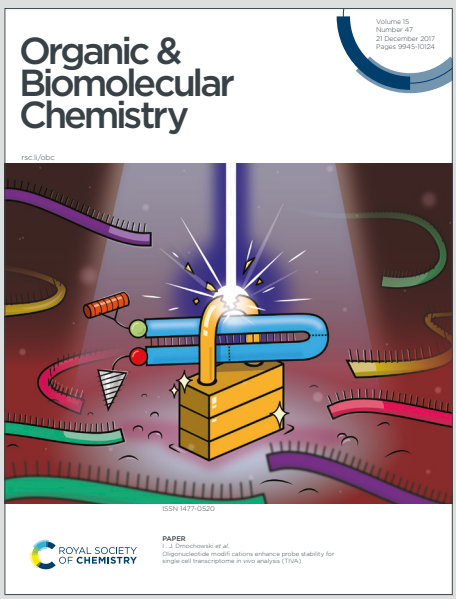
Organic & Biomolecular Chemistry

Accepted Manuscript

This article can be cited before page numbers have been issued, to do this please use: J. C. Thuan, K. Barron and J. Ohata, *Org. Biomol. Chem.*, 2026, DOI: 10.1039/D6OB00662K.



ROYAL SOCIETY OF CHEMISTRY

rsc.li/obc

View Article Online
DOI: 10.1039/D6OB00662K

Scalable mechanochemical synthesis of a cyclic dehydroalanine peptide

Jamie C. Thuan,‡ Katelyn M. Barron,‡ Jun Ohata*

Department of Chemistry, North Carolina State University, Raleigh, North Carolina 27695, United States.

Email: johata@ncsu.edu

‡: These authors contributed equally to this work.

Abstract

Dehydroalanine derivatives are valuable building blocks in organic and biomolecular chemistry fields, and their scalable synthesis represents unmet needs. This work examined previously reported *N*-to-*O* acyl transfer-based condensation reactions of a derivative of diketopiperazine (also known as cyclic glycine dimer or glycine anhydride) through a series of reaction optimization processes to identify scalable conditions. Liquid-assisted mechanochemistry proved important for the promotion of the overall efficiency and reproducibility of a condensation reaction between acetyl-diketopiperazine and paraformaldehyde.

Introduction

With the unique reactivity as an electrophile and a radical acceptor in stark contrast to typical natural amino acid residues, dehydroalanine has garnered considerable interests in various organic and biomolecular chemistry fields. Because of the nucleophilic nature of side chains of canonical amino acids and even non-canonical yet natural amino acids (e.g., post-translationally modified amino acids), studies or applications of those amino acids are often enabled by electrophilic reagents and reactants.[1,2] On the other hand, dehydroalanine bearing an α,β-unsaturated carbonyl group is a unique electrophilic and radical-acceptor amino acid and has been extensively studied during the past years including bioconjugation of polypeptides,[3–5] prebiotically relevant reactions,[6,7] and a key intermediate for total synthesis (Figure 1A).[8]

Dehydroalanine formation can be facilitated by a condensation reaction through acyl transfer mechanism in a diketopiperazine scaffold, but the efficiency and reproducibility of reactions can vary for formaldehyde substrates. We recently demonstrated formation of dehydroalanine through an *N*-to-*O* acyl transfer-based condensation reaction between *N*-acetyl-diketopiperazine (i.e., acetylated cyclic glycine dimer or acetylated glycine anhydride) and formaldehyde under prebiotically relevant mechanochemical conditions (Figure 1B).[9] The reaction proved more efficient through mechanochemistry over the solution-based ones, although the mechanochemical reaction was tested only at small scales and suffered from reproducibility of reaction yields depending on practitioners. Since the dehydroalanine-containing diketopiperazine can be a useful building block beyond prebiotic chemistry research, this work is focused on

View Article Online
DOI: 10.1039/D6OB00662K

optimization and scale increase of the mechanochemical condensation reaction for the preparative purpose. After screening of a series of additives, we discovered liquid-assisted grinding is particularly more effective to increase the reproducibility, led to a larger scale preparation of the dehydroalanine-containing cyclic peptide. In addition to the unique optimization strategies of the present work compared to a previous report,[10] utilization of mechanochemical reactions would be important in the peptide science field, as there have not been many reports on peptide substrate examples to date.[11,12]

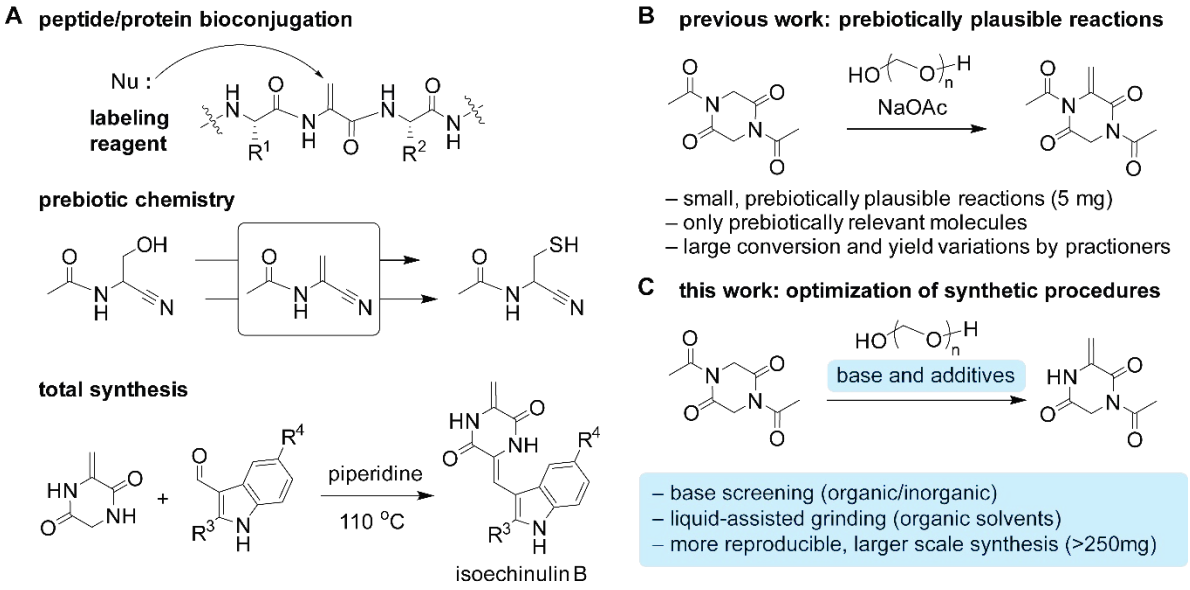


Fig. 1. Importance of dehydroalanine-containing compounds. (A) Representative examples highlighting the importance of dehydroalanine derivatives in bioconjugation, prebiotic chemistry, and total synthesis. (B) Previous work focused on the formation of dehydroalanine under prebiotically relevant, small-scale reaction conditions. (C) This work aimed at increasing the reaction scale and reproducibility for preparative synthesis purposes.

Results and discussion

The screening of metal acetate salts with varying counter cations revealed that reaction efficiency was associated with the types of cations (Fig. 2). In our previous study,[9] sodium acetate was one of the best bases identified in terms of reaction conversions but with limitations of reproducibility. Therefore, we first examined a series of acetate salts by changing the counter cations to evaluate their effects on the reaction outcome. Sodium, calcium, magnesium, indium, and bismuth acetate salts were chosen for the study, and the reaction mixtures were analyzed by ^{1}H NMR. NMR analysis of the calcium and magnesium salt reactions showed substantially less conversion than that of the sodium salt, and the indium and bismuth salts provided no detectable conversions (Figure 2B and Figure S1,S2). The lack of reactions may be due to the reported trends in metal-acetate binding strengths,[13] where interactions between acetate and a higher valent cation can reduce the availability of free acetate relative to sodium acetate ($Na^+ < Ca^{2+} < Mg^{2+} << Bi^{3+} \approx In^{3+}$). Although there

View Article Online
DOI: 10.1039/D6OB00662K

may be a possibility to slightly increase the reproducibility by further screening cation types, we decided to change other components to cause substantial improvement of reproducibility. Additional control experiments indicated that the observed product peaks did not seem to result from the lasting reaction after milling until the NMR sample preparation (aging phenomenon)[14] as well as from the reaction in a deuterated solvent for the NMR analysis (Figure S3 and S4). NMR analysis after milling at different time points may also be indicative of the necessity of mechanical inputs for this reaction (Figure S5).

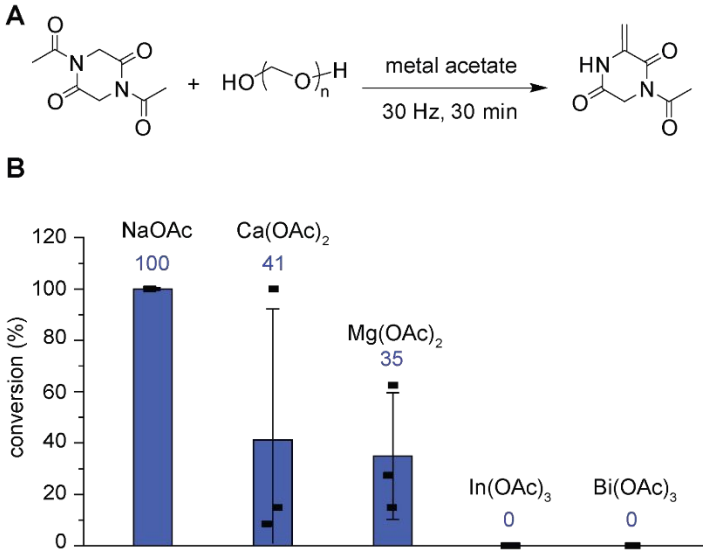


Fig. 2 Attempts to identify efficient and reproducible reaction conditions through screening of acetate base additives. Reaction conditions: acetyl DKP (50 mg, 0.25 mmol), metal acetate (0.85 mmol), and paraformaldehyde (150 mg, 5 mmol per monomeric formaldehyde) at 30 Hz for 30 min. (A) General reaction scheme. (B) Bar graph of NMR analysis of reaction mixtures with metal acetate. OAc = acetate. Salts examined include NaOAc, $Ca(OAc)_2$, $Mg(OAc)_2$, $In(OAc)_3$, and $Bi(OAc)_3$. Error bars represent standard deviation (n = 3), as more than one practitioner performed experiments to obtain the replicates. The conversion of each replicate is shown as a black rectangle in the bar graph. The averages of the conversions of the replicates are shown above each bar in dark blue. Spectra and NMR yields for the reactions analyzed with an internal standard (1,3,5-trimethoxybenzene) are available in ESI.

Analogous to the effects of cation counterpart, screening of types of carboxylate additives did not result in substantial improvement of reaction efficiency and reproducibility (Figure 3 and Figure S6 and S7). As the acetate salt was one of the best bases during our previous study,[9] our next working hypothesis was to increase the efficiency and reproducibility through structural and electronic alteration of the carboxylic acid substitution. Several commercially available sodium salts of carboxylate compounds such as propionate, lactate, benzoate, and oxalate were chosen to this end. Mechanochemical condensation with those carboxylate salts showed virtually no meaningful improvement from that with the parent acetate salt. For example, no detectable degree of product formation was observed for lactate and oxalate groups, possibly indicative of negative effects of the presence of alkylalcohol and β-keto groups on the condensation reaction. The conversion substantially dropped with the benzoate salt while use of *n*-propionate salt resulted in subtle yet probably not statistically significant increase from the acetate one.

View Article Online
DOI: 10.1039/D6OB00662K

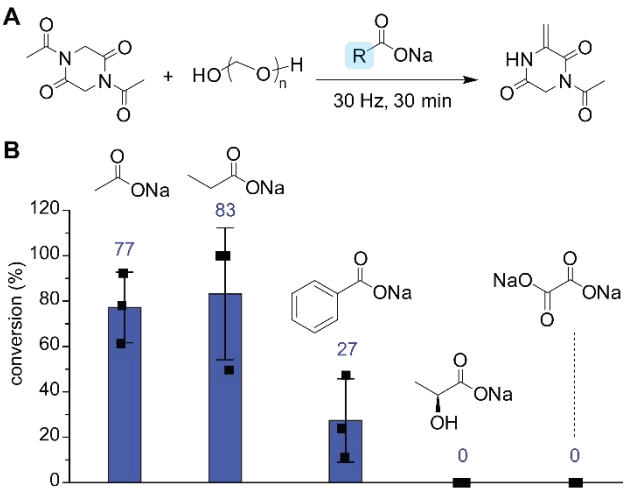


Fig. 3. Attempts to identify efficient and reproducible reaction conditions through screening of carboxylate additives. Reaction conditions: acetyl DKP (50 mg, 0.25 mmol), sodium carboxylate salt (0.85 mmol), and paraformaldehyde (150 mg, 5 mmol per monomeric formaldehyde) at 30 Hz for 30 min. (A) General reaction scheme. (B) Bar graph of NMR analysis of reaction mixtures with carboxylate salts (acetate, propionate, benzoate, lactate, and oxalate). Error bars represent standard deviation (n = 3), as more than one practitioner performed experiments to obtain the replicates. The conversion of each replicate is shown as a black rectangle in the bar graph. The averages of the conversions of the replicates are shown above each bar in dark blue. Spectra and NMR yields for the reactions analyzed with an internal standard (1,3,5-trimethoxybenzene) are available in ESI.

When using organic base additives, there was no appreciable reactivity and did not result in an increase in reaction efficiency or reproducibility. As our previous report demonstrated,[9] there is a tolerance for basicity in this reaction which provided a motivation to test a range of prebiotically irrelevant organic bases. Organic bases with a varied basicity were selected including phenazine (pKa 1.2),[15] phenanthroline (pKa 4.9),[16] methyl benzimidazole (pKa 5.6),[17] and proton sponge (pKa 12.1).[18] The mechanochemical reactions with those organic bases did not show any evidence for product formation based on the NMR analysis (Fig. 3,S8, and S9). Because the range of inorganic and organic bases did not offer the chance to increase efficiency and reproducibility dramatically, we turned out attention to a unique reactivity and selectivity alteration method called liquid-assisted grinding.

View Article Online
DOI: 10.1039/D6OB00662K

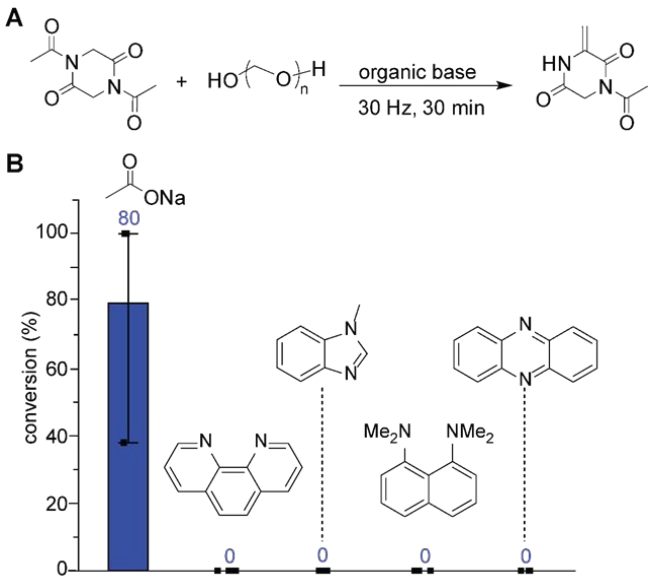


Fig. 4. Attempts to identify efficient and reproducible reaction conditions through screening of organic base additives. Reaction conditions: acetyl DKP (50 mg, 0.25 mmol), organic bases (0.85 mmol), and paraformaldehyde (150 mg, 5 mmol per monomeric formaldehyde) at 30 Hz for 30 min. (A) General reaction scheme. (B) Bar graph of NMR analysis of reaction mixtures with acetate and organic bases (1,10-phenanthroline, *N*-methyl benzimidazole, proton sponge, and phenazine). Error bars represent standard deviation (n = 3), as more than one practitioner performed experiments to obtain the replicates. The conversion of each replicate is shown as a black rectangle in the bar graph. The averages of the conversions of the replicates are shown above each bar in dark blue. Spectra and NMR yields for the reactions analyzed with an internal standard (1,3,5-trimethoxybenzene) are available in ESI.

Inclusion of a small amount of a common organic solvent, known as liquid-assisted grinding, showed more reproducible formation of the desired dehydroalanine product in a more modest conversion (Figure 5). Liquid-assisted mechanochemistry has proved its usefulness in numerous types of chemical reactions for various purposes.[19] Inspired by such a large body of literature, a set of common solvents such as water, methanol, ether, and acetonitrile was applied to the mechanochemical condensation of acetyl-diketopiperazine (Figure S10–S15). While most of those solvents caused either virtually no effects or random increase/decrease of reaction efficiency, we identified the reaction with a small amount of 1,4-dioxane tends to generate the desired product more exclusively without significant generation of unidentified side products (Figure 5C and Figure S11). To confirm the reproducibility of the result, the same experiments of the liquid-assisted mechanochemistry were repeated three times by two practitioners as the partial NMR spectra are shown in Figure 5B–F. For example, milling without liquid additives (Figure 5B) resulted in substantial consumption of the starting material (peaks labeled with gray square), though peaks of not only the desired product (peaks labeled with gray circle) but also other unidentified peaks such as those at 4.8 and 5.5 ppm were observed in a varying degree in each replicate (Figure S10). Another example is milling with acetonitrile shown in Figure 5E where the amount/ratio of remaining starting material, desired product, and random peaks after the reaction vary in every experiment (Figure S13). Because separation of the starting material is more facile than those unidentified compounds from the desired product during purification, 1,4-dioxane would offer most reliable and reproducible preparation of the condensation product among the tested liquid-assisted conditions, even if the dioxane-assisted milling would end up in a modest yield due to the relatively low conversion. While it remains unclear why 1,4-dioxane is

View Article Online
DOI: 10.1039/D6OB00662K

effective for suppression of the potential side products, it is possible that this reaction proceeds through *N*-to-*O* acyl transfer mechanism with a carboylate salt as a base (Figure S16). In order to make sure that remaining carboxylate salts are not causing any unwanted side reactions after the milling process, we also compared the reaction outcomes with and without ammonium chloride addition, but the effect of the addition does not seem very significant (Figure S17). With the dioxane-assisted procedure, we were able to scale up the reaction that produced approximately 250 mg of the desired product using either sodium acetate or sodium propionate salts (procedures available in ESI), and those procedures would be of great value for further applications of dehydroalanine-based DKPs.

View Article Online
DOI: 10.1039/D6OB00662K

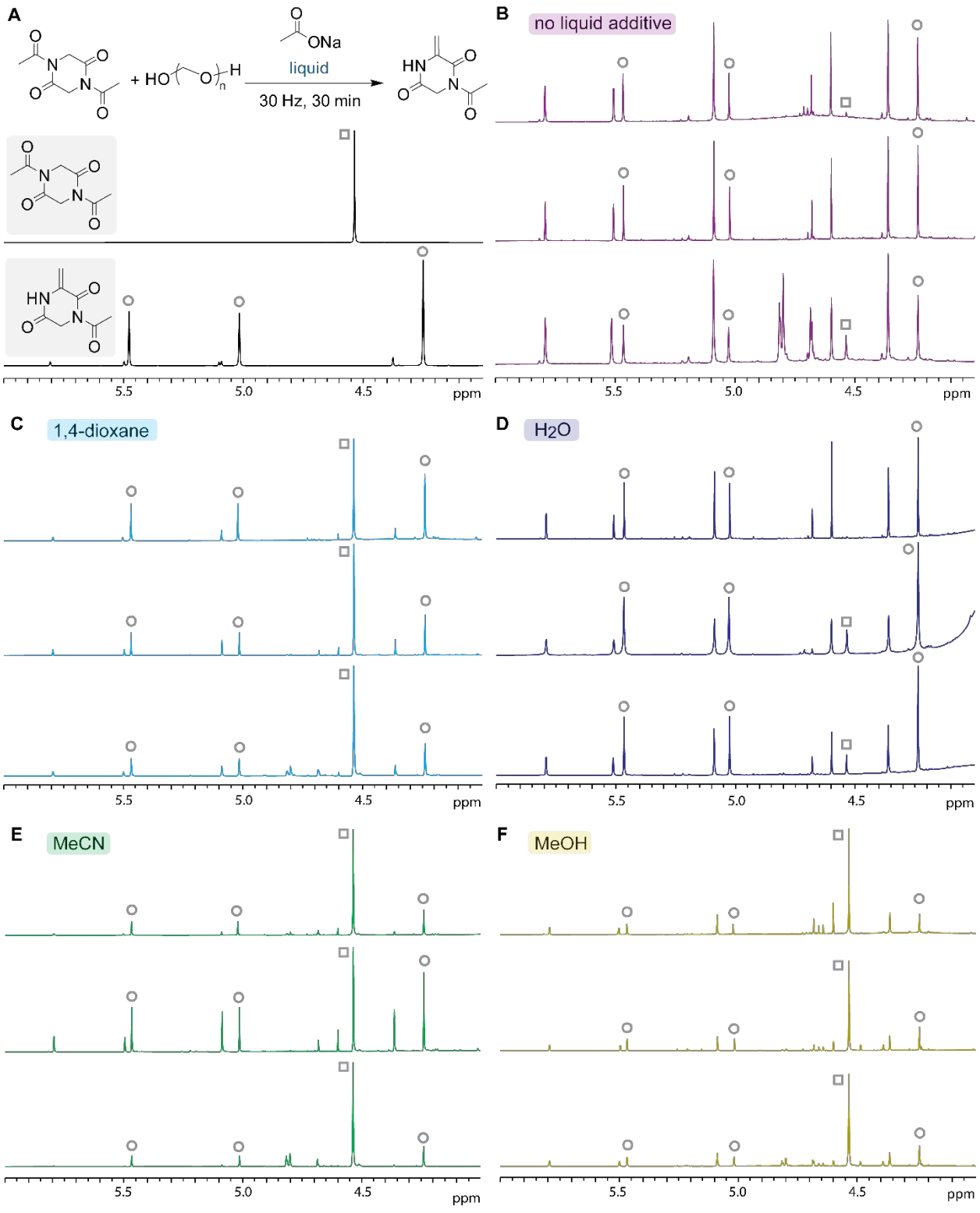


Fig. 5. NMR analysis of liquid-assisted grinding to achieve reproducible dehydroalanine formation. Gray circle: peaks of product. Gray square: a peak of starting material. Reaction conditions: acetyl DKP (50 mg, 0.25 mmol), sodium acetate (150 mg, 1.82 mmol), paraformaldehyde (150 mg, 5 mmol per monomeric formaldehyde) liquid (20 μL) at 30 Hz for 30 min. The η-value for the liquid-assisted grinding is 0.057 μL/mg.[20] (A) General reaction scheme and NMR spectra of starting material and desired product. (B–F) Partial NMR spectra (4–6 ppm) of analysis of reaction mixtures

View Article Online
DOI: 10.1039/D6OB00662K

in DMSO-d_6 after milled using no additional liquid (B), 1,4-dioxane (C), water (D), acetonitrile (E), and methanol (F). Three independent experiments by two practitioners are shown for each condition. Spectra and NMR yields for the reactions analyzed with an internal standard (1,3,5-trimethoxybenzene) are available in ESI.

Conclusions

Liquid-assisted mechanochemical aldol condensation showed its importance for dehydroalanine formation in a diketopiperazine motif. While this work focused on laboratory-scale reactions, there is a potential for the liquid-assisted mechanochemical condensation reaction to be used at an industry level. For example, paraformaldehyde is a relatively safer, solid formaldehyde donor than its aqueous solution, and the mechanochemical approach circumvent the common solubility challenges of the solid paraformaldehyde. Need of only a small amount of organic solvent is another attractive feature for industrial applications from the standpoint of safety and environment/waste as well.[21] Even if our research group's primary interest for dehydroalanine derivatives is centered on prebiotic chemistry, the cyclic dehydroalanine-acetylglycine product would be useful as a building block with nucleophilic and electrophilic reactivities in a broad chemistry community; the α,β-unsaturated carbonyl unit can be used for nucleophilic or radical addition[22] (or another reaction such as Diels-Alder reaction though stereocontrol could be a challenge)[23,24] whereas the remaining acetylglycine unit can undergo another condensation reaction with aldehyde electrophile.[25,26]

Conflict of interests

The authors declare no conflict of interests

Acknowledgements

This work was financially supported by North Carolina State University and Goodnight Early Career Innovators Award. J. C. T. and K. M. B. were financially supported by the NC State NIH T32 Chemistry of Life Training Program and NC State Provost's Professional Experience Program (PEP), respectively. We thank Comparative Medicine Institute (CMI) at NC State University for their equipment grant to purchase the ball mill equipment (MM 500). NMR was performed in the Molecular Education Technology and Research Innovation Center (METRIC) at NC State University, which is supported by the State of North Carolina.

References

(1) Boutureira, O.; Bernardes, G. J. L. Advances in Chemical Protein Modification. *Chem. Rev.* **2015**, *115* (5), 2174–2195. https://doi.org/10.1021/cr500399p.
(2) Ohata, J. Friedel–Crafts Reactions for Biomolecular Chemistry. *Org. Biomol. Chem.* **2024**, *22* (18), 3544–3558. https://doi.org/10.1039/D4OB00406J.

View Article Online
DOI: 10.1039/D6OB00662K

(3) Dadová, J.; Galan, S. R.; Davis, B. G. Synthesis of Modified Proteins via Functionalization of Dehydroalanine. *Curr. Opin. Chem. Biol.* **2018**, *46*, 71–81. https://doi.org/10.1016/j.cbpa.2018.05.022.
(4) Peng, X.; Xu, K.; Zhang, Q.; Liu, L.; Tan, J. Dehydroalanine Modification Sees the Light: A Photochemical Conjugate Addition Strategy. *Trends Chem.* **2022**, *4* (7), 643–657. https://doi.org/10.1016/j.trechm.2022.04.008.
(5) Yu, C.; Bao, G.; Sun, W. Recent Advances on Dehydroalanine-Specific Modification and Diversification of Peptides and Proteins. *ChemBioChem* **2025**, *26* (20), 202500349. https://doi.org/10.1002/cbic.202500349.
(6) Foden, C. S.; Islam, S.; Fernández-García, C.; Maugeri, L.; Sheppard, T. D.; Powner, M. W. Prebiotic Synthesis of Cysteine Peptides That Catalyze Peptide Ligation in Neutral Water. *Science* **2020**, *370* (6518), 865–869. https://doi.org/10.1126/science.abd5680.
(7) Green, N. J.; Russell, D. A.; Tanner, S. H.; Sutherland, J. D. Prebiotic Synthesis of N-Formylaminonitriles and Derivatives in Formamide. *J. Am. Chem. Soc.* **2023**, *145* (19), 10533–10541. https://doi.org/10.1021/jacs.2c13306.
(8) Kuttruff, C. A.; Zipse, H.; Trauner, D. Concise Total Syntheses of Variecolortides A and B through an Unusual Hetero-Diels–Alder Reaction. *Angewandte Chemie International Edition* **2011**, *50* (6), 1402–1405. https://doi.org/10.1002/anie.201006154.
(9) Thuan, J. C.; Koirala, A.; Pirez, J.; Ohata, J. Prebiotic Roles of Formaldehyde as an Activating Agent and a Building Block in Solid-State Peptide Modification. *ACS Bio & Med Chem Au* **2025**, *5* (6), 982–993. https://doi.org/10.1021/acsbiomedchemau.5c00167.
(10) Rodríguez, B.; Bruckmann, A.; Bolm, C. A Highly Efficient Asymmetric Organocatalytic Aldol Reaction in a Ball Mill. *Chem. Eur. J.* **2007**, *13* (17), 4710–4722. https://doi.org/10.1002/chem.200700188.
(11) Duvnjak, M.; Vidović, N.; Užarević, K.; Horvat, G.; Tomišić, V.; Speranza, G.; Cindro, N. Rapid and Green Anion-Assisted Mechanochemical Peptide Cyclization. *ACS Sustain. Chem. Eng.* **2025**, *13* (1), 30–35. https://doi.org/10.1021/acssuschemeng.4c03309.
(12) Nuruzzaman, M.; Uzoewulu, C. P.; Ghader, N. O.; Asadi, M.; Ohata, J. Harnessing Cyclohexadiene Chemistry for Chemical Modification of Unprotected Peptides. *J. Org. Chem.* **2026**, *65* (8), accepted.
(13) Jafari, M.; Zhen, L.; Song, L. F.; Sagresti, L.; Brancato, G.; Merz Jr, K. M. Thermodynamics of Metal–Acetate Interactions. *J. Phys. Chem. B* **2024**, *128* (3). https://doi.org/https://doi.org/10.1021/acs.jpcb.3c06567.
(14) Templ, J.; Borchardt, L. Experimental Isolation of Single Impact, Cumulative Energy, Aging, and Liquid-Assisted Grinding Effects in Mechanochemical Reactions. *Angew. Chem. Int. Ed.* **2026**, *65* (8), e23191. https://doi.org/10.1002/anie.202523191.
(15) Bedeković, N.; Stilinović, V.; Friščić, T.; Cinčić, D. Comparison of Isomeric *Meta* - and *Para* -Diiodotetrafluorobenzene as Halogen Bond Donors in Crystal Engineering. *New J. Chem.* **2018**, *42* (13), 10584–10591. https://doi.org/10.1039/C8NJ01368C.
(16) Sanna, D.; Buglyó, P.; Tomaz, A. I.; Pessoa, J. C.; Borović, S.; Micera, G.; Garribba, E. VIVO and CuII Complexation by Ligands Based on Pyridine Nitrogen Donors. *Dalton Trans.* **2012**, *41* (41), 12824. https://doi.org/10.1039/c2dt31109g.
(17) Kaiya, T.; Aoyama, S.; Kohda, K. Reactions of 1-Methylbenzimidazole Derivatives with m-Chloroperoxybenzoic Acid. *Bioorg. Med. Chem. Lett.* **1998**, *8* (6), 625–630. https://doi.org/10.1016/S0960-894X(98)00082-1.
(18) Hibbert, F.; Simpson, G. R. Acid–Base Properties of Highly Substituted Diaminonaphthalenes. *J. Chem. Soc., Perkin Trans. 2* **1987**, No. 2, 243–246. https://doi.org/10.1039/P29870000243.
(19) Ying, P.; Yu, J.; Su, W. Liquid-Assisted Grinding Mechanochemistry in the Synthesis of Pharmaceuticals. *Adv. Synth. Catal.* **2021**, *363* (5), 1246–1271. https://doi.org/10.1002/adsc.202001245.

View Article Online
DOI: 10.1039/D6OB00662K

(20) Friščić, T.; Childs, S. L.; Rizvi, S. A. A.; Jones, W. The Role of Solvent in Mechanochemical and Sonochemical Cocrystal Formation: A Solubility-Based Approach for Predicting Cocrystallisation Outcome. *CrystEngComm* **2009**, *11* (3), 418–426. https://doi.org/10.1039/B815174A.
(21) Salami, S. A.; Manyeruke, M. H.; Ezekiel, C. I.; Ndagano, U. N.; Safari, J. B.; Amusat, S. O.; Krause, R. W. M. Recent Advances in Liquid Assisted Grinding Chemistry: Towards Sustainable Synthesis. *Results Chem.* **2025**, *18*, 102675. https://doi.org/10.1016/j.rechem.2025.102675.
(22) Chai, C. L. L.; King, A. R. Stereocontrolled Intermolecular Radical Additions to Methylidenepiperazine-2,5-Diones. *J. Chem. Soc., Perkin Trans. 1* **1999**, No. 9, 1173–1182. https://doi.org/10.1039/A900771G.
(23) Burkett, B. A.; Chai, C. L. L.; Hockless, D. C. R. Unexpected Variations in the Reactivities and Selectivities of Acyclic and Cyclic Dehydrodipeptides in Diels-Alder Reactions. *Aust. J. Chem.* **1998**, *51* (11), 993–998. https://doi.org/10.1071/C98116.
(24) Chai, C. L. L.; Johnson, R. C.; Koh, J. The Reactivities of Dehydroalanine Derivatives towards 1,3-Dienyl Cobaloxime Complex: New Routes to Functionalised Carbocyclic Amino Acids. *Tetrahedron* **2002**, *58* (5), 975–982. https://doi.org/10.1016/S0040-4020(01)01160-7.
(25) Liao, S.; Qin, X.; Li, D.; Tu, Z.; Li, J.; Zhou, X.; Wang, J.; Yang, B.; Lin, X.; Liu, J.; Yang, X.; Liu, Y. Design and Synthesis of Novel Soluble 2,5-Diketopiperazine Derivatives as Potential Anticancer Agents. *Eur. J. Med. Chem.* **2014**, *83*, 236–244. https://doi.org/10.1016/j.ejmech.2014.06.030.
(26) Balducci, D.; Conway, P. A.; Sapuppo, G.; Müller-Bunz, H.; Paradisi, F. Novel Approach to the Synthesis of Aliphatic and Aromatic α-Keto Acids. *Tetrahedron* **2012**, *68* (36), 7374–7379. https://doi.org/10.1016/j.tet.2012.06.078.

View Article Online
DOI: 10.1039/D6OB00662K

The data supporting this article have been included as part of the Supplementary Information. Supplementary information: Supplementary figures, general procedure, equipment, synthetic procedure, and ^{1}H NMR spectrum of the final product. See DOI: [URL – format https://doi.org/DOI]