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Synthesis of Peptides by Reactive Extrusion. Application to the Continuous and Solventless Preparation of Aspartame.

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Dedicated to Professor Jean Martinez on the occasion of his 75th birthday.

The solventless synthesis of peptides from unactivated amino acids was optimized in a twin-screw extruder (TSE). The general method enabled the preparation of dipeptides and tripeptides with no epimerization. This flow mechanochemistry approach

was applied to the synthesis of aspartame, a synthetic dipeptide, prepared in a continuous mode on large scale and with high productivity.

Introduction

Peptides are of increasing interest in pharmaceuticals, cosmetics, and biology, as well as in other fields such as nutriment and material science. Remarkably, such molecules play fundamental roles in medicinal chemistry, acting as diagnostic agents or therapeutics.^[1] Yet, the accessibility to many peptidic drugs is still impeded by their expensive production, challenging purification and major health and environmental issues.^[2] Moreover, the industrial production of peptides is mainly carried out by chemical synthesis in solution or on solid supports by solid-phase peptide synthesis (SPPS), and yet falls short of green chemistry expectations. Indeed, vast amounts of toxic and hazardous organic solvents (such as *N,N*-dimethylformamide (DMF), dichloromethane (DCM), *N*-Methyl-2-pyrrolidone (NMP), tetrahydrofuran (THF) and 1,4-dioxane), coupling reagents and bases as well as large excesses of starting protected amino acids are usually required during the synthesis and isolation steps.^[3] DMF and NMP are reprotoxic, while DCM, THF and 1,4-dioxane are toxic and/or carcinogenic, and therefore their use

will be regulated in the coming years under the European Chemicals Agency (ECHA)'s REACH framework.^[4] Consequently, considerable research has been carried out in recent years to make peptide synthesis more sustainable by identifying less toxic and more environmentally benign alternative solvents and reagents, and more energy-efficient processes.^[5] Of particular interest is the synthesis of peptides using ball-mills^[6] which was pioneered by some of us.^[6a] Indeed, mechanochemistry^[7] has recently gained a remarkable interest in synthesis owing to the possibility of avoiding problematic organic solvents, reducing the reaction time and overall improving synthetic processes. However, the discontinuous batch production of ball-milling processes and the difficulty in further scaling-up, which is not straightforward, have limited their wide spreading in industrial peptide production. Extruders, which are scarcely used in the production of high-added-value chemicals,^[8] and recently included in the family of mechanochemistry tools,^[9] emerged as an equivalent to solution-based flow reactors that permit continuous and scalable syntheses, enabling the handling of solids and highly viscous mixtures.

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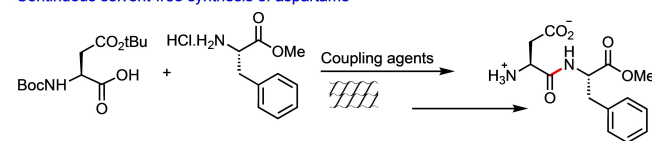
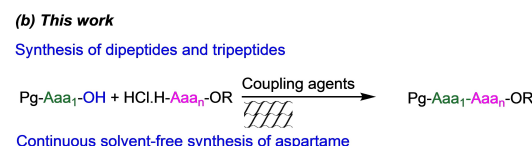
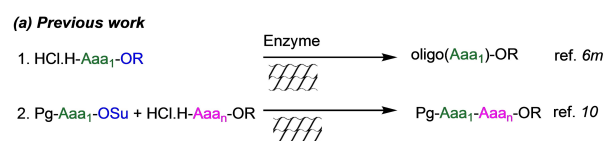
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Scheme 1. Previously reported syntheses of peptides by twin-screw extrusion and outline of this work. Generic twin screw extruder symbol:



The first example of dipeptide synthesis by reactive extrusion, including an aspartame precursor, was reported by some of us (Scheme 1a.2).^[10] Accordingly, a pre-mixed mixture of *N*-hydroxysuccinimide esters (NHS), hydrochloride salts of amino acid esters, NaHCO₃ and reagent grade acetone as liquid additive (LA) was poured into the extruder's barrel that was pre-heated to 40 °C with a speed of screws set at 150 rpm, to provide the expected dipeptide. Another report in peptide synthesis described the combination of a twin-screw extruder and enzymatic catalysis to provide a mixture of oligopeptides (Scheme 1a.1).^[6m]

Accordingly, the application of reactive extrusion in industrial peptide synthesis can be thought of to access longer peptide sequences and allow intensified and continuous production without being affected by the presence of highly concentrated reaction mixtures and the related precipitation of solids, which is usually encountered in the traditional solution-based continuous flow peptide production processes.^[11] In this context and as a continuation of our efforts in the field of peptide synthesis under solvent-free/solventless conditions by mechanochemistry, we envisioned studying the coupling of *N*-protected α -amino acids with α -amino ester salts by reactive extrusion in the presence of a coupling agent (Scheme 1b).

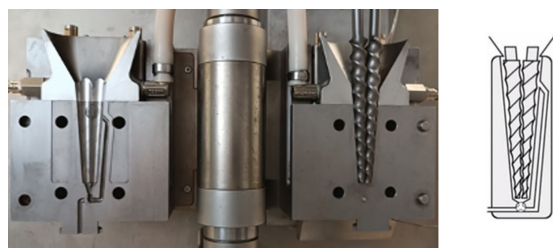


Figure 1. Picture of the open barrel (left) and schematic representation (right) of a vertical twin-screw extruder with re-circulation system.

Results and Discussion

Optimization of the Coupling Conditions

Reactive extrusion experiments were performed in a vertical parallel co-rotating twin screw extruder, with a conical shape and barrel's volume of 2 mL (Figure 1). This extruder includes a re-circulation channel with a well-defined geometry and with an outlet that can be set to continuous or batch mode. The re-circulation (batch mode) allows to maintain the reaction mixture in the extruder during an extended amount of time for optimization of the reaction conditions, simulating the use of screws of a greater length.

First, the coupling of an equimolar mixture of Fmoc-His(Trt)-OH and *S*-phenylethylamine using a co-rotating vertical twin-screw extruder was chosen as the model reaction to optimize the different parameters and evaluate the percentage of epimerization, a crucial criterion in peptide synthesis. Indeed, histidine is known as the most epimerization-prone amino acid.^[12] Thus, a small amount of DMF was used as liquid additive, diisopropylcarbodiimide (DIC) and *S*-phenylethylamine were primarily introduced into the extruder's barrel pre-heated to 40 °C with the speed of screws fixed at 200 rpm, followed by the addition of a pre-mixed mixture of Fmoc-His(Trt)-OH and Ethyl cyano(hydroxyimino)acetate (OxymaPure). Mixing inside the extruder was carried out for 4 min using the re-circulation pipe prior to recovering the crude reaction mixture as a pale brown gel. This gel was further quenched using a mixture of HCl (1 M)/MeCN (v/v, 1:1) and analyzed by HPLC, showing 99% conversion into the desired amide product **1** (Table 1, Entry 1). Moreover, epimerization was determined following the deprotection of the trityl group of **1** in an acidic medium to give **2**, for better separation of the formed diastereoisomers on the HPLC column.

As DMF is a highly problematic solvent that needs to be replaced,⁴ various less toxic/greener or bio-based solvents and

Table 1. Impact of solvents on the conversion and epimerization of Fmoc-His(Trt)-*S*-phenylethylamide.

Entry ^[a,b]	Solvent	Solvent (Equiv.)	η ^[c] (μ L/mg)	Conversion ^[d] (%)	Epimerization ^[e] (%)
1	DMF	4	0.29	99	0.41
2	Cyrene	4	0.38	74	0.46
3	DMC	4	0.31	97	0.48
4	Anisole	4	0.40	97	0.32
5	2-MeTHF	4	0.38	97	0.40
6	EtOAc	4	0.37	97	0.36
7	EtOAc	5	0.46	> 99	0.37

[a] Experiments were performed in duplicates. [b] *Reaction conditions*: DIC (1.5 equiv.), *S*-phenylethylamine (1.0 equiv.) and the solvent (4.0–5.0 equiv.) were pre-mixed inside the extruder's 2 mL barrel prior to the addition of a mixture of Fmoc-His(Trt)-OH (1.0 equiv.) and OxymaPure (1.0 equiv.). Mixing using re-circulation was carried out for 4 min at 40 °C with a speed of 200 rpm. [c] The η parameter is defined as the ratio of the volume of liquid (in μ L) to the combined weights of reactants (in mg). [d] LCAP (liquid chromatography area percent) of crude **1**. [e] LCAP of crude **2**.



Table 2. Screening of different coupling reagents and additives in the synthesis of Fmoc-His(Trt)-S-phenylethylamide.

Entry ^[a]	Reagent/Additive	Conversion ^[b] (%)	Epimerization ^[c] (%)
1 ^[d,e]	DIC/OxymaPure	> 99	0.37
2 ^[d,e]	EDC.HCl/ OxymaPure	86	0.40
3 ^[d,e]	EDC/OxymaPure	59	0.52
4 ^[f,e]	TCFH/DIEA	77	5.25
5 ^[f,e]	T ₃ P-2-MeTHF/DIEA/ OxymaPure	94	1.07
6 ^[g,h]	T ₃ P-EtOAc/DIEA/ OxymaPure	90	1.45
7 ^[g,h]	T ₃ P-MeCN/DIEA/ OxymaPure	90	1.40
8 ^[g,h]	T ₃ P-DMF/DIEA/ OxymaPure	81	2.12

[a] Experiments were performed in duplicates. [b] LCAP of crude 1. [c] LCAP of crude 2. [d] *Reaction conditions*: Coupling reagent (1.5 equiv.), S-phenylethylamine (1.0 equiv.) and EtOAc (5.0 equiv.) were pre-mixed inside the extruder's 2 mL barrel prior to the addition of a mixture of Fmoc-His(Trt)-OH (1.0 equiv.) and OxymaPure (1.0 equiv.). [e] Mixing using re-circulation was carried out for 4 min at 40 °C with a speed of 200 rpm. [f] *Reaction conditions*: DIEA (2.5 equiv.), S-phenylethylamine (1.0 equiv.) and EtOAc (5.0 equiv.) were pre-mixed inside the extruder's 2 mL barrel prior to the addition of TCFH (1.5 equiv.) and Fmoc-His(Trt)-OH (1.0 equiv.). [g] *Reaction conditions*: S-phenylethylamine (1.0 equiv.) and EtOAc (5.0 equiv.) were pre-mixed inside the extruder's 2 mL barrel prior to the addition of a mixture of Fmoc-His(Trt)-OH (1.0 equiv.) and OxymaPure (1.0 equiv.) followed by DIEA (2.5 equiv.) then T₃P (1.5 equiv.). [h] Mixing using re-circulation was carried out for 6 min at 40 °C with a speed of 200 rpm.

Table 3. Optimization of the number of DIC equivalents, re-circulation/mixing time and barrel temperature in the synthesis of Fmoc-His(Trt)-S-phenylethylamide.

Entry ^[a,d]	DIC (Equiv.)	Mixing time (min)	Barrel's temp. (°C)	Conversion ^[b] (%)	Epimerization ^[c] (%)
1	1.5	4	40	> 99	0.37
2	1.2	4	40	97	0.38
3	1.0	4	40	92	0.38
4	1.2	8	40	> 99	0.37
5	1.2	8	25	85	0.31
6	1.2	16	25	97	0.32
7	1.2	20	25	98	0.30

[a] Experiments were performed in duplicates. [b] LCAP of crude 1. [c] LCAP of crude 2. [d] *Reaction conditions*: DIC (1.0–1.5 equiv.), S-phenylethylamine (1.0 equiv.) and EtOAc (5.0 equiv.) were pre-mixed inside the extruder's 2 mL barrel prior to the addition of a mixture of Fmoc-His(Trt)-OH (1.0 equiv.) and OxymaPure (1.0 equiv.). Mixing using re-circulation was carried out for 4–20 min at 25–40 °C with a speed of 200 rpm.

solvent mixtures were screened (see Table S1 in SI for details). Bio-based cyrene gave a lower yield (Table 1, Entry 2), most probably due to its high viscosity, which rendered re-circulation more challenging. Among other greener solvents, dimethylcarbonate (DMC), anisole, 2-MeTHF and ethyl acetate (Table 1, Entries 3–6) provided essentially the same conversion (97%),

slightly inferior to the one obtained with DMF (99%, Table 1, Entry 1). From this series, ethyl acetate (EtOAc) was identified as the best liquid additive providing excellent conversion with the minimal epimerization (Table 1, Entry 6). The amount of EtOAc was further optimized with a minimum of 5.0 equivalents (0.65 mL, $\eta = 0.46 \mu\text{L}/\text{mg}$) required to attain a quantitative



conversion (Table 1, Entry 7). Of note, unlike the conversion, epimerization was unaffected by the amount of EtOAc used, except when using 1.0 equivalent, where a slightly higher epimerization (0.56%) was detected (see Table S2 in SI for details).

Trying to further lower the percentage of epimerization, other coupling reagents and additives were screened (Table 2; see Table S3 in SI for the detailed screening). Particularly, coupling reagents with sufficient thermal stability (inexplosive and shock insensitive) and which are less likely to cause safety concerns were only tested.^[13] OxymaPure was found to be the best additive with most of the coupling reagents. *N*-(3-Dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC.HCl) (Table 2, Entry 2) gave lower conversion (86%) and a similar percentage of epimerization compared to DIC (Table 2, Entry 1). Using EDC provided an even lower conversion with a slightly higher percentage of epimerization (Table 2, Entry 3).

Moreover, moderate conversion with 5% epimerization was observed when Chloro-*N,N,N',N'*-tetramethylformamidiniumhexafluorophosphate (TCFH) was used (Table 2, Entry 4). Propylphosphonic anhydride (T₃P), recently introduced as a green alternative coupling reagent,^[14] and commercially available in solution, provided good conversions ranging from 81% (T₃P solution in DMF, Table 2, Entry 8) to 94% (T₃P solution in 2-MeTHF, Table 2, Entry 5). Unfortunately, remarkable epimerization (up to 2.12%, Table 2, Entry 8) was detected using this coupling reagent. Thus, among the different reagents tested, combination of DIC and OxymaPure^[13c] provided quantitative conversion with the lowest percentage of epimerization (Table 2, Entry 1).

Next, further optimization of the number of DIC equivalents, re-circulation/mixing time and barrel's temperature, was performed (Table 3). Reducing the number of DIC equivalents from 1.5 to 1.0 resulted in a lower conversion (92%) (Table 3,

Table 4. Scope of the dipeptides synthesis by reactive extrusion.

Entry ^{b,c}	Dipeptides	Mixing time (min)	Conversion ^a (%)	Isolated yield (%)
1	Fmoc-L-Phe-L-Tyr(tBu)-OtBu	8	99	96
2	Fmoc-L-Lys(Boc)-L-Tyr(tBu)-OtBu	8	99	97
3	Fmoc-L-His(Trt)-L-Tyr(tBu)-OtBu	8	97	95
4	Fmoc-L-Leu-L-Ser(tBu)-OMe	8	98	98
5	Fmoc-L-Leu-L-Phe-OMe	8	> 99	98
6	Fmoc-Gly-Gly-OMe	4	> 99	95
7	Fmoc-L-Pro-Aib-OtBu	8	99	95
8	Fmoc-L-Pro-L-Ser(tBu)NH ₂	24	98	98
9	Fmoc-L-Gln(Trt)-L-Arg(Pbf)-OMe	8	97	95
10	Fmoc-L-Thr(tBu)-L-Met-OMe	8	99	97
11 ^d	Fmoc-L-Asn(Trt)-L-Ile-OMe	8	96	94
12 ^e	Fmoc-L-Asn(Trt)-L-Arg(Pbf)-OMe	16	97	95
13	Boc-L-Phe-L-Val-OMe	8	> 99	99
14	Boc-L-Thr(tBu)-L-Met-OMe	8	99	99
15	Boc-Aib-L-Phe-OMe	8	> 99	94
16	Boc-L-Glu(OtBu)-L-Phe-OMe	8	99	93
17	Boc-L-Arg(Tos)-L-His(Trt)-OMe	8	99	93
18	Boc-Trp(For)-His(Trt)-OMe	8	99	95
19	Boc-L-Trp(For)-L-Arg(NO ₂)-OMe	8	3	–
20	Boc-L-Asp(OBzl)-L-Arg(NO ₂)-OMe	8	32	Traces
21	Boc-L-Asp(OBzl)-L-Arg(Pbf)-OMe	8	> 99	96
22	Boc-L-Asp(OBzl)-L-Cys(Bzl)-OMe	12	> 99	90
23	Boc-Aib-L-Cys(Bzl)-OMe	8	99	84
24	Z-Phe-L-Val-OtBu	8	98	96
25	Z-Ala-Phg-OtBu	8	> 99	97

[a] LCAP of crude before purification. [b] Reaction conditions: DIC (1.2 equiv.), DIEA (1.0 equiv.) and EtOAc (5.0 equiv.) were pre-mixed inside the extruder's barrel prior to the addition of a mixture of PG-AA₁-OH (1.0 equiv.), HCl.H-AA₂-OR (1.0 equiv.) and OxymaPure (1.0 equiv.). Mixing using re-circulation was carried out for 4–24 min at 40 °C with a speed of 200 rpm. [c] Reactions were carried out in the extruder of 2 mL barrel where the total mass of reactants was 2.0 g. [d] 7.0 equiv. of EtOAc were used. [e] 2.4 equiv. of DIC were used.



Entries 1 & 3). Excellent conversion (97%) was obtained with 1.2 equivalents (Table 3, Entry 2), thus minimizing the amount of waste. In all cases, similar percentages of epimerization (0.37–0.38%) were obtained regardless of the amount of DIC used. Increasing the re-circulation time to 8 min (Table 3, Entry 4) resulted in a full conversion. With the aim of energy saving, decreasing the barrel temperature from 40 °C (Table 3, Entry 4) to 25 °C (Table 3, Entries 5–7), resulted in lower yields even with longer re-circulation time up to 20 min. This may indeed have an influence on the viscosity of the reaction mixture, with increasing mixing difficulties. At 25 °C, slightly lower epimerization values were obtained (0.30–0.32%).

Hence, optimal conditions involved 1.2 equivalents of DIC, 5.0 equivalents of EtOAc and 8 min of re-circulation at 40 °C. Thus, Fmoc-His(Trt)-*S*-phenylethylamide was obtained in 99% isolated yield (0.37% epimerization).

Synthesis of Dipeptides

The optimized reaction conditions were next applied to the synthesis of a wide range of dipeptides using 1.0 equivalent of each starting adequately protected amino acids, including challenging ones, with diisopropylethylamine as a base (DIEA, 1 equiv.), and DIC (1.2 equiv.) and OxymaPure (1.0 equiv.) as coupling reagents (Table 4). Dipeptides were isolated with a high purity after recovery with EtOAc, followed by aqueous washings. A chromatographic purification was required only in the case of Trt-protected amino acids. Fmoc-protected phenylalanine, lysine and epimerization-prone histidine were successfully coupled to tyrosine tert-butyl ester to produce Fmoc-Phe-Tyr(tBu)-OtBu, Fmoc-Lys(Boc)-Tyr(tBu)-OtBu and Fmoc-His(Trt)-Tyr(tBu)-OtBu dipeptides in excellent yields (Table 4, Entries 1–3). Sterically hindered Fmoc-Leu-OH was efficiently coupled with serine and phenylalanine methyl esters to furnish Fmoc-Leu-Ser(tBu)-OMe and Fmoc-Leu-Phe-OMe dipeptides in quantitative yields (Table 4, Entries 4–5). Notably, Fmoc-Gly-Gly-OMe was formed in 95% yield, after only 4 min of mixing (Table 4, Entry 6). The coupling of Fmoc-Pro-OH with the sterically hindered aminoisobutyric (Aib) tert-butyl ester was also fast providing the desired dipeptide in 95% yield (Table 4, Entry 7) whereas the coupling with serine amide was longer, requiring 24 min to attain 98% yield (Table 4, Entry 8).

Glutamine and threonine were also smoothly coupled with the corresponding arginine and methionine esters which are susceptible to undergoing various side reactions. So, the corresponding, Fmoc-Gln(Trt)-Arg(Pbf)-OMe and Fmoc-Thr(tBu)-Met-OMe were formed in excellent yields (Table 4, Entries 9–10) with no by-products being observed. Likewise, asparagine was efficiently coupled with the hindered β -branched isoleucine amino ester, known to undergo difficult coupling,^[15] to give Fmoc-Asn(Trt)-Ile-OMe in 94% yield (Table 4, Entry 11). Also, Fmoc-Asn(Trt)-Arg(Pbf)-OMe was obtained in 95% yield, however after 16 min of mixing while using 2.4 equiv. of DIC (Table 4, Entry 12). Boc- and Z-protected amino acids, which are commonly used in classical solution peptide synthesis, were also tested. Thus, Boc-Phe-Val-OMe, Boc-Thr(tBu)-Met-OMe,

Boc-Aib-Phe-OMe, Boc-L-Glu(OtBu)-L-Phe-OMe and Boc-Arg(Tos)-His(Trt)-OMe, Boc-Trp(For)-His(Trt)-OMe were isolated in excellent yields (93–99%, Table 4, Entries 13–18). Remarkably, Ng-Nitro-arginine methyl ester was almost unreactive when coupled to tryptophan and aspartic acid amino acids, where 3% and 32% conversions were attained, respectively (Table 4, Entries 19–20). When NO₂ protecting group of arginine was replaced by Pbf, Fmoc-L-Gln(Trt)-L-Arg(Pbf)-OMe, Fmoc-L-Asn(Trt)-L-Arg(Pbf)-OMe and Boc-Asp(OBzl)-Arg(Pbf)-OMe were provided in excellent yields (95–96%) (Table 4, Entries 9, 12 and 21). Similarly, the synthesis of Boc-Asp(OBzl)-Cys(Bzl)-OMe and Boc-Aib-Cys(Bzl)-OMe which were obtained in 90% and 84% yields, respectively (Table 4, Entries 22–23). Alternatively, the hindered valine and phenylglycine (Phg), known for difficult and slow coupling, were efficiently coupled providing Z-Phe-Val-OtBu and Z-Ala-Phg-OtBu in 96% and 97% yields, respectively (Table 4, Entries 24–25).

As mentioned before, epimerization should be as low as possible to avoid a mixture of diastereoisomers obtained during the formation of the peptide bond. To our delight, with the optimized reaction conditions, a single diastereomer was obtained for all the previously synthesized dipeptides, as confirmed by ¹H NMR data. Further, epimerization was more precisely evaluated on three dipeptides synthesized by reactive extrusion: Fmoc-L-Phe-L-Tyr(tBu)-OtBu, Fmoc-His(Trt)-Tyr(tBu)-OtBu and Boc-Phe-Val-OMe (Table 4, Entries 1, 3 and 13). For this purpose, the following standards Fmoc-D-Phe-Tyr(tBu)-OtBu, Fmoc-D-His(Trt)-Tyr(tBu)-OtBu and Boc-D-Phe-Val-OMe were synthesized by ball-milling. Analyses by chiral HPLC of the three dipeptides synthesized by twin-screw extrusion clearly showed that in each case, the diastereomeric excesses were excellent (99% and above) and no epimerization occurred (see SI for details).

This dipeptides synthesis method proved to be quite general, high yielding in most cases, essentially with no epimerization, and could be applied to a large variety of protected amino acids. In contrast to SPPS, large excesses of starting materials and coupling reagents were not necessary. This is in contrast with the related recently reported amidation methods which necessitated fine tuning of the reaction conditions depending on the starting materials involved in the reaction.^[16]

Synthesis of Tripeptides

Next, as a proof of concept, the reactive extrusion method was extended to the synthesis of two tripeptides to confirm the applicability of this approach to longer peptide fragments (Scheme 2). One tripeptide (Boc-Ala-Phe-Val-OMe) was synthe-



Scheme 2. Two tripeptides synthesized by reactive extrusion.



sized in the classical direction (C→N synthesis). Accordingly, dipeptide Boc-Phe-Val-OMe (from Table 4, Entry 13) was deprotected at its N-terminus under classical acidic conditions (HCl, dioxane), and coupled in 8 min with Boc-Ala-OH in the extruder using the aforementioned optimal conditions, to yield 97% of the corresponding tripeptide, with an excellent diastereomeric excess, evaluated by chiral HPLC (see SI for details). A second tripeptide (Z-Ala-Phg-Ile-OMe) was prepared by reverse peptide synthesis (N→C synthesis).^[17] Indeed, some of us have recently demonstrated that mechanochemistry was particularly well-adapted to this much less common approach, to provide an epimerization-free coupling.^[6q] Z-Ala-Phg-OtBu (from Table 4, Entry 25) was deprotected at its C-terminus (TFA, CH₂Cl₂) to yield Z-Ala-Phg-OH, which was then coupled in the extruder with HCl.H-Ile-OMe. This particular epimerization-prone sequence, with the presence of a Ph group^[18] on the alpha carbon of phenylglycine, and hindered Ile resulted in a slower process. Re-circulation was performed for 24 min to provide 75% yield (94% conversion) of the expected tripeptide Z-Ala-Phg-Ile-OMe. Remarkably, in this case again, no epimerization could be detected by chiral HPLC (see SI or details).

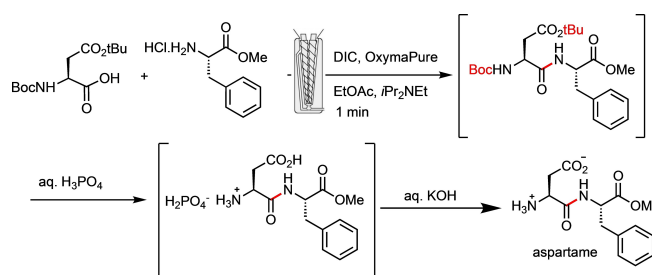
Metallic Contaminations Assessment

Applications of mechanical forces to perform chemical reactions may introduce contaminations from the material of the devices employed in the process. This issue has already been previously addressed and quantitatively evaluated when using a vibratory or planetary ball-mill, where wear from jars and balls made of various types of materials can lead to product contamination. Eventually, the products synthesized with metallic jars and balls, in the absence of purification step, may contain contaminants ranging from 642 to 42000 ppm.^[19] Contamination could also be questioned in the production of fine chemicals by reactive extrusion. For this purpose, we assessed the amount of metallic impurities, produced during the synthesis of Boc-Phe-Val-OMe in the twin-screw extruder, with an eight-minute recirculation. Since the metallic barrel and screws are made of stainless steel, three samples were analyzed by ICP-MS to detect the presence of iron, chromium and nickel: the mixture of starting materials and reactants before introduction in the TSE, the crude product recovered from the die of the extruder, and the final product after purification by extractions. The first sample, corresponding to the sum of reactants, exhibited a very low content of each metal: 2.7 ppm of iron, 0.7 ppm of chromium and 0.2 ppm of nickel. After reaction in the TSE, the crude extrudate was contaminated with metallic species but in much lower amounts than what was experienced in ball-mills: 97.5 ppm of iron, 9.5 ppm of chromium and 1.4 ppm of nickel. Notably the product of the reaction, after aqueous work-up, contained even lower amounts of the tested metals compared to the commercial reactants with iron, chromium and nickel contents of 0.75, 0.03 ppm and 0.06 ppm, respectively. Both the milling load and the way forces are applied seems to prevent excessive wear of the screws and barrel, resulting in reduced metal-leaching in the extrudate. Product analysis reveals that only

sparse quantities of metals remain after work-up, well below the permitted concentrations of elemental impurities according to the United States Pharmacopeia: 1100 ppm for chromium and 20 ppm for nickel.^[20]

Continuous Synthesis of Aspartame and Scale-Up

As mentioned in the introduction, the purpose of using a twin-screw extruder as a synthetic tool in organic chemistry is to produce molecules of interest on large scale, in a continuous and solventless mode. The synthesis of aspartame (H-Asp-Phe-OMe), an artificial sweetener, was used as a model to investigate the scaling-up of a peptide by using reactive extrusion. The mechanosynthesis of the corresponding Boc-protected dipeptide by mechanochemistry both in a ball-mill^[6a] and in an extruder^[10] was previously reported, starting with activated amino acids. In the current work, Boc-Asp(OtBu)-OH and HCl.H-Phe-OMe were first coupled on a small scale (about 2 mmoles) according to the optimized procedure, with a reduced mixing time of 1 min, to yield the expected fully-protected dipeptide in a quantitative yield after work-up (Scheme 3). Switching from 1 min re-circulation to continuous mode produced the same result. An attempt to cleave the Boc group was performed by heating the resulting Boc-Asp(OtBu)-Phe-OMe at 60 °C in the extruder during 1 h, resulting in only 14% deprotection along with the formation of diketopiperazine (DKP, 13%). Increasing the temperature and reaction time (80 °C during 5 h) provided an unsatisfactory mixture of H-Asp-Phe-OMe (54%), H-Asp-Phe-OH (15%), and DKP (30%). Noteworthy, no DKP from any dipeptide reported herein was detected when the synthesis temperature is kept at 40 °C. Subsequently, a procedure, which included deprotection in acidic conditions performed on the crude extrudate, was optimized (Scheme 3). Hence, in another experiment, the extrudate resulting from the reaction of Boc-Asp(OtBu)-OH and H-Phe-OMe provided Boc-Asp(OtBu)-Phe-OMe, with 100% conversion as assessed by liquid chromatography-mass spectrometry analysis (LC/MS) on a taken sample, and was directly poured from the die of the extruder into an acidic aqueous solution of H₃PO₄.^[21] After 5 min of stirring, LC/MS analysis of the crude mixture confirmed the full deprotection of the Boc and tBu groups of the dipeptide. pH adjustment by titrating with an aqueous KOH solution, together with filtrations and extractions, enabled the smooth removal of diisopropyl urea (from DIC) and the recovery of



Scheme 3. Continuous synthesis of aspartame.



OxyPure. Then, precipitated pure aspartame, was collected and obtained in an 82% overall yield from the starting protected amino acids.

Compared to the previously reported methods, preactivation of the starting amino acid was not needed in the current procedure. In the initial approach by ball-milling, a cumbersome synthesis of a *N*-carboxyanhydride derivative of Boc-Asp(OtBu)-OH was necessary^[6a] while the reported synthesis in the extruder required preactivation of Boc-Asp(OBn)-OH, as a hydroxysuccinimide ester.^[10] Furthermore, this latter synthetic scheme necessitated two deprotection steps, an acidic hydrolysis followed by hydrogenolysis, to provide aspartame.

Finally, scaling up of the synthesis ($\times 100$) was performed on a 0.215 mole scale by starting from 62.3 g of Boc-Asp(OtBu)-OH and 46.5 g of HCl.H-Phe-OMe, and running the reaction in the extruder in a continuous mode during 8 min and 41 seconds. Gratifyingly, after direct deprotection and isolation following the above procedure, 56.2 g (90% yield) of pure aspartame were obtained. Additionally, the space time-yield (STY), a metric which can be utilized in examining reactor efficiency and process intensification of a reaction, was estimated to be equal to $4.7 \times 10^6 \text{ kg m}^{-3} \text{ day}^{-1}$ (see SI for details). To the best of our knowledge this is the highest value obtained so far in flow mechanosynthesis of organic fine chemicals.^[6b] For comparison, a STY of $4.7 \times 10^5 \text{ kg m}^{-3} \text{ day}^{-1}$ was obtained in the previously reported peptide synthesis.^[10]

Conclusions

In summary, an unprecedented method for peptide synthesis by reactive extrusion from unactivated *N*-protected amino acid derivatives was reported. The combination of DIC and OxyPure in the presence of EtOAc used as a liquid additive, gave the best results in short reaction times. These optimized conditions are general and compatible with a wide variety of amino acids with various protecting groups. Besides dipeptides synthesis, it was also illustrated that tripeptides could be also prepared by elongation either on the N or C terminus of a dipeptide. Of note, no epimerization was observed even with “difficult” peptide sequences. Metal contamination from the TSE to the product has been assessed, and shown to be low after purification. Scaling up was facilitated by the flow chemistry approach. The dipeptide aspartame preparation in a continuous mode with a high productivity (56 g in 8 min) represents a successful example of flow mechanosynthesis. From the sustainability point of view, not only the amount of solvent used was drastically decreased but also, in sharp contrast to the classical methods in peptide synthesis, only quasi stoichiometric amount of starting materials and coupling agents were needed, facilitating the purification step, and avoiding unnecessary excess of chemicals. These results pave the way to further development in the sustainable solventless synthesis of peptides, including at larger scale in industry with a possible extrapolation on horizontal extruders with adapted screws.

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: flow synthesis · mechanochemistry · peptide · scale-up · twin-screw extrusion

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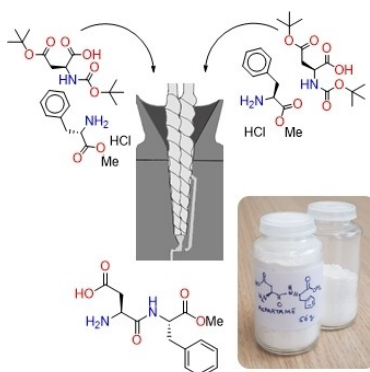


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RESEARCH ARTICLE

Peptide synthesis was performed in a twin-screw extruder. The reaction was achieved essentially in the absence of solvent, avoiding excesses of starting materials and reagents. This continuous flow mechanochemistry approach enabled the synthesis of a multi-gram quantity of sweetener aspartame, a synthetic dipeptide.



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Synthesis of Peptides by Reactive Extrusion. Application to the Continuous and Solventless Preparation of Aspartame.

