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Hydrothermal, catalyst-free production of a cyclic dipeptide from lysine

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ABSTRACT

The formation of cyclic dipeptides, 2,5-diketopiperazines (DKP), from lysine in aqueous solution was investigated at hydrothermal conditions (250–350 °C) without the addition of catalyst. The products obtained were analyzed by GC–MS combined with extensive ^{1}H,^{13}C NMR analysis, after purification via preparative chromatography. The main product of the conversion of lysine, octahydrodipyrido[1,2-a:1′,2′-*d*]pyrazine-6,12(2H,6aH)-dione, was successfully isolated and identified. The purification/separation protocol is rapid, environmentally friendly, and highly efficient with excellent selectivity (81 wt%) in the oils obtained from the conversion of lysine at 300 °C. Performing the conversion step at higher temperatures or lysine concentrations led to the formation of complicated side products. Based on the evolution of key compounds during hydrothermal conversion of lysine, we propose a tentative mechanism for the formation of diketopiperazine. The technique presented in this work provides a novel catalyst-free pathway for the synthesis of DKP.

1. Introduction

Hydrothermal conversion is subject to increasing interest in a sustainable approach, for instance in the production of platform chemicals from biomass, following the principles of bioeconomy [1]. Our previous work [2] aimed to study the effects of the Maillard reaction on the nitrogen-containing compounds during hydrothermal conversion of protein-rich biomass; and revealed the occurrence of a rapid one-step cyclization of lysine, leading to a complex mixture of cyclic N-containing heterocycles in the isolated organic products. While focusing on diketopiperazine (DKP), we envision that hydrothermal conversion of pure lysine, without employing catalysts, promoters or bases, could provide a simple pathway for the production of cyclic-dipeptide derivatives with good-to-high purity, which are interesting platform molecules for further chemical conversion.

Cyclic dipeptides, such as 2,5-diketopiperazines (DKPs) are biologically active natural products, and represent potential drug candidates owing to potential anticancer, antibacterial, and antiviral properties [3–5]. Although there exist established processes for the lab-scale synthesis of these compounds, the procedure involves extraction, chromatographic fractionation, and purification, making it labor-intensive, costly and environmentally hazardous. DKPs can be obtained in solution or solid from commercially available and appropriately protected chiral α-amino acids [6]. The self-assembly of DKPs derived from aromatic amino acids as building blocks for bio-nanostructures like peptide nanotubes has also been reported [3], describing the condensation of free secondary amino acids, such as proline and substituted proline derivatives without using any catalyst, to return pure cyclo-(-Pro-Pro)-(octahydro-dipyrrolo[1,2-a;1′,2′-*d*]pyrazine-5,10-dione) in quantitative yield.

Currently, there is increasing scientific interest in the development of green and efficient approaches to the production of application-specific DKPs from biomass using sustainable chemical pathways [7]. Besides being deliberately biologically or chemically synthesized, or isolated from various natural sources like fungi and marine organisms [8], DKPs can be formed unintentionally upon thermal treatment of organic matter containing amino acids, peptides, or proteins [9,10].

Diketopiperazines were identified as the main nitrogen-containing compounds in the liquid condensates obtained from fast pyrolysis and hydrothermal liquefaction (HTL) of microalgae [10]. Taking into account the complexity of biological matrices used in HTL processes, the main limitation of potential applications based on value-added DKP derivatives is the isolation of the final products from the complex thermal bio-oils. The formation of DKPs in these thermochemical

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processes is due to thermal cleavage of the protein chain, followed by cyclization of two adjacent amino acids retaining their side chains [10–12]. However, hydrothermal reactions utilize water as a solvent, reaction partner and catalyst, and therefore proceed via completely different pathways than in the case of dry pyrolytic reactions. The combination of elevated pressure and temperature close to the critical point of pure water (374 °C, 22 MPa) leads to a decrease in density, a weakening of the hydrogen bond networks, and an increase in the solubility of non-polar organic compounds.

Previously published articles about the formation of DKPs from amino acids have focused on the condensation of lysine with other building blocks like proline, due to high stability under pyrolysis conditions. Preliminary results indicated that only a few amino acids proved to be suitable DKP building blocks [4,10,13]. The hydrothermal formation of DKP from lysine on its own has not been described so far.

One of the objectives of the work presented in this manuscript was to evaluate the experimental conditions needed to form DKPs from lysine under hydrothermal conditions. Based on the obtained values, with support from the literature, a tentative reaction mechanism can be proposed. Finally, as an application-oriented objective, a detailed analytical investigation facilitates the identification of DKPs in complex mixtures, such as bio-oils. The combination of pre-chromatographic isolation to reduce matrix effects and Nuclear Magnetic Resonance (NMR) analysis was regarded as most promising compared to gas chromatography coupled with mass spectrometry (GC-MS) analysis.

2. Materials and methods

2.1. Materials

Analytical grade lysine (> 98 %) was purchased from Sigma-Aldrich. The aqueous solution of lysine was prepared by dissolving it in deionized water. HPLC-grade dichloromethane (DCM), methanol and tetrahydrofuran (THF) were purchased from Sigma-Aldrich. Standard octahydrodipyrido[1,2-a:1,2-*d*]pyrazine-6,12(2H,6aH)-dione (> 90 %) was supplied by Alinda Chemical Ltd.

2.2. Hydrothermal conversion

Trials were performed in micro-autoclaves with a volume of 24.5 ml made of stainless steel, which can withstand pressures of up to 40 MPa and a maximum temperature of 400 °C. The reactors were loaded with aqueous lysine solution (5–20 wt%). The micro-autoclaves were then flushed using nitrogen gas for air purging and subsequent pressurization to 2 MPa. Heating was performed in a fluidized sand bath (SBL2, Techne, Germany) at a heating rate of 36 °C/min and kept at the target temperature for defined holding times. After the reaction, the autoclaves were removed from the heating device and placed in room temperature water to quench the reaction. The quenched micro-autoclaves were opened in a gas-tight containment after purging with dry nitrogen; the gas phase was discarded without analysis. All the experiments were executed at least in duplicate to check for reproducibility.

The reaction mixture turned into a clear solution after 20 min changing from colorless to pale yellow. The solution was poured into a pre-weighted vessel. A small amount of product remained on the micro-autoclave inner walls so the use of an additional solvent, dichloromethane (DCM), was required for complete recovery of the products. The mixture obtained was then centrifuged and separated for aqueous and oily phases. DCM in the oily phases was evaporated by flushing nitrogen for 24 h. Once a constant weight was achieved, the value was considered the mass of the organic product.

2.3. Product analysis

2.3.1. GC-MS

Qualitative analysis of the organic product was carried out using an Agilent 6890 N gas chromatograph (GC) with an Agilent 5973 MSD mass spectrometry (MS) detector and a DB-5 capillary column (30 × 0.25 mm × 0.25 μm) after diluting with THF (1:10 ml/ml) and filtering with a 0.20 μm polytetrafluoroethylene (PTFE) Filter. The substances were identified using the NIST library, considering only molecules with an identification probability of more than 80 %. The relative fraction of the different compounds was estimated by the relative peak area method.

Quantitative analysis of selected chemicals identified by GC-MS was performed by GC-FID using an Agilent DB-5 capillary column (30 × 0.25 mm × 0.25 μm). The GC column was kept at 80 °C for 2 min, held at 175 °C for 5 min, after a temperature ramp of 5 °C/min, and eventually held at 250 °C for 8 min after a second temperature ramp of 30 °C/min.

2.3.2. NMR spectroscopy

The distribution of functional groups was measured by Nuclear Magnetic Resonance (NMR) analysis. The 1D NMR spectra (^{1}H: 399.81 MHz, ^{13}C: 100.54 MHz) were recorded with a Varian Inova Unity 400 spectrometer equipped with an Oxford Magnet (9.4 T) and a direct detection switchable 5 mm Probe head using the proprietary Software Package VNMRJ 3.2. The 2D spectra were recorded with a Bruker avance 400 equipped with a Spectrospin Magnet (9.4 T) and an indirect detection 5 mm Probehead (PABBO BB-1H Z-GRD) using the proprietary Software Package TopSpin. The chemical shifts δ (in ppm) are given for ^{1}H spectra relative to the residual ^{1}H signal of the solvent (3.31 ppm – quintet for CD3OD 99.8 %) and for ^{13}C, relative to the strong solvent signal with characteristic C-D coupling (septet at 49.1 ppm for CD3OD 99.8 %)). 1D NMR spectra were measured using the standard Varian pulse sequence “s2pul” with the following parameters:^{1}H (PW 6.35 μs), relaxation delay 1.0 s, number of scans 32, spectral width 6398.5 Hz, Acquired/spectral Size 16,384/65,536. ^{13}C (PW 4.2 μs), relaxation delay 1.0 s, number of scans: 2700, spectral width 25,141.4 Hz, Acquired/spectral Size 32,768/65,536.

2D NMR spectra were measured using the standard Bruker pulse sequences “hsqcedetgp” for the short range C-H correlation spectrum (HSQC) and “hmbcgplpndqf” for the long range C, H correlation spectrum (HMBC), using the following parameters: HSQC (PW 14.25 μs), relaxation delay 1.5 s, number of scans 32, spectral width (1H: 5341.9, 13C: 10,062.9) Hz, Acquired/spectral Size: 512,512/512, 512. HMBC (PW 14.25 μs), relaxation delay 1.0 s, number of scans: 32, spectral width (H: 5208.3, 13C: 21,130.5 Hz, Acquired/spectral Size: 2048, 256/2048, 1024. ^{1}H-, ^{13}C-1D and 2D data were processed using MestReNova (version 9.0).

2.3.3. Preparative column chromatography

Purification of the organic product was performed by fractionation using preparative column chromatography (PCC). The glass column (90 cm in height and 3 cm in diameter) was packed with silica gel (70–230 mesh) for up to 3/4 of the total volume. The silica gel was activated at 600 °C for 8 h before its use.

2.3.4. Lysine quantification

The total nitrogen (TN) in the aqueous phase was measured with a Dimatec® 2100 instrument. Ammonium (NH_4^+) was investigated with a Metrohm 838 advanced sample processor device. The remaining lysine is separated and determined by ion exchange chromatography and ninhydrin post-column derivatization using an amino acid analyser (Biochrom 30, USA).

2.4. Data definition

After hydrothermal conversion, the unconverted lysine remains only in the aqueous phase, therefore, lysine conversion was calculated as the reduced mass of lysine related to the initial mass of lysine, see Eq. (1).

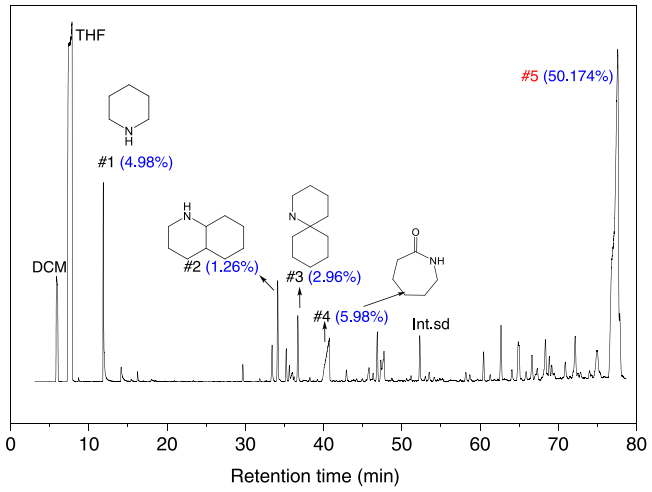

Fig. 1. Total ion chromatogram with relative peak areas obtained from GC-MS analysis of organic products from the hydrothermal conversion of lysine under 300 °C at 20 min holding time.

$$Lysine\quad conversion\quad (\%) = \frac{mass_{initial\quad lysine} - mass_{lysine\quad in\quad aqueous\quad phase}}{mass_{initial\quad lysine}} \times 100 \tag{1}$$

The selectivity of DKP and other products in the organic product is defined as the mass weight of products related to the total mass of oil, and given as an indicator to evaluate the purity, see Eq. (2),

$$Product\quad Selectivity\quad (\quad wt\%) = \frac{mass_{product}}{mass_{oil}} \times 100 \tag{2}$$

The yields of DKP and other products were calculated as the actual moles of products related to the theoretical moles,

The yield of DKP is calculated as Eq. (3), the yields of $P_{(piperidine)}$ and $C_{(caprolactam)}$ are defined as Eq. (4),

$$DKP\quad Yield\quad (\quad mol\%) = \frac{mole_{DKP}}{(mole_{\quad initial\quad lysine})/2} \times 100 \tag{3}$$

$$P\quad or\quad C\quad Yield\quad (\quad mol\%) = \frac{mole_{P\quad or\quad C}}{mole_{\quad initial\quad lysine}} \times 100 \tag{4}$$

Each data point was represented as the average of the two independent experiments, with an indicative error corresponding to its standard deviation. Whenever the deviation between duplicates surpassed 10 % between the two data sets, the experiment was repeated.

3. Results

3.1. Characterization of DKP

3.1.1. Isolation of DKP

The organic products obtained from conversion at 300 °C were firstly investigated by GC-MS, the results being depicted in Fig. 1. Hydrothermal conversion of lysine resulted in the formation of a relatively wide range of products, mostly gained via inter- or intramolecular cyclization.

One of the major products identified this way, labeled compound #5 in Fig. 1 at 77.59 min retention time accounts for ca. 50 % of the total peak area. The related mass spectrum (Fig. A1), exhibits fragment peaks at $m/z = 84$, $m/z = 110$, $m/z = 138$, $m/z = 193$ and an intense molecular peak at $m/z = 222$. Regarding the concentration of this product in the mixture, it seems to be a worthy candidate for further downstream processing. However, the elucidation of its structure is revealed to be quite challenging. Assuming as a working hypothesis that its molecular weight is 222 g/mol, the most likely molecular formula would be $C_{12}H_{18}N_2O_2$.

Taking into account the structures proposed by the database for the remaining main peaks #1, #2, #3, and #4, it is highly probable that this unidentified compound is also an N-containing heterocycle. Comparing the different fragmentation patterns found in the MS spectra database for peaks #1, #2, #3, and #4 with the mass spectrum of #5 (Figs. A2–3), it is obvious that its structure should contain a piperidine backbone (represented by the fragment ion $m/z = 84$ amu).

A wide range of ^{1}H- and ^{13}C- based NMR methods is available to fully characterize a specific molecule and ascertain its 3D structure. However, the complexity of the sample and the presence of similar molecules in the mixture hinder an unequivocal attribution of the signals and the evaluation of the structure, as in the case of the GC-MS analytics. For these reasons, a preliminary purification of the complex organic product mix is paramount to further investigate the nature of product #5.

3.1.1.1. Fractionation and purification of bio-oil. Preparative column chromatography (PCC) is often used for the fractionation and purification of complex product mixtures. In this case, 6 vol%methanol in DCM, was tested as the best eluents with pure silica as stationary phase. Each fraction was subjected to GC-MS and ^{1}H NMR, and the obtained spectra were quickly checked; whenever necessary, similar fractions were combined and concentrated, allowing an easier characterization of the main components present in these pooled fractions. In the optimal case,

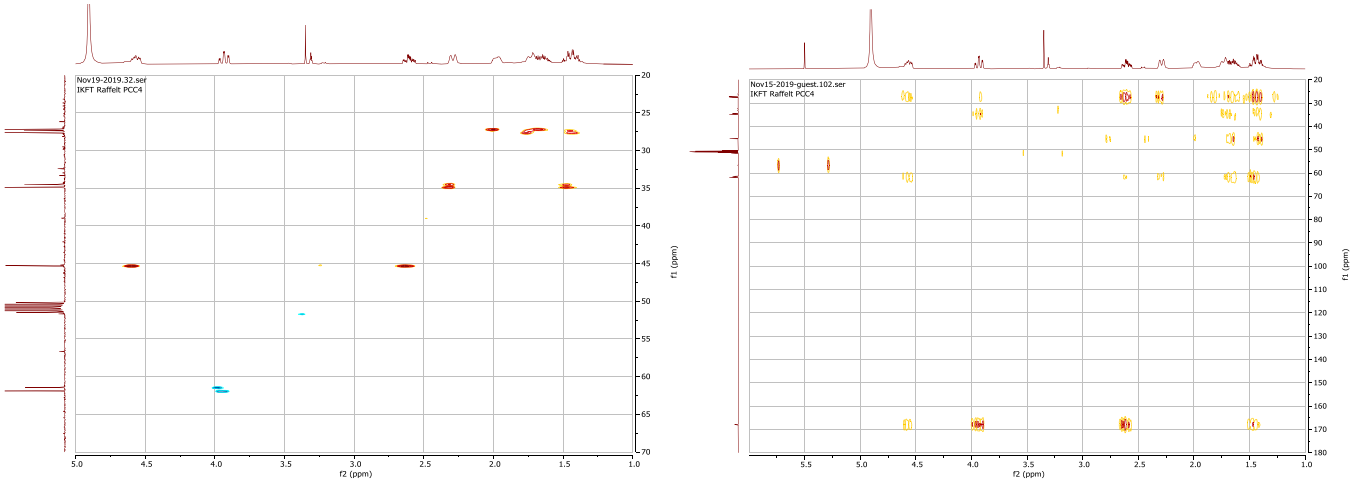

Fig. 2. HSQC spectrum (left) and HMBC spectrum (right) of the purified HTL product of lysine.

Table 1
Calculation of the molecular mass of the NMR detected building blocks.

	CH	4 CH_2	CNO (amide)	COOH (acid)	COO (ester)	Sum
Combination 1	13	56	42			111
Combination 2	13	56		45		114
Combination 3	13	56			44	113

this approach permits an easier evaluation of e.g. 2D- ^{13}C, ^{1}H NMR spectra and allows for the elucidation of the real structure of the main unidentified compound (#5). The percentage of each fraction is displayed in Fig. A4(a). More than 65 wt% of the recovered product was found in fractions 3–6, the maximum of 39 % in fraction 4 (see in Fig. A4 (b)). GC-MS was again used for qualitative analysis, to identify the distribution of the unidentified compound ($m/z = 222$) among the recovered fractions, the chromatogram of fraction 4 was shown in Fig. A5. It turns out that PCC 4 fraction, with the highest purity of DKP, is the best candidate for further analysis.

3.1.2. Characterization by NMR

The organic product obtained from the hydrothermal conversion of lysine was purified by PCC and showed two overlapping signals in the gas chromatograms with molecule ions at $m/z = 222$, which were, in a first interpretation, thought to be isomers with very similar properties. ^{1}H and ^{13}C NMR spectra is shown in Fig. A6. In the short-range ^{1}H, ^{13}C-correlated gHSQC spectrum of the final product one methine (=CH—) and four methylene (—CH_2—) groups have been detected (Fig. 2). Considering that the protons of the methylene groups in the ^{1}H spectra are chemically different, it seems plausible that they are part of a cyclic structure, a fact also supported by the relative simplicity of the ^{13}C signal set. A carbonyl group is detectable at 164 ppm in the long-range ^{1}H, ^{13}C-correlated HMBC and the 1D ^{13}C spectrum [14], which suggests three possible functional groups in the molecule: carboxylic acid, ester or amide. Neither methyl, aromatic C-H, nor pure quaternary carbons could be detected.

The attribution of the signal at 164 ppm to an amide group is logical considering the different possible combinations of the molecule fragments as summarized in Table 1. The molecular peak recorded at $m/z = 222$, as well as the high likeliness of a cyclic structure, lead to a proposed molecule that is a cyclic, symmetric molecule, and displays two identical building blocks containing each an amide (–C(=O)N=), two methane, and four methylene groups. The most logical solution is found in decahydodipyrido[1,2-a:1′2′-*d*]pyrazine-6,12-dione which displays two stereogenic centers (carbon 12a and 6a in Fig. 3) and therefore different enantiomeric and diastereomeric forms (RS, SR, RR, and SS) with RS, SR on the one hand and RR, SS, on the other hand, resulting in equal NMR spectra. The differences between the RS, SR and RR, SS diastereomers are only clearly visible on a high resolution one-dimensional ^{13}C spectrum in opposition to the lower resolved HSQC

Table 2
Chemical shifts δ in ppm of decahydrdipyrido[1,2-a:1′2′-*d*]pyrazine-6,12-dione (SR, RS and RR, SS) in methanol.

Atom numbers	^{13}C		^{1}H		^{13}C
	Dataset 1	Dataset 2	A	B	HSQC
1, 7	33.20	32.81	1.47	2.32	34.9
2, 8	25.52	25.47	2.00	1.88	27.2
3, 9	25.95	25.67	1.44	1.76	27.8
4, 10	43.61	43.65	2.62	4.59	45.4
6, 12	166.15	166.30			168
6a, 12a	60.23	59.76	3.95		62.0

Datasets 1 and 2 represent the different diastereomers SR, RS and RR, SS, which are not resolved in the HSQC data due to the measuring methodology. A and B represent axial and equatorial protons (unassigned).

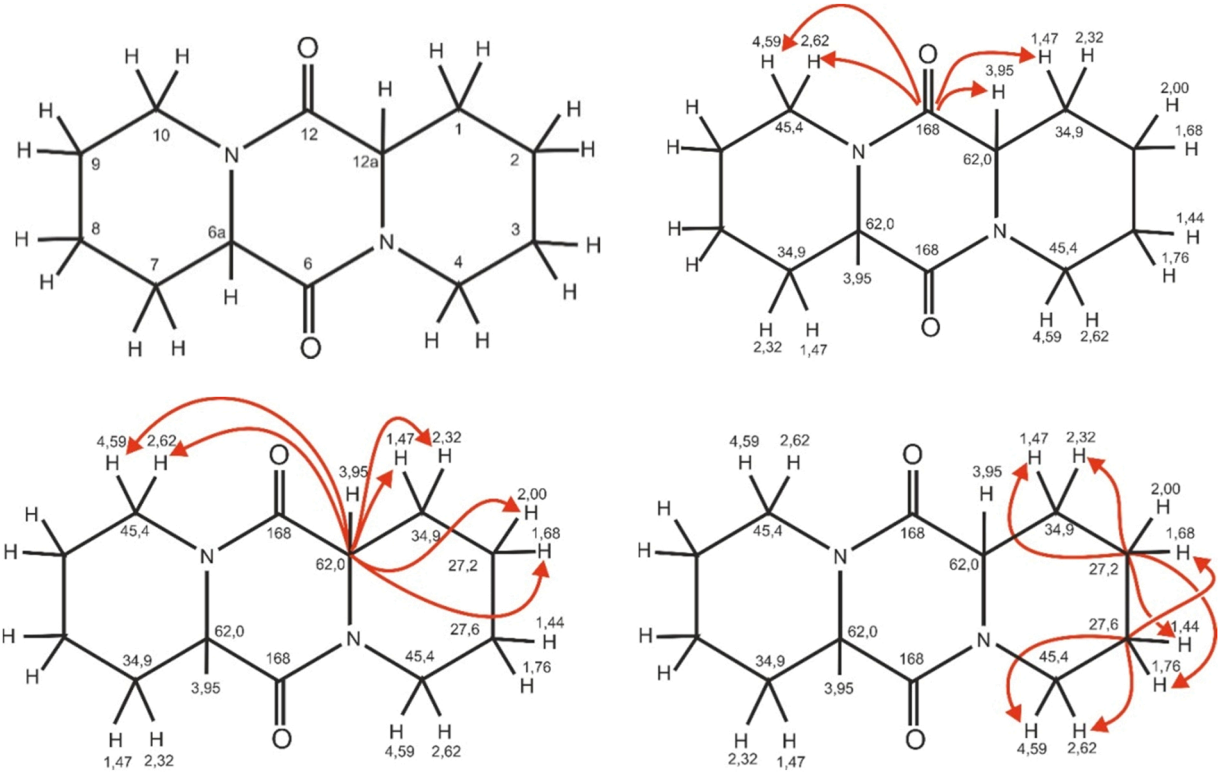

Fig. 3. Chemical structure of octahydrodipyrido[1,2-a:1′2′-*d*]pyrazine-6,12(2H,6aH)-dione and its NMR data (the red arrows represent cross-peaks in the HMBC spectrum (^{2}J, ^{3}J, and ^{4}J spin-spin couplings between ^{13}C and ^{1}H)).

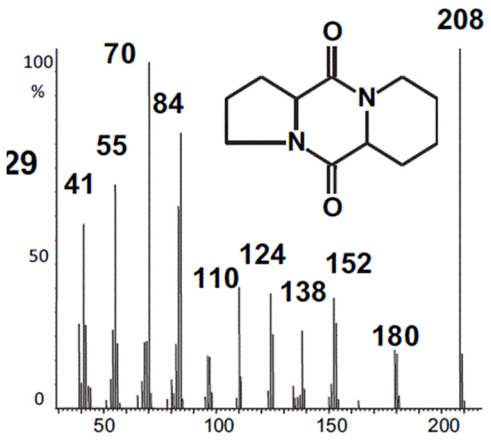

Fig. 4. Mass spectra of Cyclo(Pro-Lys), reported by Fabbri et al. [10].

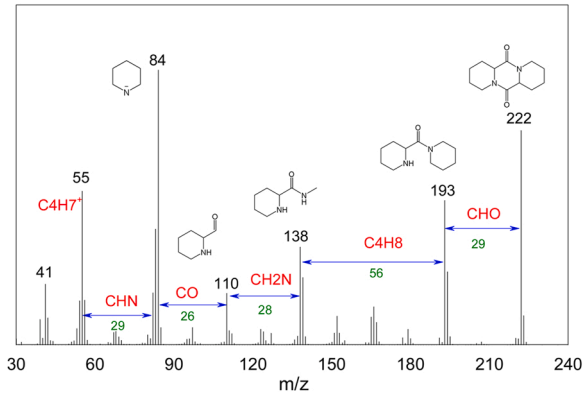

Fig. 5. Tentative characteristic fragments of DKP.

and HMBC spectra [14].

The assessment of the backbone's connectivity of the molecule using the ^{1}H, ^{13}C two-dimensional spectra can be however drawn for both diastereomers. Furthermore, RS, SR and RR, SS isomers have slightly different properties and therefore their signals overlap in the one-dimensional ^{1}H spectrum producing complex multiplets(as shown in Table 2). The ^{1}H resonances are given as the centers of cross-peaks in the HSQC spectrum, regarding that definite, sharp ^{1}H–^{1}H couplings are not achievable owing to the lower resolution of the measurements. On the other hand, the ^{13}C resonances of the one-dimensional ^{13}C spectrum are well resolved [14], allowing a distinction between RS, SR and RR, SS diastereomers. It cannot be decided, which data-set of signals to attribute to SR, RS and RR, SS, but the data sets are well distinguishable by signal intensities. Similarly, the combined presence of the characteristic ion clusters at $m/z = 41$ amu, $m/z = 55$ amu, $m/z = 84$ amu, $m/z = 110$ amu, and $m/z = 138$ amu has been detected in DKP derivatives like e.g. in the work of Fabbri et al. [10], who investigated, among others, a molecule labeled "Cyclo(Pro-Lys)" gained from the condensation of proline and lysine with a total mass of 208 as shown in Fig. 4. These typical fragmentation patterns found in DKP structures have been documented using various combinations of amino acids with proline as the set reaction partner, whereby the characteristic fragment ions $m/z = 70$ amu could be regularly found. A parallel can be drawn with the strong peak regularly found at $m/z = 84$ amu in our system, which can be logically attributed to a fragment merging a 70 amu moiety with a supplementary methylene group. This can be only translated into a symmetric, "dimeric" structure with an ion peak at m/z 222 (2 * 111), ultimately displaying three six-membered rings as described in Fig. 5.

The methylene protons in the constrained 6-membered ring systems have axial and equatorial positions, impeding a clear attribution of all the ^{1}H signals.

The presence of a cyclic structure is also supported by investigating the fragmentation patterns of the main ionic fragments and comparing them to common fragmentation rules reported in previous works of Poerschmann and Fabbri [4,10]. Additional spectra found in the literature including those given by Adamiano et al. [12] and Hendricker et al. [15] also support this assumption. An intense fragment peak measured at $m/z = 84$ amu could for instance be associated with the signature of a piperidine unit, in this case, a building block of the symmetric, dimeric structure.

Other clear-cut peaks could be consequently attributed to specific fragments. For example, the ion at $m/z = 193$ amu could be attributed to the loss of CO due to ring-opening of the original tricyclic backbone, and that at $m/z = 110$ amu resulting from the subsequent loss of a piperidine ring. Both GC-MS data and the NMR study confirm the identity of the isolated DKP derivative as octahydrodipyrido[1.2-a:1.2-*d*]pyrazine-6.12(2H.6aH)-dione. A pure DKP "standard" was characterized (Fig. A7 in Appendices), and both ion chromatography and mass spectroscopy results are fitting to the "real" component in the study.

3.2. The yields of DKP

Taking into account the "green" potential of the hydrothermal approach to produce DKP, it is imperative to investigate the DKP yields, for further comparison with conventional processes. In this section, the influence of temperature, lysine concentration and holding time on DKP selectivity and yields are investigated. Lysine conversion rate and the

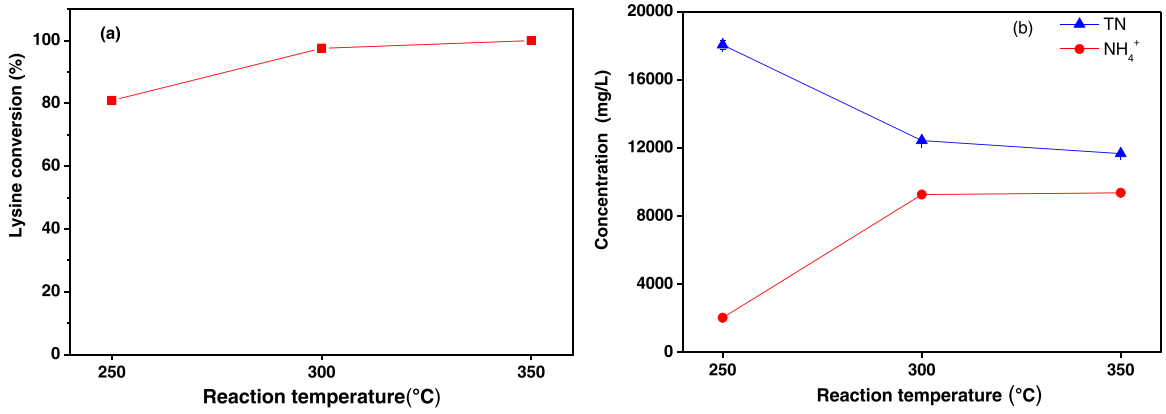

Fig. 6. The influence of temperature on hydrothermal conversion of lysine: (a) lysine conversion; (b) total nitrogen and ammonium content in the aqueous phase (10 wt% aq., holding time 20 min).

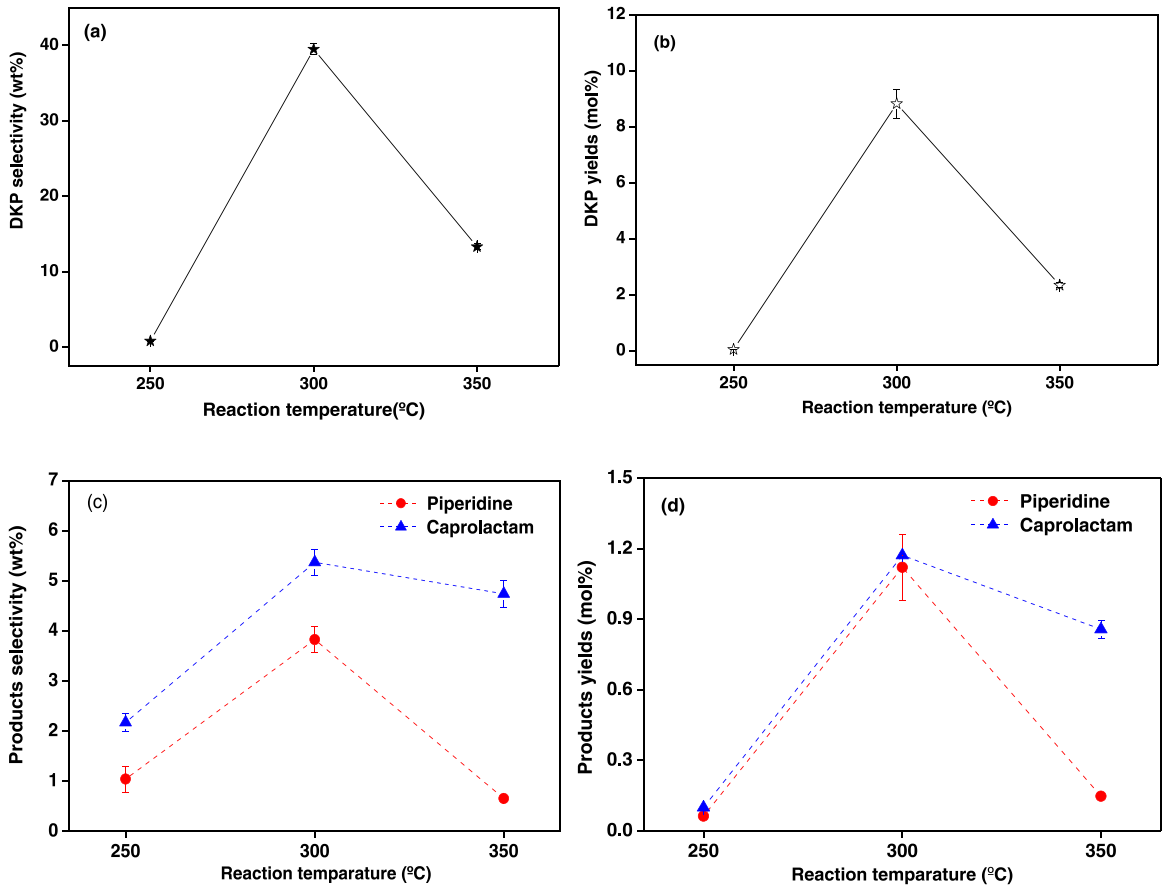

Fig. 7. Influence of temperature on products formation: (a) DKP selectivity(based on oils); (b) DKP yields; (c) side products selectivity(based on oils); (d) side products yields from the hydrothermal conversion of lysine (10 wt% aq., holding time 20 min).

generated side-products are also illustrated, aiming to speculate the reaction pathways of DKP from hydrothermal conversion of lysine.

3.2.1. *The influence of temperature*

Hydrothermal conversion can be seen as hydrous pyrolysis using subcritical water as the medium, where typical processing temperatures are between 250 °C and 370 °C, at operating pressures ranging from 4 and 22 MPa [16,17]. The influence of temperature on the conversion of lysine is depicted in Fig. 6. The conversion rate increases from 80.9 % to 99.7 % when the temperature raises from 250 °C to 350 °C. After collecting the oil products from the mixture, the total nitrogen content in the aqueous phase largely decreases from 18,405 mg/L to 11,668 mg/L, which correspond to the increase conversion rate of lysine. As a fact, the ammonium (NH_4^+) escalates and indicates the enhancement of deamination of lysine with higher temperature.

The effect of the temperature from 250 °C to 350 °C on the yields of DKP obtained from the hydrothermal conversion of lysine is illustrated in Fig. 7(a) and (b). As depicted in Eq. (2), the DKP selectivity was calculated based on the weight of DKP to the weight of oil products. The yields of oil products under all the conditions are shown in Table A1. The DKP selectivity in the oils dramatically increases from 0.78 wt% at 250 °C to reach 39.49 wt% at 300 °C, decreasing slowly to reach 13.29 wt% at 350 °C. Among the temperatures applied, 300 °C seems to be optimal to obtain maximum amounts of DKP suitable for further separation. Interestingly, the yields of DKP (Eq. (3)) obtained from the HTL of lysine follow a similar trend with a maximum yield reached at 300 °C with a value of 8.8 mol%.

As described in our previous work, the major N-containing compounds found from hydrothermal conversion of lysine are piperidine, caprolactam and un-known N-cyclic heterocycles. Using multiple analytical methods, the key N-cyclic heterocycle has been proven to be DKP. Aiming to proof the concept of sustainable way for the synthesis of DKP, piperidine and caprolactam yields have been monitored, as they can be regarded as main side products. With increasing temperature, the yields of piperidine and caprolactam first reach an optimum to decrease then around 350 °C, caprolactam yield being higher than that of piperidine.

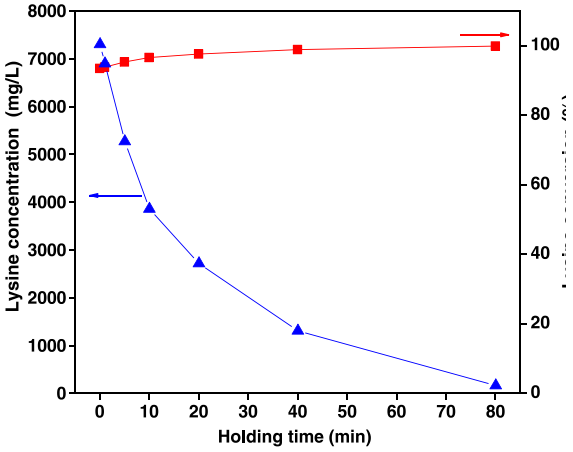

Fig. 8. The influence of holding time on lysine conversion with loading of 10 wt% at 300 °C.

3.2.2. *The influence of holding time*

Fig. 8 shows the influence of holding time on the lysine conversion at

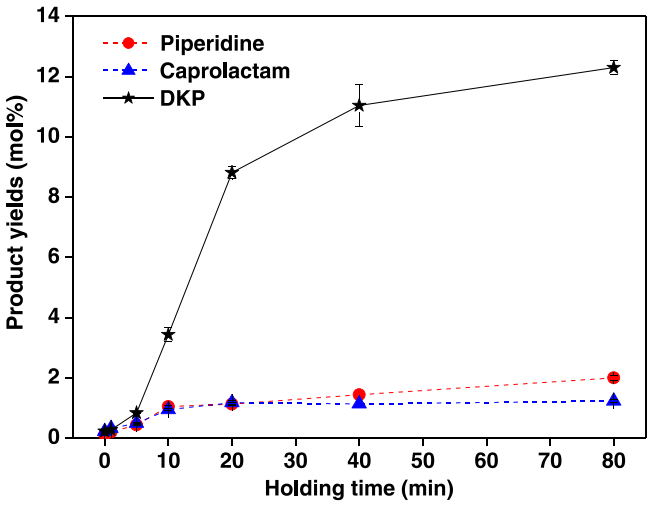

Fig. 9. The influence of holding time on the N-containing compounds from hydrothermal conversion of lysine with loading of 10 wt% at 300 °C.

the predetermined optimal temperature of 300 °C for DKP production. Lysine concentration in the aqueous phase largely decreases as the holding time increase. After 80 min, the remaining lysine concentration is 162 mg/L, leading the conversion rate of 99.9 %. Almost all the lysine is converted into other water-soluble containing compounds, NH_4^+, and the abundant N-containing species condensed in the bio-oil phase. Lysine conversion rate raises most substantially from 0 to 20 min holding time with a value of 97.6 % then does not vary as significantly after that.

Fig. 9 presents the relationship between holding time and DKP yield obtained at 300 °C using a lysine loading concentration of 10 wt%. The DKP yield shows a steep increase in the first 20 min of reaction reaching 8.8 mol% after 20 min to flatten out and reach 12.3 mol% after 80 min of reaction time. Both piperidine and caprolactam yields show first noticeably increase in the first 20 min of reaction reaching a plateau after 20 min to even out at prolonged reaction time. The yields of three major compounds are close when holding time less than 5 min. With prolonging, the yields of DKP shows 10 times higher than that of piperidine and caprolactam, indicating the selectivity of DKP is enhanced by extending the holding time.

3.2.3. *The influence of the lysine concentration*

The influence of the lysine loading concentration in the feed solution on the DKP yields is shown in Fig. 10. The reaction temperatures applied were 300 and 350 °C. It can be seen that with increasing initial lysine concentration, rising from 5 wt to 20 wt%, DKP selectivity decrease, from ca. 47 to 25 wt% at 300 °C and from 14 to 8 wt% at 350 °C, respectively. In contrast, the yields of DKP rise constantly, from ca. 3.4 to 13.7 mol% at 300 °C and from 1.2 to 4.0 mol% at 350 °C, suggesting that the enhancement of bio-oil products compensate the reduction of DKP selectivity.

With increasing initial lysine concentration, the selectivity of caprolactam in the products significantly increase from ca. 3 to 8 wt% at 300 °C, showing a small drop at higher temperature. The concentrations of piperidine based on lysine loading are strongly temperature dependent, specifically decreasing at 300 °C, while increasing and then decreasing at 350 °C. The yields of both compounds increase with lysine loading concentration, with maximum value of 3.5 mol% of caprolactam at 20 wt% of lysine loading.

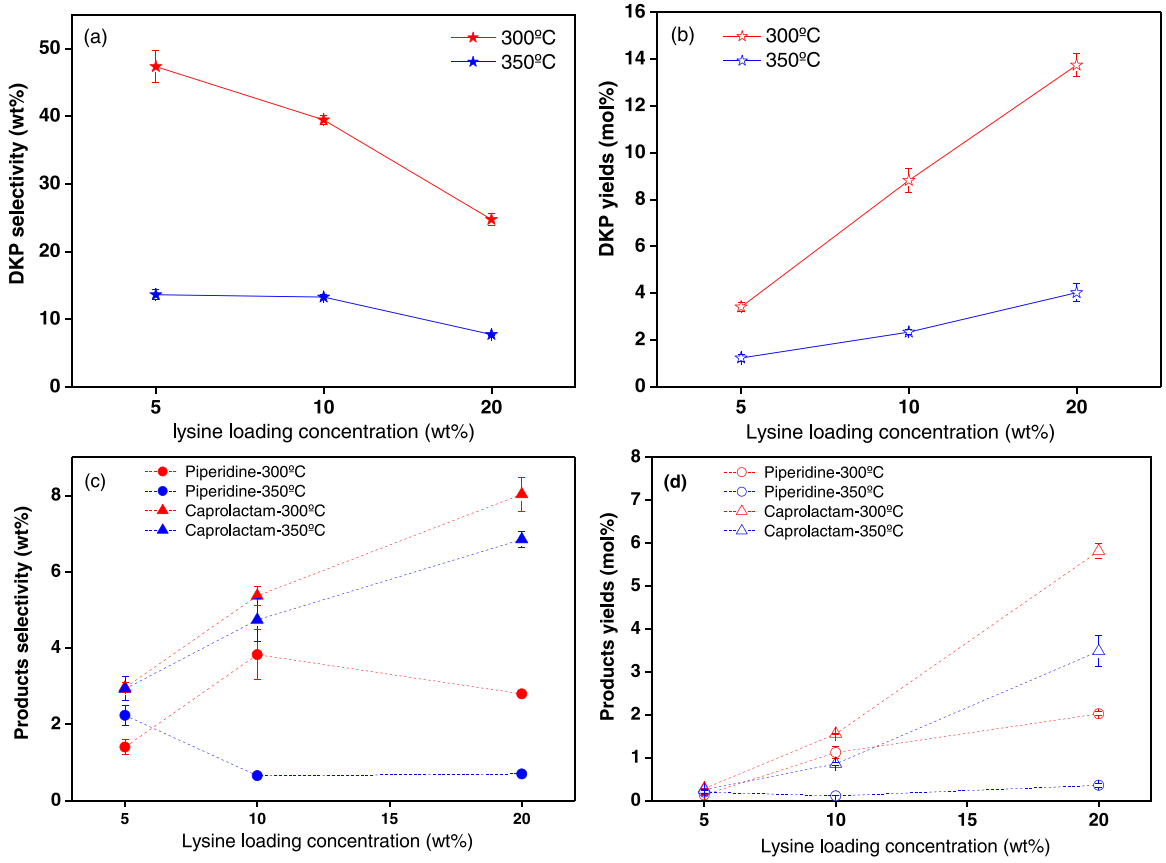

Fig. 10. Influence of the lysine loading concentration on the products formation: (a) DKP selectivity (based on oils); (b) DKP yields; (c) side products selectivity (based on oils); (d) side products yields from the hydrothermal conversion of lysine (holding time of 20 min).

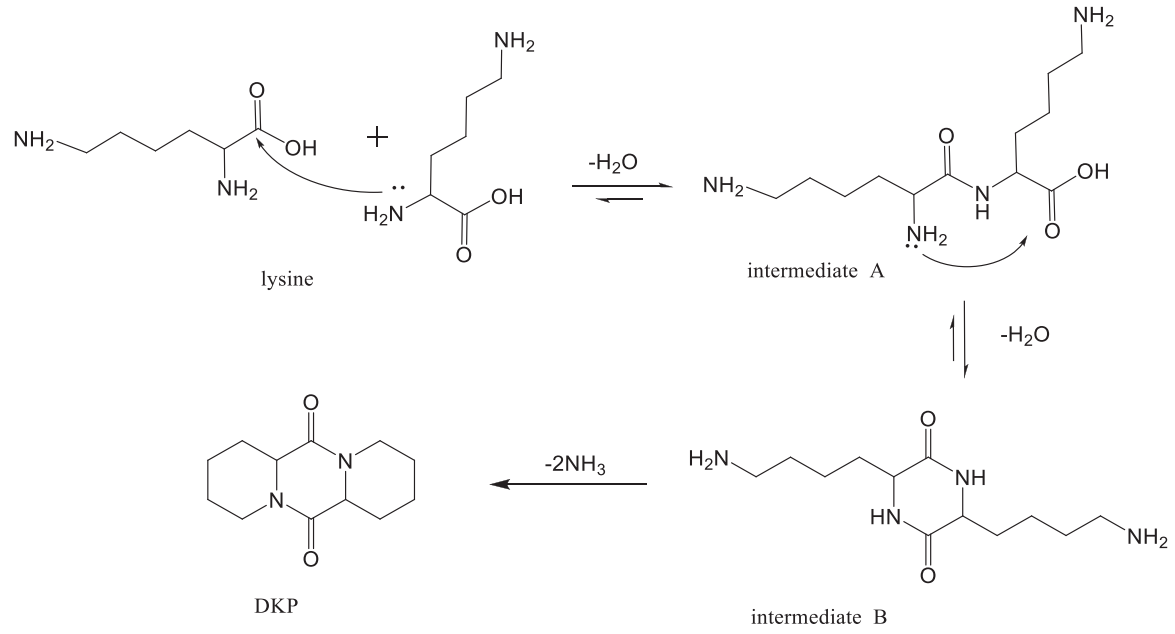

Fig. 11. Intermediacy of dipeptides via HTL of lysine and subsequent formation of DKP.

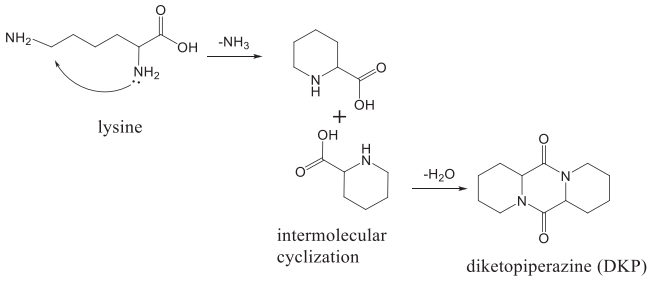

Fig. 12. Proposed pathways of DKP formation, based on the findings in this work and the reports from the cited resources [3,4].

4. Discussion

Lysine is an amino acid with a reactive ε-amino group, which can be easily degraded via decarboxylation and deamination. The main degradation products are aldehydes, short-chain carboxylic acids, small hydrocarbons, and amines [18,19]. During hydrothermal conversion, water can simultaneously be regarded as a solvent, active reactant, as well as acidic or basic catalyst [20], resulting in complex, fascinating chemistry where various reactions can concurrently happen, e.g. dimerization, cyclization, hydrolysis, and depolymerization.

Along these lines, intramolecular rearrangement might also occur, for instance, epimerization as reported by the research group of Poerschmann [4] in the case of cyclo(Pro-Val) derivatives where the *cis*- and rearranged *trans*-isomer display similar GC retention times and mass spectra. In our case, it is obvious comparing the isolated DKP that two signal sets are overlapping in the ion chromatogram of bio-oil and the condensed fraction 4. Most likely, the presence of double peaks is due to the isomerization of cis-DKP, which is exclusively formed by intramolecular cyclization of L-amino acid. Both the *cis*-isomer and rearranged *trans*-isomer showed similar retention behavior and mass, making them difficult to distinguish without resorting to additional techniques besides GC-MS.

Two main pathways leading to the formation of diketopiperazine using lysine as a starting material can be mentioned: dehydration of amino acid to form dipeptides followed by cyclization as depicted in Fig. 11, and cyclization of amino acid to form pipecolic acid followed by dimerization as depicted in Fig. 12.

Dipeptides, as reactive intermediates, have been reported to be formed via dehydration of amino acids during pyrolysis [11]. By suffering second dehydration, these dipeptides can readily form the corresponding 2.5-diketopiperazines [11,21]. In addition, Sharma et al. [22] stated that dipeptides are often regarded as non-detectable intermediates during amino acid pyrolysis because of their high thermal stability and low volatility. In our case, lysine was subjected to HTL instead of dry pyrolysis, during which dehydration commonly occurs. It is, therefore, reasonable to assume that a Lys-Lys dipeptide (intermediate A in Fig. 11) is generated as an active intermediate.

As already demonstrated with simple dipeptides, their cyclization to DKP cores proceeds more rapidly than their hydrolysis [23]. Hydrolysis of dipeptides is favored under acidic conditions [24] whereas in our case the pH of the reaction mixture is around 9.0–9.5. Similarly, the rate constants for cyclization were higher than those for hydrolysis, showing that the formation of a stable cyclic, respectively polycyclic system is thermodynamically more favorable than the hydrolysis of dipeptide (A).

The second synthetic pathway involving the cyclization of amino acids to form specifically pipecolic acid and eventually DKP via intermolecular amide formation seems to be more likely to happen if one considers the related literature [3,25].

Nonappa et al. [3] first reported on an environmentally benign catalyst-free cyclization procedure for a range of amino acids yielding exclusively DKPs in very high yields. Later on, Poerschmann et al. [4] investigated the straightforward formation of DKP derivatives via hydrothermal synthesis using free amino acids, such as proline and phenylalanine as building blocks without using any catalyst or promoter.

In comparison, Nonappa et al. [3] employed *rac*-pipecolic acid as the building block, performing the reaction under controlled microwave irradiation using N,N-dimethlyformamide as solvent. Therefore, we can envisage in our case that the formation of DKP is due to the presence of in-situ generated pipecolic acid, formed during HTL of lysine. It must be mentioned that pipecolic acid cannot be identified by GC-MS without further derivatization [26], hindering its direct characterization in complex reaction mixtures. Interestingly, the decarboxylation and dihydroxylation of commercially available pipecolic acid (R-Piperidine-2-carboxylic acid; $C_6H_{11}NO_2$; 129.16 g/mol) presents two characteristic peaks during GC-MS, $m/z = 84$ amu and $m/z = 110$ amu, via

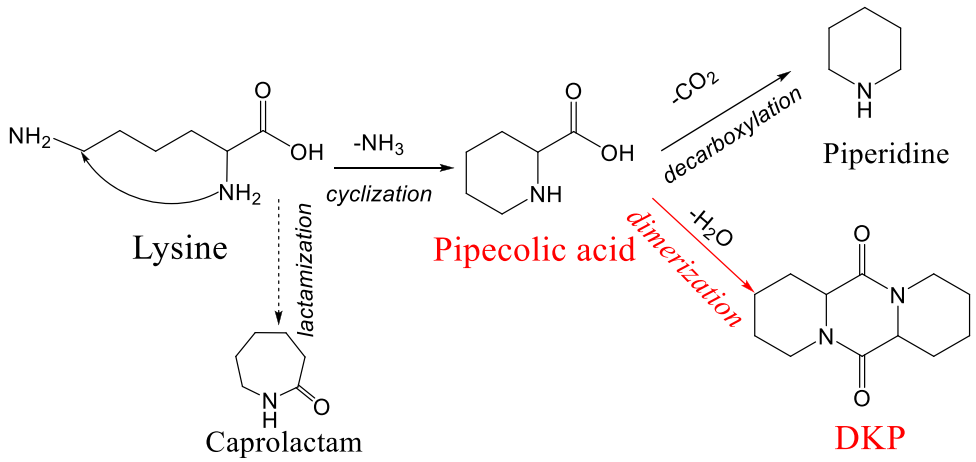

Fig. 13. A reaction scheme showing the possible products from hydrothermal conversion of lysine, partially sourced from [2].

the loss of CO_2 and OH, respectively. This fact is highly in line with our findings and substantiates the assignment of the DKP structure as shown in Fig. 5.

Chiavari et al. [11] found in their investigation of the pyrolytic fragmentation pathways of numerous amino acids using Py-GC/MS, that the main product obtained from the pyrolysis of lysine was 3-aminohexahydro-2-azepinone ($C_6H_{12}ON_2$, $m/z = 129$ amu, relative area proportion 89.6 %) formed by simple cyclization of the amino acid, followed by dehydration. This strongly suggests that the cyclization of lysine occurs more rapidly than dehydration to form the related dipeptide.

Combining the findings with those of our previous work [2], piperidine and caprolactam were detected as other major components (Fig. 1), we propose summarizing the conceivable pathways of DKP formation under hydrothermal conditions, as shown in Fig. 13. As the dominant intermediate, pipecolic acid is formed directly by cyclization of lysine, releasing ammonia (NH_3). This initial reaction was also proposed by Chen et al. [27] during pyrolysis of algae, which is also followed by the loss of COOH* and H* radicals to produce, however, pyridine as the end product from lysine. Lipids, proteins and carbohydrates present in algae greatly influence the nature of the final N-containing products, explaining the divergence from this system. Having generated the actual active intermediate in situ under hydrothermal conditions, it is either possible to simply form piperidine by decarboxylation or to form a cyclic *di-amido* species via an intermolecular condensation.

Preliminary studies addressing the yield of DKP formation provide evidence that only higher operating temperatures can lead to DKP production, indicating that the dimerization rate of amino acids increased with temperature. Presuming conversion in subcritical water, dimerization rates increase due to the proportional increase of the ion product of water, which facilitates acid and base-catalyzed reactions. Sakata et al. [28] also stated that the dimerization rate of glycine increased with operating temperature, and is generally stimulated by alkaline conditions. During hydrothermal conversion of amino acids, deamination is improved by reaction temperature [2], which can provide an in-situ foundation for self-catalyzed dimerization. As lower yields of piperidine were detected in our study, the reaction rate for dimerization seems to be higher than that for decarboxylation. Kawamura et al. [13] also stated that decarboxylation could be ignored in their system since it was much slower than the formation of DKP. The DKP yields reach an optimum at 300 °C, the high temperature being necessary for the homogeneity of the reaction mixture whereas higher temperatures decreased the DKP yields via unwanted side reactions. Likewise, increasing reaction time leads clearly to an optimum in the formation of DKP, flattening out at higher residence times. This suggests the occurrence of concurrent condensation reactions involving reactive monomers, meaning depletion of available reactants for the formation of the target product. As the lysine loading concentration increases, generated compounds with hydrophobic functional groups will expectedly concentrate in the oil phase [29], inhibiting reactions favorable to the generation of DKP, when in batch mode.

5. Conclusion

This study reported for the first time the production of 2,5-diketopiperazine (DKP) in high yields (8.8 mol%) resorting to hydrothermal conversion of lysine at 300 °C with feed concentrations (up to 10 wt%). The application of chromatographic techniques to the obtained oils allowed for the recovery of fractions containing high selectivity of DKP (81 wt%). Batch residence times and initial reagent concentration also significantly affect the yields of the desired product. A tentative reaction scheme is proposed for the formation of 2,5-diketopiperazine, in which the key step is the cyclization of lysine to form pipecolic acid via loss of NH_3, followed by a condensation/dimerization of this intermediate. In comparison, the direct formation of a dipeptide via dehydration of lysine may succeed, but the further cyclization under elimination of NH_3 is not thermodynamically likely to happen.

In this work, a novel lab-scale process, which yields a DKP-rich effluent, combines hydrothermal conversion of lysine and optimized separation techniques, without resorting to either solid or liquid catalysts. As a common amino acid, lysine is recurrent in products of hydrolysis of protein-containing feedstocks. Thus, the process here described constitutes the first step in a potential future sustainable production of DKP.

CRediT authorship contribution statement

Yujie Fan and Alexander Hoffmann performed the experiments, and analyzed the data. Yujie Fan wrote the manuscript. Klaus Raffelt and Thomas A. Zevaco performed part of data analysis and wrote in the manuscript. Ursel Hornung and Nicolaus Dahmen supervised the project, and put efforts to revise the manuscript. All authors discussed the results and commented on the manuscript. All authors have given approval to the final version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

Acknowledgements

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jaap.2022.105792.

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