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Solventless Synthesis of *N*-Protected Amino Acids in a Ball Mill

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ABSTRACT: An eco-friendly methodology for preparing carbamate *N*-protected α -amino acids is described using ball-milling technology. Some β -amino acids were also investigated in this study. This solvent-free methodology gave Boc-, Z-, and Fmoc-*N*-protected compounds in good to excellent yields with improved environmental impact compared to classical syntheses in solution. Different planetary ball mills were employed. The results are also discussed according to the different process parameters (grinding material, number of balls, rotation speed).

KEYWORDS: Amino Acids, Ball mill, Protective groups, Solvent free, E-factor



INTRODUCTION

For several years, the awareness of environmental problems caused by human activity has led scientists to change their way of “thinking chemistry”, considering the 12 Green Chemistry Principles.¹ As a result, new green and sustainable chemical processes have recently grown at a rapid pace, leading to the use of safer reagents or solvents, diminishing the generation of toxic and nontoxic waste through alternative synthetic methodologies and experimental techniques^{1–5} that limit energy consumption and solvent use.⁶ A strong emerging field of research is the employment of techniques such as mixing, grinding, or ball milling to carry out chemical reactions in the absence of solvent,^{7–10} considerably decreasing the *E*-factor¹¹ of the organic reactions. We have recently described new strategies for amide bond formation under solvent-free conditions using ball-milling technology¹² for the preparation of peptides^{13,14} or amino acid analogues.^{15,16} Usually, peptide syntheses (in solution or solid phase) are carried out from *C* to *N* direction by assembly of amino acids through a protection/deprotection coupling strategy according to well-established procedures.¹⁷ One of the most significant issues in peptide chemistry is the temporary protection of the α -amino function of amino acids^{18,19} to ensure better activation of the *C*-terminal carboxylic function and to prevent side reactions (polymerization of the amino acid or uncontrolled synthesis of peptides). Nevertheless, this kind of synthesis requires huge quantities of solvents (2000–5000 kg for a large peptide) and the use of expensive building blocks. Protecting groups such as 9-fluorenylmethyloxycarbonyl (Fmoc),²⁰ *tert*-butyloxycarbonyl (Boc),^{21,22} and benzyloxycarbonyl (Z)²³ groups are most commonly used to mask the amino group of α -amino acids. However, the majority of current synthetic procedures require the use of excess of reagents, volatile toxic organic solvents (THF, benzene, dioxane, chloroform, etc.) in dry or aqueous biphasic conditions, and long reaction times. In this contest, exploring low cost eco-friendly methods for the ease and simple preparation of *N*-protected amino acids by ball milling is a challenging task, especially to reduce reaction time and

temperature and to simplify workup procedures. Mechanochemistry has been scarcely exploited for the derivatization of biological molecules and their analogues. The only data reported so far deals with the functionalization of nucleosides^{24–27} or carbohydrates.²⁵ We describe herein a new strategy for the direct preparation of Boc-, Z-, and Fmoc-protected α -amino acids under solid-state solvent-free conditions²⁸ by using a planetary ball mill. It is worth noting that in the case of α -amino acids, the selective *N*-protection was achieved in a one-pot/two-step procedure; the α -carboxylic acid function was temporarily masked in situ to avoid dipeptide formation and at the same time to reduce the total number of steps and subsequent purification of the *C*-protected intermediate. We investigated different variables influencing the success of individual reactions that are not driven by the nature of reactants and additives. Technical and process parameters²⁹ such as type of ball mill apparatus, rotation frequency (up to 750 rpm), milling time, material of balls and jars (stainless steel or tungsten carbide), size and number of milling balls (up to 80, with a diameter of 0.5 mm), and mode of operation (continuous or cycled) were also explored in some cases.

RESULTS AND DISCUSSION

Synthesis of *tert*-Butyloxycarbonyl (Boc) *N*-Protected Amino Acids. Boc protection is classically achieved under Schotten–Baumann conditions²² (use of a biphasic system: organic solvent-aqueous basic conditions) starting from amino acids.

Recently, the solvent-free synthesis of Boc-nucleosides has been described for the protection of exocyclic amino function of nucleosides²⁶ by grinding cytidine with Boc₂O in a ball mill in the presence of DMAP as base. Solvent-free Boc-protection of primary and secondary amines has also been recently

Scheme 1. General Scheme for *N*-Boc Protection of *L*-Phenylalanine

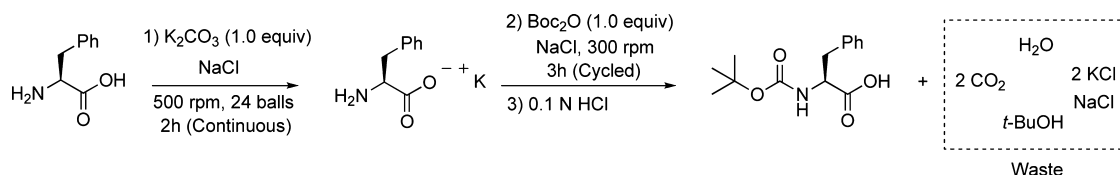
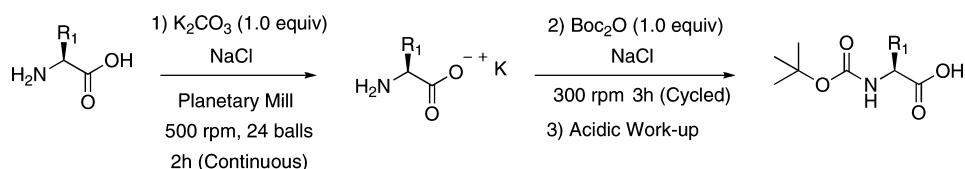


Table 1. *tert*-Butyloxycarbonyl *N*-Protected Amino Acids^{a,b}



entry	α -amino acid	<i>N</i> -protected α -amino acid	yield (%) ^c
1	H-Phe-OH	Boc-Phe-OH	100
2	H-Tyr(Me)-OH	Boc-Tyr(Me)-OH	95
3	H-Ser(Bzl)-OH	Boc-Ser(Bzl)-OH	96
4	H-Thr(Bzl)-OH	Boc-Thr(Bzl)-OH	85
5	H-Glu(Bzl)-OH	Boc-Glu(Bzl)-OH	68
6	H-His(Bzl)-OH	Boc-His(Bzl)-OH	72
7	H-Trp(For)-OH	Boc-Trp(For)-OH	95
8	H-Val-OH	Boc-Val-OH	86

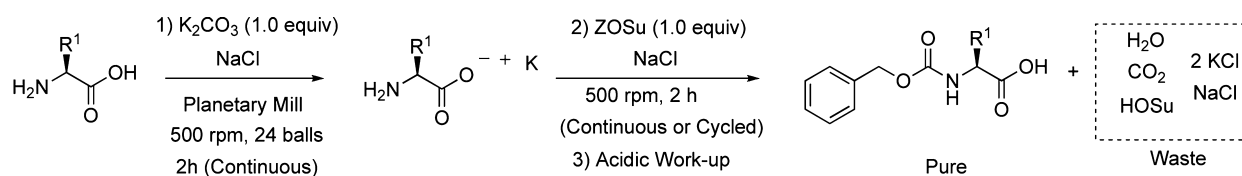
^aConfiguration of all the substrates is *L*. ^bGeneral method using 12 mL stainless steel jar in a planetary ball mill: (step 1) α -amino acid (0.605 mmol), K_2CO_3 (0.605 mmol), NaCl (quantity in grams corresponding to the total weight of the reactants), 24 steel balls (0.5 mm diameter) at 500 rpm for 2 h; (step 2) Boc_2O (0.605 mmol) and NaCl (quantity in grams corresponding to the total weight of Boc_2O introduced) at 300 rpm (three cycles of 1 h, 10 min intervals between each cycle), inverted rotation. ^cIsolated yield after liquid–liquid extraction.

performed via a carbonyldiimidazole (CDI)-mediated approach (grinding the reaction media with a spatula)³⁰ in a two-step procedure in the presence of DMAP as well. However, only one step was performed without any solvent. *L*-Phenylalanine was selected as benchmark for a one-pot/two-step protocol involving the transient α -carboxylic acid protection as the internal salt in the presence of an inorganic base (step 1), followed by the introduction of stoichiometric amounts of Boc_2O (step 2); the optimized conditions are reported in Scheme 1.

The reaction was performed in a planetary mill using a 12 mL stainless steel jar with 24 balls (0.5 mm diameter). In our first attempts, a one-step protocol was used, milling the α -amino acid directly with the base and the protecting group derivative at 500 rpm for 2 h without any additive. Monitoring the reaction by reverse phase HPLC revealed very low conversion of the starting amino acid, probably due to inefficient mixing, resulting from the formation of a sticky paste inside the reactor. Therefore, we speculated that introduction of an inert additive that is able to keep the crude as a powder during the whole mixing process could be a solution. In the presence of molecular sieves 4 Å or $MgSO_4$ (inert to the reaction), the conversion remained low (20%), and the recovery of the product after careful acidic workup was particularly difficult. In contrast, the crude resulted in a nice white powder when NaCl was used, and it was selected as the additive of choice for the next trials. At this point, we argued that catalytic amounts of free α -carboxylic acid still present in the mixture during milling could probably be responsible for the poor results. For this reason, we envisaged that a temporary salt protection of the C-terminal was suitable, directing our tests toward the conception of a two-step protocol as shown in Scheme 1. For both steps, chemical parameters were

investigated, such as different bases (KOH, sodium, potassium, or cesium hydrogenocarbonates or carbonates, triethylamine, or DMAP), variable processes, presence or not of NaCl (added in each step in a corresponding amount equal to the total weight of the reactants), different rotation speeds (from 200 up to 500 rpm), increasing number of balls (from 6 to 24), different reaction times (30 min up to 4 h for each step), and using continuous or cycled milling.

Because it was not possible to have direct evidence for the quantitative formation of the carboxylate after the first step, the efficiency of both steps was concomitantly optimized with an evaluation on the yield of the final protected amino acid. In addition, we observed that conversion and reaction time were affected by the NaCl quantity added in each step. For “highly diluted solid solutions”, the conversion remained moderate, even after extended reaction times (more than 4 h), while in “concentrated solid solutions”, the mixing was poor and the crude was a paste with all the balls agglomerated around the chemicals. Reaction conditions needed to be also adjusted to promote a fast kinetic for *N*-protection while diminishing the kinetic for Boc_2O degradation. We observed that the tuning of the mechanical energy transferred to the mixture through different combinations of rotation speed/number of balls had an influence on the reaction kinetics. As a general trend, at high rotation speed (500 or 650 rpm) using 6, 12, or 24 stainless steel balls, Boc_2O degradation remained significant, which translated again into lower conversion rates (around 40%). The better results were finally obtained with a cycled milling mode for the second step (at 300 rpm during three cycles of 1 h each, with 10 min pause in between), inverting the rotation in between two consecutive cycles, as shown in Scheme 1. The final product was recovered by liquid–liquid extraction from diethyl ether, after careful acidification of the crude with 10%

Table 2. Benzylloxycarbonyl *N*-Protected Amino Acids^a


entry	<i>N</i> -protected α -amino acids ^b	yield (%) ^c
1	Z-Phe-OH	75
2	Z-Tyr(Me)-OH	78
3	Z-Ser(Bzl)-OH	88
4	Z-Thr(Bzl)-OH	87
5	Z-Glu(OBzl)-OH ^d	95
6	Z-His(Bzl)-OH	97
7	Z-His(Trt)-OH	100
8	Z-Trp(For)-OH	73
9	Z- <i>D</i> -Lys(Z)-OH ^e	75
10	Z-Val-OH	89
11	Z-Ala-OH	85
12	Z- <i>D,L</i> -3-Aib-OH ^e	70

^aConfiguration of all the substrates is *L* except when differently stated. ^b12 mL stainless steel jar in a planetary ball mill: (step 1) α -amino acid (0.605 mmol), K_2CO_3 (0.605 mmol), NaCl (quantity in grams corresponding to the total weight of the reactants), 24 stainless steel balls (0.5 mm diameter) at 500 rpm for 2 h; (step 2): ZOSu (0.605 mmol) and NaCl (quantity in grams corresponding to the total weight of ZOSu introduced) at 500 rpm for 2 h (continuous milling). ^cIsolated yield after workup. ^dContinuous milling 500 rpm for 2 h (twice). ^eCycled milling at 500 rpm: 12 cycles of 10 min, 10 min intervals between each cycle, inversed rotation).

aqueous citric acid or 0.1 N HCl. The only byproducts obtained (Scheme 1) were removed in the aqueous phase (inorganic salts or eventually residual α -amino acid) and by simple evaporation (*t*-butanol or CO_2), while the Boc-*N*-phenylalanine amino acid was recovered in quantitative yield as a white powder without any additional purification step (Table 1, entry 1). The methodology was extended to other commercially available α -amino acids; a selection of data is given in Table 1. Noteworthy, no diacylated products could be identified in the crude.

Synthesis of Benzylloxycarbonyl (Z) *N*-protected amino acids. The preparation of Z-protected amino acids is classically achieved by the addition of benzyl chloroformate (Z-Cl) to an alkaline solution of amino acid or ester²³ or alternatively using a *N*-hydroxysuccinimide (HOSu) derivative (ZOSu) or dibenzyl dicarbonate (Z_2O) in hazardous solvents such as THF or dioxane in dry conditions. Diverse Z-protected α - and β -amino acids were prepared using a planetary ball mill (one-pot/two-step protocol), investigating the reactivity of Z-derivatives (ZCl, ZOSu, and Z_2O) as shown in Table 2. On the basis of the previous screening for Boc-protected amino acids, the same one-pot/two-step procedure was envisaged for the present scope. We realized that this approach (Table 2) was inefficient with Z-Cl or the highly reactive dibenzyl dicarbonate (Z_2O), leading to their fast decomposition into benzyl alcohol and very poor conversions (less than 10%), with most of the starting unreacted amino acid. Benzylloxysuccinimide (ZOSu) was suitable for carbamate bond formation under cycled milling, leading to various Z-*N*-protected amino acids as pure compounds after simple precipitation/filtration workup in acidic medium (Table 2).

Byproducts like inorganic salts or hydroxysuccinimide were soluble in water and easily washed from the crude. In some cases (Supporting Information), Et_2O , CHCl_3 , or AcOEt extraction was necessary. γ -*N*-Benzylloxycarbonyl-lysine was

also prepared, but it was necessary to adjust the milling conditions for the second step.

D-Lysine-OH led to traces of Z- α -aminobenzyl ester when more energetic conditions were used (36 balls at 500 or 650 rpm under continuous milling). Indeed, when the crude was grinded using cycled milling for 3 h at 500 rpm (entry 9, Table 2) with 10 min interruptions every 10 min, the reaction was energetic enough to favor amine protection in good yield without concomitant ester formation (Table 2, entry 9). We were pleased to find that the reaction was suitable also for the synthesis of the β -amino acid *H-D,L*-3-Aib-OH (entry 12, Table 2).

9-Fluorenyloxycarbonyl *N*-Protected Amino Acids.

Fmoc amino acids are commonly used for solid-phase peptide synthesis,³¹ especially with acido-labile side-chain protecting groups. They are prepared by slowly adding a solution of the amino acid in dilute sodium carbonate to a cold solution of Fmoc-Cl in dioxane, ether, or other solvent.²⁰ This order of reagent addition avoided the presence of an excess of an amino function, which might lead to premature degradation of the desired Fmoc derivative. However, with the usual synthesis, some impurities such as dibenzofulvene or its polymer can be formed, using appropriate workup conditions to recover the pure product. During our ongoing study, the synthesis of Fmoc-protected amines was reported under solvent-free conditions using Fmoc-OSu/DMAP, with an excess of starting amine under vibration milling conditions.²⁷

The methodology applied so far for the introduction of Boc- and Z-protecting groups was extended to the synthesis of Fmoc amino acid derivatives (Table 3), and a variation of energy requirements was explored using different planetary milling apparatus and jar materials (stainless steel or tungsten carbide) (Table 4).

Despite the advantages coming from the use of Fmoc-Cl (e.g., formation of inorganic salts soluble in water and easy to remove), this reagent was not suitable for the protection of α -

Table 3. Fmoc *N*-Protected Amino Acids^a and Milling Conditions with Different Apparatus/Jar Material Combinations

entry	apparatus – rpm – jar	<i>N</i> ^b	product	yield (%) ^{c,d}
1	PM1 – 650 – stainless steel	24	Fmoc–Phe–OH	87 ^e
2	PM1 – 500 – stainless steel	36	Fmoc–Tyr(Me)–OH	95 ^e
3	PM1 – 500 – stainless steel	36	Fmoc–Ser(Bzl)–OH	76 ^e
4	PM1 – 500 – WC jar	50	Fmoc–His(Trt)–OH	34 ^f
5	PM2 – 650 –	50		63 ^f
6	PM1 – 500 – WC jar	80	Fmoc–Thr(Bzl)–OH	48 ^e
7	PM2 – 750	50		64 ^g
8	PM1 – 500 – WC jar	50	Fmoc–Leu–OH	25 ^e
9	PM2 – 750	50		55 ^g

^aConfiguration of all substrates is *L*. ^bNumber of balls. ^cMethod: 12 mL stainless steel jar or 50 mL tungsten carbide (WC) jar in a planetary ball mill: (step 1) α -amino acid (1.0 equiv), K₂CO₃ (1.0 equiv), NaCl (quantity in grams corresponding to the total weight of the reactants), balls (0.5 mm diameter) at 500 rpm for 2 h; (step 2) Fmoc–OSu (1.0 equiv) and NaCl (quantity in grams corresponding to the total weight of Fmoc–OSu introduced). ^dIsolated yield after workup. ^eContinuous milling for 2 h (step 2). ^fContinuous milling for 3 h (step 2). ^gTwelve cycled milling of 10 min cycles followed by 5 min of standby in between with reverse rotation.

Table 4. Overview of Planetary Ball-Milling Apparatus and Grinding Jars for Fmoc-Protection of Amino Acid Derivatives

entry	apparatus (abbreviation)	<i>V</i> _{jar} (mL)	<i>n</i> of balls (0.5 mm \varnothing)	material
1	PM 100 (PM1)	12	12, 24, 36, 50	steel
2	PM 100 (PM1)	50	50, 80	WC
3	pulverisette 7 premium line (PM2)	20	50	steel

amino acids due to decomposing very fast to dibenzofulvene in the planetary ball mill. In the case of Fmoc–Phe–OH (entry 1, Table 3), by keeping constant the milling rotation at 500 rpm while increasing the number of milling balls (6, 12 or 24), yields were improved (45% with 6 balls to 70% with 24 balls) but still some traces of dibenzofulvene were present in the crude. In this case, more energetic conditions are achieved by increasing the rotation speed to 650 rpm, which led to a better yield (85%, entry 1, Table 3) of Fmoc–Phe–OH without dibenzofulvene formation. In this case also, the workup was really practical because the protonated water-insoluble Fmoc–Phe–OH precipitated after treatment with 10% aqueous citric acid, while water-soluble *N*-hydroxysuccinimide was washed out during filtration. Similarly, Fmoc–Tyr(Me)–OH and Fmoc–Ser(Bzl)–OH (entries 2 and 3, Table 3) were easily recovered as white powders in good to excellent yields after the usual workup.

In general, the use of Fmoc–OSu in solution can lead, together with dibenzofulvene, to in situ formation of side products (i.e., Fmoc– β -Ala–OH or dipeptides Fmoc– β -Ala–AA–OH via the Lossen rearrangement).^{32,33} In our case, dibenzofulvene was detected in a low amount as the only byproduct under unoptimized conditions (prolonged and moderate energetic milling), while no traces of byproduct

Fmoc– β -Ala–OH could be detected by mass spectrometry analyses of the crude.

Actually, we extended the study to other amino acids, speculating that the more energetic conditions applied into the grinding jar the higher the yields should be. First, this was accomplished by increasing the number of balls, rotation speed, and extending the reaction times under continuous or cycled mode. Then, stainless steel grinding jars and balls were replaced by tungsten carbide (WC). For each amino acid investigated, Table 3 (entries 4–9) summarizes the best data obtained in the optimized apparatus/grinding material/rotation speed/number of balls combinations. Despite the moderate yields, from the data shown, it seemed that rotation speed influenced yield (doubled!!!) and selectivity more than the choice of a lighter-weight (steel; PM2) or heavier milling material (tungsten carbide; PM1), while extended reaction time could be sometimes detrimental leading to side products. Typically, Fmoc–Pro–OH could be obtained (23% yield) at 750 rpm (PM2; stainless steel jar; 50 balls) after 2 h (cycles milling), while it was not formed using at lower rotation speed of 500 rpm (PM1; tungsten carbide jar; 50 balls rpm) during 3 h of continuous milling. A question remaining open and with no answer at the moment for technical reasons concerns the outcome of syntheses using a tungsten carbide jar (WC jar) in the PM2 conditions at higher rotation speed.

CONCLUSIONS

We have developed a new and advantageous synthetic procedure with low environmental impact for preparing *N*-protected amino acids under mild conditions without special precaution, which is easy to realize, keeping the quantity of solvent used for the workup to the minimum and with no need of tedious purification steps. The “greenness” of a method is evaluated considering its environmental factor³⁴ [*E*-factor = total waste (kg)/product (kg)], defined by the ratio of mass of waste per unit of product. The protection of *L*-Phe–OH with Boc, Z, and Fmoc groups was used as benchmark to compare the *E*-factors for both solution and solvent-free syntheses (Table 5).

Table 5. Comparative *E*-Factors for *N*-Protection of Amino Acids

entry	<i>N</i> -protected amino acid	<i>E</i> -factor	
		solution synthesis	solvent-free synthesis
1	Boc–Phe–OH	62	265
2	Z–Phe–OH	20	8
3	Fmoc–Phe–OH	288	6

In the majority of cases, the *E*-factor is much better compared to the reactions carried out in solvents, especially for Z- and Fmoc-protection, displaying a comparatively lower environmental. For Boc-protection, if the nature of waste is the same for both methods (in solution or solvent free), the need for liquid–liquid extraction workup is detrimental from environmental point of view.

Amino acid derivatives were synthesized in a scale from 50 mg up to 1 g of final product. The final pure protected amino acid derivatives were recovered by aqueous precipitation/filtration or liquid–liquid extraction. Different apparatus were used, and an investigation of the parameters responsible for energy efficiency was presented. Work is in progress to extend

the methodology to more complex protecting groups for amino acids and esters of biological interest and to investigate more deeply the Fmoc-protection from a process point of view.

■ EXPERIMENTAL SECTION

Representative Procedure for the Synthesis of N-Boc-Protected Amino Acids. The amino acid (1 equiv), K_2CO_3 (1 equiv), and NaCl (quantity in grams corresponding to the total weight of the reactants) were introduced in a 12 mL stainless grinding jar with 24 stainless balls (5 mm diameter). The reaction medium was ground at 500 rpm for 2 h. Then, Boc_2O (1 equiv) and NaCl (quantity in grams corresponding to the total weight of Boc_2O) were added to the reaction medium, and the latter was ground at 300 rpm for 3 h (three cycles of 1 h, intervals of 10 min between each cycle, with inverse rotation). Et_2O and water were then added to the crude. The aqueous layer was then acidified with citric acid 10% aq and extracted with $CHCl_3$. The organic layer was dried over $MgSO_4$, filtered, and concentrated under reduced pressure to give the product as a powder after drying on KOH under vacuum.

Representative Procedure for the Synthesis of Z- or Fmoc-Protected α -Amino Acids. The amino acid (1 equiv), K_2CO_3 (1 equiv), and NaCl (quantity in grams corresponding to the total weight of the reactants) were introduced in a 12 mL stainless grinding jar with 24 stainless balls (5 mm diameter). The reactants were ground at 500 rpm for 2 h. Then, the suitable protecting group (Z-OSu or Fmoc-OSu, 1 equiv) and NaCl (quantity in grams corresponding to the total weight of the protecting group precursor used) were added to the reaction medium, and the latter was ground at 500 rpm for 2 h. HCl 1 N was added to the crude (or citric acid 10% aq), leading to the precipitation of the product. The latter was filtered and washed with water until neutral pH, to afford the title compound as a white powder after drying over P_2O_5 .

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Notes

The authors declare no competing financial interest.

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