Epimerization-free C-term Activation of Peptide Fragments by Ball-Milling

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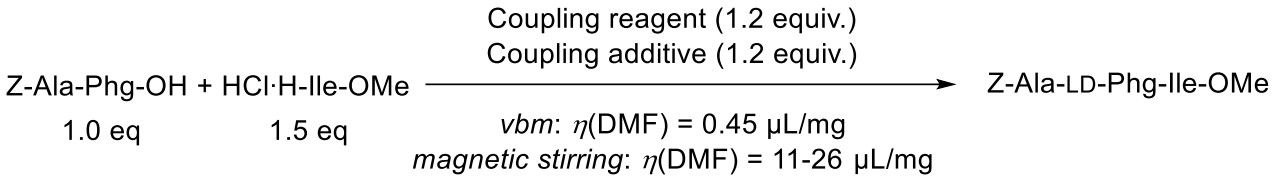
Abstract: Ball-milling enabled to perform [2+1], [2+2], and [2+3] peptide couplings with high yields and, if any, very low epimerization. Very good results were obtained with peptide fragments containing highly epimerization-prone and/or highly hindered amino acids at C-term such as phenylglycine, cysteine 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 mediated by ball-milling.

Peptides can be found in many and diverse applications as nutriments, cosmetics and pharmaceuticals. In the field of medicinal chemistry, they can act as therapeutics and diagnostic agents, including for the fight against the Covid-19 disease.[1-2] Over the years, interest in this class of compounds has increased at a steady rate,[3-7] due to their good biological activities at very low doses, high selectivity, and high probability of success at clinical trial stages.[4, 8] In 2019, the FDA has estimated that approximately 100 peptide drugs were marketed in the U.S., Europe, and Japan, accounting for annual global sales ranging between $15 billion to $20 billion.[6] Among these peptide-based drugs, we can highlight Macrilen, which was discovered in our institute (IBMM) in 1999 and that was recently approved by both FDA and EMA to be used in the diagnosis of patients with adult growth hormone deficiency.[9] When the stepwise synthesis of low molecular weight peptides do not generally present any difficulty, the chemical synthesis of longer peptides and proteins is a much more 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. These difficulties have certainly slowed down, if not prevented, commercialization of some long peptide chains, and may also explain why recombinant protein strategy is generally preferred when sequences of more than 60 amino acid residues are targeted.[10] Significant efforts have been deployed by peptide scientists to tackle this challenge, mainly by using convergent fragment coupling approaches, in particular using native chemical ligation (NCL) of peptides fragments.[11-13] Yet, these approaches suffer from a limited scope: peptide bond disconnections cannot be performed at any point of the peptide chain. For instance, a N-terminal cysteine or an amino acid modified by a thiol auxiliary are required to perform NCL. 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.[14] This hurdle can be overcome: some examples are reporting peptide fragment couplings with low to null epimerization levels.[15-20] However, these examples remain relatively scarce. In this context, and based on our knowledge of peptide synthesis under solvent-less/solvent-free conditions by mechanochemical means,[21-26] 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.

The [2+1] coupling between Z-Ala-Phg-OH and HCl·H-Ile-OMe was considered as a probe of choice.[27] Indeed, phenylglycine (Phg) is known as being one of the most epimerization-prone amino acid,[14, 17, 28-31] while isoleucine is a hindered amino acid exhibiting low coupling speeds.[32] For this study, the target dipeptide Z-Ala-Phg-OH was prepared using Z/*t*Bu orthogonal strategy (see ESI for details). Then, Z-Ala-Phg-OH was reacted with HCl·H-Ile-OMe (1.5 equiv.), in the presence of EDC·HCl (1.2 equiv.), Oxyma (1.2 equiv.) and a small amount of DMF (η = 0.45 µL/mg) in a 5 mL PTFE ball-mill reactor containing three stainless steel balls (5 mm diameter), for 10 min at 25 Hz (see ESI for details). Here, the amount of DMF was not sufficient to solubilize the reaction mixture but improved the homogeneity of the reaction mixture.[33] Although being a highly problematic solvent,[34] DMF is considered as the most widely used solvent for peptide synthesis.[35-37] 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 solvent quantities that were used.[38] After reaction mixture recovery from the milling reactor and work-up, 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 HPLC (Entry 1, Table 1; t_R(LLL) = 5.08 min and t_R(LDL) = 5.20 min, see ESI for details).



Entry	Reagents	Temp (°C)	t (min)	Yield (%)	Purity (%)[a,b]	LDL (%)[b]
1	EDC·HCl/Oxyma	33 (33)	10 (30)	93 (88)	> 99 (32)	< 1 (9)
2	EDC·HCl/HOBt·H_2O	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.)
6	HATU/Et_3N	34 (34)	10 (60)	85 (88)	88 (58)	1 (< 1)
7	HBTU/Et_3N	33 (33)	10 (20)	86 (82)	71 (55)	2 (9)

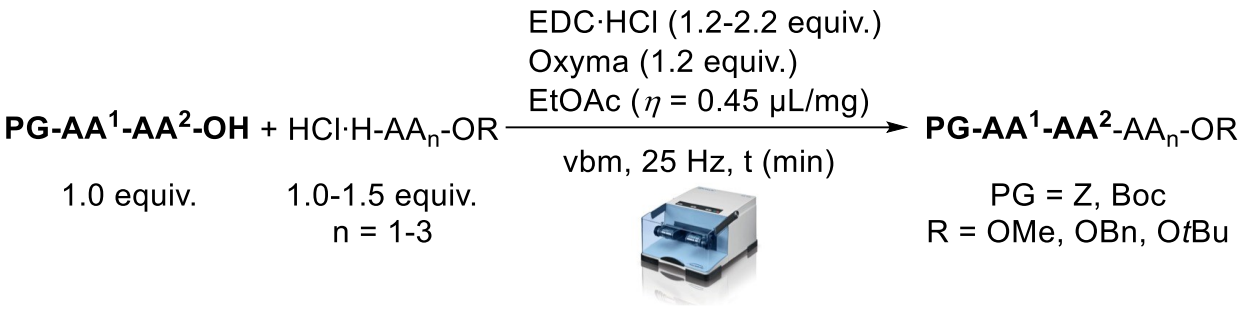
[a] Purity determined by HPLC and calculated as the surface of the LLL HPLC peak over all the other peaks, including the LDL peak. [b] Determined by HPLC. [c] n.d.: not determined.

Table 1: Comparative study between vibrating ball-milling (main figures) and solution synthesis (in parentheses) of Z-Ala-Phg-Ile-OMe

Ball-milling is known to induce a slight temperature raise of the milled materials.[39-41] As the temperature inside the reactor could have an influence on the reaction course, the temperature of the milled material was measured directly after milling. During the coupling under ball-milling conditions, the temperature reached 33 °C. 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 (η = 20 µL/mg). In these experimental conditions, Z-Ala-Phg-Ile-OMe was produced in 88% yield, 32% purity and 9% of the LDL epimer (Entry 1, figures in parentheses, Table 1). These results clearly showed that solvent-less ball-milling could minimize epimerization of a highly epimerization-prone C-term activated peptide fragment. Additionally, 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 generality of our observations. When couplings were performed in solution, the quantity of DMF was kept as low as possible to ensure maximal concentration of the reactants while enabling proper mixing under classical magnetic stirring ($11 < \eta < 26$ µL/mg). When Oxyma was replaced with HOBt·H_2O, 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 (Entry 2, Table 1). The use of HOAt gave better results than HOBt·H_2O; only 1% of the LDL epimer was produced in the ball-milling conditions while 26% LDL epimer were obtained after synthesis in solution (Entry 3, Table 1). These results were in agreement with previous publications comparing HOBt·H_2O and HOAt.[42-43] 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%) (Entry 4, Table 1). These results obtained with DIC were not improved when replacing HOAt with Oxyma (Entry 5, Table 1). Concerning the stand-alone coupling reagents HBTU and HATU, their use induced low epimerization levels both by ball-milling and in solution (Entries 6 & 7, Table 1). Yet, the HPLC purity profiles of the products obtained by ball-milling were much better than when synthesized in solution. The comparison between ball-milling and solution-phase 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.

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 (Entry 1, Table 2).



Entry	Peptides	Time (min)	Isolated yield (%)	de (%)
1	**Z-Ala-Phg**-Phe-OMe	20	89[a]	> 99
2	**Z-Ala-D-Phg**-Phe-OMe	10	92[a]	> 99
3	**Z-Ala-Cys(Bn)**-Ala-OMe	30	98[a]	> 99
4	**Z-Ala-Cys(Bn)**-Phe-OMe	20	94[a]	> 99
5	**Z-Phe-Val-Cys(Bn)**-OMe	30	98[a,b]	> 99
6	**Z-Phe-D-Val**-Cys(Bn)-OMe	30	97[a]	> 99
7	**Z-Phe-Val**-Ser(*t*Bu)-O*t*Bu	30	98[a,b]	> 99
8	**Z-Phe-D-Val**-Ser(*t*Bu)-O*t*Bu	20	97[a]	> 99
9	**Boc-Trp-Phe**-Glu(Bn)-OBn	20	92[a]	> 99
10	**Boc-Trp-Phe**-Gly-OBn	10	84[a]	> 99
11	**Boc-Trp-D-Phe**-Gly-OBn	10	95[a]	> 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

[a] 1.5 equiv. of HCl·H-AA$_n$-OR were used. [b] 1.2 equiv. of EDC·HCl were used. [c] 1.0 equiv. of HCl·H-AA$_n$-OR were used. [d] 2.2 equiv. of EDC·HCl and η (EtOAc) = 0.9 µL/mg were used.

Table 2: Scope of the peptide couplings by ball-milling

When Z-Ala-D-Phg-OH was used instead of Z-Ala-L-Phg-OH, the tripeptide Z-Ala-D-Phg-Phe-OMe was isolated with a similar yield of 92% and excellent purity (Entry 2, Table 2). Cysteine, which is another epimerization-prone amino acid,[14, 44] was next tested under

the same experimental conditions. Thus, Z-Ala-Cys(Bn)-Ala-OMe and Z-Ala-Cys(Bn)-Phe-OMe were synthesized from Z-Ala-Cys(Bn)-OH. In both cases, tripeptides were obtained in excellent yields (> 94%) without any detectable epimerization (Entries 3 & 4, Table 2). Like isoleucine, valine is a *β*-branched amino acid known to be more difficult for coupling than most of other amino acids.[32] 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 minutes ball-milling (Entry 5, Table 2). 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 (Entry 6, Table 2). 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 (Entries 7 & 8, Table 2). 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. In order to evaluate the coupling of Boc-protected fragments, Boc-Trp-Phe-Glu(OBn)-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 (Entries 9-11, Table 2). These results supported that ball-milling conditions were compatible with Boc *N*-protecting groups, without affecting the isolated yields and diastereomeric excesses of the expected products. The presence of benzyl groups was not an obstacle to the success of these syntheses.

After clearly demonstrating the efficiency of our method in epimerization-free [2+1] peptide couplings, we aimed at demonstrating that peptide fragment couplings mediated by ball-milling could be applied to longer peptide fragments. For this, [2+2] and [2+3] couplings involving peptide fragments bearing the hindered valine at C-term were performed. When the optimal experimental reaction conditions were applied to the coupling of Z-Phe-Val-OH with HCl·H-Leu$_2$-OBn, the expected tetrapeptide was obtained in 95% yield without epimerization (Entry 12, Table 2). The same conditions applied to the synthesis of the diastereomer Z-Phe-D-Val-Leu$_2$-OBn yielded the desired tetrapeptide in 91%, yet containing 2% of the epimer (Entry 13, Table 2). To our delight, when the tripeptide HCl·H-Leu$_3$-OBn was reacted with Z-Phe-Val-OH and Z-Phe-D-Val-OH, the corresponding pentapeptides Z-Phe-Val-Leu$_3$-OBn and Z-Phe-D-Val-Leu$_3$-OBn were successfully isolated with high yields (> 91%) and no to negligible level of epimerization

(Entries 14 & 15, Table 2). So far, the longest peptide synthesized by ball-milling was the pentapeptide Leu-enkephalin.[22] The successful synthesis of these two pentapeptides by a fragment coupling approach consolidates the potential of mechanochemistry to produce oligopeptides with high efficiency. Overall, the lower levels of epimerization observed under ball-milling conditions could find explanations in the concentrations of the reaction mixtures. Indeed, epimerization during peptide couplings occurs either through direct αH-abstraction in basic medium and/or through the formation of an oxazolone ring during activation.[14] Of note, the latter mechanism is an intramolecular process favored in diluted conditions, whereas “direct” peptide coupling (in other words, acylation of the free amine) is an intermolecular process, comparatively favored in concentrated conditions such as those enabled by ball-milling. Consequently, our results suggest that the absence of epimerization could be explained by the highly concentrated reaction conditions enabled by ball-milling, that would prevent the transient formation of an oxazolone ring.

To conclude, using the combination of EDC·HCl, Oxyma and small amounts of a liquid additive (DMF or EtOAc) under ball-milling conditions allowed to perform [2+1], [2+2], and [2+3] peptide 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 *ii)* applying this strategy to longer peptide fragments are currently being explored in our laboratory and will be reported in due course.

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Abbreviations

DIC	*N*,*N*′-diisopropylcarbodiimide
EDC·HCl	*N*-(3-Dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride
EMA	European Medicines Agency
FDA	Food and Drug Administration
HATU	*N*-[(Dimethylamino)-1H-1,2,3-triazolo-[4,5-b]pyridin-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate N-oxide
HBTU	*N*,*N*,*N*′,*N*′-Tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate
HOAt	1-Hydroxy-7-azabenzotriazole
HOBt·H_2O	1-Hydroxybenzotriazole hydrate
Oxyma	Ethyl hydroxyiminocyanoacetate
vbm	Vibrating ball-milling

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