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Highly Productive Continuous Flow Synthesis of Di- and Tripeptides in Water

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ABSTRACT

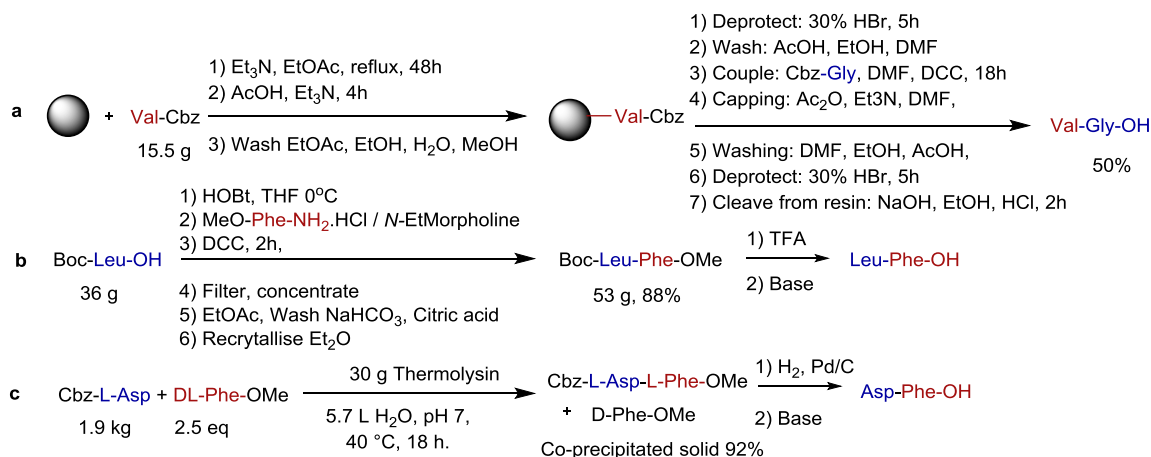
The reaction of amino acid derived *N*-carboxyanhydrides (NCAs) with unprotected amino acids under carefully controlled aqueous continuous flow conditions realized the formation of range of di- and tripeptide products in 60-85% conversion at productivities of up to 535 g.L⁻¹h⁻¹. This required a fundamental understanding of the physicochemical aspects of the reaction resulting in the design of a bespoke continuous stirred tank reactor (CSTR) with continuous solids addition, high shear mixing, automated pH control to avoid the use of buffer, and efficient heat removal to control the reaction at 1±1 °C.

KEYWORDS

Small Peptide; Leuch's NCA; Water; Continuous flow CSTR; Process control

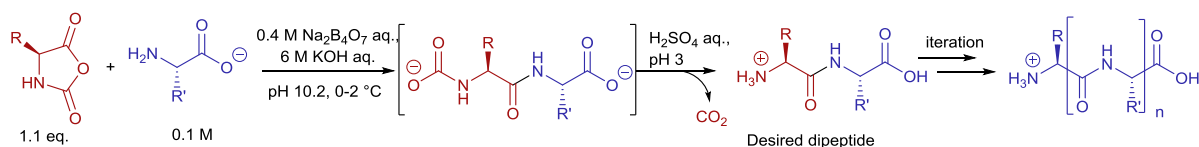
INTRODUCTION

Short peptides, that are composed of several amino acids, are important compounds within society with applications in medicine,¹ farming,² food,³ and materials,⁴ however their manufacture is both inefficient and unproductive.^{5, 6} Merrifield's solid-phase-peptide-synthesis (SPPS) is remarkably predictable and used widely for making small quantities of complex peptides.⁷ However, the scale-up of SPPS to make even kilograms of short peptides is difficult, being characterised by: sequences of excess coupling reagents; protection-deprotection steps; dilute conditions with problematic organic solvents; addition and deletion sequences caused by physical issues with washing the resin, Scheme 1a and Table 2.^{8, 9}



Scheme 1. Examples of dipeptide synthesis indicating atom economy in colored type, with waste and operations in black type. a. SPPS;⁹ b. SPS;¹⁰ c. Enzyme.¹¹

On the other hand, organic solution phase synthesis (SPS), that uses similar chemical methodology to SPPS, is the main method for small peptide manufacture. It also suffers poor green credentials: protecting groups, atom inefficiency, high E-factor and low process mass intensity, Scheme 1b, Table 2.¹²⁻¹⁴ Biosynthesis methods are leading the way in synthesis of longer peptides, for example in insulin manufacture,¹⁵ but are unproductive for short peptides. For example proteases are used to couple protected amino acids, but rely on high enzyme loadings and displacing unfavourable equilibria with either excess reagents, solubility effects or organic solvents, Scheme 1c.^{11, 16-22} Surprisingly, the pioneering work done in the 1950-60s by Bartlett and Hirschmann describing the coupling of *N*-carboxyanhydride (NCA or Leuch's anhydride) to amino acids has not been widely adopted, despite it being productive, cost effective and green, Scheme 2.²³⁻²⁷



Scheme 2. The NCA method of peptide synthesis

All twenty amino acids have been tested with several NCAs, though some require side-chain protection, and 4g of a pentapeptide has been prepared in overall 27% yield, though the purity was not measured directly.²⁴ A key enabler in this methodology is the use of water, which unlike organic solvents used in SPS is able to dissolve high concentrations of the free amino acids. In this article, we report studies to overcome the physico-chemical problems with the NCA methodology that appear to be a barrier to its exploitation, and initial development of a continuous process that enables precise control of the conditions and which required an unusual reactor design. This was used to produce a variety of di- and tripeptides in good conversion and high productivity, including an Aspartame derivative.²⁸

Hirschmann's NCA process involves the batch reaction of α -amino carboxylates with solid NCAs under carefully determined aqueous conditions: pH 10.2, controlled using a 1M sodium borate buffer with 6M KOH addition; a temperature of 0 °C maintained with ice.²⁷ Control of these are important, otherwise side reactions occur that are exacerbated by the rapid formation of acid and a strong exotherm. Our studies found that at this temperature the sodium borate buffer precipitates, furthermore, addition of ice dilutes the reaction and causes problems with mixing. Accordingly, less buffer and spatially separate cooling are preferable. It has been observed that if the NCA is dissolved in a dry organic solvent and added to a solution of the amino acid, low yields occur as a result of side reactions.²⁹ In this regard, Hirschmann recognized the need for high shear mixing to rapidly disperse the highly reactive solid NCA which, when added portion-wise into the best available equipment, a "Waring" kitchen blender, improved the selectivity.²⁵ High shear mixing is used to reduce the depth of the static boundary layer on a particle, and is a function of the Reynold's number, in particular the fluid velocity, particle shape and size, Figure 1.³⁰

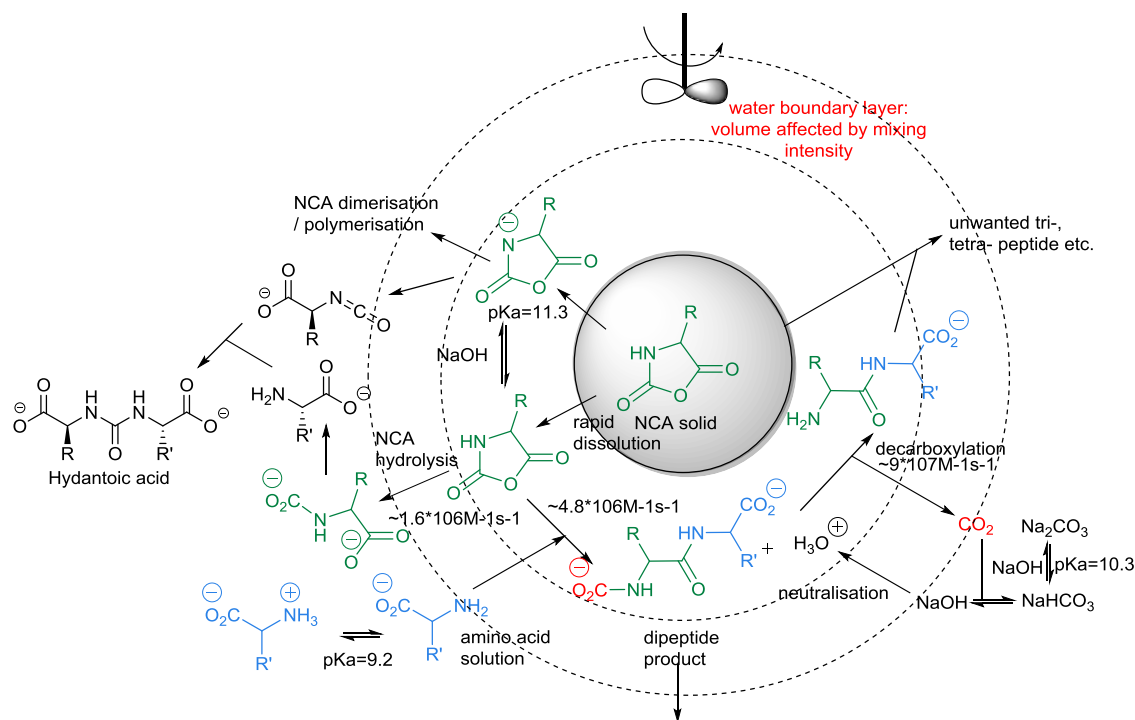


Figure 1. A model of the reactions occurring with NCA in the liquid boundary layer and bulk solution during dissolution of the solid.

The amino acid needs to be brought rapidly into contact with the NCA to compete with its hydrolysis, also to neutralise the acid to prevent decarboxylation. Any CO_2 that is generated is trapped by the base as carbonate. The reason that pH10.2 has been found to be optimal becomes clear: the NCA pKa is 11.3, so this pH keeps the reactive anion concentration low; whilst the amino acid pKa is 9.2, keeping the concentration of the free amine high. The reactivity of NCAs has been discussed in detail by Kricheldorf, and includes their use in the formation of homopolypeptides.³¹ Bartlett studied the kinetics of NCA hydrolysis and estimated the rate to be one third that of the amino acid at the same pH, both rates being above $1.6 \cdot 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$.^{32,33} Caplow and Johnson each showed the intermediate carbamic acid is unstable above 0 °C (a problem in water) and can prematurely decarboxylate to the free amino acid with a rate constant of $>9 \cdot 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$ for glycine at pH10,^{34,35} leading to an unwanted tripeptide, Figure 1. Hirschmann also proposed the formation of hydantoic acid side-products by decomposition of the NCA to

isocyanate, as well as NCA dimerization.²⁶ These side reactions lead to impurities that are difficult to separate from the desired product, Figure 1, and it is clear that carrying out the reaction in batch makes control of the conditions difficult as others have observed.²⁶ Previous reports of solution phase peptide formation in flow include Knudsen's automated sequence specific synthesis where Cbz protected NCAs formed *in-situ* are reacted with unprotected amino acids in DMF.³⁶ Whilst achieving excellent yields for a range of peptides, the process is of low productivity, requires protecting groups and excess NCA that reduce the atom efficiency. Fuse *et al.* report the reaction of *O*-protected amino acids with *N*-protected amino acids activated by *in-situ* reaction with triphosgene,³⁷ high yields are achieved with a 5 second residence time (T_{res}) over 2 steps, but with similar limitations in efficiency. Herein we describe initial studies to develop a continuous-mode version of Hirschmann's NCA process aimed at overcoming these limitations.

RESULTS AND DISCUSSION

Our study selected a single-stage CSTR in preference to a plug-flow reactor (PFR) which is incapable of providing solids addition, high intensity multiphase mixing, and pH control, Figure 2. The size and shape of the reactor were defined by the equipment needed to operate the process. A problem with the single stage CSTR is that the residence time distribution (RTD) can be wide with material passing straight through or never emerging, use of a food dye marker indicated more than 4 reactor volumes were required. A multi-stage CSTR would give a more efficient process, however with the process limitations the complexity of such a system precluded this approach. The purpose-built reactor comprised: a stainless steel vessel with overflow tube to maintain 300 mL volume; an automated powder dispenser to continuously dose the NCA (achieved with a variable speed, motor-driven, rotating angled pipe, accurate to 7 wt%, (SI, 7.2)); a high shear mixer

employed to promote rapid mixing of the reactants; an internal cooling-coil required to maintain the desired temperature of 0-2 °C; pH probe and thermocouple. The heat from the mixer at 4000 rpm along with 1.8 mL/min KOH caused the water temperature to rise at 0.85 °C.min⁻¹; and by difference, the reaction exotherm was found to be 0.54 °C.min⁻¹, 9.8 kJ.mol.⁻¹min⁻¹ (SI, Figure 1). In initial experiments, the amino acid was dissolved in sodium borate buffer at pH 10.2 and pumped at a rate stoichiometric with the continuously added, solid NCA,³⁸ which defined the residence time by the overflow pipe.

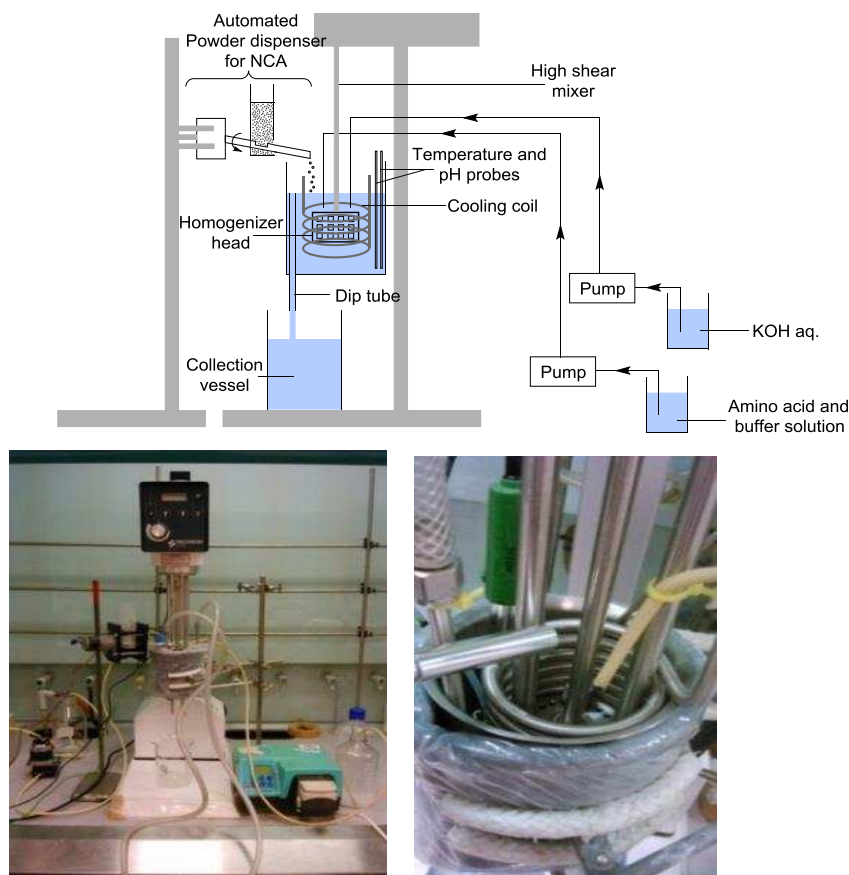


Figure 2. Diagram and photographs of the CSTR set-up used, showing high shear mixer, continuous solids addition system, internal cooling coil, and pumps.

The actual residence time was determined using a pulse of dye and found to be 8.6 minutes compared to 7 minutes theory, indicating only a small hold-up (SI 2.0). To achieve steady-state 2-3 reactor volumes (one RV is 300 mL) were required, and residence times of 5, 7 and 14 minutes were evaluated to determine the optimum (SI 2.0); equating to 10-70g of NCA per experiment. Initial experiments employed commercially available L-Ala NCA which was reacted with L-Phe, to form L-Ala-L-Phe dipeptide.³⁹ Since litres of aqueous product were generated in each experiment, the amino acid to dipeptide conversion was measured by sampling the acid quenched solutions, and determining the amino acid and peptide concentrations against authentic standards by ¹HNMR, HPLC and LCMS. The instability of the NCA meant that its conversion to dipeptide could not be measured directly. Figure 3 shows the importance of mixing, measured as mixer speed vs. conversion.

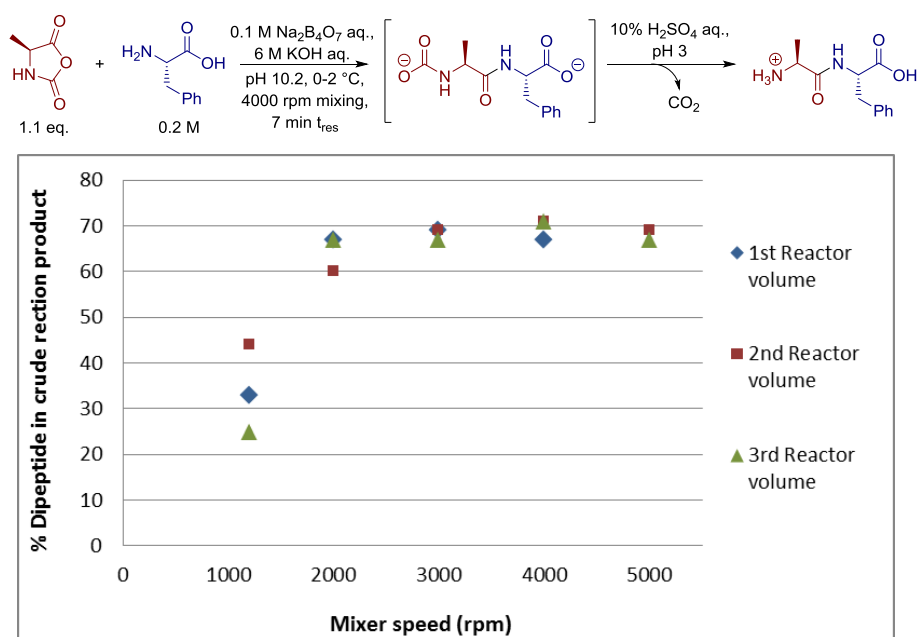


Figure 3. Reaction conditions and the effect of mixer speed on dipeptide formation

Using a residence time of 7 min, 3*300 mL reactor volumes were collected at each speed. At 1200 rpm product formation was low and inconsistent with the solid NCA building-up on the surface

(ESI Figure 4). Above 3000 rpm, 70% of the crude dipeptide was produced with little variation at steady-state, and 4000 rpm was selected for subsequent reactions. The reaction stoichiometry is important since it was found that 1.5 eq. of NCA led to over-reaction, with tripeptide and other side-products formed, whilst an undercharge caused problems with amino acid and dipeptide separation; all subsequent experiments used 1.1 eq. of NCA (ESI Figure 5). Table 1 shows the results of varying other reaction parameters.

Table 1. Optimization of reaction conditions for L-Ala-L-Phe formation.

Entry ^[a]	[Na ₂ B ₄ O ₇]aq. (M) ^[b]	Achieved pH ^[c]	Achieved temp. (°C)	% Dipeptide in crude reaction product ^[d]
1	0.4	9.9-10.3	-1.3-2.7	51
2	0.1	10-10.35	1.7-3.1	63
3	0.1	8.8-9.2 ^[e]	0.8-2.2	51
4	0.1	10.8-11.3 ^[f]	0.0-3.3	70
5 ^[g]	0.1	9.6-10.3	0.5-4.0	72
6	None	9.5-10.5	0.6-1.7	68
7 ^[h]	0.1	9.9-10.5	4.2-6.4	39
8	None	10-10.6 ^[i]	2.0-4.6	58
9	None	10.2-10.4 ^[j]	0.5-3.7	33
10 ^{[g][k]}	0.1	10.1-10.2	3.3-5.6	61
11 ^[l]	0.1	9.8-10.2	0.9-1.5	67
12 ^{[k][l]}	0.1	9.9-10.4	1.0-2.1	71

[a] Tres = 7 min. [b] With 0.2M amino acid initially at pH 10.2. [c] Manual addition of 6M KOH to target pH 10.2 [d] Calculated from the ¹HNMR ratio of dipeptide : amino acid + dipeptide + tripeptide.[e] target pH 9 [f] target pH 11. [g] Tres = 5 min. [h] NCA added as 0.22 M solution in THF. [i] Et₃N used to control pH. [j] NH₄OH used to control pH. [k] L-Ala NCA reacted with L-Leu. [l] Tres = 14 min.

Whilst 0.4 M sodium borate buffer solution enabled control of pH 10.2, its poor solubility at 0-2 °C lead to difficulties in maintaining constant addition (Entry 1). A lower buffer strength (Entry 2), or no buffer (Entry 6), gave an increase in dipeptide; however relying on manual KOH addition made it difficult to maintain a constant pH, Figure 4.

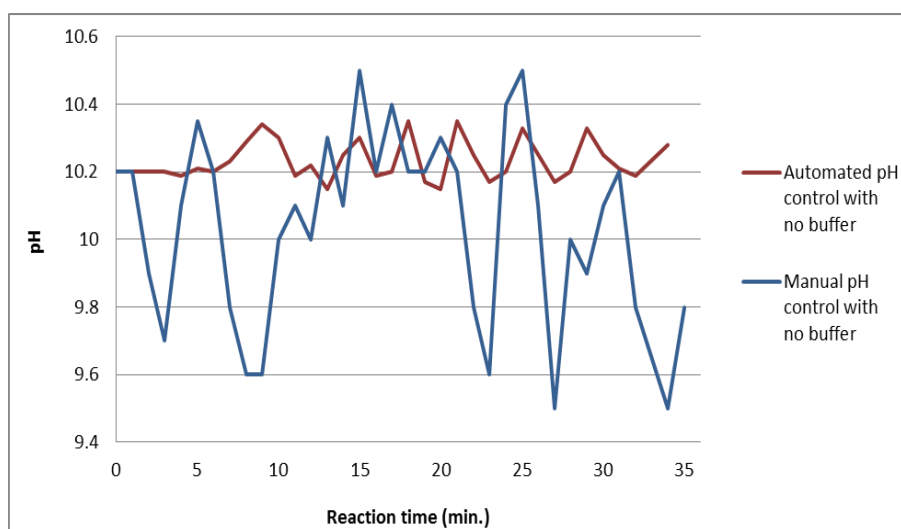


Figure 4. pH control of dipeptide formation with and without pH controller. With manual pH control the KOH flow rate was adjusted by hand to maintain pH 10.2.

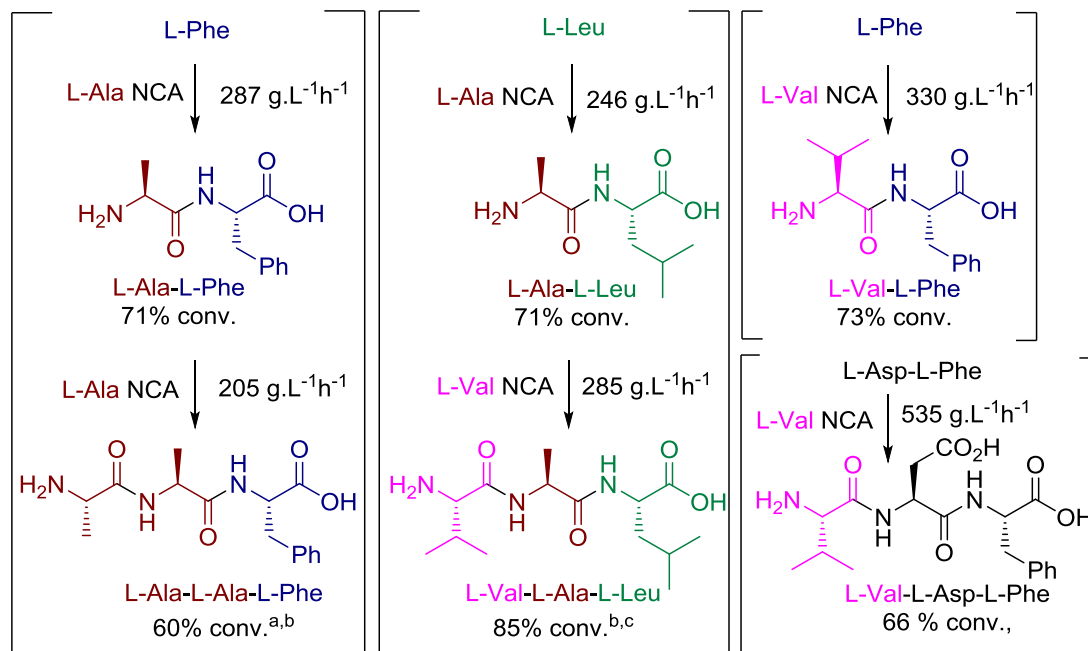
Table 1 Entry 3 shows that at pH 8.8-9.2, 20% less product is produced, whilst a higher pH of up to 11.3, was less problematic (Entry 4). Increasing the amino acid feed-rate, to reduce the residence time from 7 to 5 minutes, resulted in poor temperature control, reaching 4 °C (Entry 5) and 5.6 °C (Entry 10) because of the increased energy required to cool the ambient temperature amino acid feed. Increasing the T_{res} from 5 to 7 to 14 minutes, at similar pH and temperature ranges, changed little the steady-state conversions of 70%, 72%, 67%, (Entries 4, 5, 11) indicating the reactions were complete even at the shortest time. On the other hand, the reaction of L-Ala NCA and L-Leu, with residence times of 5 and 14 minutes, improved the conversion from 61% to 71%, that might

indicate a slower reaction or reduced mass transfer (Entries 10 and 12). The addition of L-Ala NCA dissolved in THF gave a significantly lower yield, 39% compared to 72% with solid addition, confirming previous studies.^{27, 29} The use of aqueous triethylamine or ammonia instead of KOH, gave lower yields with reduced thermal control and, in the case of the latter, a major new side-product, alanine amide (Entries 8 and 9).⁴⁰ It is interesting to note that the overall quantity of hydroxide required to maintain the reaction at pH10.2 was found to be 1.61 equivalents relative to the amino acid (See ESI, Section 1). The titration stoichiometry can be rationalised by considering the 72% conversion of L-Phe and L-Ala NCA to L-Phe-L-Ala (Entry 5), that consumes 0.72 equivalents of base. Since 1.15 equivalents of NCA are added, this means 0.43 equivalents of base are used in hydrolysis, and the remaining 0.46 probably react with the CO₂ resulting from carbamate decarboxylation, to form a 1:2 mixture of potassium bicarbonate and carbonate giving it a pKa of 10.6 in this salt solution compared with the literature pKa of 10.3 in pure water.⁴¹ Based on this, 63% of the NCA forms dipeptide product and 37% is hydrolysed. The close correlation between calculated and theory stoichiometry confirms Bartlett's observation that the relative rate of peptide formation is twice that of the NCA hydrolysis, but also indicates that the side reactions are difficult to suppress.

Isolation of small quantities of the products analysed by HPLC and ¹HNMR showed no evidence of racemisation, and this is supported by reports in the literature.^{25, 37} For purification and isolation, Hirshmann *et al.* report the use of chromatography with ion exchange resins, silica gel or carbon as the stationary phase, depending on the peptide. In our hands, ion exchange chromatography was successful in separating the salts from the reaction solution, but did not usefully separate the unreacted amino acid from the desired dipeptide. For reactions carried out without buffer, silica gel chromatographic purification was possible and gave the desired dipeptide cleanly for L-Ala-

L-Phe, and in enhanced purity for L-Val-L-Phe. All products, whether prepared with or without the presence of the buffer, could also be isolated cleanly on a small scale by use of mass directed preparative chromatography. Tripeptides could not be isolated in this way.

The defined process conditions and controls were then used to further explore the reaction scope, Figure 5.



Conversions calculated from the ¹HNMR ratios ^[a] Dipeptide starting material was 1.3 M crude solution from the L-Ala-L-Phe reaction. ^[b] Conversion given for dipeptide to tripeptide transformation. ^[c] Dipeptide starting material was 0.17 M solution

Figure 5. Peptide products synthesized, molar conversions (product / amino acid) and productivities.

L-Ala and L-Val NCAs were reacted with L-Phe and L-Leu amino acids and gave 71-73% conversions, with productivities up to 330 g.L⁻¹.h⁻¹ and unisolated dipeptide masses of 43-58g which is remarkable when compared with either SPPS or organic phase SPS. The dipeptides were then used directly as 1.3 M and 0.17 M pH 10.2 solutions and fed into the next reaction with L-Ala and L-Val NCA respectively. The L-Ala-L-Ala-L-Phe tripeptide was produced in this step

with 60% conversion and at $205 \text{ g.L}^{-1}.\text{h}^{-1}$ which over 5RV equates to 36g, whilst L-Val-L-Ala-L-Leu was formed in 85% conversion at $285 \text{ g.L}^{-1}.\text{h}^{-1}$, giving 50g of the tripeptide in solution, Figure 5.

These results show that the conversions and productivities depend upon the type of NCA and amino acids and further improvement might be achieved by optimizing each reaction separately. In the meantime, it was decided to make a more interesting tri-peptide and we chose to make an Aspartame derivative as the L-Asp-L-Phe-OMe dipeptide ester is inexpensive and commercially available in large quantities. After hydrolysing the methyl ester to give L-Asp-L-Phe it was fed continuously into the CSTR along with L-Val NCA. With good control of pH and temperature and based on $^1\text{HNMR}$ integration of a sample, the tripeptide was produced at steady-state in 66% conversion and at a rate of $535 \text{ g.L}^{-1}.\text{h}^{-1}$, providing over 5RV 94g. The ^1NMR spectrum showed 29% L-Asp-L-Phe starting material and therefore only 5% other impurities.

Presently, the difficulty in preparing longer peptides is the need to separate them from large quantities of aqueous salt solutions. The small-scale purifications using ion exchange and silica demonstrate the potential for larger scale; and since the main separation is binary (i.e. amino acid from dipeptide), continuous purification by simulated moving bed chromatography may be an option. Further work is looking at the use of membranes and affinity tags. The system is currently limited by the need for solid addition, the size of mixer and of the reactor, miniaturisation of this would allow a shorter residence time and fewer side-reactions. If quantitative conversion of reagents to the desired product could be achieved this would negate the need for intermediate purifications.

The sodium borate buffer not only poses health risks but is a significant part of the process waste. An automated pH controller was constructed in-house to allow rapid measurement and adjustment

of pH. The unit is a proportional integral controller built on an embedded electronic circuit and tuned for this application (see ESI). The controller is connected to a pH probe within the reaction solution and to a peristaltic pump with 6M KOH (aq). Figure 4 shows a comparison of manual and automated pH control for formation of a dipeptide without the use of sodium borate buffer. For manual pH control, the pH varies by 1 pH unit during the reaction, whereas with automated pH control the variation is 0.2 pH units. The conversion to product is comparable for both methods, however the pH controller allows more efficient use of KOH. The continuous process, with automated pH control allowing the removal of the buffer from the reaction not only offers high productivity and efficiency, but also avoids safety considerations and significantly reduces the chemical mass intensity of the process.

A particular focus of the study was to develop a method with improved environmental performance, so metrics of related continuous and batch processes were compared, Table 2.⁴²

Table 2. Comparison of reaction metrics for formation of dipeptide. ^[a]

Entry	Metric	SPPS	NCA method (batch)^[b]	This study: un-buffered (continuous)
	Peptide	L-Leu-L-Ala ^[c]	L-Ala-L-Phe	L-Val-L-Phe
1	Yield (%)	50	70	82 ^[d]
2	STY (g.L ⁻¹ h ⁻¹)	0.014	0.35	60.5
3	Total PMI	3165	686	28
4	RME (%)	4	57	67
5	AE (%)	10	84	86
6	Health & Safety	DMF	Na ₂ B ₄ O ₇	None

^[a] for calculations see ESI ^[b] based on reference 26 ^[c] estimated from reported tetrapeptide synthesis reference 9 ^[d] by HPLC.

Since there is no reported procedure for making the identical peptides, we based it as fairly as possible on Merrifield's SPPS description of one stage in making a tetrapeptide, and that of Hirshmann's L-Phe-L-Ala in batch.⁹ Comparing the SPPS and flow NCA method the latter gave a higher steady-state conversion of 82%, (Entry 1) and was 4600 times more productive (Entry 2), with the total mass intensity which includes solvent, a hundred-fold better (Entry 3). Reaction mass efficiency is the reciprocal of E-factor and considers atom economy, yield and stoichiometry, and this is 67% compared to 4% for the SPPS process (Entry 4). Unsurprisingly without protecting groups, the batch and flow NCA methods have much superior atom economies than SPPS (Entry 5). Meanwhile comparing the batch and flow NCA processes, the latter is 173-times more

productive (Entry 1), and has a total mass intensity 24-times better (Entry 2), mainly due to the avoidance of borate buffer which also improves health-safety aspects (Entry 6).

CONCLUSIONS

A continuous flow stirred tank reactor has been carefully designed to control within a narrow range, operating conditions that favour the reaction of NCA with amino acid, and minimise side reactions that produce impurities that are difficult to separate. The use of a cooling coil inside the reactor removes both the reaction exotherm and heat generated from the mixer to maintain the optimal of 0-2°C. This temperature range is important in lowering the rate of side-reactions relative to the desired reaction. The need for intense, high shear mixing has been shown to improve amino acid conversion to the product. This facilitates the rapid dispersion of solid NCA and concentrated hydroxide that is added to neutralise the acid produced in the reaction, that otherwise leads to product decarboxylation. Since the reactions in the system are very fast, they are likely to take place near the surface of the NCA particle, and high shear mixing reduces the size of boundary layer to facilitate transport with the bulk liquid. Automated continuous addition of NCA solid at a defined and consistent rate allowed the maintenance of the optimal 0.1 equiv. excess over the co-fed pH10.2 amino acid solution. Nevertheless, with these fast reactions precise control of stoichiometry may still be problematic. Whilst the L-Ala NCA fine white particulate material was well behaved (ensuring exclusion of moisture), the L-Val NCA particles were more cohesive, requiring regular tapping of the hopper, and making its addition less consistent. Clearly, the material properties of other NCAs may affect the ease with which they can be added. Consideration of the base stoichiometry shows the relative rates of NCA reaction with amino acid to base hydrolysis are about 2:1. Based on steady-state hydroxide and amino acid concentrations of

0.16mM and 0.1M, and assuming both reactions are first order in NCA, the rate constant for the reaction with hydroxide is 373 times that of the amino acid. Minimising hydrolysis is difficult, however if the amino acid and NCA concentrations were increased to 1M at the same hydroxide concentration, the dipeptide yield might be improved to 94%.⁴³ This would require more rapid addition of more material and would test the physical boundaries of the system, indicating that further improvements to the reactor design would be necessary.

Further work is required to determine the optimal residence time, and as the studies indicate this may vary for individual NCA and amino acid/peptide reactions. The fast reaction makes it possible to maintain a high conversion with a shorter residence time, eg. <1 minute, and this would alleviate further reaction of the product; a smaller volume multi-stage CSTR would improve the efficiency, however the need to add solids makes this difficult.⁴⁴ A homogenous reaction would considerably simplify the equipment and this approach is currently being investigated.

The productivity of the continuous process is much higher than other methods, and this could help reduce the costs of peptide production,^{45, 46} however a current problem is isolation of the peptides from large volumes of salt solutions. Work to develop continuous membrane separation and purification processes is ongoing. Realistically the flow NCA method is likely to be limited to tetra- or pentapeptides, but these might coupled by block condensation to form longer chain peptides.⁴⁷

The continuous flow synthesis of a series of di- and tri-peptides has been achieved through understanding the chemical and physical requirements of the reaction and this information used to design a multi-phasic flow reactor that minimises unwanted side-reactions. The concept of designing a reactor to meet the physicochemical requirements of a reaction can applied to other complex systems. A further useful aspect described here is the use of rapid and precise pH control

to avoid the use of a buffer thereby improving the green metrics of for example biocatalysed reactions.

EXPERIMENTAL

Commercially available chemicals used in this work were obtained from Sigma Aldrich and Fluorochem of reagent or reagent plus grade. L-Alanyl NCA and L-valyl NCA were obtained from Isochem and were stored under N₂ at -20 °C. NMR: Bruker DPX-300 (300 MHz), Bruker Ascend 400 (400 MHz) or Bruker DRX-500 (500 MHz) instruments using D₂O solvent. The HPLC employed an Agilent 1100 Series HPLC with Chemstation software for processing. LCMS was carried out using a Bruker HCT Ion trap mass spectrometer coupled with Agilent 1100 HPLC. HRMS was done with a Bruker Maxis Impact spectrometer with Ultimate 3000 UPLC. IR spectra were determined using a Bruker ALPHA FT-IR spectrometer. Melting points (Mp) with Stuart Melting Point Aparatus SMP30. Optical rotations were determined with a Schmidt and Haensch Polartronic H 352 with 1 dm cell. Mass Directed Automated preparative chromatography (MDAP) was done using Agilent Technologies 2120 Quadrupole mass spectrometer with Agilent Technologies 1260 Infinity HPLC.

A detailed description of the continuous flow equipment built and used in the study is shown in photos, diagrams and described in the ESI Section 7.3. The overflow pipe in the reactor was set at the 300 mL level. To the reactor was added 300 mL of the reaction solvent (0.1M Na₂B₄O₇ aq. or water/base) and the pH was adjusted to pH10.2 by addition of base as necessary. The solvent was stirred (4000 rpm) and cooled to 0 °C. Once at 0°C, to the reactor was then fed 0.2 M, pH 10.2 aqueous amino acid solution (41 mL/min, 8.2 mmol/min.), the required NCA (9 mmol/min.) and base, if required, at a rate necessary to maintain the reaction solution at pH 10.2. The reaction was

stirred at 4000 rpm and the temperature maintained between 0-2 °C. The reaction solution was collected *via* the overflow pipe. During the reaction, the reaction temperature, pH and flow rate of base were recorded each minute. After 7 min. (1 residence time), 300 mL (1 reactor volume) of solution had been collected. The collection vessel was replaced and the process repeated until the required number of reactor volumes had been collected. Once collected, each reactor volume was acidified with 3M sulfuric acid to allow decarboxylation of the carbamate intermediate. The products from each individual reactor volume were analysed by NMR and HPLC. For NMR, 1 mL of the reaction solution was dried by rotary evaporation and a portion of the solid was dissolved in D₂O. For the initial reactions, a known mass of NaOAc was added to the NMR sample as an external standard however results from this were found to be inconsistent and were not used further. For HPLC, 20 µL of the reaction solution was added to 10 µL of 0.1M biphenyl in MeOH (external standard). To this was added 500 µL MeOH and 470 µL H₂O. Grace Davidson Vydac C18(218TP) 250 x 4.6 mm, pore size 300 Å, particle size 5 µm, pH 2-7.5; 210 nm; 25 °C column oven; 10 µL injection volume; 1 mL/min.; solvent A: Water + 0.1% TFA; solvent B: MeCN + 0.1% TFA; Gradient: 0 min 5% B, 16 min 40% B, 18 min 40% B, 20 min 80% B, 27 min 80% B, 28 min 5% B. The retention times of amino acids and peptides were: L-Phenylalanine: 7.2 min; L-Alanyl-L-phenylalanine: 8.9 min; DL-Alanyl-DL-phenylalanine: 8.9 and 10.6 min; L-Alanyl-L-alanyl-L-phenylalanine: 14.0 min; L-Valyl-L-phenylalanine: 14.7 min; L-Alanyl-L-leucine: 10.2 min; L-Leucine: 7.0 min; L-Valyl-L-alanyl-L-leucine: 17.3; L-Valyl-L-aspartyl-L-phenylalanine: 18.0 min; L-aspartame: 16.2 min; Biphenyl (external standard): 25.7 min.

Purification was carried out by mass directed auto preparative chromatography (MDAP) or ion exchange and flash chromatography. For the former technique, small quantities (5-10 mL) of the crude reaction solutions were purified to give samples of the desired reaction products for

characterisation and to act as HPLC standards (see ESI Section 4.5 Peptide products). The MDAP method employed an Xbridge Prep C18 5 μm OBDTM 19x100 mm. 5-95% MeOH in water with 0.1% formic acid over 8 min., 20 mL/min flow rate. Purification by ion exchange chromatography was done using Dowex 50WX4 100-200 mesh ion exchange resin prewashed with water. 20 mL of the crude reaction solution was basified to pH 5.5 with 1 M KOH aq. and loaded onto the column. The products were eluted with firstly a pH 5.5 aqueous solution, followed by pH 5.7 and finally pH 6. The fractions collected were analysed by TLC (Si gel, CHCl_3 : MeOH: NH_4OH 18% aq. 50:40:10, visualisation by UV and PMA dip.) This method was found to successfully remove salts and tri-, tetra- peptide side products from buffer free reactions. In the reaction to form L-Alanyl-L-Phenylalanine, the dipeptide was successfully separated from residual phenylalanine starting material by this method alone, whilst for the other dipeptides this was not possible and subsequent silica gel flash chromatography was required. This was carried out by loading the concentrated dipeptide/amino acid mixture onto the silica gel column. The products were then eluted with DCM: MeOH: NH_4OH aq. 18% 30:60:10. The fractions were analysed by TLC using the same solvent system, and visualised by UV and PMA dip. For L-Val-L-Phe preparation, the dipeptide was obtained as an enriched mixture along with less phenylalanine than by ion exchange chromatography alone.

Characterisation data for the products is as follows.

L-Alanyl-L-Phenylalanine. Mp 242-243°C; $[\alpha]_{\text{D}}^{20} +39.3$ (589 nm, c 2, water) (lit.⁴⁸ $[\alpha]_{\text{D}}^{25} + 38.8$ (*L,L*)); IR ν_{max} 3229.8, 2923.7, 1668.0, 1522.6, 1222.5, 1076.8, 739.4, 697.5, 608.7 cm^{-1} ; ^1H NMR (500 MHz, D_2O) ^1H NMR (501 MHz, Deuterium Oxide) δ 7.43 (t, $J = 7.3$ Hz, 3H, CHAr), 7.38 – 7.31 (m, 4H, CHAr), 4.65 (dd, $J = 8.9, 5.6$ Hz, 1H, CHCH_2Ar), 4.03 (q, $J = 7.1$ Hz, 2H, CHCH_3), 3.29 (dd, $J = 14.0, 5.6$ Hz, 1H, $\text{CH}^a\text{H}^b\text{Ar}$), 3.09 (dd, $J = 14.1, 9.0$ Hz, 2H, $\text{CH}^a\text{H}^b\text{Ar}$), 1.53 (d, $J =$

7.1 Hz, 3H, CH_3); ^{13}C NMR (125 MHz, D_2O) 175.78 ($C=O$), 170.37 ($C=O$), 137.11 (C_iHAr), 129.30 (2 x C_oHAr), 128.83 (2 x C_mHAr), 127.19 (C_pHAr), 55.36 ($CHCH_2$), 49.01 ($CHCH_3$), 36.78 (CH_2), 16.49 (CH_3); LRMS m/z (ESI) 237.3 ($M^+ + H$); HRMS m/z (ESI): $M^+ + H$, 237.12342 $C_{12}H_{17}N_2O_3$ requires M 237.12337.

L-Alanyl-L-Leucine. Mp 226-227°C; $[\alpha]_D^{20}$ -30.2 (589 nm, c 1, water); IR ν_{max} 3218.1, 3067.3, 2956.8, 1667.4, 1520.7, 1049.5, 606.6 cm^{-1} ; 1H NMR (500 MHz, D_2O) δ 4.42 (t, $J=7.3$ Hz, 1H, $CHCH_2CH(CH_3)_2$), 4.15 (q, $J=7.1$ Hz, 1H, $CHCH_3$), 1.73-1.72 (m, 3H, CH_2 and $CH(CH_3)_2$ overlapping), 1.60 (d, $J=7.0$ Hz, 3H, CH_3), 0.97 (dd, $J=17.8, 6.3$ Hz, 6H, $CH(CH_3)_2$); ^{13}C NMR (125 MHz, D_2O) 176.82 ($C=O$), 170.85 ($C=O$), 52.20 ($CHCH_2(CH_3)_2$), 49.02 ($CHCH_3$), 39.41 (CH_2), 24.57 ($CH(CH_3)_2$), 22.22 (CH_3), 20.72 (CH_3), 16.57 (CH_3); LRMS m/z (ESI) 203.4 ($M^+ + H$); HRMS m/z (ESI): $M^+ + H$, 203.13897 $C_9H_{19}N_2O_3$ requires M 203.13902.

L-Valyl-L-Phenylalanine. Mp 233-234°C; $[\alpha]_D^{20}$ +70 (589 nm, c 1, water); IR ν_{max} 3218.1, 3067.3, 2956.8, 1667.4, 1520.7, 1049.5, 606.6 cm^{-1} ; 1H NMR (500 MHz, D_2O) δ 7.44-7.41 (m, 2H, $CHAr$), 7.38 – 7.35 (m, 3H, $CHAr$), 4.67 (dd, $J = 8.6$ Hz, 1H, $CHCH_2Ar$), 3.80 (d, $J = 5.6$ Hz, 1H, $CHCH(CH_3)_2$), 3.27 (dd, $J = 14.1, 5.8$ Hz, 1H, CH^aH^bAr), 3.10 (dd, $J = 14.1, 8.7$ Hz, 1H, CH^aH^bAr), 2.28 – 2.18 (m, 1H, $CH(CH_3)_2$), 1.05 – 1.01 (m, 6H, $(CH_3)_2$); ^{13}C NMR (125 MHz, D_2O) 175.78 ($C=O$), 168.93 ($C=O$), 137.07 (C_iHAr), 129.29 (2 x C_oHAr), 128.85 (2 x C_mHAr), 127.20 (C_pHAr), 58.50 ($CHCH_2CH(CH_3)_2$), 55.54 ($CHCH(CH_3)_2$), 36.88 (CH_2), 30.09 ($CH(CH_3)_2$), 17.69 (CH_3CHCH_3), 16.69 (CH_3CHCH_3); LRMS m/z (ESI) 265.3 ($M^+ + H$); HRMS m/z (ESI): $M^+ + H$, 265.15510 $C_{14}H_{21}N_2O_3$ requires M 265.15467.

L-Alanyl-L-Alanyl-L-Phenylalanine. Mp 209-210 °C; $[\alpha]_D^{20}$ -13.6 (589 nm, c 1, water); IR ν_{max} 3261.8, 3063.9, 2935.8, 1632.8, 1529.9, 1077.7, 958.5, 696.5, 575.8 cm^{-1} ; 1H NMR (500 MHz,

D₂O) δ 7.42-7.30 (m, 2H, CHAr), 4.63-4.54 (m, 1H, CHCH₂Ar), 4.34 (q, J = 7.2 Hz, 1H, CHCH₃), 4.08 (q, J = 7.1 Hz, 1H, CHCH₃), 3.24 (dd, J = 13.8, 5.4 Hz, 1H, CH^aH^bAr), 3.08 (dd, J = 13.8, 7.9 Hz, 1H, CH^aH^bAr), 1.51 (d, J = 7.0 Hz, 3H, CH₃), 1.37 (d, J = 7.1 Hz, 3H, CH₃); ¹³C NMR (125 MHz, D₂O) 176.13 (C=O), 173.87 (C=O), 170.50 (C=O), 137.00 (C_iHAr), 129.47 (2 x C_oHAr), 128.74 (2 x C_mHAr), 127.10 (C_pHAr), 55.13 (CHCH₂Ar), 49.82 (CHCH₃), 49.00 (CHCH₃), 37.17 (CH₂), 16.69 (CH₃), 16.65 (CH₃); LRMS m/z (ESI) 308.4 (M⁺ + H); HRMS m/z (ESI): M⁺ + H, 308.16069 C₁₅H₂₂N₃O₄ requires M 308.16048.

L-Valyl-L-Alanyl-L-Leucine. Mp 341-342 °C; $[\alpha]_D^{20}$ -93.4 (589 nm, c 1, water); IR ν_{\max} 3295.1, 3065.6, 2961.1, 1644.7, 1541.2, 1232.1, 1160.8, 1044.2, 605.1 cm⁻¹; ¹H NMR (500 MHz, D₂O) δ 4.48 (q, J = 7.2 Hz, 1H, CHCH₃), 4.41-4.35 (m, 1H, CHCH₂), 3.85 (d, J = 5.9 Hz, 1H, CHCH(CH₃)₂), 2.30-2.21 (m, 1H, CH(CH₃)₂), 1.74-1.69 (m, 3H, CH₂ and CH(CH₃)₂ overlapping), 1.45 (d, J = 7.2 Hz, 3H, CH₃), 1.08-1.06 (m, 6H, (CH₃)₂), 0.98 (d, J = 6.3 Hz, 3H, (CH₃)₂), 0.93 (d, J = 6.3 Hz, 3H, (CH₃)₂); ¹³C NMR (125 MHz, D₂O) 177.11 (C=O), 174.27 (C=O), 168.89 (C=O), 58.45 (CHCH(CH₃)₂), 52.04 (CHCH₂(CH₃)₂), 49.58 (CHCH₃), 39.69 (CH₂), 30.10 (CH₂CH(CH₃)₂), 24.52 (CHCH(CH₃)₂), 22.32 (CH₃), 20.71 (CH₃), 17.65 (CH₃), 16.94 (CH₃), 16.50 (CH₃); LRMS m/z (ESI) 302.4 (M⁺ + H); HRMS m/z (ESI): M⁺ + H, 302.20749 C₁₄H₂₈N₃O₄ requires M 302.20743.

L-Valyl-L-Aspartyl-L-Phenylalanine. Mp 184-186 °C; $[\alpha]_D^{20}$ -5.6 (589 nm, c 1, water); IR ν_{\max} 3193.3, 3066.0, 1713.8, 1660.3, 1517.5 1030.3, 581.8 cm⁻¹; ¹H NMR (500 MHz, D₂O) δ 7.37-7.14 (m, 5H, CHAr in tripeptide and 2H CHAr in impurity), 4.69-4.67 (m, 1H, CHCH₂CO₂H), 4.49 (dd, J = 8.0, 5.3 Hz, CHCH₂Ar), 4.40-4.38 (m, 0.4H, impurity), 4.14-4.12 (m, 0.4H, impurity), 3.69 (d, J = 5.9 Hz, 1H, CHCH(CH₃)₂), 3.16 (ddd, J = 38.2, 13.8, 4.6 Hz, 1.4H, CH^aH^bCO₂H and impurity), 2.98-2.95 (m, 1.4H, CH^aH^bCO₂H and impurity), 2.77 (dd, J = 16.7, 5.0 Hz, 1H,

CH^aH^bAr), 2.64 (dd, $J = 16.8, 8.8$ Hz, 1H, CH^aH^bAr), 2.10-2.07 (m, 0.5H, impurity), 2.04-1.99 (m, 1H, $CH(CH_3)_2$), 0.85 (t, $J = xx$ Hz, 6H, $CH(CH_3)_2$); ^{13}C NMR (125 MHz, D_2O) Complex spectra obtained due to unidentified impurity. Peaks could not be assigned; LRMS m/z (ESI) 380.4 ($M^+ + H$); HRMS m/z (ESI): $M^+ + H$, 380.18241 $C_{18}H_{26}N_3O_6$ requires M 380.18161.

ASSOCIATED CONTENT

Supporting Information. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Information on stoichiometry, heat flow, equipment design, green metrics, analytical and experimental details, product characterization NMR and HPLC spectra.

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Notes

The authors declare no competing financial interest.

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