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Solvent-Free Synthesis of Peptides**

Valérie Declerck, Pierrick Nun, Jean Martinez, and Frédéric Lamaty*

The chemistry of peptides has been continuously growing during the last few decades. Peptides are now not only considered as pharmacological tools but also as active pharmaceutical ingredients, in connection to their high therapeutic index and low toxicity.[3] The market for therapeutic bulk peptides is expected to grow rapidly in the next few years.[3] In spite of the well-established procedures[3] for peptide synthesis by chemical ways, that is, stepwise synthesis in solution and solid-phase peptide synthesis, one of the major problems related to peptide preparation concerns the huge amount of solvent needed, particularly for synthesis on solid supports (2000–5000 kg for a large peptide). There is still a need to explore efficient, convenient, and environmentally friendly methods for peptide synthesis, particularly when the time for the scale-up of peptide production comes.

The field of “green chemistry” has recently grown at a rapid pace. Some major thematic areas have emerged: use of alternative feedstock and of innocuous reagents, employment of natural processes, use of substitute solvents, development of alternative reaction conditions, and minimization of energy consumption.[4] One particularly active area is in the use of substitute solvents such as aqueous, ionic, fluororous, or supercritical fluids to replace volatile organic and chlorinated solvents and to solve the problems of treating or recycling solvent waste.[5] An alternative approach would be to carry out chemical reactions in the absence of solvent.[6] Techniques such as mixing, grinding, or ball-milling have proved their efficiency in the field of organic chemistry in the solid state.[7] We report herein a new strategy for the preparation of peptides under solvent-free conditions by using ball-milling technology.[8] This strategy has been exemplified by the synthesis of the sweetener dipeptide H–Asp–Phe–OMe (aspartame).

We have studied the coupling of urethane-protected \( \alpha \)-amino acid N-carboxyanhydride (UNCA) derivatives \( 1 \) with \( \alpha \)-amino acids, amides, or esters, while keeping in mind that all of these compounds have to remain in the solid state under the ball-milling conditions[9] (Scheme 1). UNCA derivatives are activated forms of amino acids that have proved to be useful in peptide[10] and organic synthesis.[11]

The reaction was tested for the coupling of Boc–Val–NCA \( (1a, 1 \text{ equiv}) \) with HCH–Leu–OMe \( (2a, 1 \text{ equiv}) \) in the presence of NaHCO\(_3\) \((1.5 \text{ equiv})\) in a hardened-steel vessel with steel balls. The vessel was agitated for 1 h at a frequency of 30 Hz. Analysis of the reaction mixture after this time showed the exclusive presence of the dipeptide Boc–Val–Leu–OMe, obtained in quantitative yield. To our knowledge, this represents the first example of peptide bond formation in solvent-free conditions. The results obtained with various UNCA and amino acid derivatives are presented in Table 1.

The various UNCA derivatives do not present the same reactivity profile. Boc–Val–NCA \( (1a) \) reacted quantitatively with amino acid derivatives to yield the corresponding dipeptides (Table 1, entries 1–5) whereas Fmoc–Val–NCA \( (1b) \) gave lower conversions (Table 1, entries 6, 7, 9, and 10), except when coupling with HCH–Ala–OMe \( (2c, 1 \text{ equiv}) \) (Scheme 1, entry 8). Excellent conversions and yields were achieved with Boc–Phe–NCA \( (1c) \) (Table 1, entries 12–14), except for the reaction with HCH–Phe–OMe (Table 1, entry 15). Recovery of the product from the reaction vessel was less efficient in some cases and resulted in inferior yields (Table 1, entries 12 and 14). An amino ester, AcOH–Gly–OrBu was also tested and gave satisfying results (Table 1, entry 16). By contrast, the free amino acid did not react. It is worth noting that better results were obtained with freshly prepared starting materials. By reaching the maximum capacity of the ball-mill used in this study, up to 500 mg of dipeptides could be prepared. The reaction mixture was recovered directly from the milling jar, washed with water to remove the inorganic salts, and dried to provide the clean dipeptide. In the case of an incomplete reaction, maybe due to the physicochemical state of the reaction mixture (Table 1, entry 15), addition of water to the reaction mixture resulted in the opening of the UNCA. Insoluble in water, the protected dipeptide Boc–Phe–Phe–

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[3] We thank the MENRT, the CNRS, and the Fondation d’Entreprise EADS for financial support.
Table 1: Examples of dipeptides synthesized under solvent-free conditions.

<table>
<thead>
<tr>
<th>Entry</th>
<th>UNCA</th>
<th>Amino ester</th>
<th>Dipeptide/Tripeptide</th>
<th>Conversion [%]</th>
<th>Yield [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Boc–Val–NCA (1a)</td>
<td>HCl·Leu–OMe (2a)</td>
<td>Boc–Val–Leu–OMe</td>
<td>100</td>
<td>87</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>HCl·Leu–OtBu (2b)</td>
<td>Boc–Val–Leu–OtBu</td>
<td>97</td>
<td>85</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>HCl·Ala–OMe (2c)</td>
<td>Boc–Val–Ala–OMe</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>HCl·Ala–OtBu (2d)</td>
<td>Boc–Val–Ala–OtBu</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>HCl·Phe–OMe (2e)</td>
<td>Boc–Val–Phe–OMe</td>
<td>100</td>
<td>88</td>
</tr>
<tr>
<td>6</td>
<td>Fmoc–Val–NCA (1b)</td>
<td>HCl·Leu–OMe (2a)</td>
<td>Fmoc–Val–Leu–OMe</td>
<td>90</td>
<td>–</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>HCl·Leu–OtBu (2b)</td>
<td>Fmoc–Val–Leu–OtBu</td>
<td>92</td>
<td>–</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>HCl·Ala–OMe (2c)</td>
<td>Fmoc–Val–Ala–OMe</td>
<td>100</td>
<td>76</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td>HCl·Ala–OtBu (2d)</td>
<td>Fmoc–Val–Ala–OtBu</td>
<td>78</td>
<td>–</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>HCl·Phe–OMe (2e)</td>
<td>Fmoc–Val–Phe–OMe</td>
<td>93</td>
<td>–</td>
</tr>
<tr>
<td>11</td>
<td>Boc–Phe–NCA (1c)</td>
<td>HCl·Leu–OMe (2a)</td>
<td>Boc–Phe–Leu–OMe</td>
<td>85</td>
<td>–</td>
</tr>
<tr>
<td>12</td>
<td></td>
<td>HCl·Leu–OtBu (2b)</td>
<td>Boc–Phe–Leu–OtBu</td>
<td>100</td>
<td>70</td>
</tr>
<tr>
<td>13</td>
<td></td>
<td>HCl·Ala–OMe (2c)</td>
<td>Boc–Phe–Ala–OMe</td>
<td>99</td>
<td>79</td>
</tr>
<tr>
<td>14</td>
<td></td>
<td>HCl·Ala–OtBu (2d)</td>
<td>Boc–Phe–Ala–OtBu</td>
<td>100</td>
<td>73</td>
</tr>
<tr>
<td>15</td>
<td></td>
<td>HCl·Phe–OMe (2e)</td>
<td>Boc–Phe–Phe–OMe</td>
<td>58</td>
<td>55</td>
</tr>
<tr>
<td>16</td>
<td></td>
<td>AcO·H·Gly–OtBu (2f)</td>
<td>Boc–Phe–Gly–OtBu</td>
<td>100</td>
<td>90</td>
</tr>
<tr>
<td>17</td>
<td>Boc–Val–NCA (1a)</td>
<td>HCl·Ala–Gly–OMe</td>
<td>Boc–Val–Ala–Gly–OMe</td>
<td>100</td>
<td>89</td>
</tr>
</tbody>
</table>

[a] The reaction mixture was recovered in AcOEt and washed with water and analyzed by 1H NMR spectroscopy. Calculated conversion numbers are given with a ± 2% error.

OMe was recovered by filtration and drying, with an excellent yield based on the conversion (Table 1, entry 15).

We showed unambiguously that coupling occurred in the solid state in the presence of the three solids (UNCA, amino ester, and base) and not during the analysis process. For this purpose and as an example, we analyzed in detail the ball-milling of Boc–Phe–NCA (1c), HCl·Ala–OMe (2c), and NaHCO₃ (Table 1, entry 13). Firstly, ball-milling of the starting materials in the absence of NaHCO₃ did not yield the dipeptide Boc–Phe–Ala–OMe (analyzed by IR and solid-state 13C NMR spectroscopies). Furthermore, when the UNCA was ball-milled alone in the presence of NaHCO₃, the reaction did not occur, nor was degradation observed. Consequently, all three solids were necessary for the coupling reaction. Two analytical samples that did not involve any solvent were used for analysis: the starting materials and the crude reaction mixture taken directly from the ball-mill jar. Solid-state IR analysis showed the disappearance of the characteristic bands of Boc–Phe–NCA (1c; 1871 and 1872 cm⁻¹) and the formation of the peptide bond (characteristic amide carbonyl bands in the solid state at 1624 and 1655 cm⁻¹). We then used cross-polarization/magic-angle spinning (CP/MAS) 13C NMR analysis (Figure 1). The solid-state NMR analysis of the crude mixture, with no further treatment, clearly showed signals corresponding to the exclusive formation of the expected dipeptide, with consumption of the starting materials. The carbonyl region in Figure 1c shows the disappearance of the characteristic signal of 1c (labeled as f in Figure 1a), which corresponds to the loss of CO₂ during the reaction. Complete conversion of the amino ester 2c cannot be proved from Figure 1c because of overlapping of the signal, but complementary analysis (mass spectrometry) of the same sample confirmed the absence of 2c. The dipeptide cannot be formed during this analysis because solid-state 13C NMR spectroscopy has shown the absence of 1c. Again, no reaction was detected in the absence of NaHCO₃. These results establish without any ambiguity that the reaction took place in the solid state.

The reaction kinetics were studied at different frequencies (10, 20, and 30 Hz). Independently of the frequency, all reactions were completed, albeit with an expected longer reaction time at lower speeds (5 h at 10 Hz and 2.5 h at 20 Hz, compared with 1.25 h at 30 Hz). For each vibration speed, the profile showed apparent zero-order kinetics, in agreement with a solid–solid-state reaction mechanism.[12]

The possible epimerization that could occur during the solid–solid coupling reaction was evaluated. An HPLC analysis of an authentic sample of Boc–d-Phe–t-Ala–OMe prepared under known nonracemizing conditions was compared to the HPLC profile of the dipeptide product from the reaction of 1c and 2c. No racemization was detected by HPLC analysis.

As a proof of concept and to show the suitability of this method for making larger peptides, we synthesized the tripeptide Boc–Val–Ala–Gly–OMe from HCl·Ala–Gly–OMe, which was coupled to Boc–Val–NCA (1a), as depicted in Scheme 2. Complete conversion and a quantitative yield of the expected tripeptide (Table 1, entry 17, and Scheme 2) were obtained, with the coupling step being performed without solvent and according to the standard procedure.

To demonstrate the application of this method, we prepared the commercially attractive dipeptide aspartame or α-L-aspartyl-L-phenylalanine methyl ester. Aspartame is a nutritive sweetener approximately 150 times sweeter than sucrose.[13] To the best of our knowledge, aspartame had not been previously prepared by the UNCA route, either in the presence or absence of solvent.[14]

By using the method described above, we considered the coupling of Boc–Asp(OtBu)–NCA (1d) and HCl·Phe–OMe (2e). Acid-labile Boc and tBu protecting groups were chosen because they can be simultaneously cleaved under acidic conditions.

First, UNCA 1d was prepared according to the synthetic method depicted in Scheme 3. The key step in the preparation of 1d was the cyclization of compound 3 by the Vilsmeier’s salt method (Scheme 3).[15]
The procedure described above for the preparation of dipeptides was then used for the preparation of aspartame (Scheme 4). Ball-milling of Boc-/C₀Asp(O₄Bu)/NCA (1d) and HCl·H₂N-Phe-OMe (2e) for 1 h yielded the protected dipeptide as the only product, along with inorganic salts. The Boc and tBu protecting groups were then cleaved under acidic conditions in a gas–solid reaction, without solvent. The protected dipeptide in solid form was placed on a fritted glass, and HCl gas was blown through for 3 h in the absence of solvent to yield the hydrochloride form of aspartame. The yield for the two steps is quantitative, and removal of the Boc and tBu protecting groups under acidic conditions afforded only volatile by-products. The hydrochloride of aspartame was dissolved in water and then neutralized to the isoelectric point (pH 5.0) with an aqueous solution of Na₂CO₃. The resulting aspartame precipitated as a white powder and was filtered and dried in vacuo (84% yield).

In summary, a straightforward, high-yielding method for the preparation of peptides (including dipeptides and a tripeptide) with no epimerization and in the absence of solvent has been reported. This method involves the reaction of a UNCA with an amino ester (or amino acid amide). It was illustrated by the preparation of the aspartame sweetener.
Aspartame was obtained in pure form in three steps from 
Boc–Asp(OtBu)–NCA without the use of organic solvents, 
either for the coupling reaction or the removal of the 
protecting groups, and with the generation of volatile organic 
by-products and water-soluble inorganic salts. The only 
purification step consisted of a final precipitation from 
water to recover solid aspartame. This methodology was 
also exemplified by the synthesis of the tripeptide Boc– 
Val–Ala–Gly–OMe with good purity and excellent yield.

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solid-state reactions · solvent-free reactions

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