



**HAL**  
open science

# Synthesis of peptides by reactive extrusion. Application to the continuous and solventless preparation of aspartame

Tharwat Mohy El-Dine, Matthieu Lavayssiere, H el ene Adihou, Gilles Subra, Thomas-Xavier M etro, Olivier Ludemann-Hombourger, Fr ed eric Lamaty

## ► To cite this version:

Tharwat Mohy El-Dine, Matthieu Lavayssiere, H el ene Adihou, Gilles Subra, Thomas-Xavier M etro, et al.. Synthesis of peptides by reactive extrusion. Application to the continuous and solventless preparation of aspartame. 2023. hal-04361336

**HAL Id: hal-04361336**

**<https://hal.science/hal-04361336v1>**

Preprint submitted on 22 Dec 2023

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destin ee au d ep ot et  a la diffusion de documents scientifiques de niveau recherche, publi es ou non,  emanant des  tablissements d'enseignement et de recherche fran ais ou  trangers, des laboratoires publics ou priv es.



Distributed under a Creative Commons Attribution - NonCommercial - NoDerivatives 4.0 International License

# Synthesis of peptides by reactive extrusion. Application to the continuous and solventless preparation of aspartame.

Tharwat Mohy El-Dine,<sup>\*1§</sup> Matthieu Lavayssiere,<sup>1</sup> H  l  ne Adihou,<sup>2</sup> Gilles Subra,<sup>1</sup> Thomas-Xavier M  tro,<sup>\*1#</sup> Olivier Ludemann-Hombourger,<sup>2</sup> Fr  d  ric Lamaty<sup>\*1</sup>

<sup>1</sup>IBMM, Univ Montpellier, CNRS, ENSCM, Montpellier, France

<sup>2</sup>PolyPeptide Group, 67100 Strasbourg, France

<sup>§</sup>Current address: PolyPeptide Group, 67100 Strasbourg, France

<sup>#</sup>Current address: ICGM, CNRS, UM, ENSCM, 34293 Montpellier (France)

\* tharwat.mohy-el-dine@polypeptide.com, thomas-xavier@umontpellier.fr, frederic.lamaty@umontpellier.fr

**Abstract:** The solventless synthesis of peptides from unactivated amino acids was performed in a twin-screw extruder. The general method was applied to the preparation of dipeptides and tripeptides. Aspartame, a synthetic dipeptide, was also prepared in a continuous mode on large scale.

## Introduction

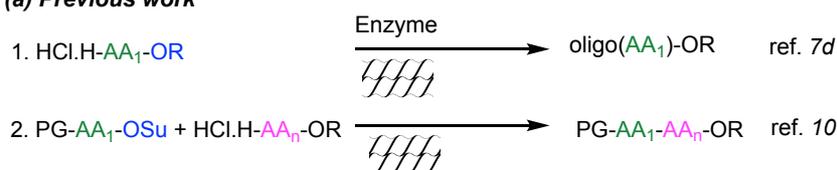
Peptides are of increasing interest in pharmaceuticals, cosmetics, and biology, as well as in other fields such as nutriment and material science. Remarkably, such molecules play fundamental roles in medicinal chemistry, acting as diagnostic agents or therapeutics.<sup>1</sup> Yet, the accessibility to many peptidic drugs is still impeded by their expensive production, challenging purification and major health and environmental issues.<sup>2</sup> 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 are usually required during the synthesis and isolation steps.<sup>3</sup> 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.<sup>4</sup> 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.<sup>5</sup> Of particular interest is the synthesis of peptides using ball-mills<sup>6</sup> which was pioneered by some of us.<sup>6a</sup> Indeed, mechanochemistry<sup>7</sup> has recently gained a remarkable interest 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,<sup>8</sup> and recently included in the family of mechanochemistry tools,<sup>9</sup> emerged as an equivalent to solution-based flow reactors that

permit continuous and scalable syntheses, enabling the handling of solids and highly viscous mixtures.

The first example of dipeptide synthesis, including an aspartame precursor, by reactive extrusion was reported by some of us (Scheme 1a.2).<sup>10</sup> Accordingly, a pre-mixed mixture of *N*-hydroxysuccinimide esters (NHS), hydrochloride salts of amino acid esters, NaHCO<sub>3</sub> 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).<sup>7d</sup>

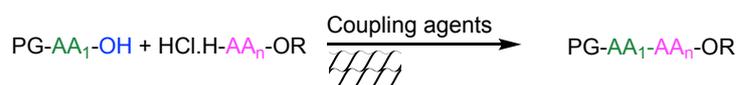
Accordingly, the application of reactive extrusion in industrial peptide synthesis can be thought of to access longer peptide sequences and allow intensified and continuous production without being affected by the presence of highly concentrated reaction mixtures and the related precipitation of solids,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

**(a) Previous work**

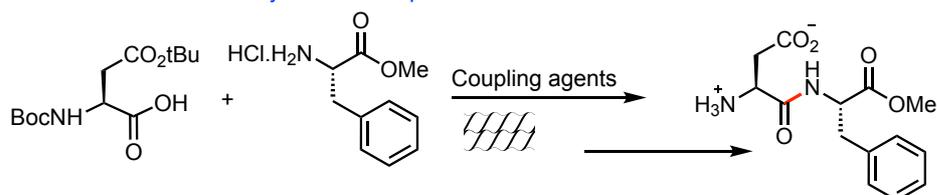


**(b) This work**

Synthesis of dipeptides and tripeptides



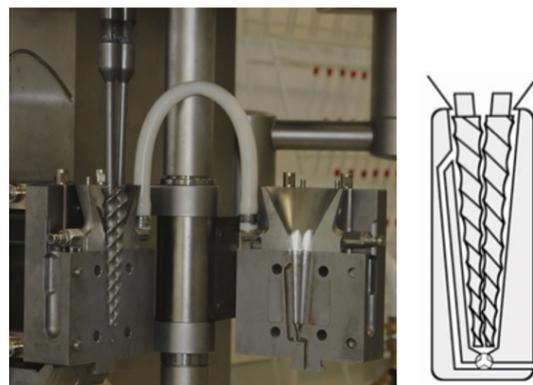
Continuous solvent-free synthesis of aspartame



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

## Results and discussion

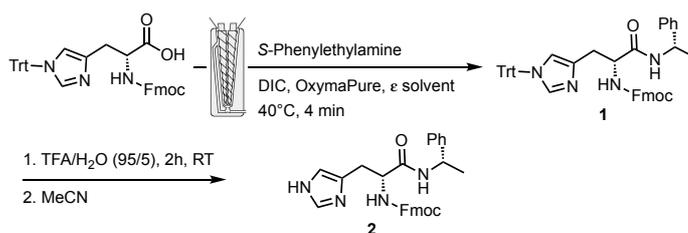
Reactive extrusion experiments were performed in a vertical parallel co-rotating twin screw extruder, with a conical shape and a volume of 2 mL (Figure 1). This extruder includes a recirculation channel and the outlet can be set to continuous or batch mode. The recirculation (batch mode) allows to maintain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



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

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.<sup>12</sup> Thus, a small amount of DMF used as liquid additive, DIC (diisopropyl carbodiimide) 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 OxymaPure. Mixing inside the extruder was carried out for 4 min using the re-circulation pipe before recovering the crude reaction mixture as a pale brown gel. This gel was further quenched using a mixture of HCl (1M)/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 in acidic medium of the trityl group of **1** in an acidic medium to give **2**, for better separation of the formed diastereoisomers on the HPLC column.

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



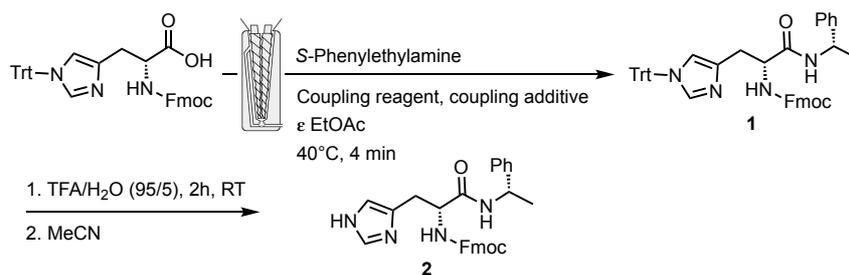
Entry <sup>a,b</sup>	Solvent	Solvent (Equiv.)	$\eta^c$ ( $\mu\text{L}/\text{mg}$ )	Conversion <sup>d</sup> (%)	Epimerization <sup>e</sup> (%)
1	DMF	4	0.29	99	0.41
2	Cyrene	4	0.38	74	0.46
3	DMC	4	0.31	97	0.48
4	Anisole	4	0.40	97	0.32
5	2-MeTHF	4	0.38	97	0.40
6	EtOAc	4	0.37	97	0.36
7	EtOAc	5	0.46	>99	0.37

<sup>a</sup>Experiments were performed in duplicates. <sup>b</sup>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. <sup>c</sup>The  $\eta$  parameter is defined as the ratio of the volume of liquid (in  $\mu\text{L}$ ) to the combined weights of reactants (in mg). <sup>d</sup>LCAP of crude **1**. <sup>e</sup>LCAP of crude **2**.

As DMF is a highly problematic solvent that needs to be replaced,<sup>4</sup> various less-toxic or bio-based solvents and 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, 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.<sup>13</sup> OxymaPure was found to be the best additive with most of the coupling reagents. EDC.HCl gave lower conversion (86%) and a similar percentage of epimerization compared to DIC (Table 2, Entry 2). 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 TCFH was used (Table 2, Entry 4). Propylphosphonic anhydride ( $\text{T}_3\text{P}$ ), recently introduced as a green alternative coupling reagent,<sup>14</sup> and commercially available in solution, provided good conversions ranging from 81% ( $\text{T}_3\text{P}$  solution in DMF, Table 2, Entry 8) to 94% ( $\text{T}_3\text{P}$  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, DIC provided quantitative conversion with the lowest percentage of epimerization (Table 2, Entry 1).

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

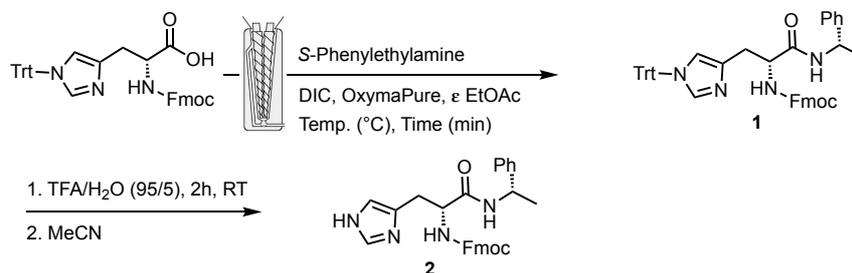


Entry <sup>a</sup>	Reagent/Additive	Conversion <sup>b</sup> (%)	Epimerization <sup>c</sup> (%)
1 <sup>d,e</sup>	DIC/OxymaPure	>99	0.37
2 <sup>d,e</sup>	EDC.HCl/OxymaPure	86	0.40
3 <sup>d,e</sup>	EDC/OxymaPure	59	0.52
4 <sup>f,e</sup>	TCFH/DIEA	77	5.25
5 <sup>f,e</sup>	T <sub>3</sub> P-2-MeTHF/DIEA/OxymaPure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

<sup>a</sup>Experiments were performed in duplicates. <sup>b</sup>LCAP of crude **1**. <sup>c</sup>LCAP of crude **2**. <sup>d</sup>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.). <sup>e</sup>Mixing using re-circulation was carried out for 4 min at 40 °C with a speed of 200 rpm. <sup>f</sup>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.). <sup>g</sup>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 T<sub>3</sub>P (1.5 equiv.). <sup>h</sup>Mixing using re-circulation was carried out for 6 min at 40 °C with a speed of 200 rpm.

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 resulted in a lower conversion (92%) (Table 3, Entries 1 & 3). Excellent conversion (97%) was obtained with 1.2 equiv. (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 recirculation time to 8 min 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%).

**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 <sup>a, d</sup>	DIC (Equiv.)	Mixing time (min)	Barrel's temperature (°C)	Conversion <sup>b</sup> (%)	Epimerization <sup>c</sup> (%)
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

<sup>a</sup> Experiments were performed in duplicates. <sup>b</sup> LCAP of crude **1**. <sup>c</sup> LCAP of crude **2**. <sup>d</sup> 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.

Hence optimal conditions involved 1.2 equiv. of DIC and 8 min of re-circulation at 40 °C. Thus, Fmoc-His(Trt)-S-phenylethylamide was obtained in 99% isolated yield (≈0.12% epimerization).

The optimized reaction conditions were next applied to the synthesis of a wide range of dipeptides using 1.0 equiv. 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). 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. 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 methyl ester was longer, requiring 24 min to attain 98% yield (Table 4, Entry 8).

**Table 4.** Scope of the dipeptide synthesis by reactive extrusion

$$\text{PG-AA}_1\text{-OH} + \text{HCl.H-AA}_2\text{-OR} \xrightarrow[40^\circ\text{C, 4-24 min}]{\text{DIC, Oxymapure, } \varepsilon \text{ EtOAc, } i\text{Pr}_2\text{NEt}} \text{PG-AA}_1\text{-AA}_2\text{-OR}$$

Entry <sup>b,c</sup>	Dipeptides	Mixing time (min)	Conversion <sup>a</sup> (%)	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 <sub>2</sub>	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 <sup>b</sup>	Fmoc-L-Asn(Trt)-L-Ile-OMe	8	96	94
12 <sup>c</sup>	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 <sub>2</sub> )-OMe	8	3	---
20	Boc-L-Asp(OBzl)-L-Arg(NO <sub>2</sub> )-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

<sup>a</sup> LCAP of crude before purification. <sup>b</sup> *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-AA<sub>1</sub>-OH (1.0 equiv.), HCl.H-AA<sub>2</sub>-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 and an acceleration of 1200 rpm/min. <sup>c</sup> Reactions were carried out in the extruder of 2 mL barrel where the total mass of reactants was 2.0 g. <sup>b</sup> 7.0 eq. of EtOAc were used. <sup>c</sup> 2.4 eq. of DIC were used.

Glutamine and threonine were also smoothly coupled with the corresponding methionine and arginine and methionine esters which are susceptible to undergoing various side reactions. 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,<sup>15</sup> 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 NO<sub>2</sub> 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 (95-97%) (Table 3, 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). Interestingly, this method for peptide synthesis proved to be quite general, high yielding in most cases, and could be applied to a large variety of protected amino acids. This is in contrast with the recently reported related methods which necessitated fine tuning of reaction conditions depending on the starting materials involved in the reaction.<sup>16</sup>

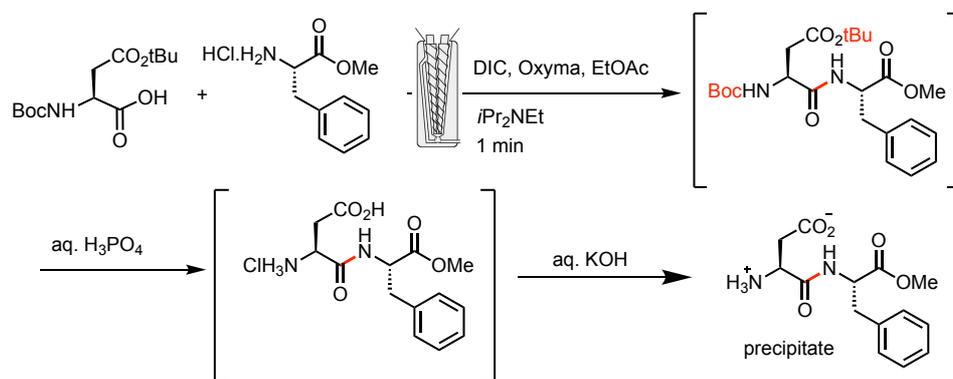
As mentioned before, epimerization should be as low as possible to avoid a mixture of diastereoisomers obtained during the formation of the peptide bond. To our delight, a single diastereomer was obtained for all the previously synthesized dipeptides, as confirmed by <sup>1</sup>H NMR data. Epimerization was also 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 clearly showed that in each case, the diastereomeric excesses were excellent (>99%) (see SI for details).

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 synthesized 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 acidic conditions (HCl, dioxane), and coupled in 8 min with Boc-Ala-OH in the extruder in the aforementioned optimal conditions, to yield 97 % of the corresponding tripeptide, with an excellent diastereomeric excess. A second tripeptide (Z-Ala-Phg-Ile-OMe) was prepared by reverse peptide synthesis (N → C synthesis).<sup>17</sup> Indeed, some of us have recently demonstrated that mechanochemistry was particularly well-adapted to this approach, to provide an epimerization-free coupling.<sup>6n</sup> Z-Ala-Phg-OtBu (from Table 4, entry 25) was deprotected on its C-terminus (TFA, CH<sub>2</sub>Cl<sub>2</sub>) 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 a Ph group<sup>18</sup> 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.



**Scheme 2.** Two examples of tripeptides synthesized by reactive extrusion

Then, 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 this dipeptide by mechanochemistry both in a ball-mill<sup>6a</sup> and in an extruder<sup>10</sup> was previously reported. In the current work, Boc-Asp(OtBu)-OH and HCl.H-Phe-OMe.HCl 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 recirculation to continuous mode produced the same result. Subsequently, a procedure, which included deprotection in acidic conditions on the crude extrudate, was optimized (Scheme 3). Hence, in another experiment, the extrudate resulting from the reaction of Boc-Asp(OtBu)-OH and HCl.H-Phe-OMe provided Boc-Asp(OtBu)-Phe-OMe, with 100 % conversion as assessed by liquid chromatography-mass spectrometry analysis (LC/MS) on a removed sample, and was directly poured from the die of the extruder into an acidic aqueous solution of  $\text{H}_3\text{PO}_4$ .<sup>19</sup> After 5 min of stirring, LC/MS analysis of the crude mixture confirmed the full deprotection of the Boc and tBu groups. pH adjustment by titrating with an aqueous KOH solution, together with filtrations and extractions, enabled the facilitated removal of diisopropyl urea (from DIC) and the recovery of OxymaPure. Then pure aspartame precipitated and was obtained in an 82 % overall yield from the starting protected amino acids.



**Scheme 3:** Continuous synthesis of aspartame

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<sup>6a</sup> while the reported synthesis in the extruder required preactivation of Boc-Asp(OBn)-OH, as a hydroxysuccinimide ester.<sup>10</sup> Furthermore, this latter synthetic scheme necessitated two deprotection steps to provide aspartame.

Finally, scaling up of the reaction ( $\times 100$ ) 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, running the reaction in the extruder in a continuous mode during 8 min and 41 seconds. Gratifyingly, after direct deprotection and isolation following the procedure described above, 56.2 g (90 % yield) of pure aspartame were obtained. This represents a high throughput rate of  $0.39 \text{ kg h}^{-1}$ . Additionally, the space time-yield (STY), a metric which can be utilized in examining reactor efficiency and process intensification of a reaction, was estimated (see SI for details) to be equal to  $4.7 \times 10^6 \text{ kg m}^{-3} \text{ day}^{-1}$ . To the best of our knowledge, this is the highest value obtained so far in flow mechanosynthesis of organic fine chemicals.<sup>8a</sup> For comparison, a STY of  $4.7 \times 10^5 \text{ kg m}^{-3} \text{ day}^{-1}$  (10 times smaller) was obtained in the previously reported peptide synthesis.<sup>10</sup>

## Conclusion

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 OxymaPure 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. It was also illustrated that tripeptides could be prepared by elongation either on the N or C terminus of a dipeptide. Of note, no epimerization was observed even with “difficult” peptide sequences. The dipeptide aspartame preparation in a continuous mode (56 g in 8 min) represents a successful example of flow mechanochemistry, with an efficient process intensification approach. From the sustainability point of view, not only the amount of solvent used was drastically decreased but, 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.

**Acknowledgments:** PolyPeptide Group, The Centre National de la Recherche Scientifique (CNRS), University of Montpellier, and Chimie Balard Cirimat Carnot Institute (through the ANR programme N°16 CARN 0008-01) are acknowledged for financial support. Marion Jean (iSm2, Marseille) is acknowledged for performing chiral HPLC analyses.

**Abbreviations:** DMF (*N,N*-dimethylformamide)

DCM (Dichloromethane)

NMP (*N*-Methyl-2-pyrrolidone)

THF (tetrahydrofuran)

Cyrene (Dihydrolevoglucosenone)

DMC (Dimethyl carbonate)

Anisole (Methoxybenzene)

DMC (Dimethyl carbonate)

2-MeTHF (2-Methyltetrahydrofuran)

DIC (*N,N'*-Diisopropylcarbodiimide)

EDC.HCl (*N*-(3-Dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride)

OxymaPure (Ethyl cyano(hydroxyimino)acetate, Ethyl cyanoglyoxylate-2-oxime)

TCFH (Chloro-*N,N,N',N'*-tetramethylformamidinium hexafluorophosphate)

T<sub>3</sub>P (2,4,6-Tripropyl-1,3,5,2,4,6-trioxatriphosphorinane-2,4,6-trioxide solution)

DIEA (Diisopropylethylamine)

LCAP liquid chromatography area percent

liquid chromatography-mass spectrometry analysis (LC/MS)

STY (space time-yield)

## References:

- (1) a. Vlieghe, P.; Lisowski, V.; Martinez, J.; Khrestchatsky, M. Synthetic therapeutic peptides: science and market. *Drug Discov. Today* **2010**, *15*, 40-56; b. Lau, J. L.; Dunn, M. K. Therapeutic peptides: Historical perspectives, current development trends, and future directions. *Bioorg. Med. Chem.* **2018**, *26*, 2700-2707; c. Muttenthaler, M.; King, G. F.; Adams, D. J.; Alewood, P. F. Trends in peptide drug discovery. *Nat. Rev. Drug Discovery* **2021**, *20*, 309-325.
- (2) Isidro-Llobet, A.; Kenworthy, M. N.; Mukherjee, S.; Kopach, M. E.; Wegner, K.; Gallou, F.; Smith, A. G.; Roschangar, F. Sustainability Challenges in Peptide Synthesis and Purification: From R&D to Production. *J. Org. Chem.* **2019**, *84*, 4615-4628.
- (3) a. Bray, B. L. Large-scale manufacture of peptide therapeutics by chemical synthesis. *Nat. Rev. Drug Discovery* **2003**, *2*, 587-593; b. Pattabiraman, V. R.; Bode, J. W. Rethinking amide bond synthesis. *Nature* **2011**, *480*, 471-479; c. Werbitzky, O.; Giraud, M. Manufacturing of venom-derived therapeutic peptides. *RSC Drug Discovery Series* **2015**, *42*, 290-305.
- (4) a. The European Chemicals Agency (ECHA) Registration, E., Authorisation and Restriction of Chemicals (REACH), 2021, <https://echa.europa.eu/legislation>. The restrictions under ECHA regulation number (EC) 1907/2006 and 276/2010 for DMF and DCM, respectively. Restrictions under commission regulation (EU) 2018/588 for NMP, accessed on Sep 13, 2021. ; b. The European Commission, O. J. o. t. E. U., 16-19; c. Bergkamp, L.; Herbatschek, N. Regulating Chemical Substances under REACH: The Choice between Authorization and Restriction and the Case of Dipolar Aprotic Solvents. *Review of European, Comparative & International Environmental Law* **2014**, *23*, 221-245.
- (5) a. Varnava, K. G.; Sarojini, V. Making Solid-Phase Peptide Synthesis Greener: A Review of the Literature. *Chem. Asian J.* **2019**, *14*, 1088-1097; b. Jad, Y. E.; Kumar, A.; El-Faham, A.; de la Torre, B. G.; Albericio, F. Green Transformation of Solid-Phase Peptide Synthesis. *ACS Sustain. Chem. Eng.* **2019**, *7*, 3671-3683; c. Martin, V.; Egelund, P. H. G.; Johansson, H.; Thordal Le Qument, S.; Wojcik, F.; Sejer Pedersen, D. Greening the synthesis of peptide therapeutics: an industrial perspective. *RSC Adv.* **2020**, *10*, 42457-42492; d. Ferrazzano, L.; Catani, M.; Cavazzini, A.; Martelli, G.; Corbisiero, D.; Cantelmi, P.; Fantoni, T.; Mattellone, A.; De Luca, C.; Felletti, S.; Cabri, W.; Tolomelli, A. Sustainability in peptide chemistry: current synthesis and purification technologies and future challenges. *Green Chem.* **2022**, *24*, 975-1020.
- (6) a. Declerck, V.; Nun, P.; Martinez, J.; Lamaty, F. Solvent-free synthesis of peptides. *Angew. Chem., Int. Ed.* **2009**, *48*, 9318; b. Hernández, J. G.; Juaristi, E. Green Synthesis of  $\alpha,\beta$ - and  $\beta,\beta$ -Dipeptides under Solvent-Free Conditions. *J. Org. Chem.* **2010**, *75*, 7107-7111; c. Štrukil, V.; Bartolec, B.; Portada, T.; Đilović, I.; Halasz, I.; Margetić, D. One-pot mechanosynthesis of aromatic amides and dipeptides from carboxylic acids and amines. *Chem. Commun.* **2012**, *48*, 12100-12102; d. Bonnamour, J.; Métro, T.-X.; Martinez, J.; Lamaty, F. Environmentally benign peptide synthesis using liquid-assisted ball-milling: application to the synthesis of Leu-enkephalin. *Green Chem.* **2013**, *15*, 1116-1120; e. Duangkamol, C.; Jaita, S.; Wangngae, S.; Phakhodee, W.; Pattarawarapan, M. An efficient mechanochemical synthesis of amides and dipeptides using 2,4,6-trichloro-1,3,5-triazine and PPh<sub>3</sub>. *RSC Adv.* **2015**, *5*, 52624-52628; f. Porte, V.; Thioloy, M.; Pigoux, T.; Métro, T.-X.; Martinez, J.; Lamaty, F. Peptide Mechanosynthesis by Direct Coupling of N-Protected  $\alpha$ -Amino Acids with Amino Esters. *Eur. J. Org. Chem.* **2016**, *2016*, 3505-3508; g. Maurin, O.; Verdié, P.; Subra, G.; Lamaty, F.; Martinez, J.; Métro, T.-X. Peptide synthesis: ball-milling, in solution, or on solid support, what is the best strategy? *Beilstein J. Org. Chem.* **2017**, *13*, 2087-2093; h. Pétry, N.; Benakki, H.; Clot, E.; Retailleau, P.; Guenoun, F.; Asserar, F.; Sekkat, C.; Métro, T.-X.; Martinez, J.; Lamaty, F. A mechanochemical approach to access the proline-proline diketopiperazine framework. *Beilstein J. Org. Chem.* **2017**, *13*, 2169-2178; i. Gonnet, L.; Tintillier, T.; Venturini, N.; Konnert, L.; Hernandez, J.-F.; Lamaty, F.; Laconde, G.; Martinez, J.; Colacino, E. N -Acyl Benzotriazole Derivatives for the Synthesis of Dipeptides and Tripeptides and Peptide Biotinylation by Mechanochemistry. *ACS Sustain. Chem. Eng.* **2017**, *5*, 2936-2941; j. Landeros, J. M.; Juaristi, E. Mechanochemical Synthesis of Dipeptides Using Mg-Al Hydrotalcite as Activating Agent under Solvent-

Free Reaction Conditions. *Eur. J. Org. Chem.* **2017**, 2017, 687-694; k. Bolm, C.; Hernández, J. G. From Synthesis of Amino Acids and Peptides to Enzymatic Catalysis: A Bottom-Up Approach in Mechanochemistry. *ChemSusChem* **2018**, *11*, 1410-1420; l. Anselmi, M.; Stavole, P.; Boanini, E.; Bigi, A.; Juaristi, E.; Gentilucci, L. Green synthesis of bioactive oligopeptides promoted by recyclable nanocrystalline hydroxyapatite. *Future Med. Chem.* **2020**, *12*, 479-491; m. Stolar, T.; Grubešić, S.; Cindro, N.; Meštrović, E.; Užarević, K.; Hernández, J. G. Mechanochemical Prebiotic Peptide Bond Formation. *Angew. Chem. Int. Ed.* **2021**, *60*, 12727-12731; n. Yeboue, Y.; Jean, M.; Subra, G.; Martinez, J.; Lamaty, F.; Metro, T.-X. Epimerization-Free C-Term Activation of Peptide Fragments by Ball Milling. *Org. Lett.* **2021**, *23*, 631-635; o. Yeboue, Y.; Rguioueg, N.; Subra, G.; Martinez, J.; Lamaty, F.; Metro, T.-X. Gram-scale synthesis of a hexapeptide by fragment coupling in a ball mill. *Eur. J. Org. Chem.* **2022**, 2022, e202100839; p. Hernández, J. G.; Ardila-Fierro, K. J.; Crawford, D.; James, S. L.; Bolm, C. Mechanoenzymatic peptide and amide bond formation. *Green Chem.* **2017**, *19*, 2620-2625.

(7) a. Friscic, T.; Mottillo, C.; Titi, H. M. Mechanochemistry for Synthesis. *Angew. Chem. Int. Ed.* **2020**, *59*, 1018-1029; b. Pérez-Venegas, M.; Juaristi, E. Mechanoenzymology: State of the Art and Challenges towards Highly Sustainable Biocatalysis. *ChemSusChem* **2021**, *14*, 2682-2688; c. Bento, O.; Luttringer, F.; Mohy El Dine, T.; Petry, N.; Bantreil, X.; Lamaty, F. Sustainable Mechanochemistry of Biologically Active Molecules. *Eur. J. Org. Chem.* **2022**, 2022, e202101516; d. Ardila-Fierro, K. J.; Crawford, D. E.; Körner, A.; James, S. L.; Bolm, C.; Hernández, J. G. Papain-catalysed mechanochemical synthesis of oligopeptides by milling and twin-screw extrusion: application in the Juliá-Colonna enantioselective epoxidation. *Green Chem.* **2018**, *20*, 1262-1269; e. Metro, T.-X.; Colacino, E.; Martinez, J.; Lamaty, F. Amino acids and peptides in ball milling. *RSC Green Chem. Ser.* **2015**, *31*, 114-150.

(8) a. Bolt, R. R. A.; Leitch, J. A.; Jones, A. C.; Nicholson, W. I.; Browne, D. L. Continuous flow mechanochemistry: reactive extrusion as an enabling technology in organic synthesis. *Chem. Soc. Rev.* **2022**, *51*, 4243-4260; b. Crawford, D. E.; Miskimmin, C. K. G.; Albadarin, A. B.; Walker, G.; James, S. L. Organic synthesis by Twin Screw Extrusion (TSE): continuous, scalable and solvent-free. *Green Chem.* **2017**, *19*, 1507-1518; c. Atapalkar, R. S.; Kulkarni, A. A. Batch and continuous flow mechanochemical synthesis of organic compounds including APIs. *React. Chem. Eng.* **2023**.

(9) Martín-Matute, B.; Meier, M. A. R.; Métro, T.-X.; Koenig, S. G.; Sneddon, H. F.; Sudarsanam, P.; Watts, P. Sustainable Chemistry and Engineering in Pharma. *ACS Sustain. Chem. Eng.* **2021**, *9*, 13395-13398.

(10) Yeboue, Y.; Gallard, B.; Le Moigne, N.; Jean, M.; Lamaty, F.; Martinez, J.; Métro, T.-X. Peptide Couplings by Reactive Extrusion: Solid-Tolerant and Free from Carcinogenic, Mutagenic and Reprotoxic Chemicals. *ACS Sustain. Chem. Eng.* **2018**, *6*, 16001-16004.

(11) Gordon, C. P. The renaissance of continuous-flow peptide synthesis—an abridged account of solid and solution-based approaches. *Org. Biomol. Chem.* **2018**, *16*, 180-196.

(12) Yang, Y.: *Side reactions in peptide synthesis*; Academic Press, 2015.

(13) a. Sperry, J. B.; Minter, C. J.; Tao, J.; Johnson, R.; Duzguner, R.; Hawksworth, M.; Oke, S.; Richardson, P. F.; Barnhart, R.; Bill, D. R.; Giusto, R. A.; Weaver, J. D., III. Thermal Stability Assessment of Peptide Coupling Reagents Commonly Used in Pharmaceutical Manufacturing. *Org. Process Res. Dev.* **2018**, *22*, 1262-1275; b. Albericio, F.; El-Faham, A. Choosing the Right Coupling Reagent for Peptides: A Twenty-Five-Year Journey. *Org. Process Res. Dev.* **2018**, *22*, 760-772.

(14) Al Musaimi, O.; Wisdom, R.; Talbiersky, P.; De La Torre, B. G.; Albericio, F. Propylphosphonic Anhydride (T3P®) as Coupling Reagent for Solid-Phase Peptide Synthesis. *ChemistrySelect* **2021**, *6*, 2649-2657.

(15) Ostresh, J. M.; Winkle, J. H.; Hamashin, V. T.; Houghten, R. A. Peptide libraries: Determination of relative reaction rates of protected amino acids in competitive couplings. *Biopolymers* **1994**, *34*, 1681-1689.

(16) Lavayssiere, M.; Lamaty, F. Amidation by reactive extrusion for the synthesis of active pharmaceutical ingredients teriflunomide and moclobemide. *Chem. Commun.* **2023**, 59, 3439-3442.

(17) Suppo, J.-S.; Subra, G.; Bergès, M.; Marcia de Figueiredo, R.; Campagne, J.-M. Inverse Peptide Synthesis via Activated  $\alpha$ -Aminoesters. *Angew. Chem. Int. Ed.* **2014**, *53*, 5389-5393.

(18) a. Elsayy, M. A.; Hewage, C.; Walker, B. Racemisation of N-Fmoc phenylglycine under mild microwave-SPPS and conventional stepwise SPPS conditions: attempts to develop strategies for overcoming this. *J. Pept. Sci.* **2012**, *18*, 302-311; b. Fuse, S.; Mifune, Y.; Nakamura, H.; Tanaka, H. Total synthesis of feglymycin based on a linear/convergent hybrid approach using micro-flow amide bond formation. *Nat. Commun.* **2016**, *7*, 13491; c. Liang, C.; Behnam, M. A. M.; Sundermann, T. R.; Klein, C. D. Phenylglycine racemization in Fmoc-based solid-phase peptide synthesis: Stereochemical stability is achieved by choice of reaction conditions. *Tetrahedron Lett.* **2017**, *58*, 2325-2329.

(19) Li, B.; Berliner, M.; Buzon, R.; Chiu, C. K.-F.; Colgan, S. T.; Kaneko, T.; Keene, N.; Kissel, W.; Le, T.; Leeman, K. R.; Marquez, B.; Morris, R.; Newell, L.; Wunderwald, S.; Witt, M.; Weaver, J.; Zhang, Z.; Zhang, Z. Aqueous Phosphoric Acid as a Mild Reagent for Deprotection of Tert-Butyl Carbamates, Esters, and Ethers. *J. Org. Chem.* **2006**, *71*, 9045–9050.