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Environmentally benign peptide synthesis using liquid-assisted ball-milling: application to the synthesis of Leu-enkephalin

Julien Bonnamour, Thomas-Xavier Métro,* Jean Martinez and Frédéric Lamaty*

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This paper describes an original methodology for peptide bond synthesis avoiding toxic solvents and reactants. Ball-milling stoichiometric amounts of Boc-protected α -amino acid *N*-carboxyanhydrides (Boc-AA-NCA) or Boc-protected α -amino acid *N*-hydroxysuccinimide esters (Boc-AA-OSu) with α -amino acid alkyl ester salts in the presence of NaHCO₃ and a minimal quantity of EtOAc led to the production of di- to penta-peptides in an efficient and environmentally benign manner. This approach was successfully applied to the synthesis of Leu-enkephalin.

Synthetic therapeutic peptides have recently regained interest as promising drug candidates, due to the rise of alternative routes of administration and development of new strategies to improve both their *in vivo* stability and bioavailability.¹ Besides this high potential, peptide production techniques suffer from the huge amount of solvent required during the synthesis and purification steps. In order to allow suitable solubilisation of the reactants as well as high reaction speed, solvents such as DMF, DMAc, NMP or DCM are commonly used.^{2,3} Unfortunately, these solvents present either high boiling point or high toxicity. In addition, peptide synthesis often requires the use of soluble organic bases such as triethylamine, *N,N*-diisopropylethylamine or piperidine for deprotonation of ammonium salts or for removal of the Fmoc protecting group. While being chemically efficient, these bases are flammable, corrosive and toxic. Thus, finding a convenient, efficient and environmentally friendly procedure is of high interest.⁴

While being focused on the complete elimination of problematic organic solvents,⁵ our group recently published a solvent-free procedure for the synthesis of di- and tri-peptides starting from Boc-protected α -amino acid *N*-carboxyanhydrides (Boc-AA-NCA), α -amino acid alkyl ester hydrochlorides (HCl·H-AA-OR) and NaHCO₃.⁶ In this strategy, the reactants were chosen for their low toxicity and homogenisation of the reaction mixture was established *via* ball-milling.⁷ This approach allowed the production of di- and tri-peptides in high yields, but with relatively low milling loads (ML).^{8,9} In a typical experiment, Boc-Phe-NCA, HCl·H-Leu-OMe and NaHCO₃ were placed in a 10 mL stainless steel jar with one 10 mm diameter ball, accounting for a milling load of 5.9 mg/mL, and after 20 min of vigorous agitation, 28 mg of the dipeptide Boc-Phe-Leu-OMe could be isolated.

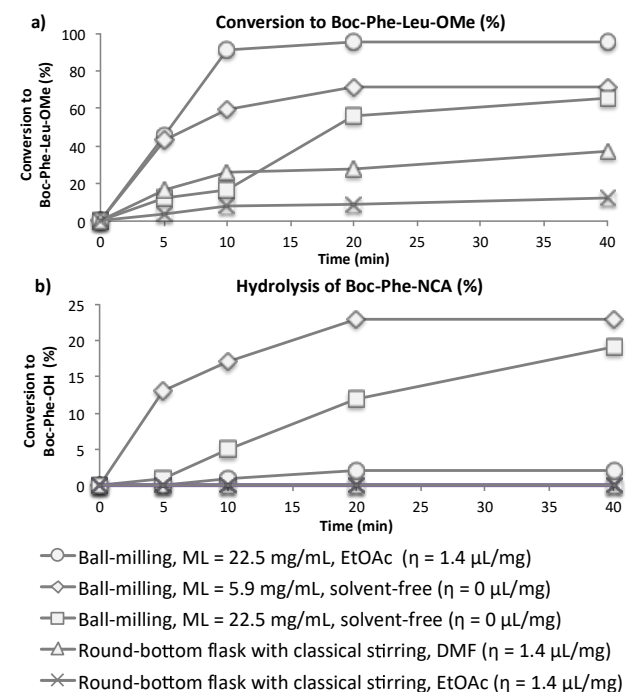
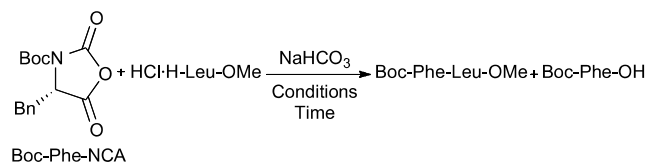


Fig. 1 Effects of agitation conditions on the reaction of Boc-Phe-NCA with HCl·H-Leu-OMe. Conversion into Boc-Phe-Leu-OMe and Boc-Phe-OH was determined by HPLC by quenching the reaction medium with BuNH₂. ML stands for milling load (mg/mL) and η for the ratio of added liquid volume to the mass of solid reactants ($\mu\text{L}/\text{mg}$).

In our quest to synthesise longer peptides in an environmentally benign fashion, we needed to set up conditions allowing the scale-up of small peptide production. The more straightforward way was to raise the milling load in the ball-mill reactor, that is to perform the reaction with more material in an identical reactor volume. Unfortunately, raising the milling load resulted in a sharp decrease in the speed of dipeptide formation. Indeed, while 43% conversion into the dipeptide Boc-Phe-Leu-OMe was obtained after 5 min agitation with a milling load of 5.9 mg/mL, only 12% conversion was reached after the same amount of time with a milling load of 22.5 mg/mL (Fig. 1a). This disappointing result could be explained by the highly viscous and sticky reaction media that would lead to mass transfer limitations when excessive material was in the reactor, thereby dramatically hampering the global speed of dipeptide formation. This hypothetical mass transfer limitation may also account for the formation of Boc-Phe-OH resulting from Boc-Phe-NCA hydrolysis (23% conversion into Boc-Phe-OH after 20 min

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reaction; Fig. 1b). At this point of the study, the sharp decrease in the speed of formation of Boc-Phe-Leu-OMe while raising up the milling load, along with the partial hydrolysis of Boc-Phe-NCA, did not support the use of this approach in the synthesis of longer peptides.

To overcome these limitations, we envisioned the use of a liquid grinding assistant that could promote the mass transfer of the reactants within the reactor.¹⁰ Following our general approach based on the twelve principles of green chemistry,¹¹ we selected ethyl acetate due to its low toxicity (EtOAc has been approved by the U. S. Food & Drug Administration as a food additive).^{12,13} While 17% conversion into the dipeptide was obtained after 10 min in solvent-free conditions with a milling load of 22.5 mg/mL, performing the same reaction in the presence of a small amount of EtOAc (1.4 μ L EtOAc per mg of reactants; $\eta = 1.4 \mu\text{L}/\text{mg}$)¹⁴ led to 91% conversion into the dipeptide after the same amount of time (Fig. 1a). Interestingly, the use of EtOAc as a grinding assistant also led to a strong minimisation of Boc-Phe-NCA hydrolysis, as less than 2% conversion to Boc-Phe-OH was observed after 20 min reaction (Fig. 1b). Besides, using EtOAc as a grinding auxiliary changed the visual aspect of the reaction medium from a highly sticky paste to a white cream-like texture. We assumed this was related to a better mixing of the reactants in the jar, resulting in a higher speed of dipeptide formation and a reduction of Boc-Phe-NCA hydrolysis. To control that EtOAc itself was not the only responsible for increasing the speed of dipeptide formation, the reaction was performed with an identical η ratio in a round-bottom flask equipped with a magnetic stirrer and placed under vigorous agitation over a magnetic stirring plate. In this case, less than 10% conversion was obtained after 20 min (Fig. 1a), indicating it was the combination of ball-mill-induced vigorous agitation and use of EtOAc as the grinding assistant that was necessary to produce the dipeptide efficiently.

Table 2 Synthesis of dipeptides applying liquid-assisted ball-milling strategy

Entry	Boc-AA-NCA or Boc-AA-OSu	AH·H-AA-OR ³	Dipeptides	Yield [%] ^a
1	Boc-Leu-NCA	<i>p</i> -TsOH·H-Leu-OBn	Boc-Leu-Leu-OBn	93
2	Boc-Val-NCA	HCl·H-Phe-OMe	Boc-Val-Phe-OMe	84
3	Boc-Leu-NCA	HCl·H-Pro-OBn	Boc-Leu-Pro-OBn	90
4	Boc-Ile-NCA	HCl·H-Ile-OMe	Boc-Ile-Ile-OMe	95
5	Boc-Gly-OSu	HCl·H-Phe-OMe	Boc-Gly-Phe-OMe	96
6	Boc-Gly-OSu	HCl·H-Pro-OBn	Boc-Gly-Pro-OBn	97
7	Boc-Tyr(Bn)-OSu	HCl·H-Leu-OMe	Boc-Tyr(Bn)-Leu-OMe	98 ^b
8	Boc-Phe-OSu	HCl·H-Phe-OMe	Boc-Phe-Phe-OMe	92
9	Boc-Phe-OSu	HCl·H-Leu-OMe	Boc-Phe-Leu-OMe	83 ^c
10	Boc-Phe-OSu	<i>p</i> -TsOH·H-Leu-OBn	Boc-Phe-Leu-OBn	90 ^d 80 ^e

^a Isolated yield. 22.0 < ML < 28.4 mg/mL and 1.1 < η < 1.4 $\mu\text{L}/\text{mg}$ unless otherwise noted. ^b Completed in 1 h. ^c > 98% diastereomeric excess determined by chiral HPLC. ^d ML = 28.4 mg/mL. $\eta = 1.1 \mu\text{L}/\text{mg}$. ^e ML = 188.8 mg/mL. $\eta =$ of 1.1 $\mu\text{L}/\text{mg}$. Completed in 2 h.

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The optimised conditions were then applied to the synthesis of a wide range of dipeptides that were obtained with good to excellent yields (Table 2, entries 1–10). α -Amino benzyl ester

Since the majority of the published peptide syntheses are performed in DMF, another assay using this solvent was realised under classical magnetic stirring with identical η ratio. After mixing the reaction medium for 20 min, 28% conversion into Boc-Phe-Leu-OMe was obtained (Fig. 1a), indicating that combination of DMF with classical mixing was less efficient than utilising EtOAc under ball-milling agitation.

Table 1 Influence of η ratio and number and size of balls on the yield

Entry	η ($\mu\text{L}/\text{mg}$) ^a	Number of balls	Yield (%) ^b
1	1.4	1 (10 mm)	95 ^c
2	0.7	1 (10 mm)	76
3	1.4	2 (10 mm)	85
4	1.4	3 (5 mm)	93

^a η = ratio of added liquid volume to the mass of solid reactants ($\mu\text{L}/\text{mg}$).

^b Isolated yield. Realised on a 0.38 mmol scale. ML = 22.5 mg/mL.

^c > 98% diastereomeric excess determined by chiral HPLC.

After having determined the optimal combination of agitation mode and grinding auxiliary for rapid formation of Boc-Phe-Leu-OMe, the latter could be isolated after 20 min of ball-milling with 95% yield (Table 1, entry 1). It is worth noting that no racemisation could be observed as the diastereomeric excess of Boc-Phe-Leu-OMe was found to be superior to 98% (Table 1, entry 1). Trying to reduce the solvent proportion to a η ratio of 0.7 $\mu\text{L}/\text{mg}$ resulted in a lower yield of 76% (Table 1, entry 2). Besides, performing the reaction with two 10 mm or three 5 mm diameter balls did not improve the process. In these conditions, Boc-Phe-Leu-OMe was isolated in 85% and 93% yield respectively (Table 1, entries 3 and 4).

⁶⁵ *para*-tosylate salts could be used as the nucleophilic counterpart instead of α -amino methyl ester hydrochlorides as Boc-Leu-Leu-OBn was isolated in 93% yield starting from *p*-TsOH·H-Leu-OBn (Table 2, entry 1). Besides, Boc-Val-Phe-OMe and Boc-Leu-Pro-OBn were synthesised in 84% and 90% yield

respectively (Table 2, entries 2 and 3), showing this approach allowed the use of bulky electrophiles and nucleophiles such as Boc-Val-NCA and HCl·H-Pro-OBn. We then envisioned to broaden the scope of this methodology by replacing the Boc-AA-NCA derivatives by the commercially available Boc-protected α -amino acid *N*-hydroxysuccinimide esters (Boc-AA-OSu).¹⁵ Due to its high water solubility, the low toxic by-product *N*-hydroxysuccinimide (HOSu) could be easily separated from the reaction media by simple aqueous extraction. Good to excellent yields were obtained when Boc-protected α -amino acid *N*-hydroxysuccinimide esters were used as starting material (Table 2, entries 5–10). While Boc-Gly-NCA was not a suitable substrate for the synthesis of dipeptides in previously described conditions,⁶ Boc-Gly-OSu furnished Boc-Gly-Phe-OMe and Boc-Gly-Pro-OBn with 96% and 97% yield respectively when treated with HCl·H-Phe-OMe and with the structurally constrained HCl·H-Pro-OBn (Table 2, entries 5 and 6). Similarly, Boc-Tyr(Bn)-OSu furnished Boc-Tyr(Bn)-Leu-OMe with 98% yield but required an hour of reaction (Table 2, entry 7). Likewise Boc-Phe-NCA, no racemisation could be observed when utilising

Boc-Phe-OSu, as the diastereomeric excess of Boc-Phe-Leu-OMe was found to be superior to 98% (Table 2, entry 9). When treating Boc-Phe-OSu with stoichiometric amounts of *p*-TsOH·H-Leu-OBn and NaHCO₃ in the presence of EtOAc, accounting for a η ratio of 1.1 μ L/mg and a milling load of 28.4 mg/mL, Boc-Phe-Leu-OBn was isolated with 90% yield (Table 2, entry 10). Interestingly, keeping an identical η ratio of 1.1 μ L/mg while raising the milling load up to 188.8 mg/mL did not dramatically hamper the reaction as Boc-Phe-Leu-OBn could be isolated in 80% yield after 2 h of ball-milling (Table 2, entry 10). By proceeding this way, 796 mg of the dipeptide could be produced using a simple vibrational ball-mill equipped with a small 10 mL jar perfectly adapted to a classical laboratory fume hood. This result clearly opened the path to the synthesis of longer peptides using only the non-hazardous reactants¹⁶ that are Boc-protected α -amino acid *N*-hydroxysuccinimide esters (Boc-AA-OSu), α -amino acid alkyl ester salts and NaHCO₃, combined with minimal amounts of EtOAc or *t*BuOAc which are considered as preferred solvents according to the sustainable chemistry standards.¹²

Table 3 Synthesis of tri-, tetra- and penta-peptides applying liquid-assisted ball-milling strategy

Entry	Boc-AA-NCA or Boc-AA-OSu	HCl·H-(AA) _x -OR ²	Product	Yield [%] ^a
1	Boc-Leu-NCA	HCl·H-Leu-Leu-OBn	Boc-Leu-Leu-Leu-OBn	79
2	Boc-Phe-OSu	HCl·H-Phe-Phe-OMe	Boc-Phe-Phe-Phe-OMe	86
3	Boc-Lys(Boc)-OSu	HCl·H-Gly-Pro-OBn	Boc-Lys(Boc)-Gly-Pro-OBn	79
4	Boc-Trp-NCA	HCl·H-Val-Phe-OMe	Boc-Trp-Val-Phe-OMe	86
5	Boc-Ile-NCA	HCl·H-Leu-Pro-OBn	Boc-Ile-Leu-Pro-OBn	86
6	Boc-Ile-NCA	HCl·H-Ile-Ile-OMe	Boc-Ile-Ile-Ile-OMe	86
7	Boc-Gly-OSu	HCl·H-Gly-Phe-OMe	Boc-Gly-Gly-Phe-OMe	74
8	Boc-Gly-OSu	HCl·H-Phe-Leu-OBn	Boc-Gly-Phe-Leu-OBn	93 ^b (94) ^c
9	Boc-Leu-NCA	HCl·H-Leu-Leu-Leu-OBn	Boc-Leu-Leu-Leu-Leu-OBn	96
10	Boc-Gly-OSu	HCl·H-Gly-Phe-Leu-OBn	Boc-Gly-Gly-Phe-Leu-OBn	96 ^d (90) ^e
11	Boc-Tyr(Bn)-OSu	HCl·H-Gly-Gly-Phe-Leu-OBn	Boc-Tyr(Bn)-Gly-Gly-Phe-Leu-OBn	88 ^f

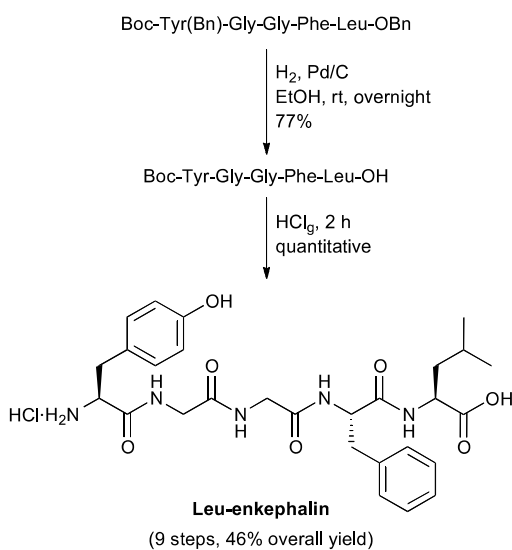
^a Isolated yield. 6.6 < ML < 8.4 mg/mL and 1.3 < η < 1.6 μ L/mg unless otherwise noted. ^b ML = 22.9 mg/mL. ^c ML = 152.7 mg/mL. Completed in 40 min with *t*BuOAc as the grinding auxiliary. ^d ML = 22.2 mg/mL, η = 1.1 μ L/mg. ^e ML = 60.6 mg/mL, η = 1.1 μ L/mg. Completed in 40 min with *t*BuOAc as the grinding aid. ^f Completed in 1h.

To evaluate the environmental impact of this methodology, the Ecoscale score of Boc-Tyr(Bn)-Leu-OMe synthesis was calculated and compared to the score of traditional protocols used to synthesise peptides.¹⁷ When realised starting from Boc-Tyr(Bn)-OSu using Et₃N as the base and DMF as the solvent, the synthesis of Boc-Tyr(Bn)-Leu-OMe obtained an Ecoscale score of 69, which corresponds to an acceptable synthesis.^{18,19} When using our methodology, that is replacement of Et₃N and DMF with NaHCO₃ and EtOAc combined with a ball-mill induced homogenisation of the reaction mixture, the Ecoscale score reached 84, which corresponds to an excellent synthesis.¹⁷ As a comparison, the commonly used protocol using Boc-Tyr(Bn)-OH, EtN(*i*Pr)₂, BOP reagent and HCl·H-Leu-OMe in DMF under classical agitation accounted for an Ecoscale score of 58.²⁰

In order to synthesise tripeptides using this methodology,²¹ previously obtained Boc-dipeptides required to be deprotected.

By blowing gaseous HCl directly on Boc-dipeptides without using any solvent,⁶ the corresponding dipeptide hydrochloride salts could be obtained in quantitative yields. The latter were then treated with a variety of either Boc-AA-NCA or Boc-AA-OSu to furnish tripeptides in good to excellent yields (Table 3, entries 1–8). The apolar tripeptides Boc-Leu-Leu-Leu-OBn and Boc-Phe-Phe-Phe-OMe were isolated in 79% and 86% yield respectively (Table 3, entries 1 and 2). Besides, Boc-Lys(Boc)-Gly-Pro-OBn and Boc-Trp-Val-Phe-OMe were produced in 79% and 86% yield, showing that side chain heteroatom-containing tripeptides could also be synthesised in good yields (Table 3, entries 3 and 4). Utilising bulky electrophiles was not an obstacle for the synthesis of tripeptides as treating HCl·H-Leu-Pro-OBn and HCl·H-Ile-Ile-OMe with Boc-Ile-NCA furnished both Boc-Ile-Leu-Pro-OBn and Boc-Ile-Ile-Ile-OMe with 86% yield (Table 3, entries 5 and 6). Less encumbered Boc-Gly-OSu furnished Boc-

Gly-Gly-Phe-OMe and Boc-Gly-Phe-Leu-OBn with 74% and 93% yield respectively (Table 3, entries 7 and 8). It is worth noting that Boc-Gly-Phe-Leu-OBn was synthesised with a high milling load of 152.7 mg/mL, furnishing 937 mg (94% yield) of the tripeptide (Table 3, entry 8). After transient HCl_g-mediated deprotection, the two tetrapeptides Boc-Leu-Leu-Leu-Leu-OBn and Boc-Gly-Gly-Phe-Leu-OBn were both isolated with an excellent yield of 96% (Table 3, entries 9 and 10). Finally, treatment of the tetrapeptide HCl-H-Gly-Gly-Phe-Leu-OBn with Boc-Tyr(Bn)-OSu furnished the pentapeptide Boc-Tyr(Bn)-Gly-Gly-Phe-Leu-OBn in 88% yield (Table 3, entry 11). To the best of our knowledge, these are the first syntheses of tetra- and pentapeptides based on the use of small amounts of green solvents with stoichiometric amounts of non-hazardous reactants.^{12,16} Besides, the pentapeptide Boc-Tyr(Bn)-Gly-Gly-Phe-Leu-OBn corresponds to the primary structure of Leu-enkephalin, which is an endogenous ligand binding the opioid receptors and has a salient role in processing sensory information in the brain.²² It has been isolated and synthesised for the first time by Hughes and coworkers in 1975.²³ For our part, we completed the Leu-enkephalin total synthesis by hydrogenation of the benzyl moieties under H₂ atmosphere followed by gaseous HCl-mediated deprotection of the Boc group (Scheme 1).



Scheme 1 Synthesis of Leu-enkephalin

Using this strategy, the synthesis of Leu-enkephalin hydrochloride salt was achieved in nine steps starting from Leucine benzyl ester *p*-tosylate salt, with an overall yield of 46%. Leu-enkephalin synthesis was realised based on:

- Coupling steps using only non-hazardous reactants¹⁶ and small amounts of EtOAc or *t*BuOAc, which are considered as preferred solvents according to green chemistry standards.¹²

- Solvent-free Boc deprotection steps.

In conclusion, the presented methodology based on liquid-assisted ball-milling is a very efficient and environmentally benign route for the synthesis of di-, tri-, tetra- and pentapeptides. Optimisation of the reaction conditions has enabled the synthesis of a tripeptide and a tetrapeptide close to the gram scale, allowing the synthesis of the pentapeptide Leu-enkephalin in high overall yield.

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Notes and references

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† Electronic Supplementary Information (ESI) available: General experimental procedures and characterisation data of all compounds. See DOI: 10.1039/b000000x/

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