNH_3^+, 91.7 2xNH_3^+



Supplementary figure S30: ^{1}H-NMR spectrum of imidazolidinones **10c**/**10d** 67:33 mixture (600 MHz)



Supplementary figure S31: ^{13}C-NMR spectrum of imidazolidinones **10c**/**10d** 67:33 mixture (600 MHz)

7-3 Structure elucidation of imidazolinones 10a-d

Combination of 1D and 2D ^{1}H, ^{13}C and ^{15}N experiments allowed us to conclude on the *N*-acyl-imidazolidin-4-ones structure of **10a-d**.

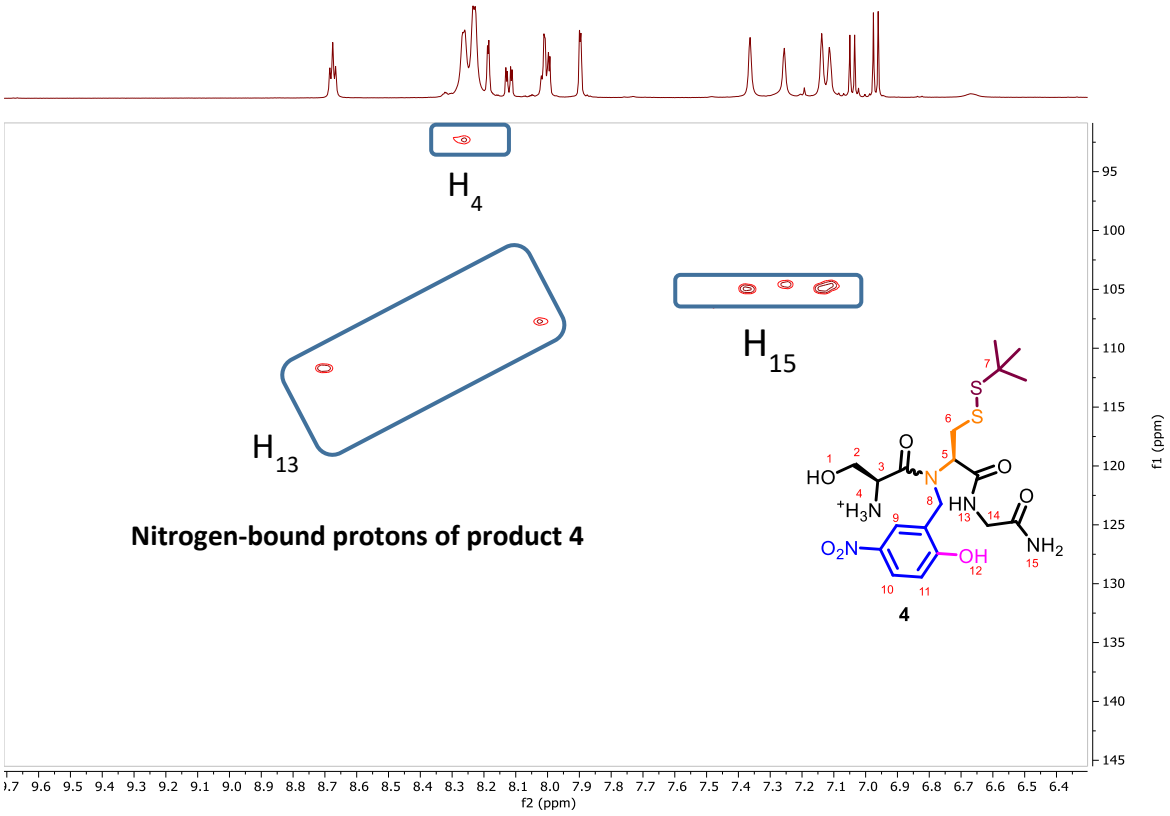
1- We observed for the four products the disappearance of the benzylic CH_2 signals observed in both **4** and **5**, accompanied with the apparition of new CH singlets at 6.0-6.7 ppm. This suggested the formation of compounds C-disubstituted in α of the nitrophenol ring.

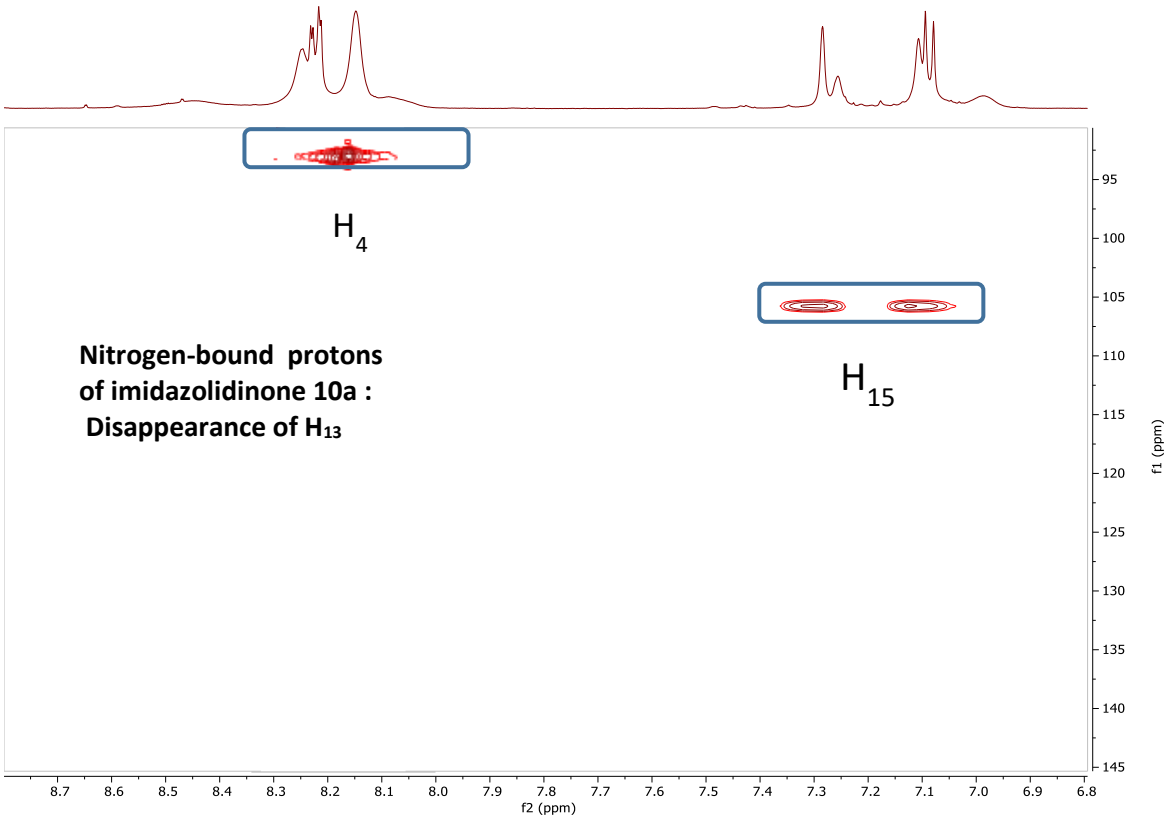
2- ^{1}H-^{15}N HSQC spectra of **10a-d** showed the disappearance of the NH amide signal of the Cys-Gly peptide bond, while glycine C-terminal $CONH_2$ and serine NH_3^+ (TFA salt) remained essentially unchanged: this suggested an *N*-substitution of the Cys-Gly amide nitrogen.

We consequently deducted the probable *N*-acyl imidazolidinone structures shown in scheme 5 (see main article).

- Compound **10a**

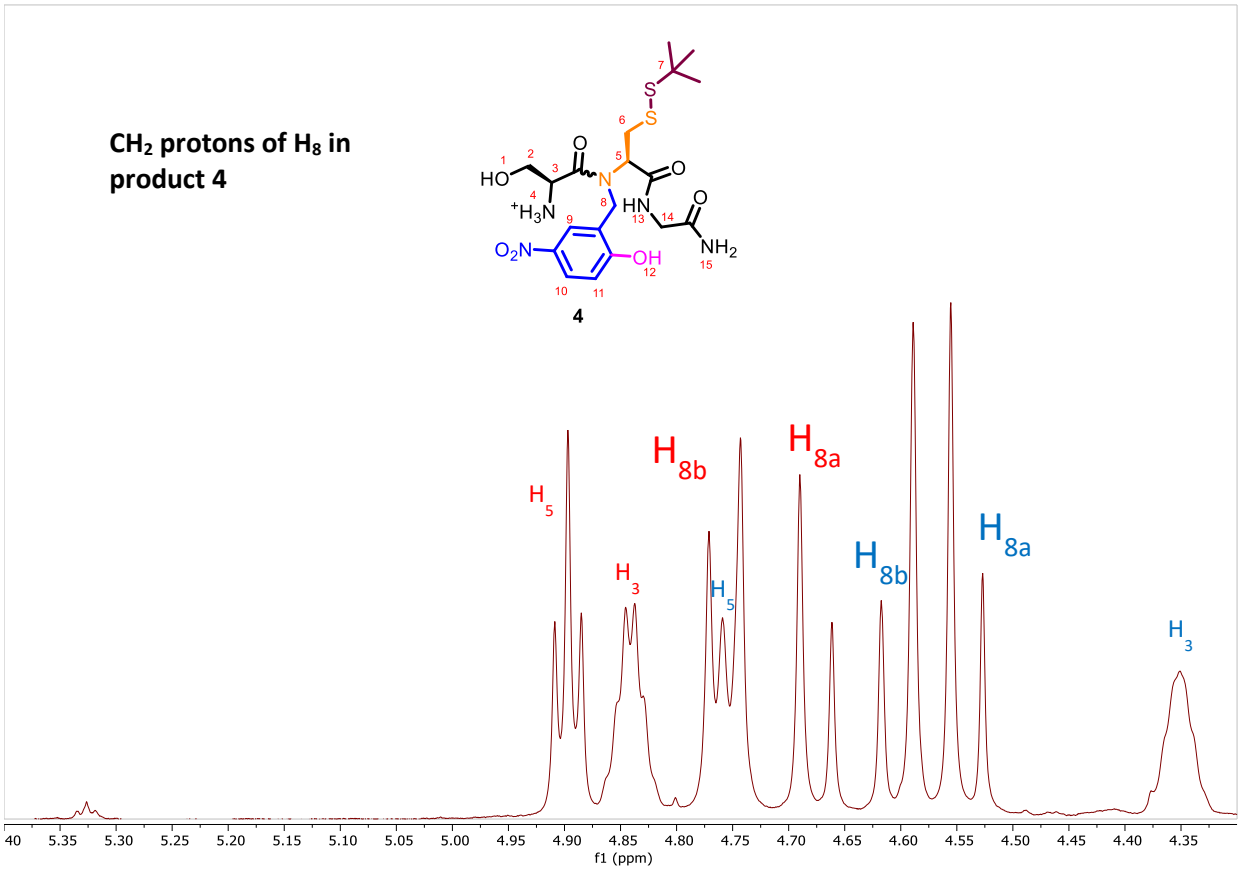
➢ Comparison between ^{1}H-^{15}N HSQC spectra of **4** and imadizolidinone **10a**

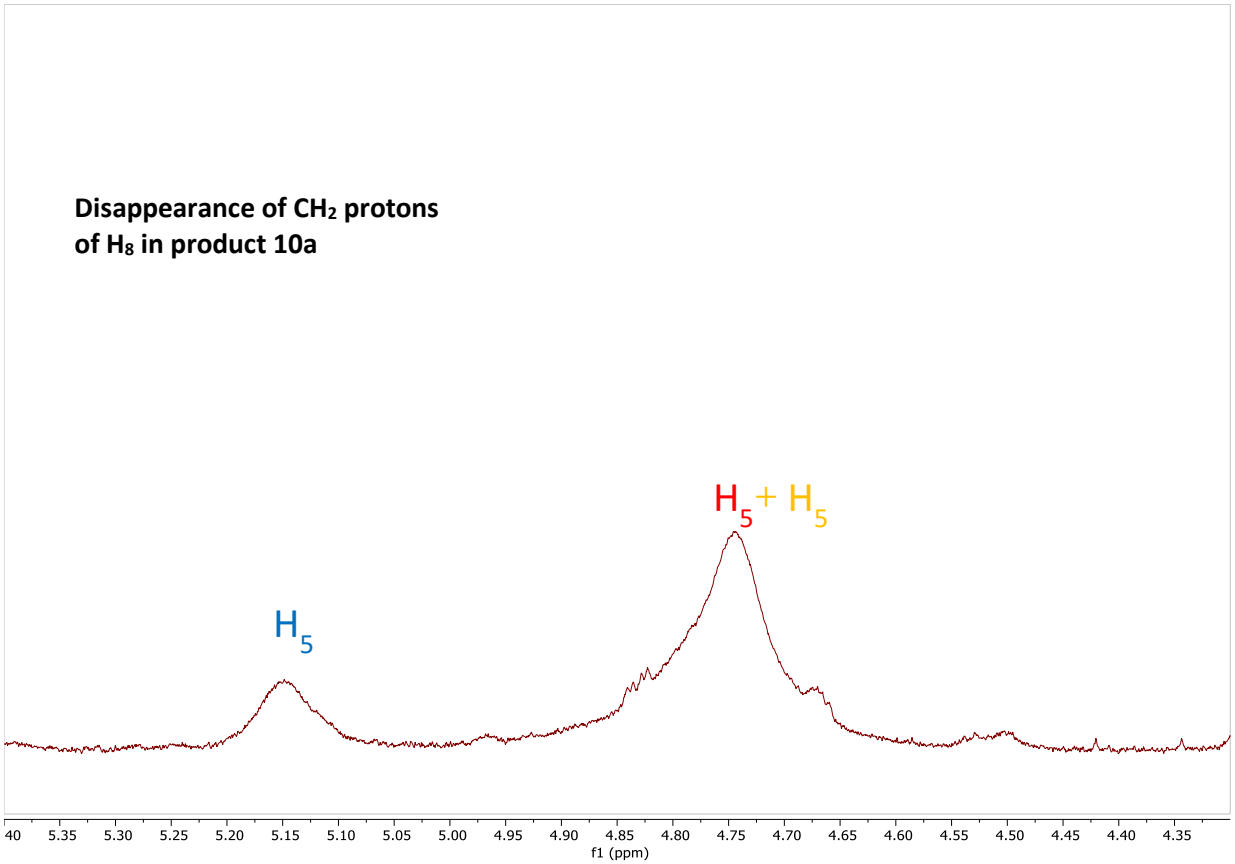




Supplementary figure S32: Comparison of ^{1}H-^{15}N HSQC NMR spectra of **4** and **10a** (600 MHz) in DMSO-*d*6

➢ Comparison between ^{1}H spectra of **4** and imidazolidinone **10a**

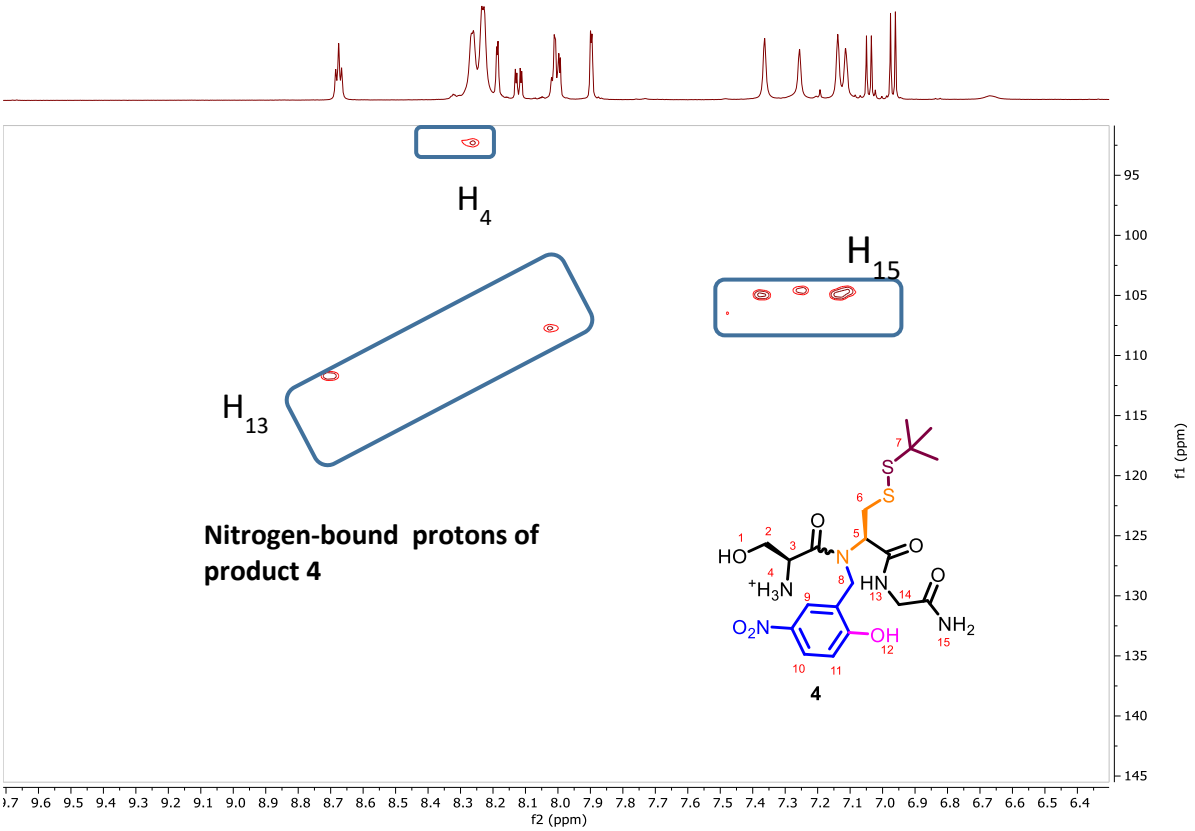


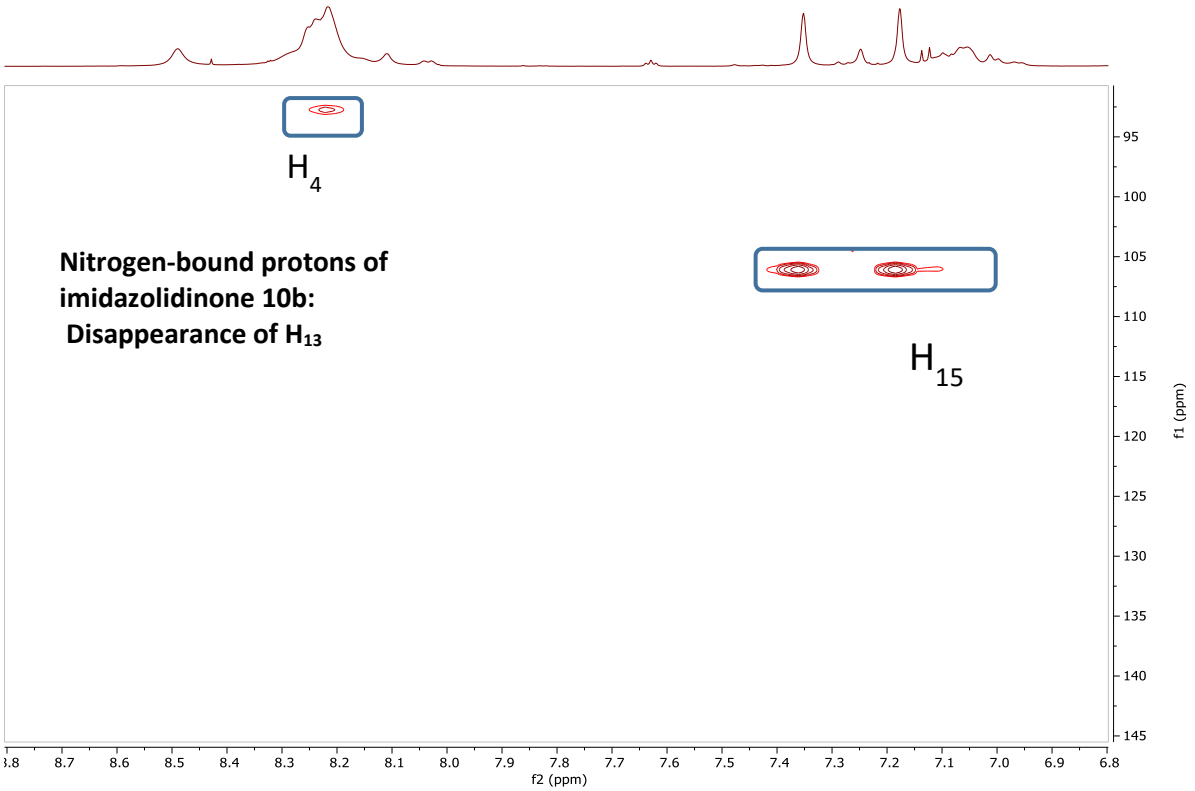


Supplementary figure S33: zoom on ^{1}H-NMR spectra of **4** and **10a** (600 MHz) in DMSO-*d*6

- **Compound 10b**

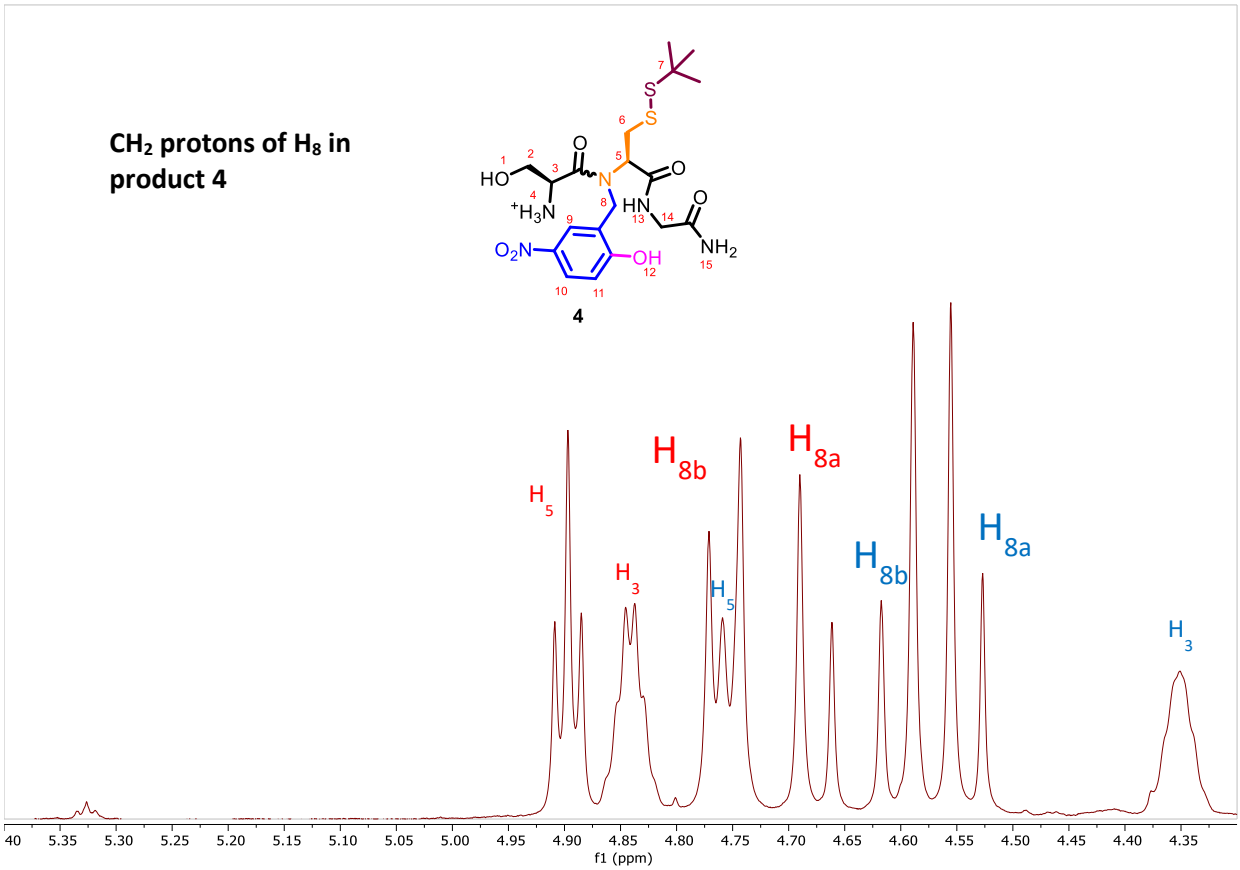
➢ Comparison between ^{1}H-^{15}N HSQC spectra of **4** and imadazolidinone **10b**

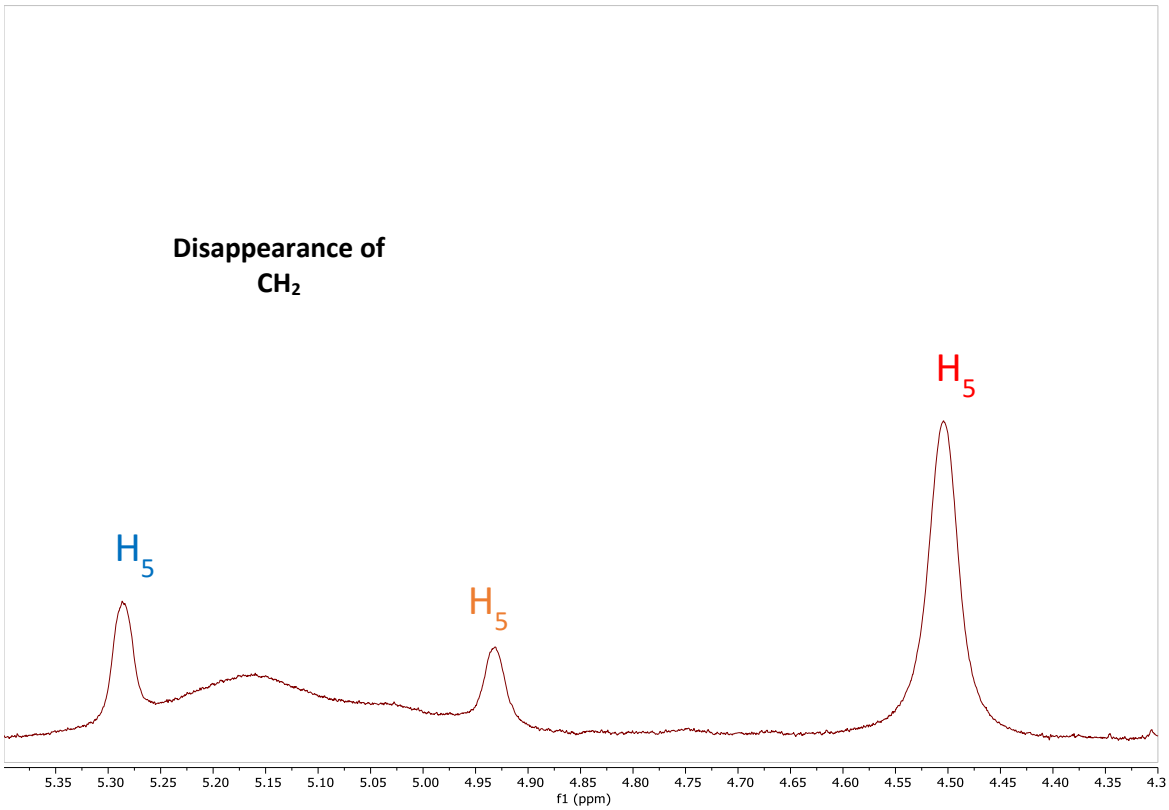




Supplementary figure S34: comparison of ^{1}H-^{15}N HSQC spectra of **4** and **10b** (600 MHz) in DMSO-*d*6

➢ Comparison between ^{1}H spectrums of **4** and imidazolidinone **10b**

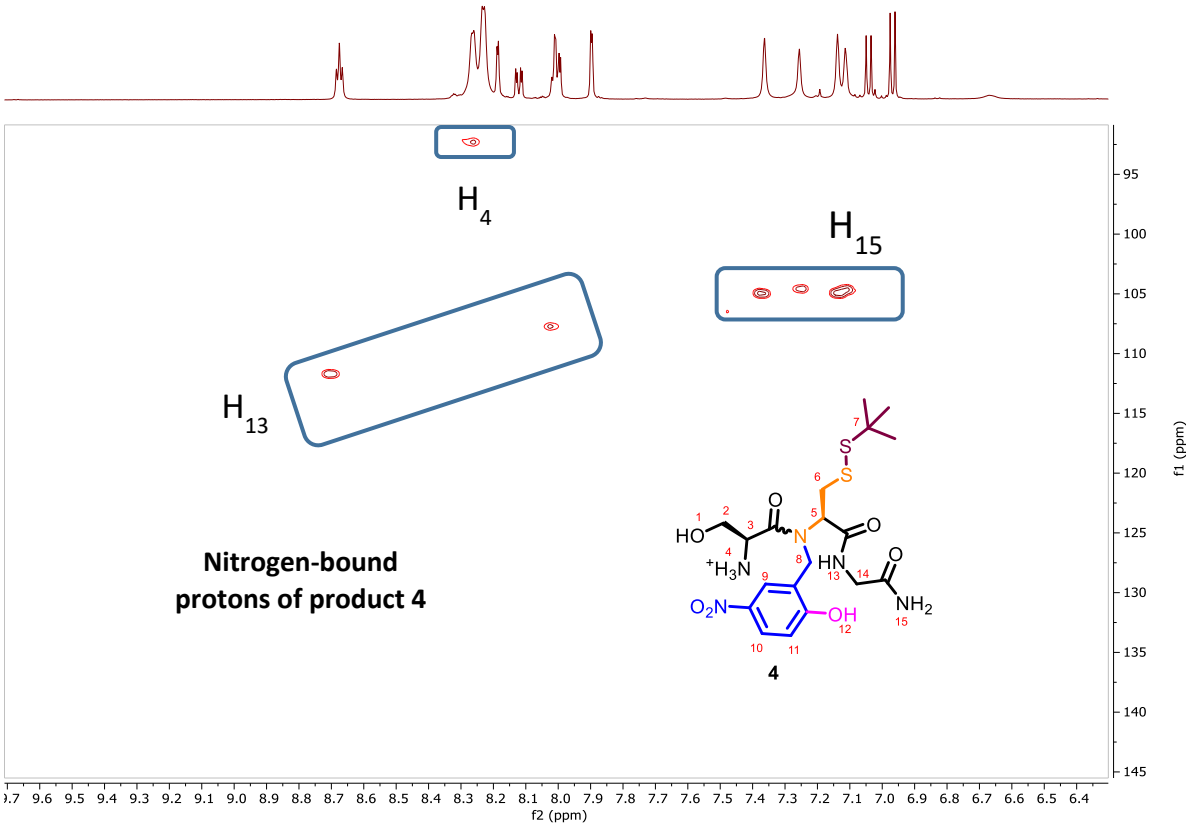


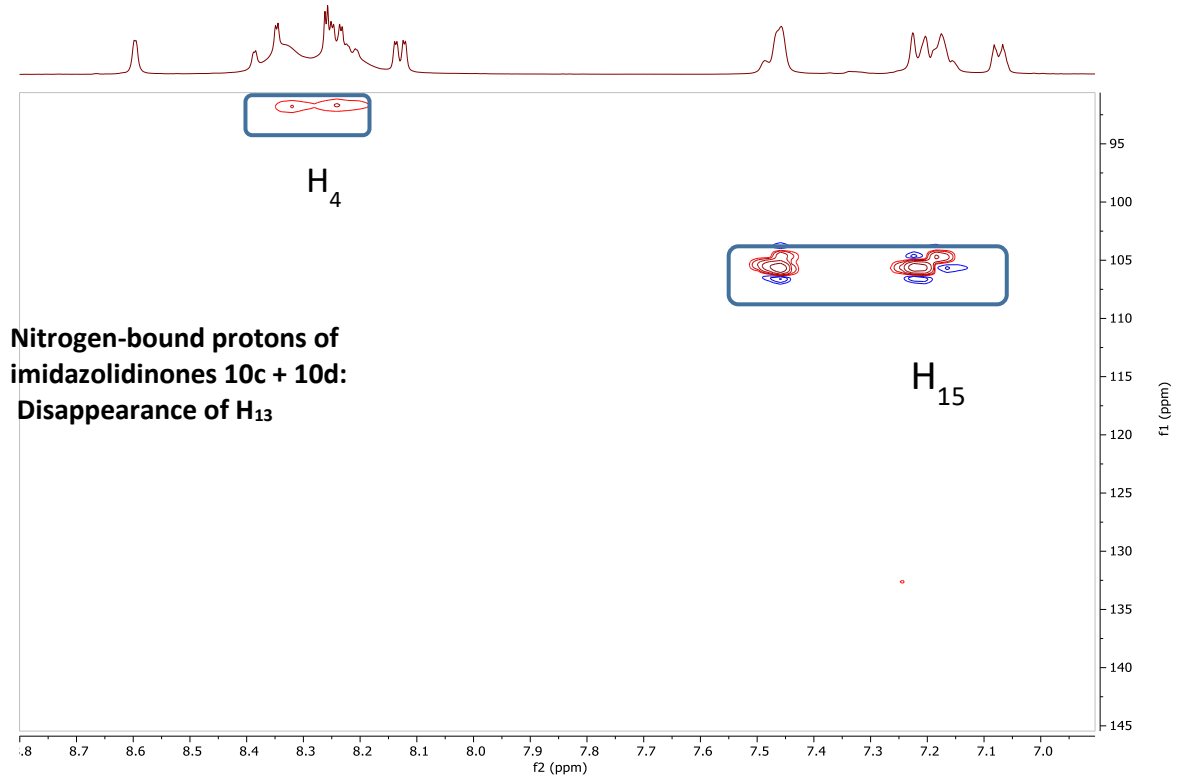


Supplementary figure S35: zoom on ^{1}H-NMR spectrum of product **4** and **10b** (600 MHz) in DMSO-*d6*

- **Compounds 10c/10d 77:33 mixture**

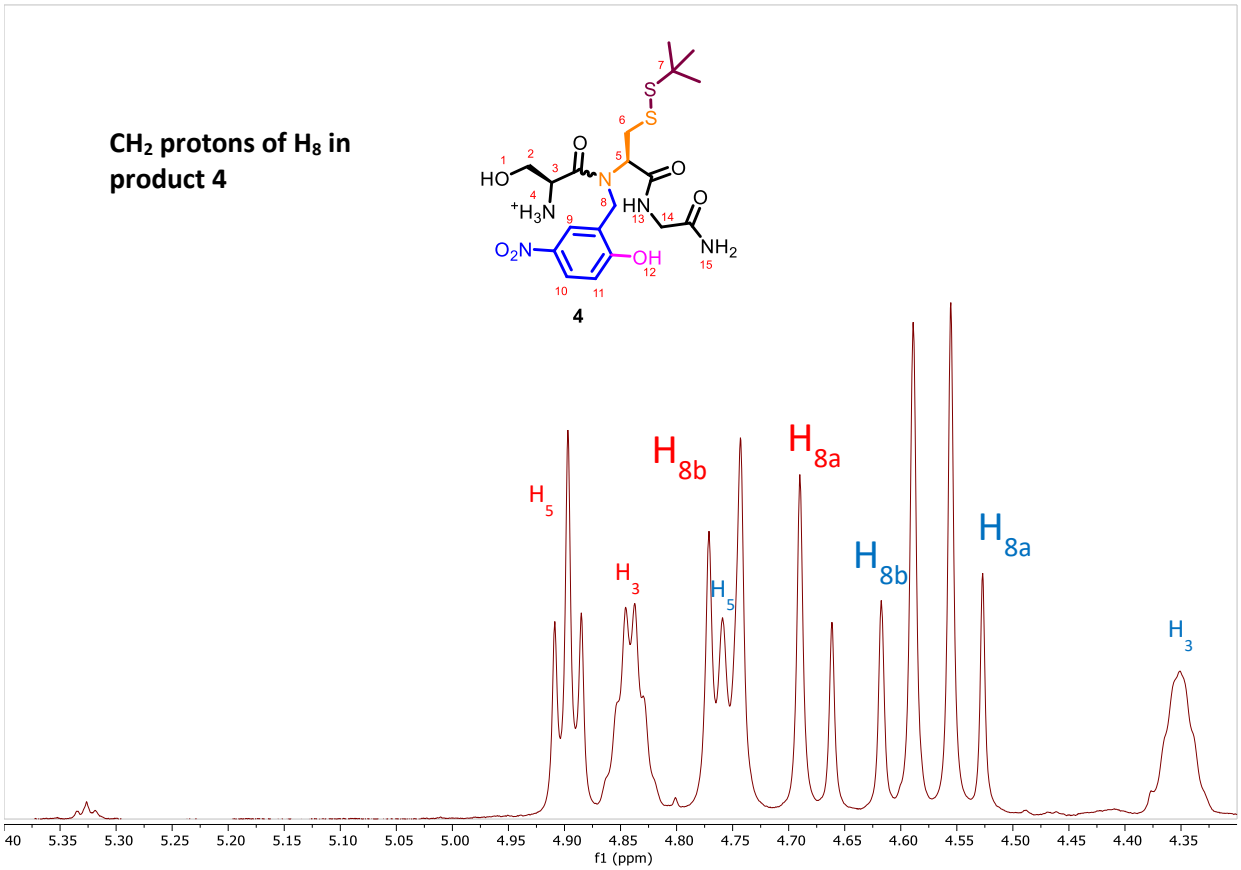
➢ Comparison between ^{1}H-^{15}N HSQC spectra of **4** and imidazolidinone **10c** + **10d**

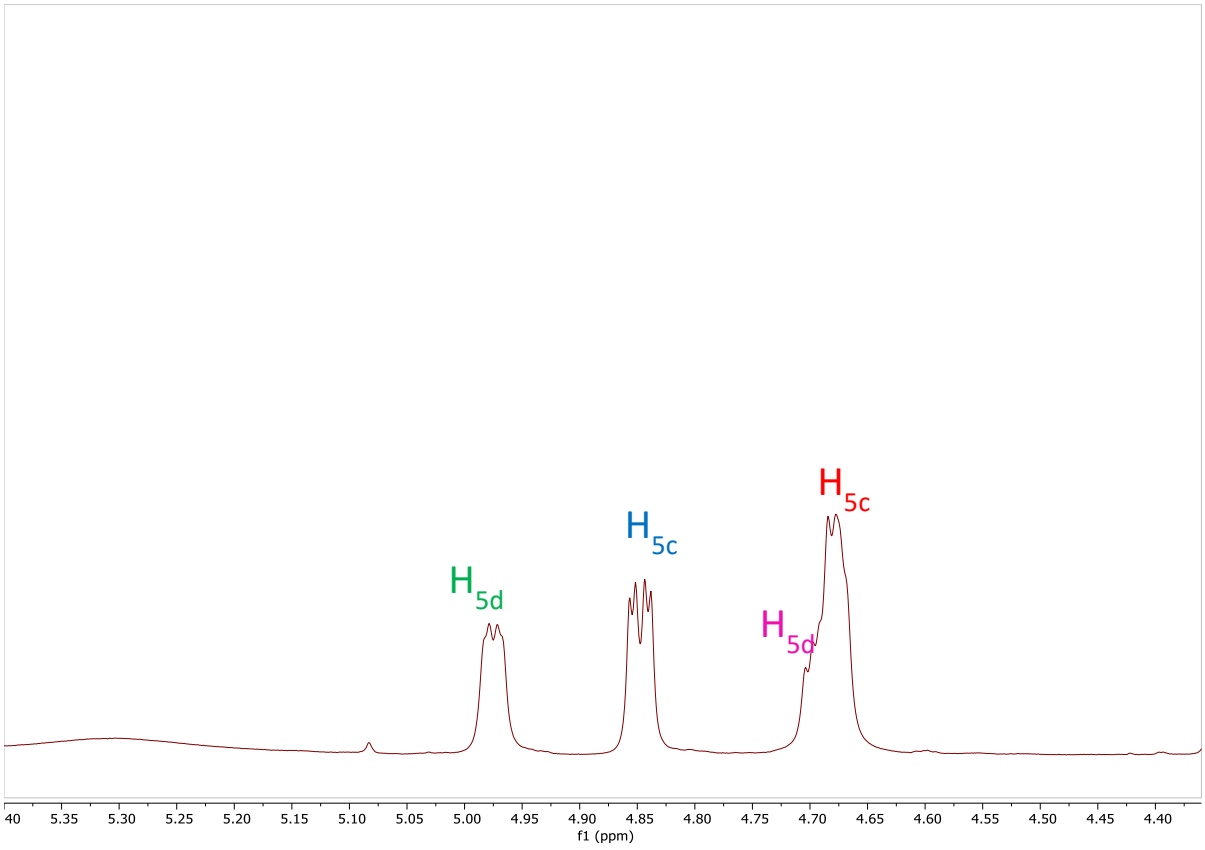




Supplementary figure S36: comparison of ^{1}H-^{15}N HSQCspectra of **4** and **10c**/**10d** (600 MHz) in DMSO-*d6*

➢ Comparison between ^{1}H spectrums of **4** and imidazolidinones **10c** + **10d**



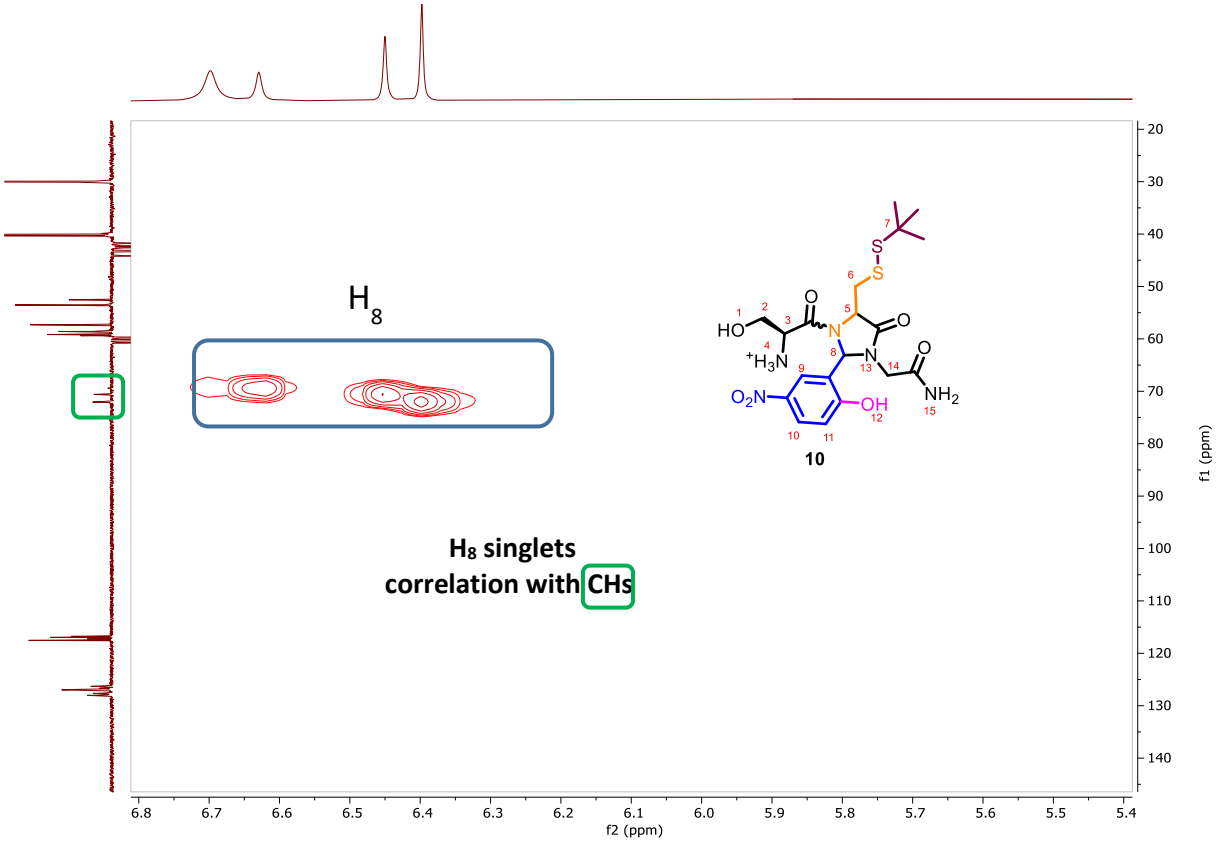


Supplementary figure S37: zoom on ^{1}H-NMR spectra of **4** and **10c** + **10d** (600 MHz) in DMSO-*d6*

The fact that we collected the mixture of **10c** and **10d** for NMR analysis yielded in bigger amount thus we could run additional analysis like DEPT 135 or HMBC. As explained above, in the case of **10c** and **10d**, only two sets of peaks were obtained (presumably *cis-trans* amide bond rotamers), yielding much simpler spectra than for **10a** and **10b**, where at least three sets of peaks corresponding to different conformers were identified.

- ^{1}H-^{13}C HSQC and DEPT-135 spectra of imidazolidinones **10c**/**10d** 67:33 mixture

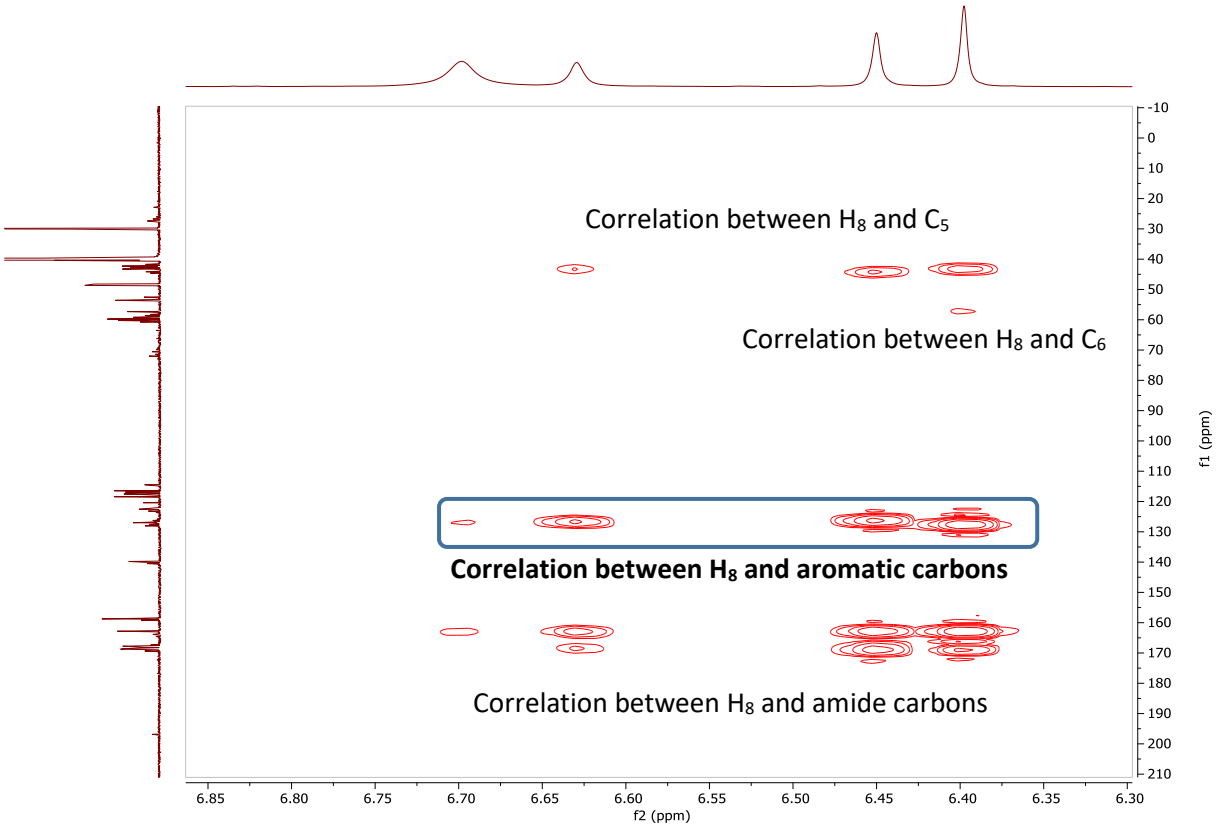
In the ^{1}H-^{13}C HSQC and DEPT-135 NMR spectra we can clearly see the correlation between the newly formed H8 singlets at 6.4-6.7 ppm and CH carbons at 70-75 ppm fig S44).



Supplementary figure S38: ^{1}H-^{13}C HSQC and DEPT-135 NMR spectra of imidazolidinones **10c** + **10d** (600 MHz) in DMSO

- ^{1}H-^{13}C HMBC spectrum of imidazolidinones **10c**/**10d** 67:33 mixture

The HMBC spectra validates our attribution of these newly formed singulets as H8 protons since it clearly shows Correlation between H8 and respectively: C5, C6, aromatic carbons and amide carbons.

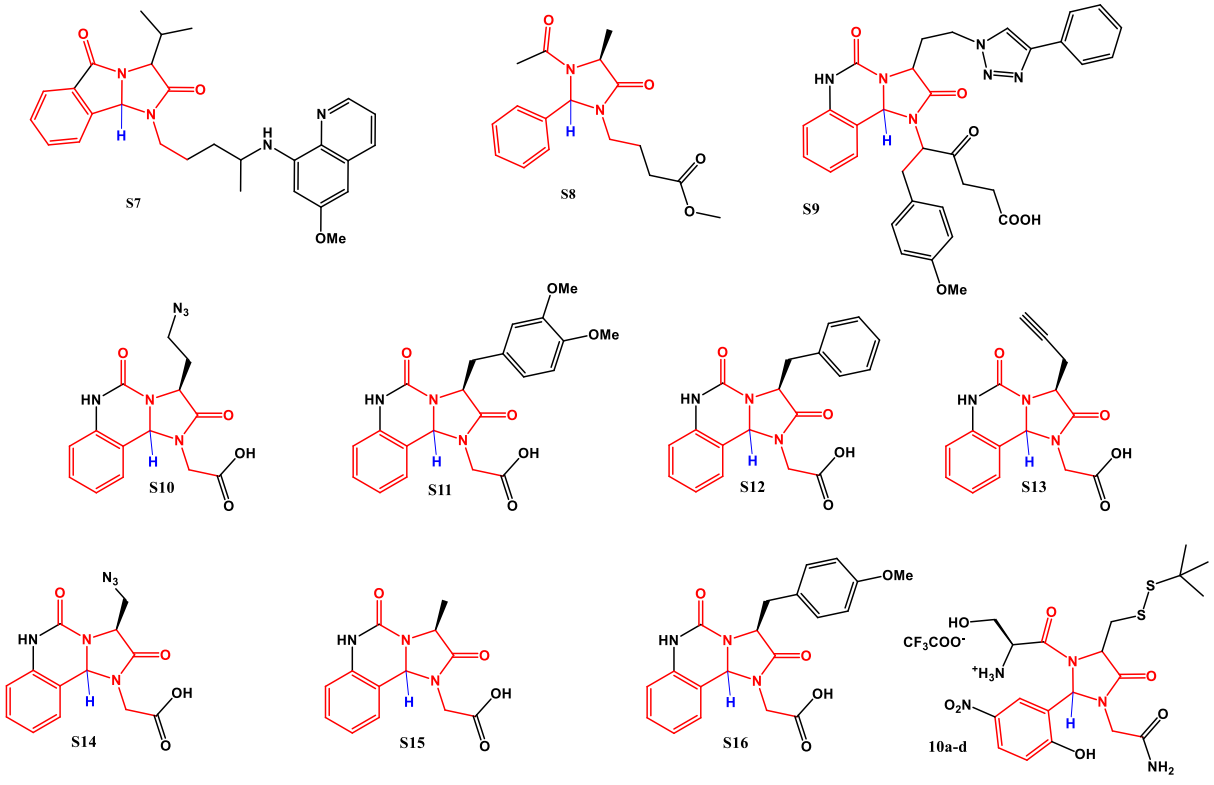


Supplementary figure S39: ^{1}H-^{13}C HMBC NMR spectrum of imidazolidinones **10c** + **10d** (600 MHz) in DMSO-*d6*

7-4- Comparison with ^{1}H and ^{13}C spectra of previously reported N^{1}-acyl-N^{3}-alkyl-2-aryl-imidazolidin-4-ones

❖ Table S4: Chemical shift of H2 and C2 of N^{1}-acyl-N^{3}-alkyl-2-aryl-imidazolidin-4-ones previously described in the literature

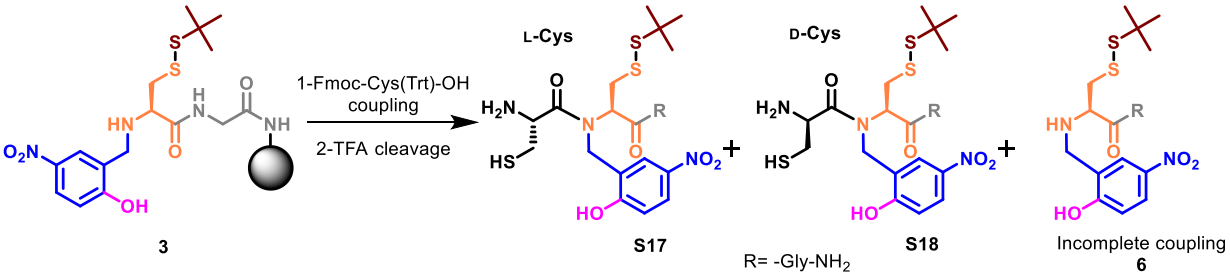
Compound	Reference	^{1}H ppm	^{13}C ppm
S7	[10]	5 .76 and 5.59	74.1 and 73.9
S8	[11]	5.98-6.23	74.1 and 73.9
S9	[12]	6.15	71.0
S10	[12]	6.27	70.1
S11	[12]	6.00	70.7
S12	[12]	6.03	70.3
S13	[12]	6.36	70.8
S14	[12]	6.34	71.0
S15	[12]	6.41	69.3
S16	[12]	5.97	70.3
10a	This work	6.56, 6.45 and 6.36	76.0, 74.1 and 69.6
10b	This work	6.66, 6.56 and 6.32	75.4, 74.2 and 69.0
10c	This work	6.70 and 6.63	69.5 and 69.4
10d	This work	6.45 and 6.40	71.6 and 70.8



Supplementary figure S40: Structures of N^{1}-acyl-N^{3}-alkyl-2-aryl-imidazolidin-4-ones previously reported in the literature (H8 protons highlighted in blue)

8-Optimization of the coupling of the first amino acid

8-1- Cysteine



- H-Cys-(Hnb)Cys(S*t*Bu)-Gly-NH_2 (**S17**)

ESI-MS (*m/z*): $[MH]^+$ calcd. for $C_{19}H_{30}N_5O_6S_3$: 520.1, found: 520.1

HPLC analysis: t_R = 8.2 min (Aeris Widepore XB-C18 2, gradient: 3-50% B'/A' over 15 min)

- H-Cys-(Hnb)Cys(S*t*Bu)-Gly-NH_2 (**S18**)

ESI-MS (*m/z*): $[MH]^+$ calcd. for $C_{19}H_{30}N_5O_6S_3$: 520.1, found: 520.1

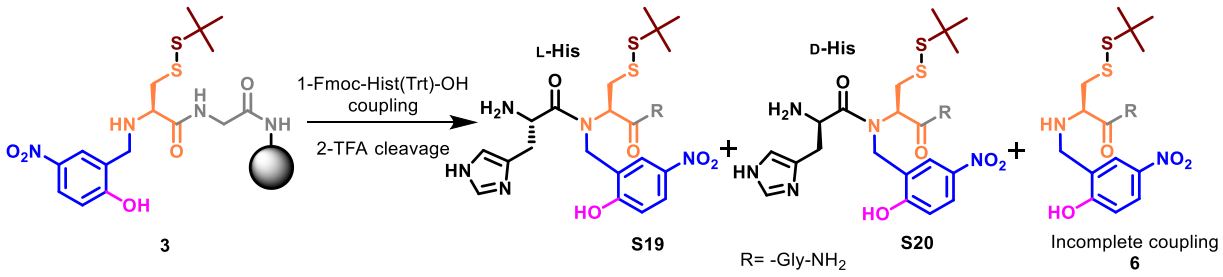
HPLC analysis: t_R = 7.4 min (Aeris Widepore XB-C18 2, gradient: 3-50% B'/A' over 15 min)

❖ Table S5: Optimization of the coupling of cysteine

Entry	AA coupled	Coupling conditions	**S17** + **S18** (%)[b]	**S17:S18 ratio** (%)[b], [c]	**6** (%)[b]
1[a]	Cys	HCTU/DIEA	91.4	96.5 : 3.5	9.6
2[a]	Cys	HATU/DIEA	97.9	96.7 : 3.3	2.1
3[a]	Cys	DIC/Oxyma	98.3	99.2 : 0.8	1.7
4[a]	Cys	DIC/HOBT	93.2	98.4 : 1.6	6.8

[a] Coupling conditions : 18 h coupling in NMP, 10 mM peptidyl resin, RT, 10 equiv. Fmoc-Cys(Trt)-OH, 9.5 coupling agent, 20 equiv. DIEA and 10 equiv. Oxyma or HOBt; [b] Relative rates determined by HPLC peak integration at λ = 320 nm; [c] **S18** is coeluted with its enantiomer arising from Cys(S*t*Bu) epimerization during reductive amination under optimized conditions, determined to be 0.3%. Therefore, this amount was deducted from the **S18** rate relative to **S17** showed in this table, in order to show only Cys(Trt) epimerization.

8-2- Histidine



- H-His-(Hnb)Cys(S*t*Bu)-Gly-NH_2 (**S19**)

ESI-MS (*m/z*): $[MH]^+$ calcd. for $C_{22}H_{32}N_7O_7S_2$: 554.2, found: 554.1

HPLC analysis: t_R = 5.8 min (Aeris Widepore XB-C18 2, gradient: 3-50% B'/A' over 15 min)

- H-His-(Hnb)Cys(S*t*Bu)-Gly-NH_2 (**S20**)

ESI-MS (*m/z*): $[MH]^+$ calcd. for $C_{22}H_{32}N_7O_7S_2$: 554.2, found: 554.1

HPLC analysis: t_R = 5.7 min (Aeris Widepore XB-C18 2, gradient: 3-50% B'/A' over 15 min)

❖ Table S6: Optimization of the coupling of histidine

Entry	AA coupled	Coupling conditions	**S19 + S20** (%)[b]	**S19/S20 ratio** (%)[b] [c]	**6** (%)[b]
1[a]	His	HCTU/DIEA	99.3	98.7 : 1.3	0.7
2[a]	His	DIC/Oxyma	99.6	99.8 : 0.2	0.4

[a] Coupling conditions : 18 h coupling in NMP, 10 mM peptidyl resin, RT, 10 equiv. Fmoc-Cys(Trt)-OH, 9.5 coupling agent, 20 equiv. DIEA and 10 equiv. Oxyma [b] Relative rates determined by HPLC peak integration at λ= 320 nm. [c] Relative rate determined by coupling of an extra Trp residue and integration of HPLC peaks at λ= 280 nm; [c] **S20** is coeluted with its enantiomer arising from Cys(S*t*Bu) epimerization during reductive amination under optimized conditions, determined to be 0.3%. Therefore, this amount was deducted from the **S20** rate relative to **S19** showed in this table, in order to show only Cys(Trt) epimerization.

8-3 Serine

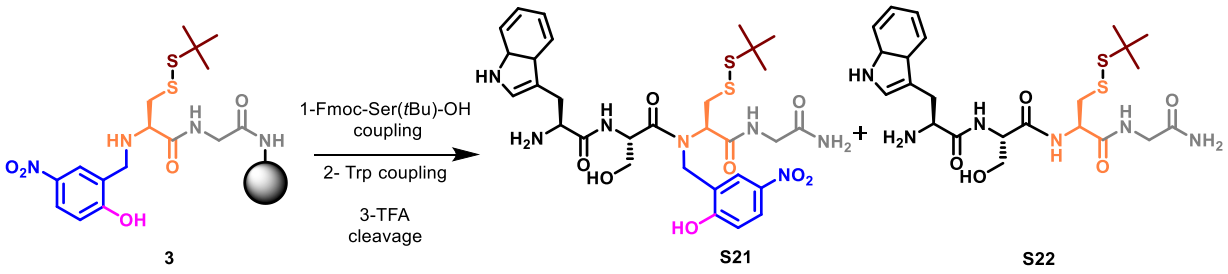
Table S7: Optimization of the coupling of serine

Entry	AA coupled	Coupling conditions	**4 + 5** (%)[b]	**4/5a** ratio (%)[b] [c]	**6** (%)[b]
1[a][d]	Ser	DIC/Oxyma	99.5	99.1 : 0.9	0.5

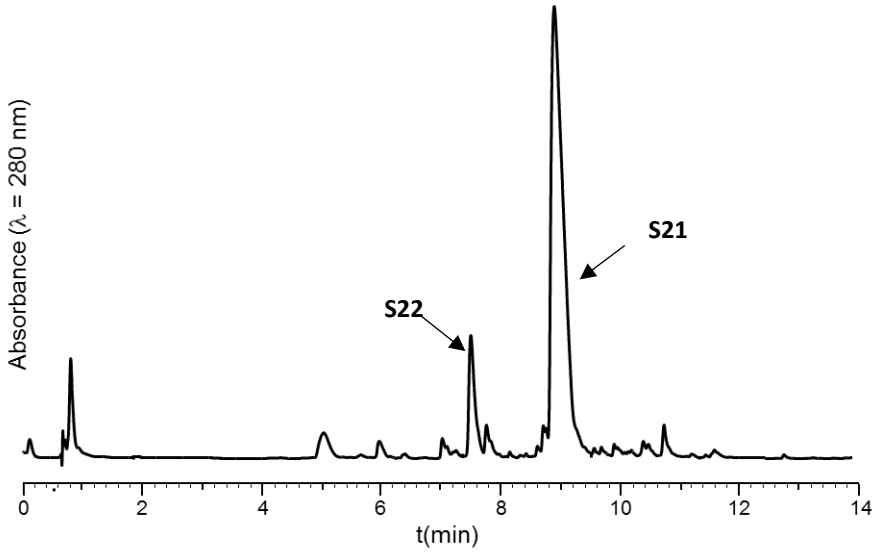
[a] Coupling conditions : 18 h coupling in NMP, 10 mM peptidyl resin, RT, 10 equiv. Fmoc-Cys(Trt)-OH, 9.5 DIC, 10 equiv. Oxyma; [b] Relative rates determined by HPLC peak integration at λ= 320 nm; [c] **5a** is coeluted with its enantiomer **5a** arising from Cys(S*t*Bu) epimerization during reductive amination under optimized conditions, determined to be 0.3%. Therefore, this amount was deducted from the **5a** rate relative to **4** showed in this table, in order to show only Cys(Trt) epimerization; [d] Reaction performed on three different resins: tentagel, ChemMatrix and polystyrene with similar results;

9-Quantification of the reductive amination yield

The coupling of a Trp residue allows the rate quantification of the non-Hnb bearing compound **S22** by integration of HPLC peaks at λ = 280 nm taking into account the molar absorption coefficient of Trp and Hnb at 280 nm: ε_{Trp} = 5500 L.mol^{-1}.cm^{-1} ε_{Hnb} = 2395 L.mol^{-1}.cm^{-1}



Fmoc-Trp(Boc)-OH (10 equiv.) was coupled for 1 h on H-Ser(*t*Bu)-(Hnb)Cys(S*t*Bu)-Gly-Rink ChemMatrix followed by piperidine treatment and TFA cleavage using general protocol PS1.



Supplementary figure S41: HPLC trace (Aeris Widepore XB-C18 2, gradient: 3-50% B'/A' over 15 min) of H-Trp-Ser-(Hnb)Cys(S*t*Bu)-Gly-NH_2 (**S21**) and H-Trp-Ser-Cys(S*t*Bu)-Gly-NH_2 (**S22**)

- H-Trp-Ser-(Hnb)Cys(S*t*Bu)Gly-NH_2 (**S21**)

ESI-MS (*m/z*): $[MH]^+$ calcd. for $C_{30}H_{40}N_7O_8S_2$: 690.2, found: 690.2

HPLC analysis: t_R = 8.6 min (Aeris Widepore XB-C18 2, gradient: 3-50% B'/A' over 15 min)

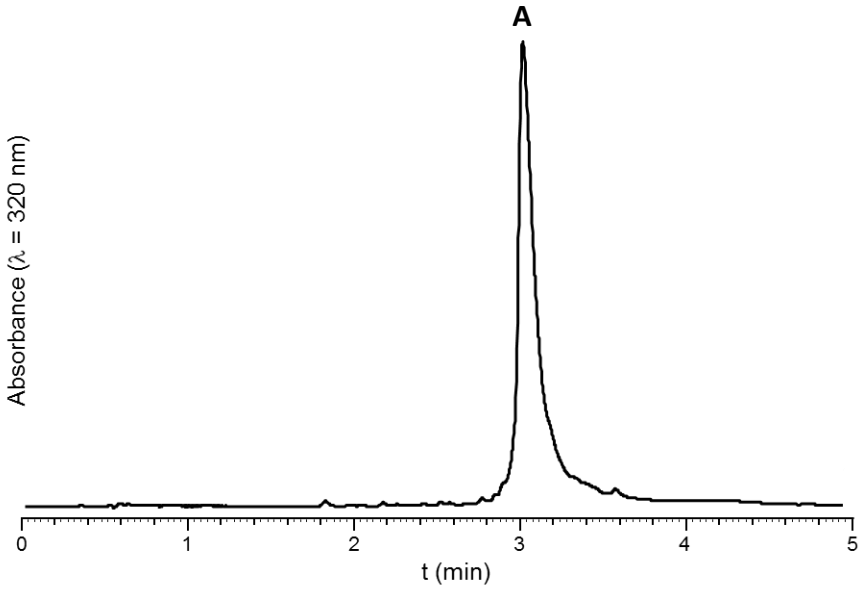
- H-Trp-Ser-Cys(S*t*Bu)Gly-NH_2 (**S22**)

ESI-MS (*m/z*): $[MH]^+$ calcd. for $C_{30}H_{40}N_7O_8S_2$: 690.2, found: 690.2

HPLC analysis: t_R = 7.2 min (Aeris Widepore XB-C18 2, gradient: 3-50% B'/A' over 15 min)

10- Examples of crypto-thioesters synthesized with the optimized method

- LYRAA-(Hnb)Cys(S*t*Bu)-Gly-NH_2 (**S23**)

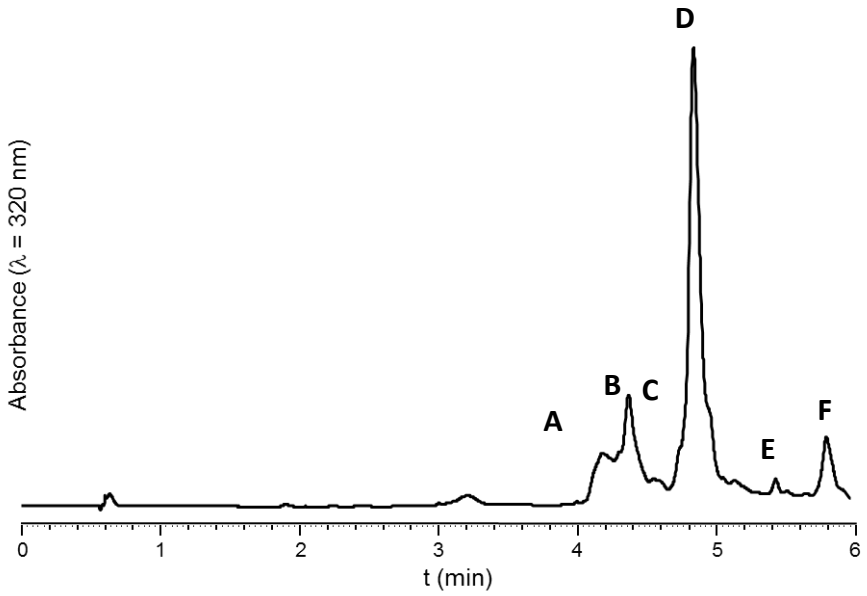


Supplementary figure S42: HPLC trace (Chromolith, gradient: 20-50% B/A over 5 min) of crude **S23**

Peak (t_R (min))	$[MH]^+$ (*m/z*) calcd.	$[MH]^+$ (*m/z*) found	Attributed to
A (3.29)	991.4	991.4	**S23**

- $(MUC1)_3$-(Hnb)Cys(S*t*Bu)-Gly-NH_2 (**S24**)

$(Muc1)_3$ = APDTRPAPGSTAPPAHGVTSAPDTRPAPGSTAPPAHGVTSAPDTRPAPGSTAPPAHGVTS

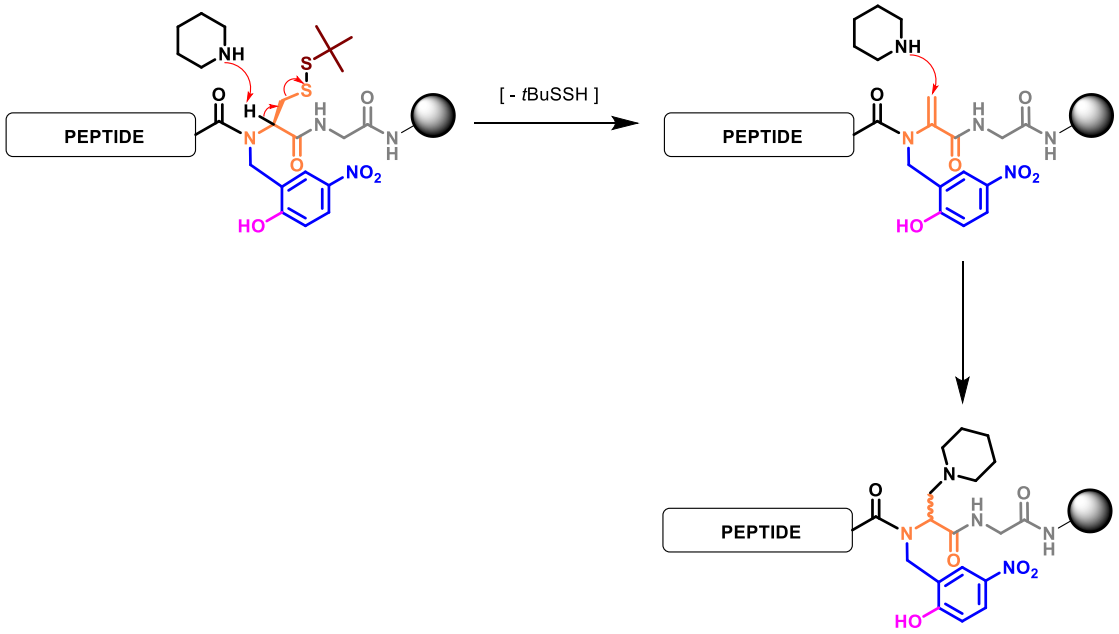


Supplementary figure S43: HPLC trace (Chromolith, gradient: 20-50% B/A over 5 min) of crude **S24**

Peak (t_R (min))	$[MH]^+$ (*m/z*) calcd.[a]	$[MH]^+$ (*m/z*) Found[a] [b]	Attributed to
A (4.22)	5681.2	5680.5	Ac-RPAPGSTAPPAHGVTSAPDTRPAPGSTAPPAHGVTSAPDTRPAPGSTAPPAHGVTS-(Hnb)Cys(S*t*Bu)-Gly-NH_2
B (4.42)	5986.4	5985.8	β-elimination + piperidine addition
C (4.61)	5923.5	5923.2	Ac-RPAPGSTAPPAHGVTSAPDTRPAPGSTAPPAHGVTSAPDTRPAPGSTAPPAHGVTS-(Hnb)Cys(S*t*Bu)-Gly-NH_2 + Trt
D (4.91)	6023.5	6022.8	**S24**
E (5.5)	2977.2	2976.7	Ac-PAHGVTSAPDTRPAPGSTAPPAHGVTS-(Hnb)Cys(S*t*Bu)-Gly-NH_2
F (5.89)	1618.8	1617.7	Ac-PGSTAPPAHGVTS-(Hnb)Cys(S*t*Bu)-Gly-NH_2

[a]: Average masses; [b] The multiply-charged envelope was deconvoluted using the charge deconvolution tool in Agilent OpenLab CDS ChemStation software.

As mentioned in the experimental part of the article, small amount of byproducts whose mass is consistent with β-elimination of the Cys(S*t*Bu) followed by Michael-type addition of piperidine is sometimes observed (figure S50). The amount was particularly high in the case of $(MUC1)_3$-(Hnb)Cys(S*t*Bu)-Gly-NH_2 **S33** (peak B, ~ 6 % based on the integration of extracted ion chromatogram for the most abundant multicharged species $[M+7H]^{7+}$).



Supplementary figure S44: putative mechanism of β-elimination followed by piperidine addition

11-Investigation of the formation of piperidinylalanine byproduct

In many cases of peptides of peptides as long as, or longer than **S24**, no piperidylalanine byproduct could be detected, which led us to question if this reaction was sequence-dependant, and not caused the presence of the *N*-Hnb group.

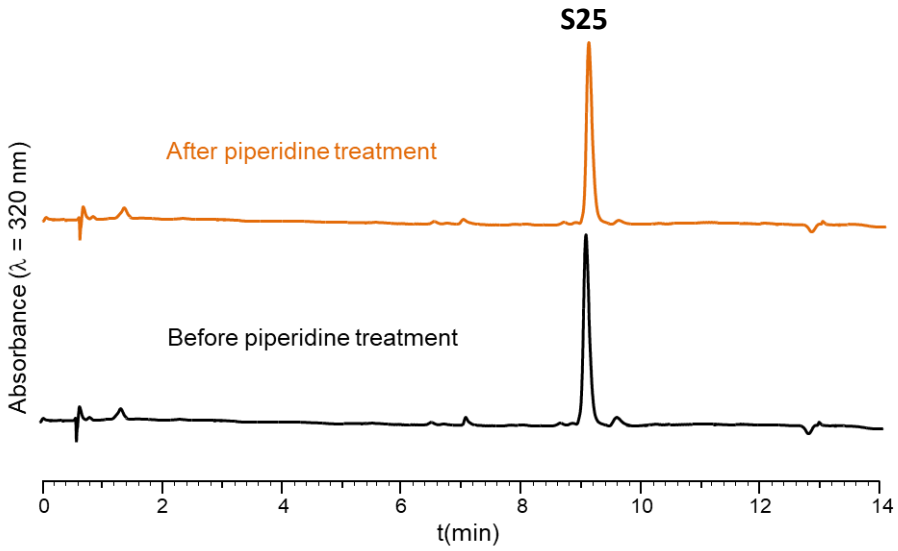
Peptidyl resin Ac-Ser(*t*Bu)-(Hnb)Cys(S*t*Bu)-Gly-Rink ChemMatrix (**S25**) was treated with 20% piperidine in NMP for 42 hours at room temperature (equivalent to 280 deprotection cycles of 3 x 3 min). TFA cleavage using protocol PS1-small peptide cleavage gave tripeptide Ac-Ser-(Hnb)Cys(S*t*Bu)-Gly-NH2 (**S26**). No trace of a β-elimination/piperidine addition by-product could be detected by LC-MS analysis. Moreover, a sample not treated with piperidine gave an essentially identical chromatogram.

This means that the formation of the β-elimination byproduct is very probably sequence-dependant, and not a general side reaction associated with the *N*-Hnb-Cys device.

- Ac-Ser-(Hnb)Cys(S*t*Bu)-Gly-NH_2 (**S25**)

ESI-MS (*m/z*): $[MH]^+$ calcd. for $C_{21}H_{32}N_5O_8S_2$: 546.16, found: 546.1

HPLC analysis: t_R = 9.03 min (Aeris Widepore XB-C18 2, gradient: 3-50% B'/A' over 15 min)



Supplementary figure S45: HPLC traces (Chromolith, gradient: 5-50% B/A over 5 min) of **S25** before and after piperidine treatment

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