



HAL
open science

Epimerization-Free C-Term Activation of Peptide Fragments by Ball Milling

Yves Yeboue, Marion Jean, Gilles Subra, Jean Martínez, Frédéric Lamaty,
Thomas-Xavier Métro

► **To cite this version:**

Yves Yeboue, Marion Jean, Gilles Subra, Jean Martínez, Frédéric Lamaty, et al.. Epimerization-Free C-Term Activation of Peptide Fragments by Ball Milling. *Organic Letters*, 2021, 23 (3), pp.631-635. 10.1021/acs.orglett.0c03209 . hal-03228800

HAL Id: hal-03228800

<https://hal.science/hal-03228800v1>

Submitted on 18 May 2021

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Epimerization-Free C-Term Activation of Peptide Fragments by Ball Milling

Yves Yeboue, Marion Jean, Gilles Subra, Jean Martinez, Frédéric Lamaty,* and Thomas-Xavier Métro*



Cite This: *Org. Lett.* 2021, 23, 631–635



Read Online

ACCESS |



Metrics & More

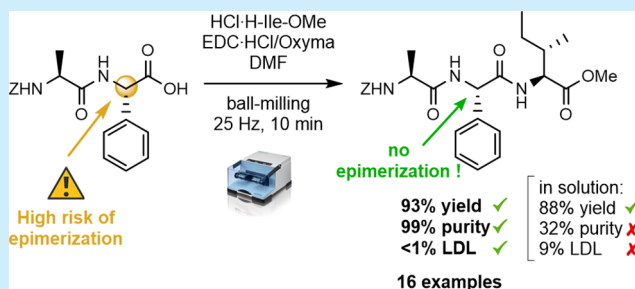


Article Recommendations



Supporting Information

ABSTRACT: Peptides were produced in high yields and, if any, very low epimerization, by mechanochemical coupling of peptide fragments containing highly epimerization-prone and/or highly hindered amino acids at C-term. Ball milling was clearly identified as the key element enabling one to obtain such results.



Thanks to their wide range of structural and physicochemical properties, as well as their diverse biological activities, peptides found numerous applications in material and life sciences. In healthcare, they play a key role in the design of active biological molecules, targeting agents, vectors and diagnostic tools, such as that recently demonstrated in the fight against COVID-19.^{1,2} Over the years, interest in this class of compounds has increased,^{3–6} because of their good biological activities at very low doses, high selectivity, and high probability of success at clinical trial stages.³ Yet, the chemical synthesis of long peptides and proteins is a highly challenging task. Indeed, the efficiency of coupling and deprotection reactions decreases with the length of the peptide chain, leading to amino acid deletions and uncompleted sequences. Significant efforts have been deployed by peptide scientists to tackle this challenge, mainly by using convergent fragment coupling approaches, such as native chemical ligation (NCL).^{7,8} These approaches suffer from a limited scope: peptide bond disconnections cannot be performed at any point of the peptide chain. Limitations are even more constraining when coupling a C-term activated peptide fragment with a free N-term fragment; the risk of epimerization can be extremely high, leading to the production of highly undesirable diastereomers.⁹ Some examples are reporting peptide fragment couplings with low to null epimerization levels, but they remain scarce.^{10–15} In this context, and based on our knowledge and on previous work in the literature on peptide synthesis under solvent-less/solvent-free conditions by mechanochemical means,^{16–25} we considered evaluating the capacity of ball mills to reduce or eliminate epimerization during C-term activation of peptide fragments, and to compare these results with the classical synthesis in solution.²⁶

The coupling between Z-Ala-Phg-OH and HCl·H-Ile-OMe was considered as a probe of choice.⁴ Indeed, phenylglycine

(Phg) is known as being one of the most epimerization-prone amino acid,^{9,12,27–29} while isoleucine (Ile) is a hindered amino acid exhibiting low coupling speeds. Thus, Z-Ala-Phg-OH was reacted with HCl·H-Ile-OMe (1.5 equiv) in the presence of EDC·HCl (*N*-(3-(dimethylamino)propyl)-*N*'-ethylcarbodiimide hydrochloride, 1.2 equiv), Oxyma (ethyl cyano(hydroxyimino)acetate, 1.2 equiv) and a small amount of DMF ($\eta = 0.45 \mu\text{L}/\text{mg}$),^{b,30} in a 5 mL polytetrafluoroethylene (PTFE) vibrating ball mill (vbm) reactor containing three stainless steel balls (5 mm diameter), for 10 min at 25 Hz.³¹ Here, the amount of dimethyl formamide (DMF) was not sufficient to solubilize the reaction mixture but improved its homogeneity. Although being a highly problematic solvent,^c DMF is the most widely used solvent for peptide synthesis. It appeared as the liquid additive of choice to ensure a rigorous comparison between the ball-milling and the classical solution-phase approach. Following the same idea, the η parameter was calculated to facilitate the comparison of the quantity of solvent that were used.^b After workup, the corresponding tripeptide Z-Ala-Phg-Ile-OMe was isolated in 93% yield, with excellent purity and no detectable traces of the Z-Ala-D-Phg-Ile-OMe epimer as indicated by high-performance liquid chromatography (HPLC) (Table 1, entry 1; $t_{\text{R}}(\text{LLL}) = 5.08$ min and $t_{\text{R}}(\text{LDL}) = 5.20$ min; see the Supporting Information for details).

During the coupling under ball-milling conditions, a slight temperature increase was observed: right after milling was

Received: September 24, 2020

Published: January 4, 2021

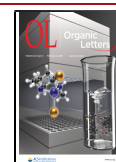
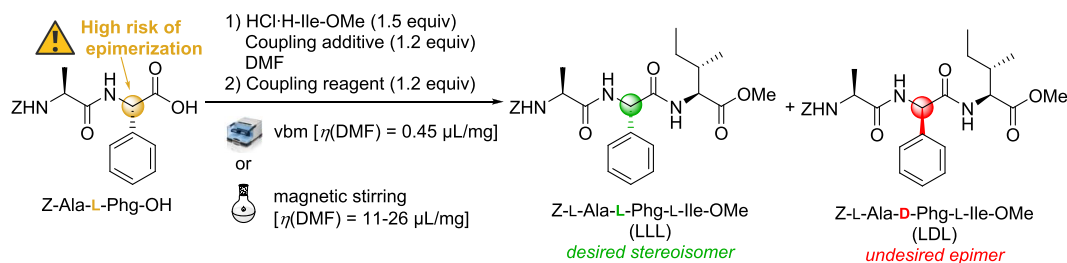


Table 1. Comparative Study between Vibrating Ball Milling and Solution Synthesis of Z-Ala-Phg-Ile-OMe^a

entry	reagents	temperature (°C)	time, <i>t</i> (min)	yield (%)	purity ^b (%)	LDL ^b (%)
1	EDC·HCl/Oxyma	33 (33)	10 (30)	93 (88)	>99 (32)	<1 (9)
2	EDC·HCl/HOBt·H ₂ O	34 (34)	10 (30)	90 (90)	70 (48)	25 (35)
3	EDC·HCl/HOAt	34 (34)	10 (30)	88 (90)	95 (59)	<1 (26)
4	DIC/HOAt	30 (31)	10 (40)	n.d. (n.d.) ^c	67 (39)	17 (33)
5	DIC/Oxyma	31 (31)	10 (40)	n.d. (n.d.) ^c	46 (<10)	<1 (n.d.) ^c
6	HATU/Et ₃ N	34 (34)	10 (60)	85 (88)	88 (58)	1 (<1)
7	HBTU/Et ₃ N	33 (33)	10 (20)	86 (82)	71 (55)	2 (9)
8	EDC·HCl/Oxyma ^d	n.d. ^c	30	96	>99	<1

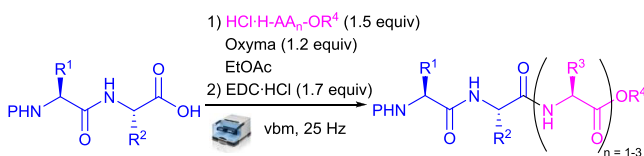
^aMilling reactions were performed in a 5 mL PTFE jar with three stainless steel balls (5 mm diameter) at 25 Hz. The total mass of reactants was 50 mg. The values in parentheses correspond to solution reactions. ^bDetermined by HPLC. ^cn.d. = not determined. ^dReaction performed on a total mass of 250 mg of reactants in a 15 mL PTFE jar with one stainless steel ball (10 mm diameter) at 25 Hz for 30 min using 1.7 equiv of EDC·HCl. EtOAc was used as a liquid additive instead of DMF.

stopped, the temperature of the milled material reached 33 °C.³² For the sake of comparison, the same temperature was applied to the synthesis of Z-Ala-Phg-Ile-OMe under classical magnetic stirring conditions, while using the minimal amount of DMF enabling proper mixing ($\eta = 20 \mu\text{L}/\text{mg}$). Under these experimental conditions, Z-Ala-Phg-Ile-OMe was produced in 88% yield, 32% purity, and 9% of the LDL epimer (Table 1, entry 1; see values given in parentheses). These results clearly showed that liquid-assisted ball milling could minimize epimerization of a highly epimerization-prone C-term activated peptide fragment. In addition, the coupling was complete after 10 min of ball milling, while 30 min were required under classical solution conditions. This latter observation could be explained by the higher concentration of the reaction mixture in the ball mill, compared to that in solution. Other coupling reagents and additives were screened to establish the broadness of our observations. When couplings were performed in solution, the quantity of DMF was kept as low as possible while enabling proper mixing under classical magnetic stirring ($11 \mu\text{L}/\text{mg} < \eta < 26 \mu\text{L}/\text{mg}$). When Oxyma was replaced with HOBt·H₂O, ball milling conditions led to 25% of the LDL epimer and 70% purity, while 35% of the same epimer and 48% purity were obtained for the synthesis in solution (Table 1, entry 2). The use of HOAt gave better results than HOBt·H₂O: only 1% of the LDL epimer was produced in the ball-milling conditions while 26% LDL epimer were obtained after synthesis in solution (Table 1, entry 3). Using DIC with HOAt under ball milling conditions furnished the tripeptide with 67% purity along with 17% of the LDL epimer, while synthesis in solution led to lower purity (39%) and higher epimerization (33%) (Table 1, entry 4). These results obtained with DIC were not improved when replacing HOAt with Oxyma (Table 1, entry 5). Concerning the stand-alone coupling reagents HBTU and HATU, their use induced low epimerization levels both by ball milling and in solution (Table 1, entries 6 and 7). Yet, the HPLC purity profiles of the products obtained by ball milling were much better than when synthesized in solution. The comparison between the ball milling and solution-phase

processes clearly showed the superiority of the mechanochemical approach over the classical synthesis in solution, since lower epimerization rates and better purity profiles were obtained with almost all tested coupling agents and additives, while yields remained comparable. In addition, performing the coupling with the best coupling agent and additive (EDC·HCl/Oxyma) on a larger scale (250 mg total mass instead of 50 mg) with larger reactor and ball (15 mL reactor with one 10-mm-diameter ball) did not affect the yield (96%) or epimerization rate (<1% LDL; Table 1, entry 8) (see the Supporting Information for performances of other combinations of jar and balls).

We next applied the best ball-milling experimental conditions (EDC·HCl/Oxyma) to the synthesis of other challenging peptides. Here, EtOAc was used instead of DMF to improve the overall environmental impact of our ball-milling conditions. Thus, ball milling Z-Ala-Phg-OH with HCl·H-Phe-OMe produced the tripeptide Z-Ala-Phg-Phe-OMe in 89% yield with full conservation of the stereogenic centers (Table 2, entry 1). When Z-Ala-D-Phg-OH was used, the tripeptide Z-Ala-D-Phg-Phe-OMe was isolated with a similar yield of 92% and excellent purity (Table 2, entry 2). Cysteine, which is another epimerization-prone amino acid,^{9,33} was not racemized during the synthesis of Z-Ala-Cys(Bn)-Ala-OMe and Z-Ala-Cys(Bn)-Phe-OMe under our conditions. In both cases, tripeptides were obtained in excellent yields (>94%) and diastereomeric excesses (>99%) (Table 2, entries 3 and 4). Like isoleucine, valine is a β -branched amino acid known to be more difficult for coupling than most other amino acids.³⁴ Yet, the reaction between Z-Phe-Val-OH and HCl·H-Cys(Bn)-OMe led to the desired tripeptide, Z-Phe-Val-Cys(Bn)-OMe, in almost quantitative yield after 30 min of ball milling (Table 2, entry 5). Importantly, no trace of the LDL epimer could be detected by HPLC analysis.

Similar results were obtained starting with the Z-Phe-D-Val-OH (Table 2, entry 6). When HCl·H-Cys(Bn)-OMe was replaced with HCl·H-Ser(*t*Bu)-O*t*Bu, excellent yields were obtained, again with no epimerization (Table 2, entries 7 and

Table 2. Scope of the Peptide Couplings by Ball Milling^a

entry	peptides	time (min)	isolated yield (%)	de (%)
1	Z-Ala-Phg-Phe-OMe	20	89	>99
2	Z-Ala-D-Phg-Phe-OMe	20	92	>99
3	Z-Ala-Cys(Bn)-Ala-OMe	30	98	>99
4	Z-Ala-Cys(Bn)-Phe-OMe	20	94	>99
5	Z-Phe-Val-Cys(Bn)-OMe	30	98 ^b	>99
6	Z-Phe-D-Val-Cys(Bn)-OMe	30	97	>99
7	Z-Phe-Val-Ser(<i>t</i> Bu)- <i>o</i> TBu	30	97 ^b	>99
8	Z-Phe-D-Val-Ser(<i>t</i> Bu)- <i>o</i> TBu	20	97	>99
9	Boc-Trp-Phe-Glu(Bn)-OBn	15	92	>99
10	Boc-Trp-Phe-Gly-OBn	10	84	>99
11	Boc-Trp-D-Phe-Gly-OBn	10	95	>99
12	Z-Phe-Val-Leu ₂ -OBn	25	95 ^c	>99
13	Z-Phe-D-Val-Leu ₂ -OBn	25	91 ^c	98
14	Z-Phe-Val-Leu ₃ -OBn	30	93 ^{c,d}	>99
15	Z-Phe-D-Val-Leu ₃ -OBn	30	91 ^{c,d}	99

^aMilling reactions were performed in a 15-mL PTFE jar with one stainless steel ball (10 mm in diameter). The total mass of reactants was 250 mg. ^b1.2 equiv of EDC·HCl were used. ^c1.0 equiv of HCl·H-AA_n-OR were used. ^d2.2 equiv of EDC·HCl, η (EtOAc) = 0.9 μ L/mg and total mass = 267 mg.

8). Of note, the *t*-butyl ester was stable in these experimental conditions. In classical peptide synthesis in solution, Z and Boc *N*-protecting groups are commonly used. Thus, Boc-Trp-Phe-Glu(Bn)-OBn, Boc-Trp-Phe-Gly-OBn, and Boc-Trp-D-Phe-Gly-OBn were synthesized and successfully isolated in good to excellent yields (84%–95%), without any detectable epimerization (Table 2, entries 9–11). These results supported that the presence of Boc and benzyl groups was not an obstacle to the success of these syntheses. We next aimed at demonstrating that peptide fragment couplings mediated by ball milling could be applied to longer peptide fragments. For this, activation of dipeptides, bearing the hindered valine at C-term, and couplings with dipeptides and tripeptides were performed. When the optimal experimental reaction conditions were applied to the coupling of Z-Phe-Val-OH with HCl·H-Leu₂-OBn, the expected tetrapeptide was obtained in 95% yield without epimerization (Table 2, entry 12). The same conditions applied to the synthesis of the diastereomer Z-Phe-D-Val-Leu₂-OBn yielded the desired tetrapeptide in 91%, yet containing 2% of the epimer (Table 2, entry 13). To our delight, when the tripeptide HCl·H-Leu₃-OBn was reacted with Z-Phe-Val-OH and Z-Phe-D-Val-OH, the corresponding pentapeptides Z-Phe-Val-Leu₃-OBn and Z-Phe-D-Val-Leu₃-OBn were successfully isolated with high yields (>91%) and had negligible level of epimerization (Table 2, entries 14 and 15). So far, the longest peptide synthesized by ball milling was the pentapeptide Leu-enkephalin.¹⁷ The successful synthesis of these two pentapeptides by a fragment coupling approach consolidates the potential of mechanochemistry to produce oligopeptides with high efficiency. In classical solvent-based conditions, epimerization is often attributed to the intramolecular formation of an oxazolone ring during activation,⁹ which is a process that is favored under diluted conditions. Our

results suggest that the absence of epimerization could be explained by the highly concentrated reaction conditions enabled by ball milling, which would prevent the transient formation of an oxazolone ring (see the Supporting Information for a scheme of the proposed mechanism).

To conclude, using the combination of EDC·HCl, Oxyma, and small amounts of a liquid additive (DMF or EtOAc) under ball-milling conditions permitted us to perform peptide fragment couplings with high yields and, if any, very low epimerization. Noteworthy, very good results were obtained with peptide fragments containing highly epimerization-prone and/or highly hindered amino acids at C-term, such as phenylglycine, cysteine, and valine. Ball milling was clearly identified as the key element to obtain both high yield and purity, along with low epimerization. Indeed, the ball milling conditions proved to be superior to the classical solution synthesis approach on a various array of widely used coupling agents. These results open avenues for the development of highly efficient, convergent and flexible peptide synthesis strategies based on peptide fragment couplings. Further investigations aiming at (i) deciphering the mechanism enabling epimerization suppression during ball milling, when compared to synthesis in solution and (ii) applying this strategy to longer peptide fragments are currently being explored in our laboratory and will be reported in due course.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.orglett.0c03209>.

Operating procedures and characterization of all compounds, including NMR spectra and HPLC chromatograms (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

Frédéric Lamaty – IBMM, Univ Montpellier, CNRS, ENSCM, Montpellier, France; Email: frederic.lamaty@umontpellier.fr

Thomas-Xavier Métro – IBMM, Univ Montpellier, CNRS, ENSCM, Montpellier, France; orcid.org/0000-0003-2280-3595; Email: thomas-xavier.metro@umontpellier.fr

Authors

Yves Yeboue – IBMM, Univ Montpellier, CNRS, ENSCM, Montpellier, France

Marion Jean – Aix-Marseille Université, CNRS, Centrale Marseille, iSm2, Marseille, France; orcid.org/0000-0003-0524-8825

Gilles Subra – IBMM, Univ Montpellier, CNRS, ENSCM, Montpellier, France; orcid.org/0000-0003-4857-4049

Jean Martinez – IBMM, Univ Montpellier, CNRS, ENSCM, Montpellier, France; orcid.org/0000-0002-4551-4254

Complete contact information is available at: <https://pubs.acs.org/doi/10.1021/acs.orglett.0c03209>

Author Contributions

Y.Y. performed the syntheses and the chemical analyses. Y.Y. and M.J. performed the HPLC analyses aiming at measuring diastereomeric excesses. T.X.M. devised the presented idea, obtained the funding, supervised the project and wrote the

manuscript. Y.Y., G.S., J.M., and F.L. discussed the results and commented on the manuscript.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

Université de Montpellier, Centre National de la Recherche Scientifique (CNRS), and LabEx ChemISys (through ANR program ANR-10-LABX-05-01) are acknowledged for financial support. Monique Calmès (CNRS, Univ Montpellier) is acknowledged for assistance in chiral HPLC analysis.

ADDITIONAL NOTES

^aFor sake of clarity, absolute configuration indications of L amino acids have been omitted throughout the manuscript. Only D configurations have been indicated. Thus, Z-Ala-Phg-OH corresponds to Z-L-Ala-L-Phg-OH.

^bAdding a liquid additive to a mechanochemical reaction, known as liquid-assisted grinding (LAG), is known to have beneficial effects during peptide couplings under mechanochemical conditions. (See refs 17 and 18.) The η parameter is defined by the ratio of the volume of liquid (in μL) to the total mass of solids (in mg). (See ref 30.)

^cN,N-dimethylformamide (DMF) is classified as presenting reproductive toxicity, according to Regulation (EC) No. 1272/2008.

REFERENCES

- (1) Xia, S.; Liu, M.; Wang, C.; Xu, W.; Lan, Q.; Feng, S.; Qi, F.; Bao, L.; Du, L.; Liu, S.; Qin, C.; Sun, F.; Shi, Z.; Zhu, Y.; Jiang, S.; Lu, L. Inhibition of SARS-CoV-2 (previously 2019-nCoV) infection by a highly potent pan-coronavirus fusion inhibitor targeting its spike protein that harbors a high capacity to mediate membrane fusion. *Cell Res.* **2020**, *30*, 343–355.
- (2) Liu, C.; Zhou, Q.; Li, Y.; Garner, L. V.; Watkins, S. P.; Carter, L. J.; Smoot, J.; Gregg, A. C.; Daniels, A. D.; Jervey, S.; Albaiu, D. Research and Development on Therapeutic Agents and Vaccines for COVID-19 and Related Human Coronavirus Diseases. *ACS Cent. Sci.* **2020**, *6*, 315–331.
- (3) Lau, J. L.; Dunn, M. K. Therapeutic peptides: Historical perspectives, current development trends, and future directions. *Bioorg. Med. Chem.* **2018**, *26*, 2700–2707.
- (4) Henninot, A.; Collins, J. C.; Nuss, J. M. The Current State of Peptide Drug Discovery: Back to the Future? *J. Med. Chem.* **2018**, *61*, 1382–1414.
- (5) *Impact Story: Developing the Tools to Evaluate Complex Drug Products: Peptides*. Available via the Internet at: <https://www.fda.gov/drugs/regulatory-science-action/impact-story-developing-tools-evaluate-complex-drug-products-peptides> (accessed Nov. 5, 2020).
- (6) Al Shaer, D.; Al Musaimi, O.; Albericio, F.; de la Torre, B. G. 2019 FDA TIDES (Peptides and Oligonucleotides) Harvest. *Pharmaceuticals* **2020**, *13*, 40.
- (7) Conibear, A. C.; Watson, E. E.; Payne, R. J.; Becker, C. F. W. Native chemical ligation in protein synthesis and semi-synthesis. *Chem. Soc. Rev.* **2018**, *47*, 9046–9068.
- (8) Agouridas, V.; El Mahdi, O.; Diemer, V.; Cargoët, M.; Monbaliu, J.-C. M.; Melnyk, O. Native Chemical Ligation and Extended Methods: Mechanisms, Catalysis, Scope, and Limitations. *Chem. Rev.* **2019**, *119*, 7328–7443.
- (9) Yang, Y. In *Side Reactions in Peptide Synthesis*; Academic Press, 2016; pp 257–292, DOI: 10.1016/B978-0-12-801009-9.00011-2.
- (10) Hiebl, J.; Baumgartner, H.; Bernwieser, I.; Blanka, M.; Bodenteich, M.; Leitner, K.; Rio, A.; Rovenszky, F.; Alberts, D. P.; Bhatnagar, P. K.; Banyard, A. F.; Baresch, K.; Esch, P. M.; Kollmann, H.; Mayrhofer, G.; Weihtrager, H.; Welz, W.; Winkler, K.; Chen, T.; Patel, R.; Lantos, I.; Stevenson, D.; Tubman, K. D.; Undheim, K. Large-scale synthesis of hemoregulatory nonapeptide SK&F 107647 by fragment coupling. *J. Pept. Res.* **1999**, *54*, 54–65.
- (11) Spengler, J.; Ruíz-Rodríguez, J.; Yraola, F.; Royo, M.; Winter, M.; Burger, K.; Albericio, F. A Novel Protecting/Activating Strategy for β -Hydroxy Acids and Its Use in Convergent Peptide Synthesis. *J. Org. Chem.* **2008**, *73*, 2311–2314.
- (12) Popovic, S.; Bieräugel, H.; Detz, R. J.; Kluwer, A. M.; Koole, J. A. A.; Streefkerk, D. E.; Hiemstra, H.; van Maarseveen, J. H. Epimerization-Free C-Terminal Peptide Activation. *Chem. - Eur. J.* **2013**, *19*, 16934–16937.
- (13) Zhang, T.; Chen, Z.; Tian, Y.; Han, B.; Zhang, N.; Song, W.; Liu, Z.; Zhao, J.; Liu, J. Kilogram-Scale Synthesis of Osteogenic Growth Peptide (10–14) Using a Fragment Coupling Approach. *Org. Process Res. Dev.* **2015**, *19*, 1257–1262.
- (14) Zhang, T.; Song, W.; Zhao, J.; Liu, J. Full Solution-Phase Synthesis of Acetyl Hexapeptide-3 by Fragments Coupling Strategy. *Ind. Eng. Chem. Res.* **2017**, *56*, 11697–11704.
- (15) Gurjar, M. K.; Tripathy, N. K.; Pramanik, C. M.; Deshmukh, S. S.; Mahajan, U. S.; Deshpande, A. P. *Process for preparation of octreotide acetate via an improved 4 + 4 soln. phase synthesis of octreotide*. Int. Patent No. WO 2017175107 A1, 2017.
- (16) Declerck, V.; Nun, P.; Martinez, J.; Lamaty, F. Solvent-Free Synthesis of Peptides. *Angew. Chem., Int. Ed.* **2009**, *48*, 9318–9321.
- (17) Bonnamour, J.; Métro, T.-X.; Martinez, J.; Lamaty, F. Environmentally benign peptide synthesis using liquid-assisted ball-milling: application to the synthesis of Leu-enkephalin. *Green Chem.* **2013**, *15*, 1116–1120.
- (18) Porte, V.; Thioly, M.; Pigoux, T.; Métro, T.-X.; Martinez, J.; Lamaty, F. Peptide Mechanochemistry by Direct Coupling of N-Protected α -Amino Acids with Amino Esters. *Eur. J. Org. Chem.* **2016**, *2016*, 3505–3508.
- (19) Maurin, O.; Verdié, P.; Subra, G.; Lamaty, F.; Martinez, J.; Métro, T.-X. Peptide synthesis: ball-milling, in solution, or on solid support, what is the best strategy? *Beilstein J. Org. Chem.* **2017**, *13*, 2087–2093.
- (20) Pétry, N.; Benakki, H.; Clot, E.; Retailleau, P.; Guenoun, F.; Asserar, F.; Sekkat, C.; Métro, T.-X.; Martinez, J.; Lamaty, F. A mechanochemical approach to access the proline–proline diketopiperazine framework. *Beilstein J. Org. Chem.* **2017**, *13*, 2169–2178.
- (21) Yeboue, Y.; Gallard, B.; Le Moigne, N.; Jean, M.; Lamaty, F.; Martinez, J.; Métro, T.-X. Peptide couplings by reactive extrusion: solid-tolerant and free from carcinogenic, mutagenic and reprotoxic chemicals. *ACS Sustainable Chem. Eng.* **2018**, *6*, 16001–16004.
- (22) Hernández, J. G.; Juaristi, E. Green Synthesis of α,β - and β,β -Dipeptides under Solvent-Free Conditions. *J. Org. Chem.* **2010**, *75*, 7107–7111.
- (23) Anselmi, M.; Stavole, P.; Boanini, E.; Bigi, A.; Juaristi, E.; Gentilucci, L. Green synthesis of bioactive oligopeptides promoted by recyclable nanocrystalline hydroxyapatite. *Future Med. Chem.* **2020**, *12*, 479–491.
- (24) Bolm, C.; Hernández, J. G. From Synthesis of Amino Acids and Peptides to Enzymatic Catalysis: A Bottom-Up Approach in Mechanochemistry. *ChemSusChem* **2018**, *11*, 1410–1420.
- (25) Métro, T.-X.; Colacino, E.; Martinez, J.; Lamaty, F. Amino Acids and Peptides in Ball Milling, in *Ball Milling Towards Green Synthesis: Applications, Projects, Challenges*. *The Royal Society of Chemistry* **2014**, 114–150.
- (26) Yves, Y.; Marion, J.; Gilles, S.; Jean, M.; Frédéric, L.; Thomas-Xavier, M. Epimerization-free C-term Activation of Peptide Fragments by Ball-Milling. *ChemRxiv* **2020**, DOI: 10.26434/chemrxiv.12593825.v1.
- (27) Elsayy, M. A.; Hewage, C.; Walker, B. Racemisation of N-Fmoc phenylglycine under mild microwave-SPPS and conventional stepwise SPPS conditions: attempts to develop strategies for overcoming this. *J. Pept. Sci.* **2012**, *18*, 302–311.
- (28) Fuse, S.; Mifune, Y.; Nakamura, H.; Tanaka, H. Total synthesis of feglymycin based on a linear/convergent hybrid approach using micro-flow amide bond formation. *Nat. Commun.* **2016**, *7*, 13491.

(29) Liang, C.; Behnam, M. A. M.; Sundermann, T. R.; Klein, C. D. Phenylglycine racemization in Fmoc-based solid-phase peptide synthesis: Stereochemical stability is achieved by choice of reaction conditions. *Tetrahedron Lett.* **2017**, *58*, 2325–2329.

(30) 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*, 418–426.

(31) For recent reviews on mechanochemistry, see: (a) Andersen, J.; Mack, J. Mechanochemistry and organic synthesis: From mystical to practical. *Green Chem.* **2018**, *20*, 1435–1443. (b) Friščić, T.; Mottillo, C.; Titi, H. M. Mechanochemistry for Synthesis. *Angew. Chem., Int. Ed.* **2020**, *59*, 1018–1029.

(32) For a recent example of temperature rise during ball milling, see: Ardila-Fierro, K. J.; Lukin, S.; Etter, M.; Užarević, K.; Halasz, L.; Bolm, C.; Hernández, J. G. Direct Visualization of a Mechanochemically Induced Molecular Rearrangement. *Angew. Chem., Int. Ed.* **2020**, *59*, 13458–13462.

(33) Han, Y.; Albericio, F.; Barany, G. Occurrence and Minimization of Cysteine Racemization during Stepwise Solid-Phase Peptide Synthesis. *J. Org. Chem.* **1997**, *62*, 4307–4312.

(34) Ostresh, J. M.; Winkle, J. H.; Hamashin, V. T.; Houghten, R. A. Peptide libraries: Determination of relative reaction rates of protected amino acids in competitive couplings. *Biopolymers* **1994**, *34*, 1681–1689.

■ NOTE ADDED AFTER ASAP PUBLICATION

References 22–25 were added January 25, 2021.