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RESEARCH ARTICLE

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The stereodynamics of macrocyclic succinimide-thioethers

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Maleimide-thiol coupling is a popular bioconjugation strategy, but little is known about the stereoselectivity and the stereodynamics of the succinimide thioether formed in a biopolymer environment. We used thiol 1,4-addition for the macrocyclisation of 5 designed pentapeptides with the ringsize of hexapeptides because they incorporate the succinimide thioether (**4**-**8**). Both succinimide diastereomers are observed in the constrained macrocyclic rings in each case. In spite of the low diastereoselectivity of the macrocyclisation reaction, there is a significant influence of the amino acid environment on the epimerization rate of the succinimide. Its half life can be as short as several hours at room temperature when Gly is the amino acid following the succinimide (peptide **8**). On the contrary, no epimerization is detectable even after several weeks in the case of D-Phe C-terminal to the succinimide in peptide **4**. Already the small selection of examples shows how big the differences in epimerization rates can be and that the local environment has a significant influence. The variation of amino acids in the vicinity of the ligation site points the way towards the synthesis of bioconjugates which are obtained as stable and separable diastereomers.

1 | INTRODUCTION

The formation of succinimide thioethers via thiol 1,4-addition is frequently used to form bioconjugates or to immobilize bioactive compounds on surfaces.[1-12] It is the preferred method for the formation of antibody-drug conjugates.[5,13] Yet, only few studies are dedicated to the construction of homogeneous bioconjugates which do not exist as mixtures of diastereomers at the ligation site.[14,15] Our study identifies the stereodynamic and conformational properties of pairs of diastereomeric macrocyclic pentapeptides containing a succinimide thioether where the ring closure is achieved by a thiol 1,4-addition reaction. Figure 1 shows how the ligation site fits the 3-atom register of α-amino acids and thus can be seen as an isosteric replacement within the peptide backbone. The ligation has structural analogy to aspartimides formed spontaneously by Asp-Gly sequences as a side reaction in peptide synthesis.[16] This reaction is also known from proteins, where in the case of lysozyme the resulting succinimide kinks and restricts the turn region.[17]

We use the 3-letter code "Suc" for succinimide to underline the similarity of the ligation site with the 3-atom repeat of the peptide backbone. The ring-closing acylation of the amino acid following Suc is indicated by a double hyphen "=" instead of a hyphen "-" to show the restriction by the 5-membered ring.[18] Accordingly, the maleimide precursor Mal=Gly (**1**, Figure 2) reacts with Cys-OMe to the succinimide containing dipeptide Cys(OMe)-Suc=Gly (**2**). ^{1}H NMR identifies a nearly equimolar mixture of the diastereomeric dipeptides Cys(OMe)-(*R*)-Suc=Gly and Cys(OMe)-(*S*)-Suc=Gly (**3** DS1 and **3** DS2).

Three different protected amino acid building blocks of the type Mal=Xaa, wherein Xaa is either Gly, D-Ala, or D-Phe were synthesized and used in solid phase peptide synthesis according to methods recently published.[19-22] With the intention to minimize the conformational mobility of the macrocyclic peptides, we introduced the bicyclic *β-turn* mimic Hot=Tap (Hot: Hydroxythreonine, "=" backbone cyclisation, Tap: Thiaproline)[23] oppositely to the ligation site of the macrocyclic peptides as shown in Figure 3. The oligocyclic environment restricts the conformational mobility of the cyclopeptide and was expected to slow down the H/D exchange and the epimerization in solution with the aim of studying the processes by NMR spectroscopy.

The linear peptide precursors of the type Mal=Xaa-Gly-Hot=Tap-Yaa, wherein Yaa is either D-, L-Cys, or Tht, were cyclized in a 1-pot retro Diels-Alder, thiol 1,4-addition reaction yielding 2 diastereomers which were separated by preparative HPLC. The unequivocal assignment of *R/S* stereochemistry of the newly formed stereocenter at the cyclisation site was not possible by NOE and ^3J coupling data. Therefore, we distinguished the 2 diastereomers *cyclo*[-(*R*)-Suc=Xaa-Gly-Hot=Tap-Yaa] and *cyclo*[-(*S*)-Suc=Xaa-Gly-

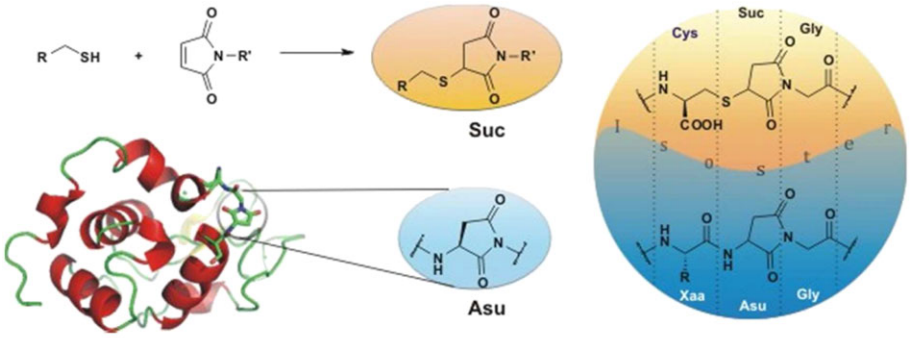


FIGURE 1 Two stereoisomers are formed by the succinimide-thioether (Suc) which closely resembles naturally occurring amino succinimides (Asu) formed by Asp-Gly sequences for the well-studied example is lysozyme (PDB-code: 1AT5). Both succinimides fit the 3-atom register of the peptide backbone (right)

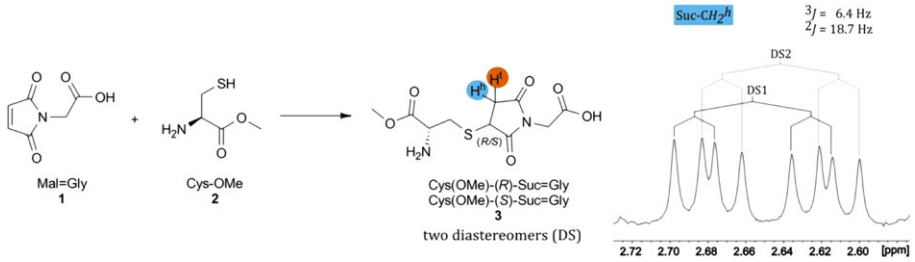


FIGURE 2 The addition reaction of Mal=Gly (**1**) and Cys-OMe (**2**) in a thiol 1,4-addition yields both diastereomeric succinimide dipeptides Cys(OMe)-(*R*)-Suc=Gly and Cys(OMe)-(*S*)-Suc=Gly (**3** DS1 and **3** DS2). The expansion from the ^{1}H NMR shows different chemical shifts for the high-field proton of the (*R*)-Suc/(*S*)-Suc methylene group of **3** in the ^{1}H NMR. The complete ^{1}H NMR is shown in the Supporting Information

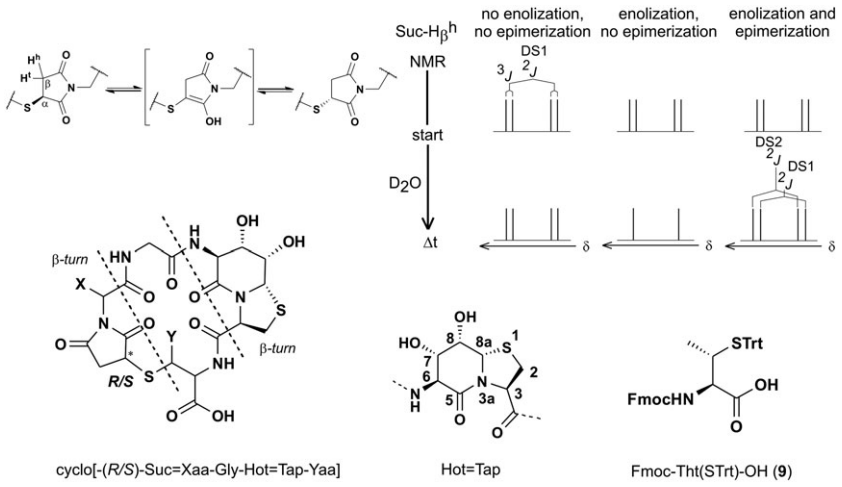


FIGURE 3 The proposed mechanism of the succinimide epimerization is accompanied by de- and reprotonation of C_α (upper left). Three different scenarios should be distinguishable by ^{1}H NMR (right). The schematic line spectra of the dd signal pattern of one of the $SucH_\beta$ either remains unchanged in the presence of D_2O, the enolisation simplifies the signal pattern without epimerization, or the enolization is accompanied by epimerization observable as a second signal set with simplified signal pattern. The general structure of the succinimide thioether containing tetracyclic pentapeptides *cyclo*[-(*R*/*S*)-Suc=Xaa-Gly-Hot=Tap-Yaa] and the nomenclature of the *β-turn* mimic Hot=Tap is shown at lower left

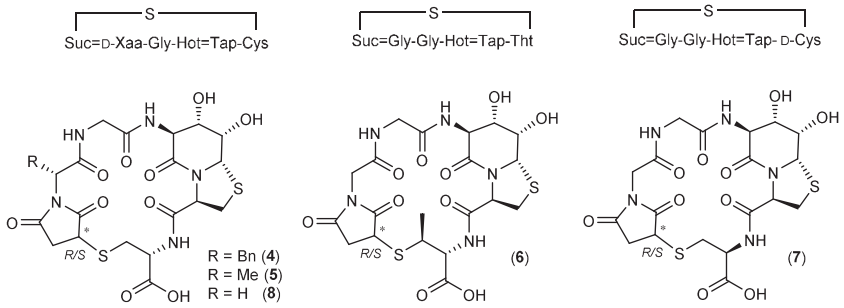
Hot=Tap-Yaa] as DS1 and DS2 according to their retention times on the HPLC. The tetracyclic peptides showed sufficient ^{1}H NMR signal dispersion to allow a complete signal assignment by 2D NMR. In order to differentiate whether the N- or the C-terminal amino acid next to the cyclisation site has a bigger influence on the cyclisation stereochemistry and on the epimerization rate, we varied both sites and separated the diastereomers. All 5 pairs of cyclic peptides **4** to **8** are shown in Scheme 1.

Peptides **4**, **5**, and **8** differ in the amino acids of the Mal=Xaa building block. Mal=D-Phe in the case of **4**, Mal=D-Ala in **5**, and Mal=Gly in the case of **8**. Peptides **6** and **7** differ in the amino acids bearing the thiol group which is L-*allo*-thiothreonine (Tht) in the case of **6** and D-Cys in the case of **7**. Fmoc-L-*allo*-trityl-thiothreonine (Fmoc-Tht(Trt)-OH, **9**) was synthesized in 7 steps from Thr in an overall yield of 20%. Detailed reaction conditions can be found in the Supporting Information.

2 | MATERIALS AND METHODS

2.1 | Reagents

All chemicals and solvents used for the synthesis of peptides were purchased from Iris Biotech and were not purified further.



SCHEME 1 The 18-membered macrocyclic ring is a common feature of all cyclic peptide analogs **4** to **8** which differ only at the amino acids N- or C-terminal to the succinimide cyclisation site. Each peptide exists as a pair of diastereomers DS1 and DS2

2.2 | Resin loading

2-Chlorotritylchloride resin (1.60 mmol/g) was loaded with Fmoc-Xaa(Trt)-OH (Xaa = Cys, D-Cys, Thiothreonine) by adding the protected amino acid (1.50 eq) and DIPEA (6.00 eq) in DMF (10 mL/g resin) and stirring for 4 hours. After washing the resin with DMF, methanol, and DCM several times, the resin was treated with a mixture of DCM/methanol/DIPEA (80:15:5) 2 times for 30 minutes and washed several times with DMF, methanol, and DCM before it was dried under vacuum. The loading of the used resins was estimated to be between 0.30 and 0.40 mmol/g by UV-Vis spectroscopy at 289 and 300 nm after cleaving the Fmoc protecting group with 20% piperidine in DMF for 20 minutes.

2.3 | Solid phase peptide synthesis

Linear precursor peptides were synthesized on a microwave assisted peptide synthesizer. Fmoc-strategy was applied. The 2CTC-resins loaded with Fmoc-Xaa(Trt)-OH (Xaa = Cys, D-Cys, Thiothreonine; scheduled quantity: 0.1 mmol, 1.00 eq) ran through the following cycles of Fmoc-deprotection and amino acid coupling:

- Fmoc-deprotection: T = 50°C, $P_{microwave}$ = 30 W, t = 210 seconds with piperidine (20 w% in DMF, 3.00 mL/deprotection)
- Amino acid coupling: T = 50°C, $P_{microwave}$ = 30 W, t = 600 seconds

with Fmoc-protected amino acid and maleimde building blocks (0.2 M in DMF, 5.00 eq, 2.5 mL/coupling), DIC (0.5 M in DMF, 5.00 eq, 1 mL/coupling), and Oxyma (1 M in DMF, 5.00 eq, 0.5 mL).

2.4 | Resin cleavage

Resin cleavage was performed with a mixture of TFA/TIPS (96:4) for 240 minutes. Peptides were precipitated from cold diethylether (40 mL), washed 2 to 3 times with diethylether and lyophilised from water.

2.5 | Cyclization of peptides

The crude linear precursor peptides were used without further purification and dissolved in 100 mL of *n*-butanol to reach a peptide concentration of c = 1 mM. After refluxing for 4 hours, the solvent was evaporated, and the resulting diastereomeric mixture of the peptide was purified by preparative HPLC.

2.6 | HPLC

Analytical HPLC was done on a Thermo Fisher Ultimate 3000 LC System. The cyclic peptides were purified by semi-preparative reversed-phase HPLC on a Thermo Fisher Ultimate 3000 LC System.

2.7 | Mass spectrometry

Mass spectra (ESI+) were acquired on a Thermo Fisher Scientific LTQ-FT.

2.8 | NMR measurement

NMR spectra (1H, TOCSY, COSY, ROESY, ^{1}H-^{13}C-HSQC) were acquired on a 500-MHz spectrometer, and the kinetic measurements were performed on a 300-MHz spectrometer, both Bruker AV series.

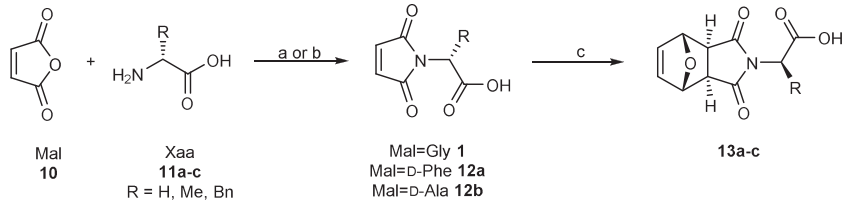
2.9 | Molecular modeling

Molecular modeling and molecular dynamics calculations were performed using Molecular Operating Environment software version 2014.0901 (Chemical Computing Group, Montreal, Canada). The structure was assembled using the Protein Builder program; later, the Suc=Gly building block was modeled starting from cysteine using the builder interface of the Molecular Operating Environment program and energy minimized using the Amber12EHT force field. The peptide was afterwards analyzed through multiple simulated annealing processes in implicit solvent using Born as the dielectric constant with extended cutoff. Several iterations were performed, and the structure with the lowest energy was further minimized.[24]

3 | RESULTS AND DISCUSSION

3.1 | Synthesis of the Mal=Gly and Mal=D-Xaa building blocks

The maleimide Mal=Gly **1** was obtained by condensation of malic anhydride (**10**) with Gly in refluxing acetic acid.[25,26] Chiral amino acids are expected to show significant racemization under these reaction conditions. Therefore, the chiral maleimide building blocks Mal=D-Phe **12a** and Mal=D-Ala **12b** were synthesized in a 2-step process according to Toru et al[27] (Scheme 2) although alternative synthesis at room temperature or with microwave irradiation exist.[28-30] To prevent side reactions of the maleimide double bond in peptide synthesis, a dihydrofuran protecting group was used to obtain the building blocks **13a** to **13c** which are compatible to the reaction conditions used for



SCHEME 2 Synthesis of maleimide building blocks compatible for SPPS. Reaction conditions for Gly: (A) HOAc, 120°C, 8 hours; (B) reaction conditions for chiral amino acids: 1) HOAc, RT, 12 hours; 2) $ZnBr_2$, HMDS, MeCN, 80°C, 3 hours; (C) furan, $CHCl_3$, 36°C, 4 days

solid phase peptide synthesis. This method has been implied by Grandas et al.[31,32] The retro Diels-Alder deprotection was described to be complete at 120°C.

3.2 | Synthesis of cyclic peptides

The linear precursor peptides were synthesized on a semi-automatic microwave-assisted peptide synthesizer in a 0.1 millimolar scale. The first amino acid loaded on a 2-CTC resin was the selectively protected thiol amino acid to obtain resins **14** to **16**. The solid-phase peptide synthesis (SPPS) was performed according to standard protocols for the coupling with DIC/Oxyma and the Fmoc deprotection with 20% piperidine in DCM. Cyclisation of these peptides takes place in a 1-pot retro DA reaction with an additional thiol 1,4-addition. To prevent oligomerisation, the cyclisation was carried out at dilute conditions with a peptide concentration of 1 mM. The solvent of choice for the cyclisation was *n*-butanol because its boiling point at 118°C fits the temperature for the retro DA reaction. Its relatively low vapor pressure simplified workup (Scheme 3). All cyclization experiments yielded 2 diastereomers in a close to equimolar mixture of both diastereomers (Scheme 3). There were no directing effects observed of the other stereo centers within the linear precursor peptide. The cyclic peptide was the only observed product without dimer formation.

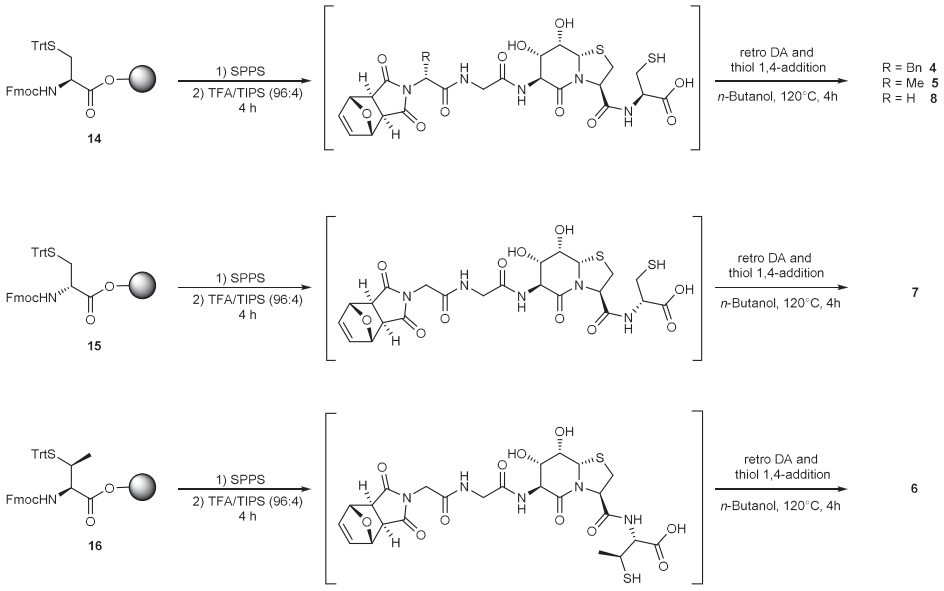
The 2 possible diastereomers of the cyclic peptides were differentiated according to their retention times on the reversed-phase HPLC chromatogram. The more polar peptide with shorter retention time is diasteromer 1 (DS1) and the second diastereomer is DS2 (Figure 4).

The diastereomers were separated by preparative reversed-phase HPLC and analyzed by NMR spectroscopy. The ^{1}H NMR spectra of DS1 are shown in Figure **5** and the spectra of DS2 in Figure **6**. In spite of the significant signal dispersions shown by both sets of spectra, there are no characteristic chemical shifts, coupling, or NOE patterns which would allow an unequivocal stereochemical assignment of the (*R*)-Suc/(*S*)-Suc stereochemistry.

3.3 | Stereodynamic behavior of the cyclic succinimide thioether containing pentapeptides

The pure peptide **8** showed the slow formation of a second signal set in the course of the NMR experiments which was identified as the second diastereomer. The same was observed for cyclopeptide **7** (Figure 7).

In order to determine the mechanism behind this epimerization process, the epimerization was followed in the presence of D_2O with the aim to estimate the enolization rate of the succinimde (Figure 8). Peptide **7** was dissolved in D_2O where H/D exchange of the slightly acidic stereocenter of the succinimide ring (green) ceased the corresponding ^3J coupling to the neighboring diastereotopic methylene group (CH_2, blue, H^h). This observation connects the epimerization process between DS1 and DS2 and enolization of the succinimide ring.



SCHEME 3 Synthesis of the cyclic peptides **4** to **8**, starting from preloaded CTC-resin. SPPS was carried out on a microwave-assisted peptide synthesizer. For complete deprotection of the Trt-protecting group, the TFA cleavage time was extended to 4 hours. The linear precursor peptide was used without further purification. The retro DA reaction of the dihydrofuryl protected *N*-terminal maleimide at 120°C under high dilution conditions in *n*-butanol is followed by the macrocyclisation of the peptide

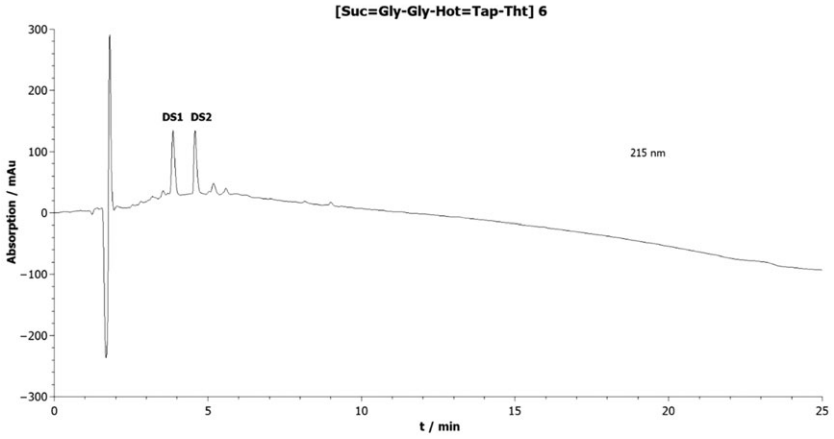


FIGURE 4 The HPLC chromatogram of the cyclisation reaction shows the crude cyclic pentapeptide **6** containing a succinimide thioether neighbouring L-*allo*-thiothreonine as a nearly 1:1 ratio of both diastereomers DS1 and DS2. Similar chromatograms were observed for all investigated cyclic peptides

The epimerization rate was investigated by NMR spectroscopy for all cyclic peptides (**4**-**8**). The well-separated Hot = Tap-3*H* or 8a*H* proton served as reference signal for the integration. The cyclic pentapeptides which contained the Suc=Gly motif (**6**-**8**) showed a ratio of approximately 3:1 DS1/DS2 after reaching the thermodynamic equilibrium.

The epimerization equilibrium and velocity strongly depend on the configuration of the stereogenic centers of the peptide backbone close to the succinimide. The epimerization velocity of peptide **8** is 4 times faster than peptide **7** (Figure 9). The additional methyl group of L-*allo*-thiothreonine (**6**) halves the epimerization velocity relative to peptide **8** (see Table 1).

Altogether, there is a strong conformational influence of the macrocyclic on the acidity of the succinimide ring and its tendency for enolization.

Cyclopeptide **4** and **5** with the D-configurated amino acid at the C-terminal position relative to the succinimide ring showed significant resistance to epimerization. The NMR kinetics of the separated diastereomers of peptide **4** in DMSO-d_6 in the presence of small amounts of D_2O showed no epimerization for more than 60 days (Figure 10). Remarkably, DS2 shows complete H/D exchange in this measuring window due to enolisation, while H/D exchange is completely absent for DS1. This is the first time that stable succinimide epimers could be separated and analyzed.

The wide ^{1}H signal dispersion allows a complete signal assignment and a closer look at the conformational properties of the cyclopeptides. The temperature dependence of the secondary amide NH protons differentiated the internal or external (solvent accessible) orientation of the amide protons. Sequential NOEs characterize the peptide backbone and identify the dominating rotamer about the thiol-succinimide linkage was identified from a combination of ^3J and NOE data.

In spite of the significant chemical shift differences, the relevant NOE and coupling data were surprisingly similar for both diastereomers,

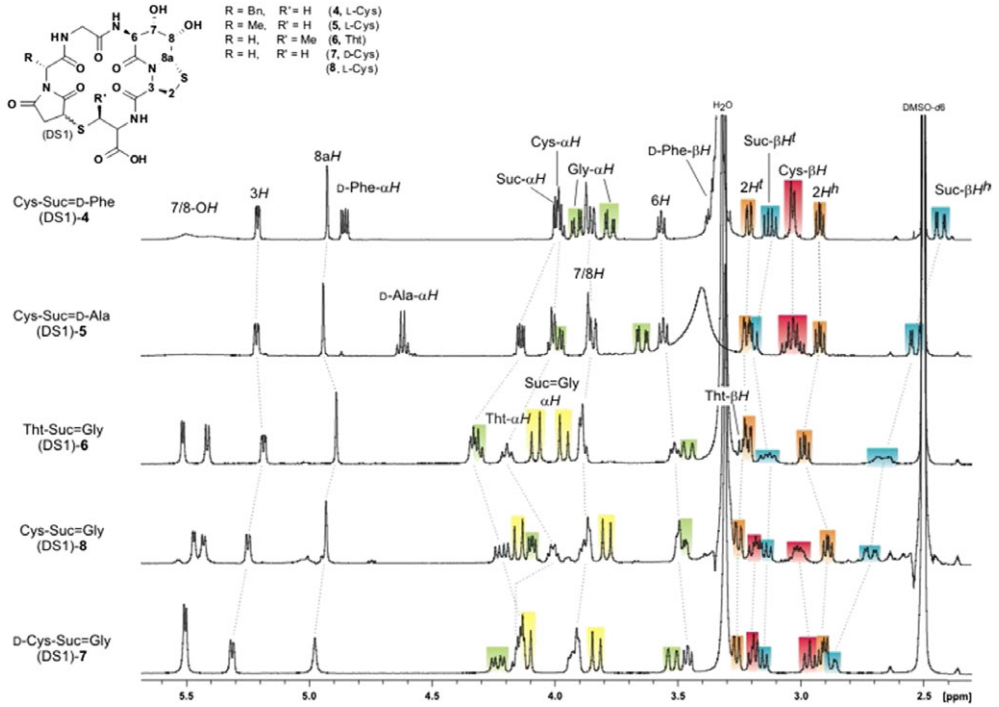


FIGURE 5 ^{1}H NMR (500 MHz, 300 K, DMSO-d_6) of the aliphatic region of the DS1 of all cyclic peptides. The diastereotopic proton pairs are colored and show characteristic signal dispersions.

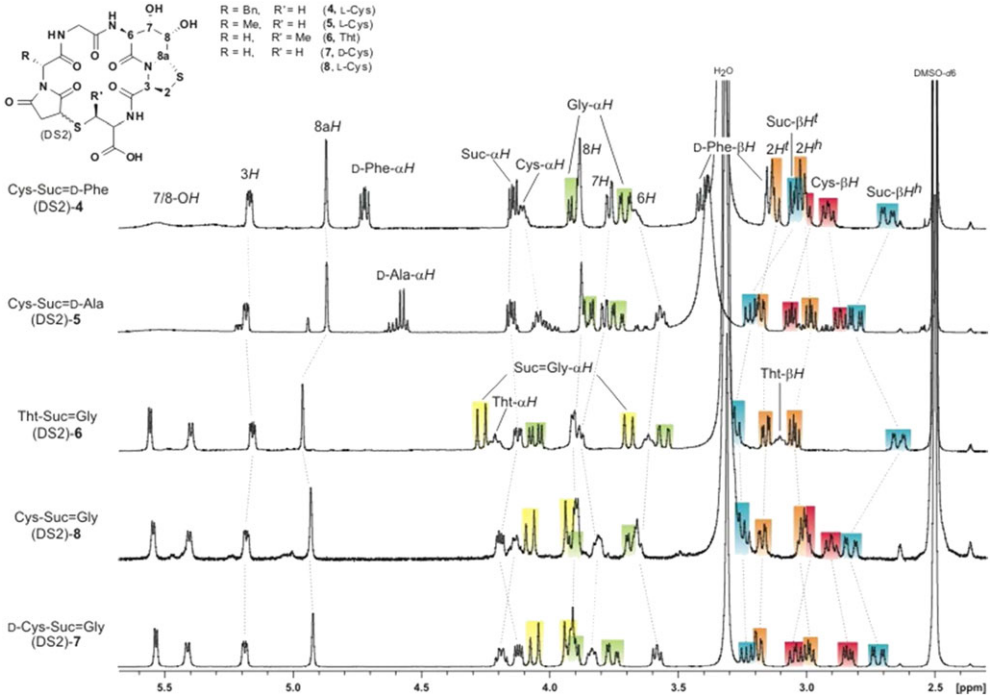


FIGURE 6 ^{1}H NMR (500 MHz, 300 K, DMSO-d_6) of the aliphatic region of the DS2 of all cyclopeptides. The diastereotopic proton pairs are colored and show characteristic signal dispersions.

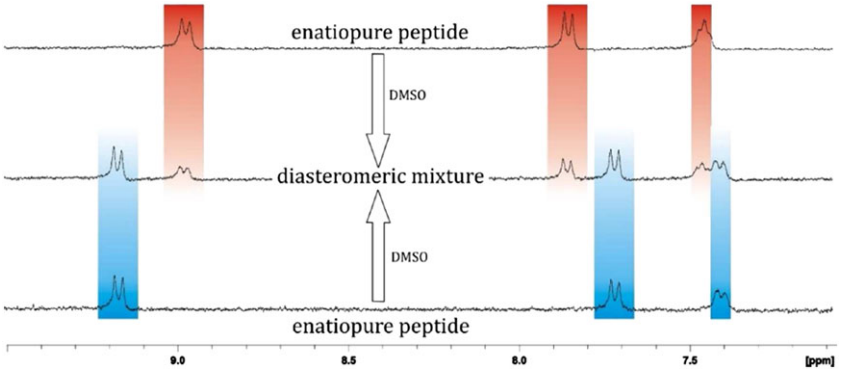


FIGURE 7 Epimerization (^{1}H NMR, 500 MHz, 300 K, DMSO-d_6) of the cyclic peptides **7** DS-2 (top) and DS-1 (below) leads to the identical mixture of both diastereomers as before the separation by preparative HPLC

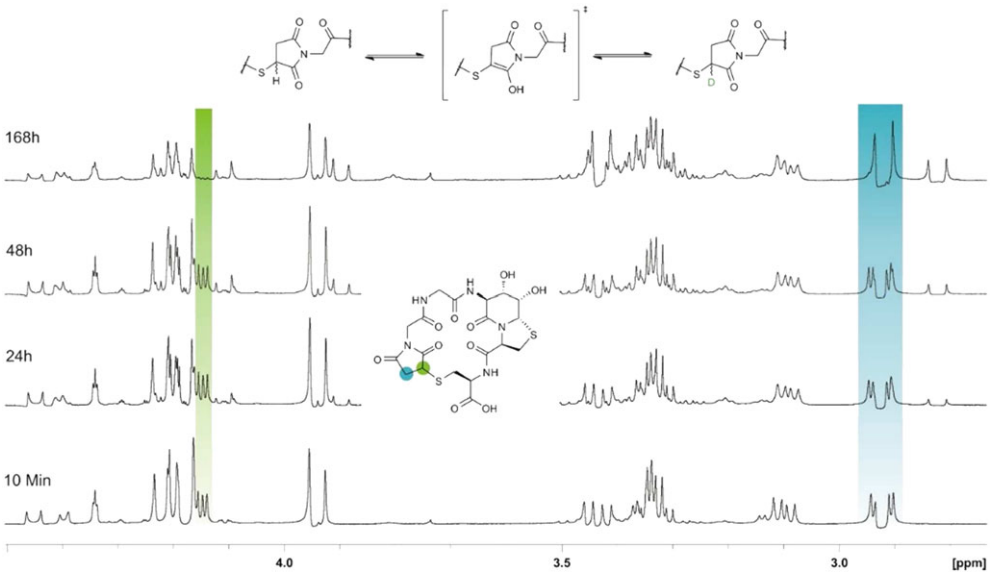


FIGURE 8 ^{1}H NMR (600 MHz, 300 K, D_2O) of peptide **7** (DS1), time-dependent H/D exchange of the acidic proton of the succinimide ring (green) is accompanied by the loss of ^{3}J coupling of the methylene group (CH_2, blue)

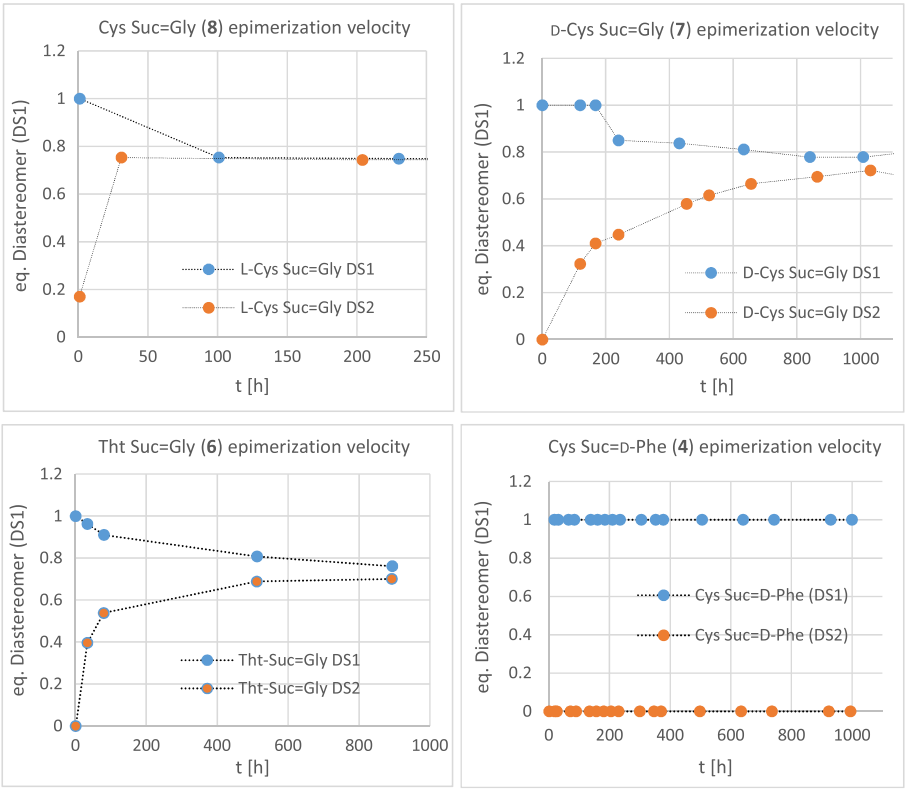


FIGURE 9 The equilibration of DS1 and DS2 was studied by NMR for 4 different peptides at room temperature. The ^{1}H NMR integrals of well-separated signals served as reference. Epimerization was the only process which explained the changes in signal intensity and multiplicity. A retro Michael-type ring opening was not observed. Cyclopeptide **5** behaves similar to **4** (Supporting Information)

TABLE 1 Comparison of the epimerization time needed for each diastereomer to reach a 50% extent

Peptide	$t_{1/2}$ DS1, h	$t_{1/2}$ DS2, h
cyclo[-(*R*/*S*)-Suc=Gly-Gly-Hot=Tap-L-Cys] (**8**)	50	15
cyclo[-(*R*/*S*)-Suc=Gly-Gly-Hot=Tap-Tht] (**6**)	100	30
cyclo[-(*R*/*S*)-Suc=Gly-Gly-Hot=Tap-D-Cys] (**7**)	200	140
cyclo[-(*R*/*S*)-Suc=D-Xaa-Gly-Hot=Tap-L-Cys] (**4, 5**)	>>1000	>>1000

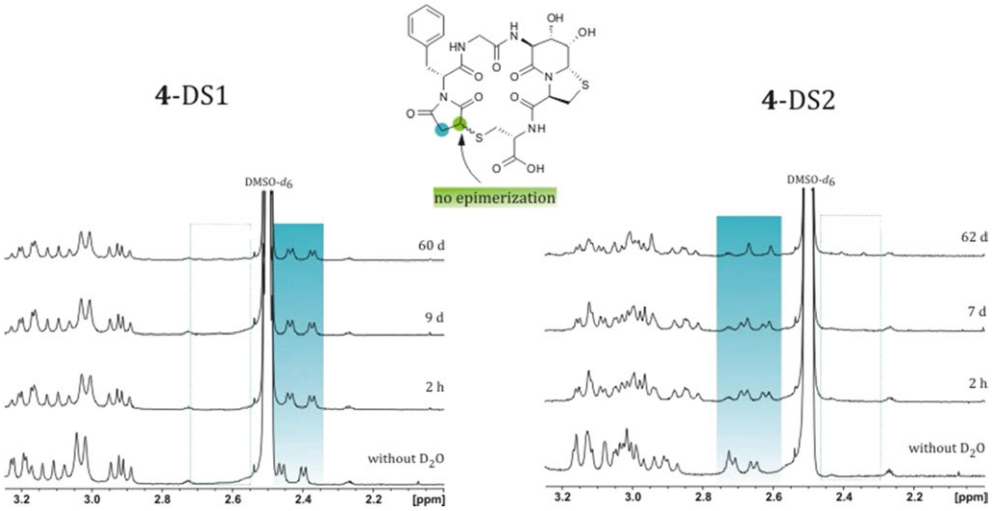


FIGURE 10 ^{1}H NMR (first: 300 MHz, 300 K, DMSO-d_6; above: 300 MHz, 300 K, D_2O/DMSO-d_6, 1:12), time-dependent H/D exchange for the stable diastereomers of cyclic peptide **4**. After the addition of D_2O to the NMR sample, only the second diastereomer (DS2, right) undergoes enolization at the succinimide ring, which is determined by loss of the ^{3}J coupling of the methylene group. For the 2 diastereomers of the Suc=D-Phe containing cyclopeptide **4** no epimerization is detected over a time period of 60 days

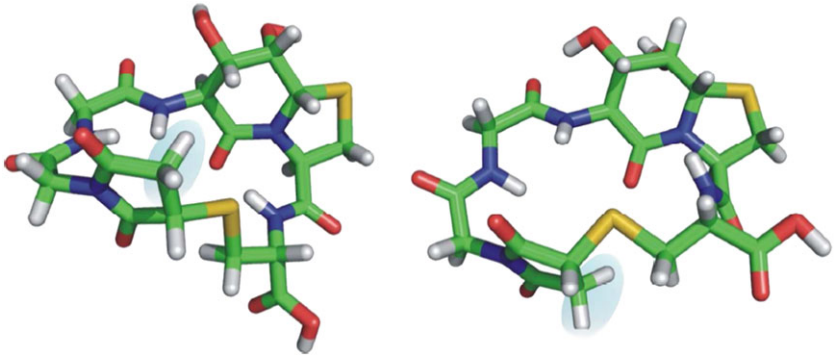


FIGURE 11 Simulated annealing calculations for both diastereomers of peptide **8** show that the main difference between the 2 diastereomers is the alignment of the succinimide ring. Left: Suc-CH_2 above the macrocyclic plane; right: Suc-CH_2 under the plane. This leads to the conclusion that enolization of the succinimide-ring facilitates the rotation around the C-S- and C-N bond, and therefore epimerization is accompanied by a ring flip (180° rotation)

and therefore it was not possible to assign the succinimide stereochemistry unequivocally. Molecular modeling of the 2 diastereomers of peptide **8** shows that the main difference between the 2 diastereomers is the alignment of the succinimide ring (Figure 11).

4 | CONCLUSION

It was shown that the synthesis of cyclic peptides with a native backbone register is possible by ring closure in a thiol 1,4 addition of a Cys side chain with a maleimide. NMR was used to quantify the stereodynamic properties of the obtained succinimide thioether. Epimerization of the cyclic peptides occurs by enolization of the succinimide ring whereby the configuration of the stereocenter at the succinimide ring and the conformation of the macro cyclic ring has a major influence on the ring acidity and epimerization velocity of the corresponding diastereomers. The epimerization velocity is strongly influenced by neighbouring stereo centers of the peptide backbone. In the case studied, introducing a D-Xaa C-terminal to the succinimide suppresses enolization and epimerization of the peptide backbone.

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SUPPORTING INFORMATION

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