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# N-Acyl Benzotriazole Derivatives for the Synthesis of Dipeptides and Tripeptides and Peptide Biotinylation by Mechanochemistry

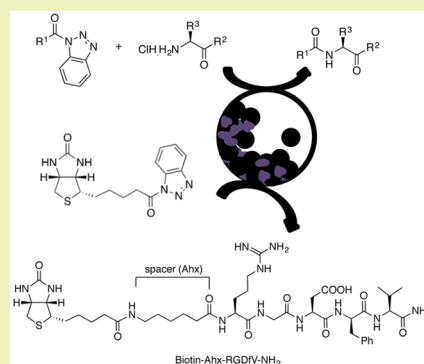
Lori Gonnet,<sup>†</sup> Thibault Tintillier,<sup>‡</sup> Natacha Venturini,<sup>†</sup> Laure Konnert,<sup>§,†</sup> Jean-François Hernandez,<sup>‡</sup> Frédéric Lamaty,<sup>\*,†</sup> Guillaume Laconde,<sup>\*,‡</sup> Jean Martinez,<sup>†,‡</sup> and Evelina Colacino<sup>\*,†</sup> 

<sup>†</sup>Université de Montpellier, Institut des Biomolécules Max Mousseron (IBMM), UMR 5247 CNRS-UM-ENSCM, Place E. Bataillon, Campus Triolet, cc 1703, 34095 Montpellier, France

<sup>‡</sup>Institut des Biomolécules Max Mousseron, UMR 5247 CNRS-Université de Montpellier-ENSCM, Faculté de Pharmacie, 15 avenue Charles Flahault, 34093 Montpellier Cedex 5, France

**ABSTRACT:** An eco-friendly methodology for preparing Fmoc-, Z-, and Boc-N-protected dipeptides and tripeptides is described, from the corresponding N-protected- $\alpha$ -aminoacyl benzotriazoles and  $\alpha$ -amino acid derivatives, with different C-terminal functionalities such as esters or amides, using vibrational ball-mill (VBM). The reactivity of a  $\beta$ -amino ester was also investigated. In some cases, the coupling was achieved by liquid-assisted grinding (LAG).  $\alpha,\alpha$ - and one  $\alpha,\beta$ -dipeptide were obtained in good to excellent yields mainly by precipitation in water, resulting in an improved environmental impact compared to classical peptide synthesis in solution, as shown by green metric calculations. The method was extended to the biotinylation, via an aminohexanoyl spacer, of the pentapeptide RGDfV, which contains the well-known integrins recognition site arginine–glycine–aspartic acid (RGD) motif.

**KEYWORDS:** Mechanochemistry, Ball-milling, Liquid-assisted grinding (LAG), Solvent-free, Benzotriazole, Amino esters, Peptides, Biotine, RGD



## INTRODUCTION

One possible way to prepare organic molecules, pharmaceutically relevant fragments, and functionalities by green methods and technologies is based on the use of mechanochemistry, which is a successful approach to perform solvent-free chemical synthesis.<sup>1–6</sup> Furthermore, ball-milling technology has been efficiently applied to the preparation of active pharmaceutical materials and ingredients (API),<sup>7</sup> but scarcely exploited for the derivatization of biological molecules and their analogues. This approach points into the direction of a modern and sustainable pharmaceutical industry, aiming to perform medicinal synthesis via mechanochemical procedures, an emerging field termed “medicinal mechanochemistry”.<sup>7</sup>

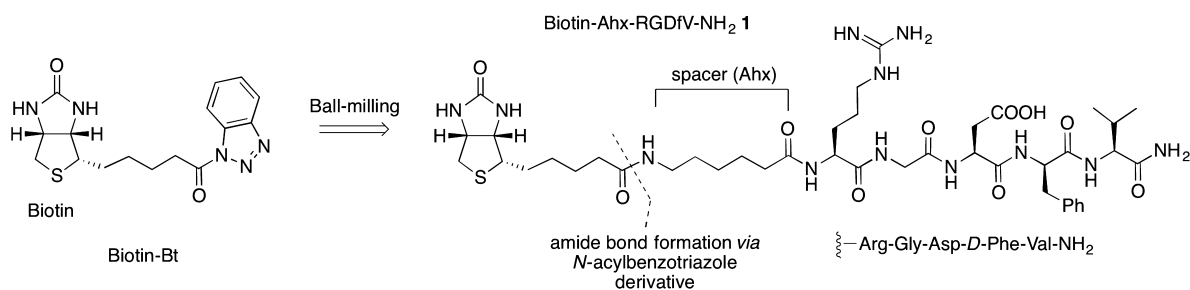
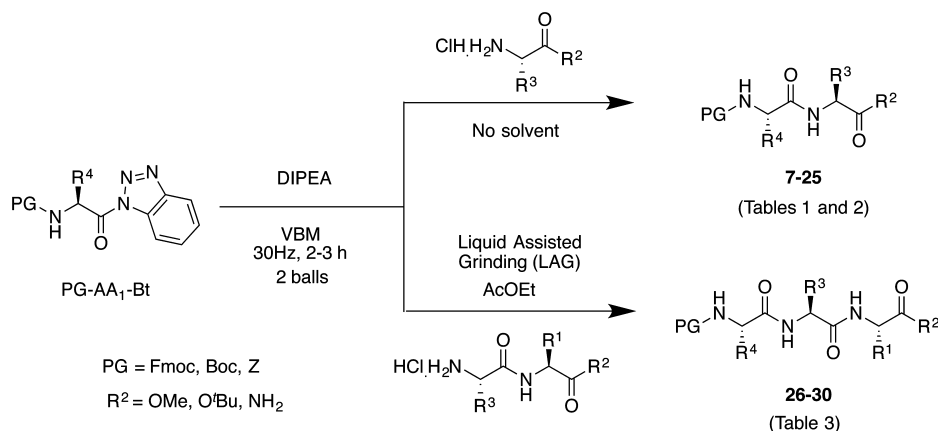
Since our earlier paper on the solvent-free synthesis of the antiaging additive for the cosmetics industry (phenyl-*tert*-butyl-nitron, PBN),<sup>8</sup> we have recently disclosed new pathways for the mechanochemical preparation of dipeptide mimics, hydantoin-based drugs: the antiepileptic phenytoin (Phenytek),<sup>9</sup> the anticonvulsant Ethotoin (Peganone), or an antibacterial agent for polymer textiles,<sup>10</sup> starting from  $\alpha$ -amino acid derivatives. In our ongoing interest in the development of eco-friendly procedures for the selective protection of  $\alpha$ -terminal<sup>11–13</sup> or  $\alpha$ -carboxylic<sup>12</sup> groups of amino acid derivatives, yielding useful building blocks for peptide synthesis in solution,<sup>14</sup> 1,1'-carbonyldiimidazole (CDI)

proved to be a safe, versatile acyl transfer agent for the N-derivatization of amino esters by mechanochemistry.<sup>10,13,15–17</sup>

Similarly to CDI, acylbenzotriazoles are also versatile reagents in which the benzotriazol-1-yl (Bt) group serves as a substitute agent for halogen and is easily displaced by nitrogen, sulfur, oxygen, or carbon nucleophiles. Therefore, N-acylbenzotriazoles offer numerous advantages over their halogen analogues, since they are isolated in high yields, in crystalline form, are usually stable in air (and even to water for short periods at 20 °C), and are more reactive than the corresponding N-acylimidazoles.<sup>18–20</sup> Recently, diverse benzotriazole amino acids and derivatives such as biotin-benzotriazole became commercially available.<sup>21,22</sup> Peptides,<sup>23,24</sup> pseudo-peptides,<sup>25</sup> and cyclic peptides<sup>26</sup> have been synthesized in solution and on solid support, using amino acid benzotriazoles as active derivatives.

In this context, and having acquired expertise on the mechanochemical reactivity of CDI to prepare hydantoin<sup>10,16</sup> or carbamates,<sup>13</sup> the reactivity of N-protected  $\alpha$ -aminoacyl benzotriazole derivatives toward amino acid or peptide derivatives was investigated in a ball mill (Scheme 1). To the best of our knowledge, the mechanochemical reactivity of N-

**Scheme 1. General Approach for the Synthesis of Peptides from N-(Protected- $\alpha$ -Amino Acyl) Benzotriazoles**



**Figure 1.** Biotinylated linear pentapeptide amide RGDfV (**1**), incorporating an aminohexanoyl (Ahx) spacer.

**Table 1. Screening of Mechanochemical Conditions for the Acylation Reaction of Amino Esters by Acyl-Bt Derivatives<sup>a</sup>**

entry	R <sup>1</sup>	H-AA-R <sup>2</sup>	base	product	time, <i>t</i> (h)	yield <sup>b</sup> (%)
1	PhCH <sub>2</sub> CH <sub>2</sub> ( <b>2</b> )	H-Phe-OMe		<b>3</b>	2	71
2			Et <sub>3</sub> N	<b>3</b>	2	71
3		H-Phe-O'Bu	Et <sub>3</sub> N	<b>4</b>	1	88
4			DIPEA	<b>4</b>	3	77
5		H-Leu-O'Bu	NaHCO <sub>3</sub>	<b>5</b>	1	n.r. <sup>c</sup>
6			Et <sub>3</sub> N	<b>5</b>	1	88
7			DIPEA	<b>5</b>	3	99
8		H-Ser( <sup>t</sup> Bu)-O'Bu	caffeine	<b>6</b>	1	n.r. <sup>c</sup>
9			K <sub>2</sub> CO <sub>3</sub>	<b>6</b>	2	99
10	Fmoc-Phe-	H-Phe-O'Bu	DIPEA	<b>7</b>	3	23
11	Fmoc-Leu-	H-Phe-O'Bu	DIPEA	<b>8</b>	2.5 ( <b>2</b> ) <sup>d</sup>	77 (83) <sup>d</sup>

<sup>a</sup>Typical procedure: *N*-acyl-benzotriazole (R<sup>1</sup>COBt, 1 equiv) HCl·H-AA-R<sup>2</sup> (1 equiv) and base (2 equiv) were milled using 5 mL stainless steel jar and 2 stainless steel balls (5 mm diameter) in a vibrational ball-mill (VBM) at 30 Hz. <sup>b</sup>Yields of isolated products after workup (see the [Supporting Information](#)). <sup>c</sup>n.r. = no reaction. <sup>d</sup>The reaction was performed in a planetary ball mill (PBM) in a 12 mL stainless steel jar with 25 balls (5 mm in diameter) at 450 rpm.

acyl-benzotriazole is still largely unexplored and only bis-(benzotriazolyl)methanethione<sup>27</sup> and thiocarbonyl benzotriazoles<sup>27,28</sup> were reported to react with amines to yield thioureas. After having explored the mechanochemical reactivity of benzotriazole amino acid derivatives for the preparation of dipeptides or tripeptides, the methodology was extended to the preparation of a biotinylated peptide (Biotin-Ahx-RGDfV-NH<sub>2</sub> (**1**), see [Figure 1](#)), containing the well-known arginine–glycine–aspartic acid (RGD)-motif, which is the main recognition site of

the cell adhesion integrin receptors.<sup>29</sup> This unprecedented mechanochemical approach opens up new perspectives for the derivatization of biological molecules.

## RESULTS AND DISCUSSIONS

**Screening of the Reaction Conditions.** Considering the complexity of the Biotin-Ahx-RGDfV-NH<sub>2</sub> (**1**), optimization of the reaction conditions was investigated starting from a model involving easily available reactants. The stoichiometric reaction

**Table 2. Reaction Scope for the Preparation of Protected Dipeptides in a Vibrational Ball Mill (VBM)<sup>a</sup>**

entry	PG-AA <sub>1</sub> -Bt	HCl·H-AA <sub>2</sub> -R <sup>2</sup>	product	time, <i>t</i> (h)	yield <sup>b</sup> (%)
1	Fmoc-Leu-Bt	H-Leu-O <sup>t</sup> Bu	9	2	78
2		H-Ser( <sup>t</sup> Bu)-O <sup>t</sup> Bu	10	2	80
3		H-Phe-NH <sub>2</sub>	11	1	91
4		H-β-Ala-O <sup>t</sup> Bu	12	2	67
5	Fmoc-Phe-Bt	H-Leu-O <sup>t</sup> Bu	13	2 <sup>c</sup>	58 <sup>c</sup>
6		H-Ser( <sup>t</sup> Bu)-O <sup>t</sup> Bu	14	2	42 (33) <sup>c</sup>
7	Fmoc-Trp(Boc)-Bt	H-Phe-O <sup>t</sup> Bu	15	2	52
8		H-Leu-O <sup>t</sup> Bu	16	2	56 (19) <sup>c</sup>
9		H-Ser( <sup>t</sup> Bu)-O <sup>t</sup> Bu	17	2	37 (37) <sup>c</sup>
10	Fmoc-Cys(Trt)-Bt	H-Ser( <sup>t</sup> Bu)-O <sup>t</sup> Bu	18	2	95
11	Z-Phe-Bt	H-Phe-O <sup>t</sup> Bu	19	2	81
12		H-Ser( <sup>t</sup> Bu)-O <sup>t</sup> Bu	20	2	79
13	Boc-Phe-Bt	H-Phe-O <sup>t</sup> Bu	21	3	58
14		H-Leu-O <sup>t</sup> Bu	22	3	30
15		H-Ser( <sup>t</sup> Bu)-O <sup>t</sup> Bu	23	3	20
16	Boc-Leu-Bt	H-Phe-O <sup>t</sup> Bu	24	3	67
17	Boc-Trp(CHO)-Bt	H-Ser( <sup>t</sup> Bu)-O <sup>t</sup> Bu	25	3.5 <sup>d</sup>	98 <sup>d</sup>

<sup>a</sup>Typical procedure: PG-AA<sub>1</sub>-Bt (1 equiv) HCl·H-AA<sub>2</sub>-R<sup>2</sup> (1 equiv) and DIPEA (2 equiv) were milled using 5 mL stainless steel jar and two stainless steel balls (5 mm in diameter) in a vibrational ball-mill (VBM) at 30 Hz. <sup>b</sup>Isolated yields after workup (see the Supporting Information). <sup>c</sup>Milling was performed by adding 15 μL of AcOEt. <sup>d</sup>The reaction mixture was milled twice (2 h, then 1.5 h).

between benzotriazolyl activated hydrocinnamic acid **2** and *L*-phenylalanine methyl ester hydrochloride was investigated for optimization studies (see Table 1).

Full conversion of the substrates was observed in two parallel experiments that were performed either in the absence of base (entry 1), or using triethylamine (entry 2), leading to good yields after workup. The final product **3** was easily recovered after precipitation in water and filtration, allowing the easy elimination of benzotriazole (HBt) (entries 1 and 2) and triethylamine hydrochloride (entry 2) as the only byproducts of the reaction. Considering that no difference in yields was observed, and to minimize the amount of reactants, the study was pursued without any base.

Disappointingly, the reaction was slow when amino acid *tert*-butyl esters were used, instead of methyl ester hydrochlorides (entries 3–9 in Table 1), in the absence of a base. Thus, a base was necessary for the *in situ* formation of the nucleophilic amine from its hydrochloride salt. This result is in agreement with our previous findings using CDI,<sup>13</sup> excluding the possibility of an autocatalyzed/base regenerating system relying on the progressive liberation of benzotriazole (HBt) during the milling. Several bases were tested while screening various amino esters: no reaction was observed with sodium bicarbonate (entry 5) or caffeine (entry 8), while triethylamine (entries 3 and 6), *N,N*-diisopropylethylamine (DIPEA, entries 4 and 7), and potassium carbonate (entry 9) were similarly effective, affording good yields of compounds 4–6.

Moreover, when *N*-(Fmoc- $\alpha$ -amino acyl) benzotriazole derivatives such as Fmoc-Phe-Bt (entry 10) or Fmoc-Leu-Bt (entry 11 in Table 1) were used, in the presence of DIPEA, the corresponding dipeptides **7** and **8** were obtained in moderate to acceptable yields. Indeed, incomplete conversion of substrate Fmoc-Phe-Bt was observed in the presence of triethylamine, while the use of K<sub>2</sub>CO<sub>3</sub>, provoked partial deprotection of the Fmoc-protecting group, not observed in the presence of DIPEA. In the case of Fmoc-Leu-Phe-O<sup>t</sup>Bu (**8**), the reaction was investigated using both a vibrational ball mill (VBM) and a planetary ball mill (PBM) (entry 11): a slightly better yield was obtained using the planetary milling for reduced reaction times.

Different mechanical stress (horizontal or circular movements) and higher stress frequency for the planetary milling could be the reason for an improved reactivity.

**Reaction Scope. Synthesis of Dipeptides.** The investigation of the reactivity profile for the *N*-protected- $\alpha$ -amino acyl benzotriazole derivatives (PG-AA<sub>1</sub>-Bt, with PG = Fmoc-, Boc-, or Z-protecting groups) under mechanochemical conditions was investigated in the presence of several  $\alpha$ - or  $\beta$ -amino acid hydrochlorides (HCl·H-AA<sub>2</sub>-R<sup>2</sup>) (see Scheme 1 and Table 2).

For *N*-Fmoc- $\alpha$ -amino acyl benzotriazole substrates (Fmoc-AA<sub>1</sub>-Bt), the better yields were obtained using the Fmoc-Leu-Bt (see entry 11 in Table 1 and entries 1–3 in Table 2) and Fmoc-Cys(Trt)-Bt (entry 10 in Table 2) series, leading to dipeptides **8–11** and **18** in good yields (77%–95%). When Fmoc-Leu-Bt was reacted with H-Phe-NH<sub>2</sub>, Fmoc-Leu-Phe-NH<sub>2</sub> (**11**; see entry 3 in Table 2) was obtained in an excellent yield, compared to the corresponding dipeptide having a C-terminal *tert*-butyl ester functionality (**8**; see entry 11 in Table 1), suggesting that the reaction, as observed earlier, was usually less efficient when using O<sup>t</sup>Bu ester.

The  $\alpha,\beta$ -dipeptide Fmoc-Leu- $\beta$ -Ala-O<sup>t</sup>Bu was also obtained, under the same reaction conditions, in good yield (**12**; see entry 4 in Table 2). Generally speaking, the successful preparation of PG-AA<sub>1</sub>-AA<sub>2</sub>-R<sup>2</sup> dipeptides **7–25** seemed to be dependent on both the nature of the *N*-protected- $\alpha$ -amino acyl benzotriazole derivative and on the second partner, the amino acid amide or ester (PG-AA<sub>1</sub>-Bt)/H-AA<sub>2</sub>-R<sup>2</sup>.

Indeed, by reaction of H-Ser(<sup>t</sup>Bu)-O<sup>t</sup>Bu (entries 2, 6, 9, 10, 12, and 15 in Table 2) and H-Phe-O<sup>t</sup>Bu (entries 10 and 11 in Table 1 and entries 7, 11, 13, and 16 in Table 2) with diverse Fmoc-, Boc-, or Z-AA<sub>1</sub>-Bt, the corresponding dipeptides were obtained in variable yields (20%–98% and 23%–81%, respectively). The outcome of the reaction was also influenced by the nature of the protecting group (PG), the lowest yields being obtained using Boc-Phe-Bt derivatives (Table 2, entries 13–15). Indeed, PG-Phe-Phe-O<sup>t</sup>Bu dipeptides (**7**, **19**, and **21**; see entry 10 in Table 1 and entries 11 and 13 in Table 2) and PG-Phe-Ser(<sup>t</sup>Bu)-O<sup>t</sup>Bu dipeptides (**14**, **20**, and **23**; see entries

6, 12, and 15 in Table 2) were obtained in variable yields (20%–81%), the better results being usually observed using Z-protected residues in each series. It was also observed that, for the same protecting group (Fmoc- or Boc-), different results were obtained when switching the order of the amino acid residues in the sequence. Therefore, dipeptides such as Fmoc-Leu-Phe-O<sup>t</sup>Bu and Boc-Leu-Phe-O<sup>t</sup>Bu (8 and 24, respectively) generally led to better yields, compared to the corresponding Fmoc-Phe-Leu-O<sup>t</sup>Bu and Boc-Phe-Leu-O<sup>t</sup>Bu dipeptides (13 and 22, respectively), presenting a switched sequence. It was observed that a Fmoc-deprotection reaction was observed during milling under liquid-assisted grinding (LAG) conditions (entries 6, 8, and 9 in Table 2), and side reactions were not detected when neat conditions were explored.

**Synthesis of Tripeptides.** With the final goal being the preparation of longer peptides as well as peptide tools such as biotinylated peptides (i.e., synthesis of Biotin-Ahx-RGDfV-NH<sub>2</sub> (1)), we investigated the limits of the methodology by increasing complexity of the coupling. First, the preparation of tripeptides (Scheme 1) was carried out selecting N-protected- $\alpha$ -amino acyl benzotriazoles (PG-AA<sub>1</sub>-Bt) having shown a good reactivity in the preparation of dipeptides, as starting materials (Table 3).

**Table 3. Reaction Scope for the Preparation of N,C-Protected Tripeptides in a Vibrational Ball Mill (VBM)<sup>a</sup>**

entry	Fmoc-AA <sub>1</sub> -Bt	TFA-H-AA <sub>2</sub> -AA <sub>3</sub> -R <sup>2</sup>	product	yield <sup>b</sup> (%)
1	Fmoc-Phe-Bt	H-Phe-Ala-NH <sub>2</sub>	26	61
2		H-Phe-Val-OMe	27	58
3	Fmoc-Leu-Bt	H-Phe-Ala-NH <sub>2</sub>	28	56
4	Fmoc-Cys(Trt)-Bt	H-Phe-Val-OMe	29	50
5		H-Val-Ala-OMe	30	52

<sup>a</sup>Typical procedure: Fmoc-AA<sub>1</sub>-Bt (1 equiv), TFA-H-AA<sub>2</sub>-AA<sub>3</sub>-R<sup>2</sup> (1 equiv), DIPEA (2 equiv), and AcOEt (15  $\mu$ L) were milled for 3 h at 30 Hz in a 5 mL stainless steel jar with two stainless steel balls (5 mm in diameter). <sup>b</sup>Isolated yields after workup (see the Supporting Information).

It was observed that reaction times could be shortened and yields improved by operating under wet grinding conditions, using 15  $\mu$ L of AcOEt during milling (LAG).<sup>30,31</sup> The presence of AcOEt allowed homogeneous mixing of the reactants, while also avoiding reactants being stuck to the wall of the grinding jar. Although it was not the case for dipeptides (entries 6, 8, and 9 in Table 2), LAG was beneficial for the preparation of tripeptides (Table 3).

In contrast to dipeptide synthesis, where the yields were variable, depending on the nature of PG-AA<sub>1</sub>-Bt reagent, C-terminal functionality, or the nature of  $\alpha$ -amine protection, yields of tripeptides were quite similar for all PG-AA<sub>1</sub>-Bt/H-

AA<sub>2</sub>-AA<sub>3</sub>-R<sup>2</sup> combinations. This result is quite promising; indeed, activated N-acyl benzotriazolyl derivatives of amino acids could be also suitable to access tripeptides, independently of the sequence.

As for the majority of dipeptides, the final products 26–30 could be also recovered by precipitation in water. No striking workup was necessary to eliminate the water-soluble benzotriazole and the DIPEA·TFA salt.

In contrast to solution synthesis of tripeptides, where N-protected dipeptidoyl benzotriazoles were coupled with unprotected amino acids (at 0 °C for 3–4 h in a water/acetonitrile mixture),<sup>24</sup> the mechanochemical preparation of such compounds involved only a few microliters of organic solvent (AcOEt), including workup, with improved safety<sup>32</sup> and environmental profiles.

**Green Chemistry Metrics for Mechanochemical and Solution-Based Reactions.** Compounds PhCH<sub>2</sub>CH<sub>2</sub>CO-Leu-O<sup>t</sup>Bu 5 (entry 7 in Table 1), Fmoc-Leu-Phe-NH<sub>2</sub> 11 (entry 3 in Table 2), and Fmoc-Phe-Phe-Val-OMe 27 (entry 3 in Table 3) were also prepared in solution<sup>33</sup> to evaluate and benchmark the more sustainable process through the calculation of the green chemistry metrics for both mechanochemical- and solvent-based procedures (Tables 4 and 5).

The environmental factor, E-factor, which is defined as<sup>34</sup>

$$E\text{-factor} = \frac{\text{total waste (kg)}}{\text{product (kg)}}$$

and the process mass intensity (PMI), which is defined as<sup>35</sup>

$$\text{PMI} = \frac{\text{total mass (kg) used in the process}}{\text{mass of product (kg)}}$$

the latter being widely used in the pharmaceutical industry, were calculated using the EATOS software<sup>36</sup> (see Table 4).

If metrics such as carbon economy ( $E_c$ ), atom economy (AE), the stoichiometric factor (SF), and the nature of waste are the same for both solution and ball-milling processes, reactions activated by mechanochemistry were faster (1–3 h, instead of 24 h), the E-factor, the PMI (Table 4), and the reaction mass efficiency (RME) metrics were better, compared to the reactions in solution. In addition, the workup was easier to realize, keeping the amount of solvent to the minimum. Indeed, there was no need for purification by preparative HPLC, which is detrimental from the environmental point of view (see E-factors and PMI in Table 4) and leads to high calculated costs for 1 g of final product (Table 5).

**Synthesis of Biotin-Ahx-RGDfV-NH<sub>2</sub> Linear Peptide (1).** Finally, we assessed the usefulness of this approach by the direct coupling of biotin (1H-benzo[d][1,2,3]triazol-1-yl) (Biotine-Bt, see Figure 1) derivative with a pentapeptide

**Table 4. Comparative Green Metrics for Amide Bond Formation Using N-acyl Benzotriazolyl Derivatives**

compound		In Solution <sup>b,c</sup> /By Mechanochemistry				
		time, t (h)	yield (%) <sup>a</sup>	E-factor <sup>b</sup>	E-factor <sup>c</sup>	PMI <sup>d</sup>
PhCH <sub>2</sub> CH <sub>2</sub> CO-Leu-O <sup>t</sup> Bu	5	24/3	56/99	50213/303	375/303	50214/304
Fmoc-Leu-Phe-NH <sub>2</sub>	11	24/1	55/91	59678/401	442/401	59679/402
Fmoc-Phe-Phe-Val-OMe	27	24/3	48/58	232052/535	1716/535	232053/536

<sup>a</sup>Isolated yields after workup (see the Supporting Information). <sup>b</sup>The E-factor for synthesis in solution includes the quantities of solvents used during the purification by preparative HPLC. <sup>c</sup>The E-factor for synthesis in solution was calculated by neglecting the quantities of solvents used for the purification by preparative HPLC. <sup>d</sup>PMI = E-factor + 1.



**Table 5. Comparative Green Metrics for Amide Bond Formation Using *N*-acyl Benzotriazolyl Derivatives**

compound	In Solution/by Mechanochemistry					
	carbon economy, $E_c$	atom economy, AE	stoichiometric factor, SF	reaction mass efficiency, RME	costs <sup>a</sup> (€ g <sup>-1</sup> )	
PhCH <sub>2</sub> CH <sub>2</sub> CO–Leu–O <sup>t</sup> Bu	5	0.58	0.53	1.23	0.24/0.43	157/71
Fmoc–Leu–Phe–NH <sub>2</sub>	11	0.68	0.65	1.20	0.29/0.49	298/54
Fmoc–Phe–Phe–Val–OMe	27	0.73	0.59	1.02	0.22/0.34	407/110

<sup>a</sup>Reaction costs in euros per gram of product, excluding postreaction treatments.

amide to prepare peptides labeled with biotin. Biotinylated peptides or ligands are quite useful tools for the detection and immobilization via the highly specific and affine interaction of biotin with avidin/streptavidin.<sup>37</sup> In addition, such labeled ligands can be used to selectively purify their receptor by affinity chromatography from a complex biological medium. Thus, the linear RGDfV peptide amide (H-Ahx-Arg-Gly-Asp-D-Phe-Val-NH<sub>2</sub>) was selected as a model to be linked to biotin on its *N*-terminus via an aminohexanoyl spacer (Figure 1).<sup>29</sup> In particular, it is the linear analogue of potent cyclic peptide antagonists of  $\alpha_v\beta_3$  integrin.<sup>38</sup>

The linear RGDfV peptide was synthesized using the Fast Parallel Peptide Synthesis (FPPS) technology used in our laboratory that allows one to obtain peptides of high purity within a relatively short time.<sup>39</sup> Then, biotin-Bt and the peptide amide were ground for 10 h at 30 Hz. LAG conditions were fundamental to afford an almost-quantitative conversion of substrates. The biotinylated-Ahx-RGDfV-NH<sub>2</sub> (1) was obtained in 36% yield, after precipitation from EtOAc, and preparative HPLC purification to eliminate traces of the unreacted peptide.

## CONCLUSION

The association between the performances of the *N*-acyl-benzotriazolyl derivatives, together with mechanochemical procedures, is a valid strategy not only for the preparation of peptides, but also for their widespread application as practical synthetic substrates to functionalize biomolecules; the last approach still needs major development in the field of mechanochemistry. In addition, we have demonstrated that *N*-protected  $\alpha$ -aminoacyl benzotriazoles are useful building blocks, allowing the synthesis of peptides by mechanochemistry, complementing the “tool box” already available, mainly based on the use of (i) preactivated amino esters [in the form of urethane-protected amino acid *N*-carboxyanhydrides (NCA)<sup>40,41</sup> and hydroxysuccinimide esters,<sup>42</sup>], and (ii) by *in situ* activation of the carboxylic acid [with 2,4,6-trichloro-1,3,5-triazine (TCT)<sup>43</sup> and by *N*-ethyl-*N'*-(3-(dimethylamino)propyl)carbodiimide (EDC),<sup>44</sup> used alone and in combination with ethylhydroxyiminocyno acetate (Oxyma)<sup>45</sup>].

Indeed, this strategy presents an improved environmental footprint, uses and generates substances with little or no toxicity to either humans or the environment, does not use any solvent, and avoids the formation of side products.

## AUTHOR INFORMATION

### Corresponding Authors

\*Tel.: +33(0)4 67 144285. Fax: +33(0)4 67 144866. E-mail: [evelina.colacino@umontpellier.fr](mailto:evelina.colacino@umontpellier.fr) (E. Colacino).

\*Tel.: +33(0)4 11 759605. Fax: +33(0)4 11 759641. E-mail: [guillaume.laconde@umontpellier.fr](mailto:guillaume.laconde@umontpellier.fr) (G. Laconde).

\*Tel.: +33(0)4 67 143847. Fax: +33(0)4 67 144866. E-mail: [frederic.lamaty@umontpellier.fr](mailto:frederic.lamaty@umontpellier.fr) (F. Lamaty).

### ORCID

Evelina Colacino: [0000-0002-1179-4913](https://orcid.org/0000-0002-1179-4913)

### Present Address

<sup>§</sup>Institut of Organic Chemistry, Landoltweg 1, 52074 Aachen, Germany.

### Author Contributions

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