Green Chemistry

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DOI: 10.1039/C7GC00615B

Journal Name

ARTICLE

Mechanoenzymatic peptide and amide bond formation

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Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

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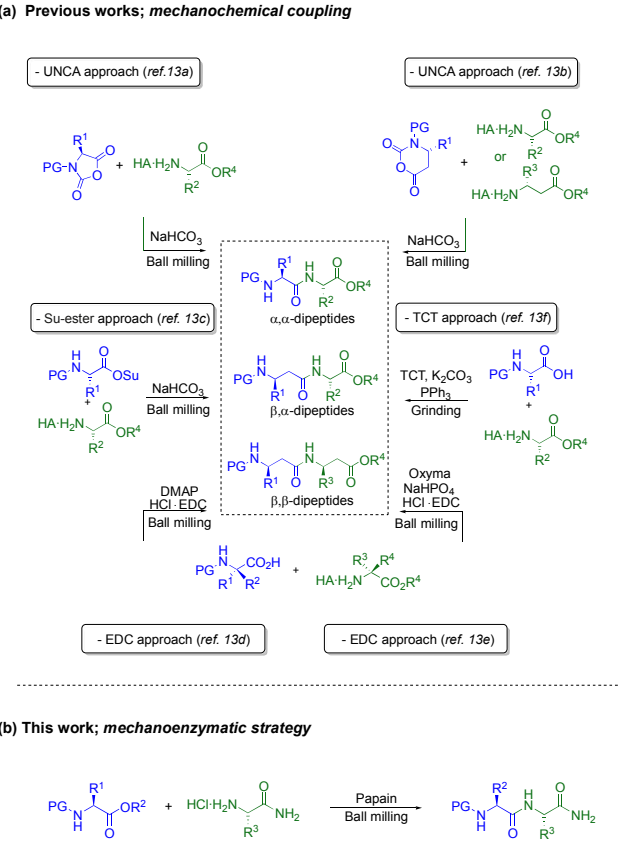
Mechanochemical chemoenzymatic peptide and amide bond formation catalysed by papain was studied by ball milling. Despite the high-energy mixing experienced inside the ball mill, the biocatalyst proved stable and highly efficient to catalyse the formation of α,α- and α,β-dipeptides. This strategy was further extended to the enzymatic acylation of amines by milling, and to the mechanosynthesis of a derivative of the valuable dipeptide L-alanyl-L-glutamine.

Introduction

The use of mechanochemical activation by milling, grinding or shearing have experienced a steady growth in recent years.[1] The increasing popularity and success of mechanosynthesis within the scientific community - particularly from the green chemistry perspective - has to do with the fact that mechanochemical reactions are often conducted under solventless conditions or only in the presence of catalytic volumes of organic solvents [liquid-assisted grinding (LAG)],[2] thereby drastically minimizing waste production by having a direct impact in the *E* factor of chemical processes.[3] Moreover, a variety of other advantages of mechanochemistry have also been experienced by scientists in fields covering organic,[4] inorganic,[5] organometallic,[6] medicinal[7] and supramolecular chemistry[8] among others, where the possibility of bypassing solubility concerns in chemical reaction design, together with the precise stoichiometry control, shorter reaction times and higher yielding procedures have encouraged the utilization of ball-milling techniques to develop more beneficial protocols compare to the solution-based counterparts. Besides offering an alternative for conducting more sustainable chemical syntheses, mechanochemical activation has also led to the discovery of new chemical reactivity, affording materials and products difficult or impossible to access in solution.[9]

Peptide and amide bond formation is a fundamental synthetic tool in chemical and biosynthesis,[10] which has been widely utilised by organic and medicinal chemists in fields such as pharmaceutical chemistry.[11] Hence, it is not surprising that syntheses of amides[12] and peptides[13] have also been the focus of systematic mechanochemical studies (Scheme 1a).

For instance, coupling of several amino acids has been successfully achieved by ball milling activated urethane-protected α- or β-amino acid N-carboxyanhydrides (UNCAs)



Scheme 1 Mechanochemical peptide bond formation: (a) selected chemical approaches; (b) chemoenzymatic strategy.

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Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/x0xx00000x

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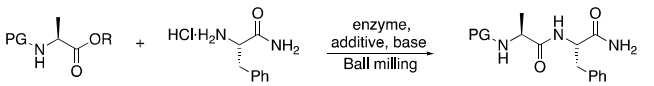
with hydrochloride salts derived from α- and β-amino esters (UNCA approach; Scheme 1a).[13a-b] Up to the present time, the UNCA strategy has enabled the solvent-free mechanochemical preparation of diverse short peptides, including illustrative applications such as the mechanosyntheses of the sweetener aspartame (Asp-Phe-OMe),[13a] and the protected derivative of the natural dipeptide L-carnosine (Boc-β-Ala-His-OMe).[13b] Furthermore, mechanical milling of N-Boc-protected α-amino acid N-hydroxysuccinimide esters and amino esters led to the high-yielding formation of peptides (Su-ester approach; Scheme 1a), including the relatively complex natural peptide Leu-enkephalin.[13c] Similarly, screening by milling of commonly used peptide coupling agents such as carbodiimides[13d-e] and benzotriazoles[13f] revealed that those popular coupling reagents also tolerated mechanochemical conditions, and could be implemented into solvent-free or LAG peptide synthesis protocols (EDC approach; Scheme 1a). Analogously, in the presence of triphenylphosphine, 2,4,6-trichloro-1,3,5-triazine (TCT) was also found to activate N-protected α-amino acids by manual grinding to generate activated TCT-esters, which upon further grinding with amino esters afforded a library of α,α-dipeptides (TCT approach; Scheme 1a).[13g]

Despite all the advantages of the aforementioned mechanochemical protocols, including solvent waste reduction, rapid coupling reactions and broad reaction scope, most of the approaches shown in Scheme 1a still suffer from drawbacks such as the use of harmful reagents and additives (DMAP, DCM, $MeNO_2$, EDC, HOBt, cyanuric chloride, PPh_3 etc.)[14] or cumbersome and time-consuming synthetic procedures to access the activated starting materials (e.g. β-UNCAs).[13b] As an alternative, chemoenzymatic catalysis has been widely utilised in solution and solid-phase peptide chemistry due to the mild reaction conditions required, lower use of toxic chemicals, higher yields, minimal side-chain protection, and the possibility to strictly control the stereoselectivity of the peptide formed. In that sense, esterases and proteases have displayed outstanding peptide and amide bond formation activity,[15] despite in nature these biocatalysts carry out completely different tasks. For instance, under standard conditions the main role of proteases consists in hydrolysing peptide bonds (proteolysis), nevertheless by tuning of the reaction conditions, proteases can exhibit peptide bond activity under thermodynamic or kinetic conditions.[15a,16]

Recently, we have reported the compatibility between lipases and mechanochemical activation.[17] The high catalytic activity and exceptional resilience by enzymes under high mechanical stress, made us wonder about the potential to utilise proteases in the ball mill to mediate the formation of peptide bonds (Scheme 1b). Therefore, motivated by our initials findings and the previous reports on biocatalysis under solvent-free conditions[18] we focused our attention on studying in depth the use of papain, a cysteine protease found in papaya latex (*Carica papaya*), to catalyse peptide and amide formation under kinetically controlled conditions. Synthesis of

Table 1 Screening of the reaction conditions for the mechanoenzymatic synthesis of PG-L-Ala-L-Phe-NH_2 (**4a**-**5a**)[(a)]



1a-c; 2a-c **3a** **4a**; PG = Z **5a**; PG = Boc

PG = Z, Boc; R = Me, Et, Bn

Entry	PG-L-Ala-OR	Enzyme/additive/base	PG-L-Ala-L-Phe-NH_2 (%)
1	Z-L-Ala-OEt (**1b**)	–/–/Na_2CO_3 or $NaHCO_3$	(**4a**) 0
2	Z-L-Ala-OEt (**1b**)	Papain/–/Na_2CO_3	(**4a**) 57
3	Z-L-Ala-OEt (**1b**)	Papain/L-Cys[(b)]/Na_2CO_3	(**4a**) 63
4	Z-L-Ala-OEt (**1b**)	Papain/L-Cys/ $Na_2CO_3·H_2O$	(**4a**) 82
5	Z-L-Ala-OEt (**1b**)	Papain/L-Cys/ $Na_2CO_3·10H_2O$	(**4a**) 90
6	Z-L-Ala-OMe (**1a**)	Papain/L-Cys/ $Na_2CO_3·10H_2O$	(**4a**) 89
7	Z-L-Ala-OBn (**1c**)	Papain/L-Cys/ $Na_2CO_3·10H_2O$	(**4a**) 93
8	Boc-L-Ala-OMe (**2a**)	Papain/L-Cys/ $Na_2CO_3·10H_2O$	(**5a**) 59
9	Boc-L-Ala-OEt (**2b**)	Papain/L-Cys/ $Na_2CO_3·10H_2O$	(**5a**) 86
10	Boc-L-Ala-OBn (**2c**)	Papain/L-Cys/ $Na_2CO_3·10H_2O$	(**5a**) 67
11	Z-L-Ala-OEt (**1b**)	Papain[(c)]/L-Cys/ $Na_2CO_3·10H_2O$	(**4a**) 99
12	Z-L-Ala-OEt (**1b**)	Papain latex/L-Cys/ $Na_2CO_3·10H_2O$	(**4a**) 89
13	Z-L-Ala-OEt (**1b**)	Papain[(c)]/L-Cys/ $Na_2CO_3·10H_2O$	(**4a**) 93[d] (23)[e]

(a) Reaction conditions: a mixture of **1b** (40 mg; 0.16 mmol), **3a** (48 mg; 0.24 mmol), enzyme (10 mg), additive (3.9 mg; 0.032 mmol) and the base (0.24 mmol) was milled in a mixer mill at 25 Hz, using a 10 mL ZrO_2 milling jar with one ZrO_2 ball of 10 mm in diameter for 2h; (b) the use of HCl·L-Cys·H_2O gave **4a** in 58%; (c) 20 mg of papain and (7.7 mg; 0.064 mmol) of L-Cys were used; (d) after 30 min of milling; (e) the mixture was ground in a mortar with a pestle for 30 min.

dipeptides mediated by cysteine proteases initiates with the formation of an acyl-enzyme intermediate with an amino acid (aa_1-E), which is later transfer to a nucleophile (aa_2) to form the dipeptide (aa_1-aa_2). In peptide synthesis, papain is known for having flexibility with respect to the nature of aa_1, but it exhibits a preference for bulky hydrophobic amino acid residues in the aa_2 position (e.g. aa_2 = Leu, Ile, Phe).

Results and discussion

To test the idea of a mechanoenzymatic formation of the peptide bond, we selected L-Ala esters (**1**-**2**) and the HCl·L-Phe-NH_2 (**3a**) as benchmark system to be studied in the ball mill. To begin, we milled a mixture of Z-L-AlaOEt (**1b**), **3a** and solid bases commonly used under milling conditions (Na_2CO_3 or $NaHCO_3$) (1.0:1.5:1.5 equiv).[19] After 2 h of mixing at 25 Hz, the analysis of the reaction mixture by ^{1}H NMR spectroscopy showed just the presence of the reactants and none of the product **4a** (entry 1 in Table 1). In contrast, repeating the reaction in the presence of papain from *Carica papaya* powder and Na_2CO_3 as the base, pleasingly afforded the dipeptide Z-L-Ala-L-Phe-NH_2 (**4a**) in 57% yield after filtration (entry 2 in Table

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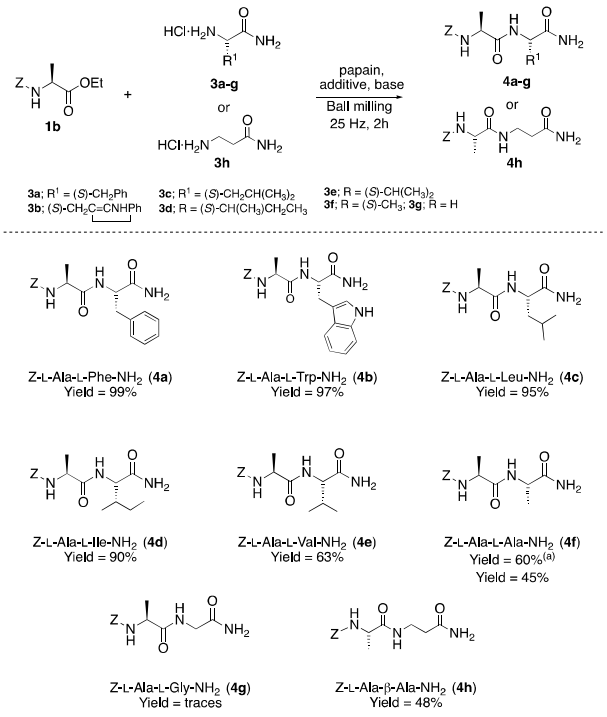
1). In peptide synthesis, low-molecular-mass thiol compounds, such as L-Cysteine are known to activate cysteine proteases,[20] improving its activity towards the peptide bond formation. Indeed, adding L-Cys to the reaction was observed to have a positive effect on the activity of papain raising the yield of **4a** from 57% to 63% (entries 2 and 3 in Table 1). Similarly, the presence of water is recognised to be vital in enzymatic synthesis to guarantee the correct enzyme structural activity. However, in a system containing a protease, an excess of water could compete with the nucleophile **3a** for the acyl-enzyme intermediate (aa_1-E), and lead to the formation of the hydrolysis product **1b**-OH. Experimentally, in comparison to the use of anhydrous Na_2CO_3 (entry 3 in Table 1), milling of **1b**, **2a**, papain, L-Cys and Na_2CO_3 monohydrate led to a substantial rise in the yield of **4a** (82%; entry 4 in Table 1). Correspondingly, the use of $Na_2CO_3{\cdot}10H_2O$ had a more pronounced impact on the yield giving the dipeptide **4a** in 90%, showing than under the applied mechanochemical conditions, aminolysis had outdone the unwanted hydrolysis background reaction (entry 5 in Table 1). Besides Z-L-AlaOEt (**1b**), the methyl and benzyl ester derivatives **1a** and **1c** where also found suitable as acyl donors, exhibiting similar reactivity under mechanochemical conditions (entries 6 and 7 in Table 1). Next, Boc-L-Ala-OR acyl donors were tested in the ball mill. After 2 h of milling, the reaction between Boc-amino esters, papain, base and the activator did generate the dipeptide Boc-L-Ala-L-Phe-NH_2 (**5a**), although to a lesser extend compare to the Z-L-Ala-derivatives, revealing a higher preference of the biocatalyst for the benzyloxycarbonyl (Z) moiety (entries 8-10 in Table 1). Then, in order to improve the yield of **4a**, the protease loading was doubled in an experiment using **1b**. After this adjustment in the reaction conditions, milling and posterior filtration of the reaction mixture led to the recovery of the Z-L-Ala-L-Phe-NH_2 (**4a**) quantitatively (entry 11 in Table 1). Subsequently, papain from papaya latex (crude powder) was also tested as mediator for the peptide bond formation. After 2 h of milling, this lower-cost papain source did favour the formation of **4a**, although less successfully compare to papain from *Carica papaya* powder (entries 11 and 12 in Table 1). Finally, manual grinding was explored as an alternative to carry out the enzymatic reaction. Pleasingly, this simpler approach also generated product **4a**. As expected, however, the automated ball milling was more efficient, outperforming the grinding by hand (entry 13 in Table 1).

With the optimized reaction condition, the influence of the nucleophile in the acyl acceptor residue (aa_2) was studied experimentally. For this, Z-L-AlaOEt (**1b**) was chosen again as acyl donor (aa_1) and it was reacted with various α-amino amides (**3a**-**g**) bearing diverse side chains. After 2 h of milling HCl·L-tryptophanamide (**3b**) and HCl·L-leucine amide (**3c**) proved to be excellent nucleophiles and afforded the dipeptides **4b** and **4c** in high yields, 97% and 95% respectively (Scheme 2).

The abovementioned preference of papain for bulky lipophilic or aromatic aa_2 residues was clearly observed when HCl·L-Ile-NH_2 (**3d**), HCl·L-Val-NH_2 (**3e**) and HCl·L-Ala-NH_2 (**3f**) were used in the mechanochemical coupling. Although, in all the cases



Reaction conditions: a mixture of **1b** (40 mg; 0.16 mmol), **3** (0.24 mmol), papain (20 mg), L-Cys (0.064 mmol) and $Na_2CO_3{\cdot}10H_2O$ (0.24 mmol) was milled in a mixer mill; (a) determined by ^1H NMR spectroscopy with 1,3,5-trimethoxy-benzene as the internal standard.

Scheme 2 Mechanochemical enzymatic peptide bond formation between Z-L-Ala-OEt (**1b**) and various amino amides (**3a-h**) in the ball mill.

the corresponding dipeptides **4d**-**f** where obtained, these were afforded in lower yields compare to the more hydrophobic or bulkier counterparts (Scheme 2). Next, HCl·L-glycine amide (**3g**) was also reacted with **1b** in the ball mill. After the standard milling time just trace quantities of the dipeptide Z-L-Ala-L-Gly-NH_2 (**4g**) were detected by ^1H NMR spectroscopy, along with small quantities of the hydrolysis product Z-L-Ala-OH. On the other hand, the HCl·β-alanine amide (**3h**) showed to be a better nucleophile giving the α,β-dipeptide **4h** in 48% yield (Scheme 2).

Next, we focused on studying the effect of the acyl donor aa_1 on the mechanoenzymatic peptide formation. For this, amino acid methyl esters derived from L-Phe (**1d**), L-Leu (**1e**), L-Ala (**1a**) and L-Gly (**1f**), were subjected to mechanical milling in the presence of HCl·L-Phe-NH_2 (**3a**), papain and the corresponding additives (Scheme 3). After 2 h of milling at 25 Hz, all the above amino esters **3**, bearing side-chains of different bulkiness and hydrophobicity were positively recognized by the biocatalyst and transferred to the acyl acceptor affording the α,α-dipeptides (**4a**, **4i**-**k**) in yields up to 98 % (Scheme 3). These results agree with the observation made in solution, that papain exhibits higher flexibility with regard to the nature of the acyl donor aa_1.[21]

Subsequently, the scope of the mechanochemical peptide bond formation reaction was expanded. The enzymatic coupling between Z-L-Phe-OMe (**1d**) and Z-L-Leu-OMe (**1e**) with

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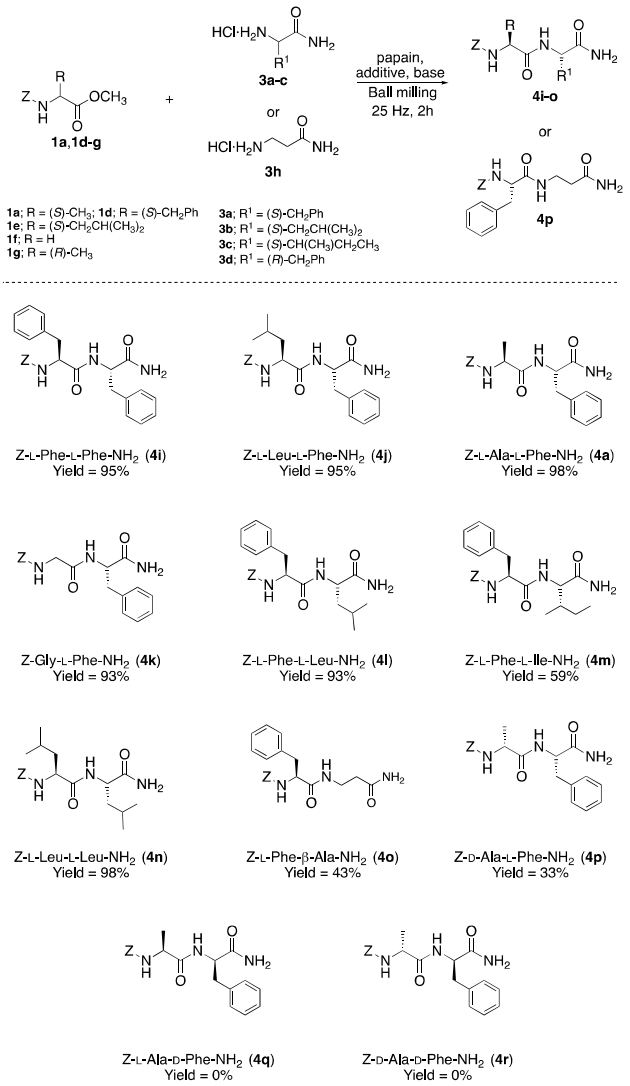
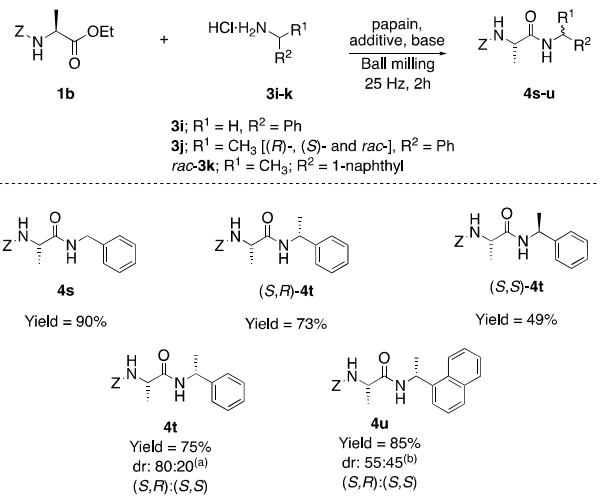


Reaction conditions: a mixture of **1** (0.16 mmol), **3** (0.24 mmol), papain (20 mg), L-Cys (0.064 mmol) and $Na_2CO_3 \cdot 10H_2O$ (0.24 mmol) was milled in a mixer mill.

Scheme 3 Scope of the mechanoenzymatic peptide bond approach.

amino amides HCl·L-Leu-NH_2 (**3b**) and HCl·L-Ile-NH_2 (**3c**) generated three α,α-dipeptides (**4l**-**n**) in yields ranging from 59% to 98% (Scheme 3). Once again, the use of the HCl·L-isoleucine amide (**3d**) as nucleophile proved challenging, generating the product **4m** in lower yield compare to the reaction with the isomeric leucine derivative (**3b**) (Scheme 3). On the other hand, the amino amide derived from the unnatural β-alanine (**3h**) proved also suitable as acyl acceptor in the coupling with **1d**. After the mechanical milling in the presence of papain, the α,β-dipeptide **4o** was separated in 43% yield (Scheme 3).

Finally, in order to study the preference of the enzyme for natural L-amino acids, a set of experiments using D-amino acids and D-amino amides was conducted. Reacting Z-D-AlaOMe (**1g**) with HCl·L-Phe-NH_2 (**3a**) in the ball mill afforded the Z-D- Ala-L-Phe-NH_2 (**4p**) in lower yield compare to the reaction with the natural methyl ester derivative **1a** (33% vs. 98%, Scheme 3).



Reaction conditions: a mixture of **1b** (40 mg; 0.16 mmol), **3** (0.24 mmol), papain (20 mg), L-Cys (0.064 mmol) and $Na_2CO_3 \cdot 10H_2O$ (0.24 mmol) was milled in a mixer mill; (a) determined by CSP-HPLC; (b) determined by ^1H NMR spectroscopy.

Scheme 4 Amide bond formation catalysed by papain in the ball mill.

This result clearly indicates the preference of papain for natural L-amino acids, but at the same time it evidences a degree of flexibility exhibited by the enzyme with regard to the stereochemistry of the aa_1 residue. In contrast, experiments utilizing the HCl·D-Phe-NH_2 (**3d**) as nucleophile, either with L- or D-alanine methyl ester failed to afford the corresponding dipeptides **4q** or **4r**, highlighting the higher specificity of papain for natural acyl acceptors (Scheme 3). With the aim to test a potential mechanochemical acylation of amines with *N*-protected amino acid ester as acyl donors, we studied the coupling between Z-L-AlaOMe (**1a**) and benzylamine hydrochloride (**3i**) in the ball mill. Delightfully, after 2 h of milling the reaction afforded the product **4s** in 90% yield, proving the benzylamine as an appropriate nucleophile that also fulfilled the steric demands by the papain (Scheme 4). Then, chiral amine hydrochlorides such as (*S*)- and (*R*)-**3j** were subjected to the mechanochemical enzymatic coupling with **1a**. As expected from the previous results using the L- or D-phenylalanine amides **3a** and **3d** (Scheme 3), both enantiomers of **3j** exhibited different chemical reactivity in the presence of the protease. For example, the HCl·(*R*)-1-phenylethylamine (**3j**) reacted faster with the protected L-alanine methyl ester **1a** to give the product (*S*,*R*)-**4t** in 73 % yield, whereas the (*S*)-**3j** enantiomer was recognized to a lesser extent by papain, yielding the diastereoisomer (*S*,*S*)-**4t** in 49% (Scheme 4). This difference in reactivity among the enantiomers of **3j** made us wonder about the potential of applying the mechanochemical protocol to resolve a racemic mixture of HCl·1-phenylethylamine **3j** in the ball mill. Indeed, under the standard reaction conditions, milling a mixture of *rac*-**3j**, Z-L-AlaOMe (**1a**), papain, base, and L-Cys afforded the product **4t** as a diastereomeric mixture where the (*S*,*R*)-stereoisomer was favoured [(*S*,*R*):(*S*,*S*)-**4t**, 80:20; Scheme 4]. Further modification of the reaction conditions e.g. stoichiometry, enzyme loading

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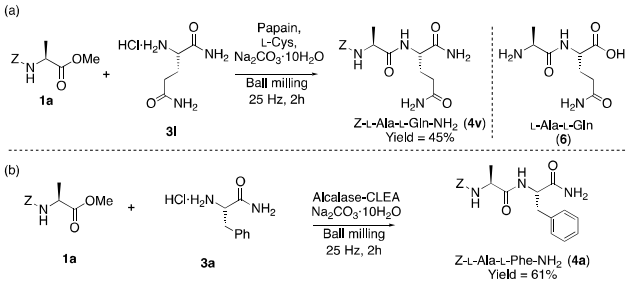

Scheme 5 (a) Papain catalysed mechanosynthesis of Z-L-Ala-L-Gln-NH_2 (**4v**); (b) mechanochemical enzymatic synthesis of **4a** catalysed by the protease alcalase.

or milling time had a negligible impact on the diastereoselectivity of the process. On the other hand, the bulkier *rac*-1-(1-naphthyl)ethylamine salt (**3k**) was also found suitable for the mechanoenzymatic acylation yielding the product **4u** in 85%, although with lower diastereoselectivity (Scheme 4).

Next, in order to demonstrate the applicability of the mechanoenzymatic peptide synthesis we attempted the preparation of the Z-$_L$-Ala-$_L$-Gln-NH_2 (**4v**), a derivative of the dipeptide L-alanyl-L-glutamine (**6**), a product widely used as an important nutrient supplement and in cell culture (Scheme 5a).[22] To test this idea, five solids (**1a**, **3l**, papain, L-Cys, $Na_2CO_3{\cdot}10H_2O$) were ground in the ball mill for 2 h. After this time, the analysis of the reaction mixture by ^{1}H NMR spectroscopy using internal standard revealed the formation of the product **4v** in 45% (Scheme 5a). The moderate yield in the formation of **4v** could be associated with a lower degree of recognition of the glutamine amide (**3l**) by the enzyme.[22b] Besides, trace quantities of unidentified by-products in the reaction mixture could have been the result of background reactions caused by the presence of the unprotected glutamine side-chain.[23]

Finally, with the aim of evaluating the generality of the mechanoenzymatic approach, the reaction between **1a** and **3a** was again conducted. This time, however, the reactants were milled in the presence of the protease alcalase, a serine protease which has been used in solution for chemoenzymatic peptide and ester formation.[24] Pleasingly, under the standard mechanochemical conditions, this new enzyme did also favour the formation of the dipeptide Z-$_L$-Ala-$_L$-Phe-NH_2 (**4a**), proving the compatibility of biocatalysts and ball milling (Scheme 5b).

Conclusions

In summary, we have studied the mechanoenzymatic peptide bond formation catalysed by the cysteine protease papain. Under mechanochemical conditions, the biocatalyst proved to be not only resilient to the mechanical stress experienced inside the ball mill, but also very effective to mediate the peptide and amide bond formation. The excellent mixing of the solid reactants in the milling vessels, in combination with the presence of the crystalline water contained in the $Na_2CO_3{\cdot}10H_2O$ led to the formation of a more homogenous molten state,[25] which enabled the papain to perform in short milling times to generate a library of α,α and α,β-dipeptides in good to high yields, and without the need for using organic solvents. The value of the mechanochemical approach was also demonstrated in the acylation of amines by milling and during the mechanosynthesis of the valuable L-alanyl-L-glutamine derivative **4v**. In order to improve our current mechanochemical approach, studies with immobilised enzymes to facilitate the easy recovery of the biocatalyst is on going in our laboratories. Finally, from a more general perspective, we believe the compatibility between mechanochemistry and enzymes (e.g. lipases,[17] proteases and potentially many others), will have a significant impact in increasing the complexity in chemical reaction design by complementing, and connecting biocatalysts with the well-established metal-[4d] and organocatalysed[26] mechanochemical approaches.

Acknowledgements

We thank RWTH Aachen University for support from the Distinguished Professorship Program funded by the Excellence Initiative of the German federal and state governments. We kindly acknowledge Hannah Blasius and Chenlu Li for their experimental work in this project, and Marcus Frings for the HPLC analysis of the sample **4t** (RWTH Aachen University). D.C. and S.L.J. thank the agency EPSRC, grant no. EP/L019655/1.

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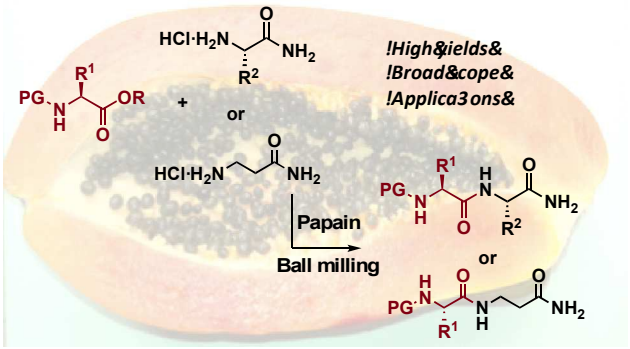

Graphical abstract