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# Pilot-Scale Enzymatic Synthesis of Bioactive Oligopeptides in Eutectic-Based Media

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## Abstract:

**The bioactive peptide precursors $N_\alpha$-Cbz-L-Lys($N_\zeta$-Cbz)-Gly-OEt (1), $N_\alpha$-Cbz-L-Lys($N_\zeta$-Cbz)-Gly-L-Asp(OAll)-OAll (2), and $N_\alpha$-Cbz-L-Lys($N_\zeta$-Cbz)-Gly-L-Asp(OAll)-L-Glu(OAll)OEt (3) were synthesized via a sequential N-to-C strategy in heterogeneous solid−liquid mixtures of substrates using Celite-deposited chymopapain and subtilisin as catalysts. The substrate pairs displayed eutectic formation, and in the presence of 16−20% w/w of water and ethanol furnished low-melting point systems that provided substrate concentrations of 0.81−1.01 M, and sustained protease catalysis. The di-, tri-, and tetra-peptide were obtained in yields of 73, 74, and 67% (yield per step) respectively, and product concentrations of 0.34, 0.48, and 0.45 g per gram of reaction mixture were achieved with these systems. The syntheses were scaled up using wiped-blade and rotating-drum reactors, and 1, 2, and 3 were produced in yields of 71, 73, and 71%, at product concentrations of 0.36, 0.49, and 0.48 kg kg$^{-1}$, on a reaction scale of 11.34, 6.30, and 5.83 kg (11.54, 6.17, and 4.96 mol), respectively. The corresponding space−time yields were 0.30, 0.64, and 0.30 kg kg$^{-1}$ d$^{-1}$, and biocatalyst reuse provided productivities of 166, 235, and 312 kg-product kg-enzyme$^{-1}$. Furthermore, a sequential one-pot, three-step protocol was readily applied to the synthesis of the tetrapeptide 3 which was obtained with an overall yield of 34%, product concentration of 0.25 kg per kg, and space−time yield of 0.07 kg kg$^{-1}$ d$^{-1}$.**

## Introduction

With the increasing focus on the structures, functions, and medical applications of bioactive oligopeptides and peptide bioconjugates, there has been great interest in developing new methods suitable for the economic and large-scale synthesis of these molecules.[1−11] Enzymatic approaches have been applied to the total synthesis of small peptides, the condensation of oligopeptide fragments produced by chemical protocols, and the ligation/modification of proteins generated by recombinant methods, as discussed in recent papers and reviews.[11−25] The advantages of using protease-mediated protocols have been well recognized, with the avoidance of racemization, dispensation with side-chain protection, decreased requirement for *N*-protection, utilization of cheap and readily available substrates, and the use of mild, nonhazardous operating conditions being the most notable features.[11,12] Although numerous enzymatic syntheses have been devised for peptides, only one process, namely the thermolysin-catalysed synthesis of Aspartame is operating on the large scale. Critical hurdles which have blocked the industrial transfer of lab-scale protocols is the requirement for bulk organic solvents (which complicates reactor design and operation and requires solvent recovery and disposal) and low substrate concentrations which invariably lead to low space−time yields and a lack of economic feasibility.

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In the context of “Green Process” development and high-productivity biocatalysis, two novel approaches have recently been introduced, namely peptide synthesis in heterogeneous solid−liquid systems comprised of mixtures of substrates which undergo eutectic formation[23,26−29] and solid-to-solid conversions in aqueous suspensions of substrates.[19,30−38] The former method relies upon the observation that amino acid and peptide derivatives commonly employed as substrates in peptide synthesis form low-melting point mixtures (completely liquid eutectic or liquid eutectic plus excess solid substrate) when combined together in the absence of bulk solvents, while the latter utilizes concentrated aqueous suspensions of solid substrates in which reactions appear to take place in a saturated aqueous phase rather than a liquid eutectic. The selection of substrate combinations with or without the judicious addition of small amounts (0−20% w/w) of water or polar solvents (“adjuvants”, defined as solvents used in quantities far below those required for the dissolution of the solid substrates to form conventional solutions) capable of depressing the melting points of the mixtures leads to homogeneous liquids or heterogeneous liquid−solid systems which readily sustain enzymatic activity and enable the synthesis of oligopeptides. Such liquid−solid systems provide very high substrate concentrations approaching the theoretical maximum, typically being in the range of 0.8−3.1 M, together with high synthetic rates and yields, leading to product concentrations and productivities which are typically 5−13-fold higher than those obtained in conventional solution systems. This, together with the dispensation with bulk solvent and the lower reaction volume provides a facile, efficient, and scalable methodology which can be readily applied to the multikilogram-scale synthesis of peptides and complements the scale-up of enzymatic peptide synthesis using the solid-to-solid conversion approach.[30−38]

The present work discloses the first example of the scale-up of peptide synthesis in such low-melting point mixtures derived from eutectic interactions and describes the development of pilot processes for the kilogram-scale production of the flavor-active peptide precursors $N_\alpha$-Cbz-L-Lys($N_\zeta$-Cbz)-Gly-OEt, $N_\alpha$-Cbz-L-Lys($N_\zeta$-Cbz)-Gly-L-Asp(OAll)-OAll, and $N_\alpha$-Cbz-L-Lys($N_\zeta$-Cbz)-Gly-L-Asp(OAll)-L-Glu(OAll)OEt using a stepwise N-to-C protocol conducted totally in heterogeneous, low-solvent mixtures.

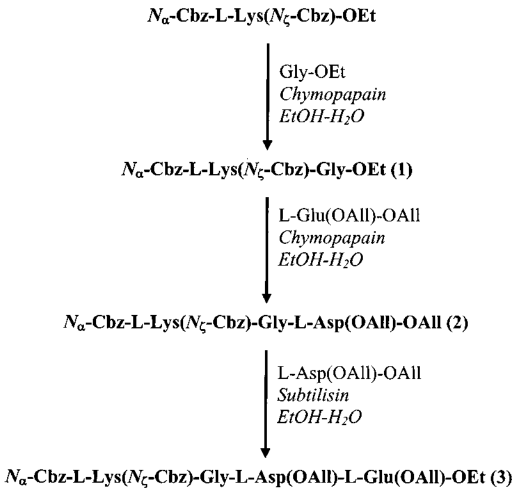

***Figure 1.*** **N-to-C synthesis of $N_\alpha$-Cbz-L-Lys($N_\zeta$-Cbz)-Gly-L-Asp(OAll)-L-Glu(OAll)-OEt.**

## Results and Discussion

The synthesis of $N_\alpha$-Cbz-L-Lys($N_\zeta$-Cbz)-Gly-L-Asp(OAll)-L-Glu(OAll)OEt (**3**) was based around a convergent N-to-C protocol devised for the solution-phase synthesis of the octapeptide L-Lys-Gly-L-Asp(OH)-L-Glu(OH)-L-Glu(OH)-L-Ser-L-Leu-L-Ala-$NH_2$ (Figure 1).[22] The strategy employs stepwise N-to-C assembly, utilizing the selectivities of the proteases employed rather than differential protection to achieve the required amino acid couplings. For large-scale production, particularly from the point of view of reaction control, reactor volume, and productivity, it was highly desirable to translate the solution-phase protocol to a synthesis based upon heterogeneous, low-solvent reaction media. For this purpose a screening program was initiated to determine if the requisite substrate combinations formed eutectics and whether the couplings could be efficiently performed in such homogeneous/heterogeneous media. Chymopapain and subtilisin deposited onto Celite were chosen as the biocatalysts, Cbz *N*-terminal protection, together with ethyl ester or allyl ester donors, was chosen for the synthesis of peptides **1**, **2**, and **3**, and the reactions were examined under a variety of conditions (Table 1). In addition to water, ethanol was chosen as a solvent (adjuvant) in view of its efficiency in promoting the formation of low-melting point multicomponent mixtures,[26−29] its low toxicity, and its ready recovery and reuse in large-scale processes.

Neat combinations of the pure substrates in the complete absence of water/solvent (adjuvant) provided simple heterogeneous systems consisting of eutectic plus excess solid substrate, with the eutectic compositions displaying eutectic melting points ($T_e$’s) of 58, 16, and 18 °C for the respective substrate combinations of **1**, **2**, and **3**. It should be noted from Table 1 that the volume fractions of the liquid phases were lower than expected for full eutectic formation, wherein one substrate goes completely into the liquid phase. This is because the substrate mixtures were held at 70 °C for only a short period of time (5 min) to avoid decomposition of

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***Table 1.*** **Protease-mediated synthesis of peptides 1, 2, and 3 in heterogeneous solid−liquid mixtures**

| substrates (mol ratio) (enzyme) | adjuvant[a] |  | $T_e$ [b] (°C) | phase fraction[c] (% v/v, $t = 0$) |  | concn[d] (M) | initial rate[e] (mol kg$^{-1}$ h$^{-1}$) | phase changes[f] extent (% v/v), time (h) |  | [prod.][g] (g g$^{-1}$) | yield (%) (40 h) |
|---|---|---|---|---|---|---|---|---|---|---|---|
|  | $H_2O$ (% w/w) | EtOH (% w/w) |  | liquid | solid |  |  | liquefaction | solidification |  |  |
| dipeptide (**1**): | 0 | 0 | 58 | 0 | 100 | 1.20 | 3.6 | 8, 9.0 h | 98, 14.5 h | 0.04 | 7 |
| $N_\alpha$-Cbz-L-Lys-OEt | 2 | 0 | 36 | 24 | 76 | 1.18 | 19.3 | 37, 7.5 h | 83, 19.0 h | 0.18 | 31 |
| + | 4 | 0 | 36 | 27 | 73 | 1.15 | 37.2 | 44, 7.0 h | 87, 20.5 h | 0.30 | 56 |
| Gly-OEt·HCl | 6 | 0 | 36 | 32 | 68 | 1.13 | 51.7 | 51, 5.0 h | 78, 22.0 h | 0.28 | 50 |
| + | 4 | 4 | 23 | 37 | 63 | 1.10 | 39.8 | 61, 4.0 h | 63, 18.0 h | 0.32 | 58 |
| DIPEA | 4 | 8 | 23 | 41 | 59 | 1.06 | 38.0 | 84, 3.5 h | 57, 21.5 h | 0.34 | 64 |
| (1:1.5:1.5) | 4 | 12 | 23 | 48 | 52 | 1.01 | 33.2 | 94, 4.5 h | 51, 23.0 h | 0.37 | 73 |
| (chymopapain) | 4 | 16 | 23 | 53 | 47 | 0.96 | 30.8 | 90, 4.0 h | 53, 23.0 h | 0.34 | 70 |
|  | 6 | 12 | 23 | 60 | 40 | 0.98 | 45.3 | 93, 3.0 h | 45, 26.0 h | 0.31 | 64 |
|  | 6 | 16 | 23 | 69 | 31 | 0.94 | 41.1 | 90, 2.5 h | 41, 28.5 h | 0.32 | 68 |
| tripeptide (**2**): | 0 | 0 | 16 | 26 | 74 | 1.22 | 9.8 | 29, 7.5 h | 82, 9.0 h | 0.13 | 16 |
| **1** | 2 | 0 | 8, 11 | 28 | 72 | 1.20 | 42.6 | 50, 7.0 h | 73, 11.0 h | 0.32 | 40 |
| + | 4 | 0 | 8, 11 | 33 | 67 | 1.17 | 64.1 | 57, 5.5 h | 70, 11.5 h | 0.41 | 52 |
| L-Asp(OAll)-OAll | 6 | 0 | 8, 11 | 37 | 63 | 1.15 | 83.0 | 63, 6.0 h | 65, 13.0 h | 0.41 | 53 |
| (1: 1.5) | 4 | 4 | 2, 7 | 48 | 52 | 1.12 | 67.6 | 75, 5.0 h | 69, 10.5 h | 0.43 | 57 |
| (chymopapain) | 4 | 8 | 2, 7 | 57 | 43 | 1.07 | 62.8 | 81, 6.0 h | 63, 13.5 h | 0.46 | 65 |
|  | 4 | 12 | 2 | 68 | 32 | 1.03 | 61.8 | 89, 5.0 h | 75, 13.5 h | 0.47 | 68 |
|  | 4 | 16 | 2 | 73 | 27 | 0.98 | 58.3 | 100, 4.5 h | 78, 14.5 h | 0.48 | 74 |
|  | 6 | 12 | 2 | 70 | 30 | 1.00 | 74.1 | 98, 5.5 h | 70, 12.5 h | 0.43 | 64 |
|  | 6 | 16 | 2 | 77 | 23 | 0.95 | 65.3 | 90, 5.0 h | 72, 13.0 h | 0.42 | 67 |
| tetrapeptide (**3**): | 0 | 0 | 18 | 33 | 67 | 0.99 | 9.4 | 44, 8.5 h | 67, 12.5 h | 0.19 | 23 |
| **2** | 1 | 0 | 12, 17 | 36 | 64 | 0.98 | 38.1 | 49, 7.0 h | 61, 15.0 h | 0.30 | 37 |
| + | 2 | 0 | 12, 17 | 39 | 61 | 0.96 | 60.4 | 53, 7.0 h | 63, 16.5 h | 0.34 | 43 |
| L-Glu(OAll)-OAll | 4 | 0 | 12, 17 | 43 | 57 | 0.95 | 79.2 | 61, 6.0 h | 70, 14.0 h | 0.35 | 46 |
| (1:1.5) | 2 | 4 | 5, 14 | 57 | 43 | 0.93 | 82.5 | 73, 7.0 h | 60, 12.0 h | 0.41 | 55 |
| (subtilisin) | 2 | 6 | 5 | 63 | 37 | 0.91 | 83.0 | 80, 6.5 h | 65, 16.0 h | 0.42 | 58 |
|  | 2 | 8 | 5 | 68 | 32 | 0.89 | 78.6 | 86, 6.5 h | 69, 15.5 h | 0.42 | 62 |
|  | 2 | 12 | 5 | 70 | 30 | 0.85 | 73.1 | 86, 6.0 h | 76, 14.0 h | 0.42 | 67 |
|  | 4 | 8 | 5 | 73 | 27 | 0.87 | 79.0 | 95, 5.0 h | 71, 16.0 h | 0.45 | 65 |
|  | 4 | 16 | 5 | 77 | 23 | 0.77 | 75.5 | 98, 5.5 h | 75, 13.5 h | 0.39 | 61 |

[a] Ethanol and water were used as solvents (adjuvants). [b] Eutectic temperature, determined by DSC. A second figure indicates the observation of two low-melting temperature peaks in the DSC thermogram, the second probably due to hydrate formation. [c] The volume fractions of the solid and liquid phases, determined by centrifugation analysis at 40 °C. It should be noted that to avoid decomposition of the acyl acceptors, the substrate mixtures were held at 70 °C for only a short period of time (5 min), which was insufficient for the formation of equilibrium eutectics, and thus the measured volume fractions of the liquid phases were lower than expected. [d] The concentration of the acyl donor (limiting component) in the reaction mixture at $t = 0$. [e] The initial rate of product synthesis, determined at 10 min, and quoted as mol per kg of Celite-deposited biocatalyst per h. [f] The extent and time of the maximum (net) liquefaction observed during the reaction are given. Similarly, the degree of solidification observed at the end of the reaction (40 h), and the time at which solidification began are also given. [g] Product concentration attained at the end of the reaction. Reactions were conducted on a 10−15-g scale in open beakers (150 mL) incubated at 40 °C, 150 rpm. Substrates and solvents (adjuvants) were mixed at room temperature in a sealed vial, heated at ca. 5 °C min$^{-1}$ to 70 °C, held at this temperature for 5 min, cooled at ca. 5 °C min$^{-1}$ to 40 °C, and maintained at this temperature for 10 min prior to addition of the biocatalyst. Chymopapain and subtilisin deposited onto Celite (at 10% w/w) were used as catalysts at 10% w/w of the reaction mixtures. DTT (0.1% w/w) was added as activator when chymopapain was used as a catalyst. Weighed samples were taken at 10 min, then at 0.5 h intervals for centrifugation and HPLC analysis.

the acyl acceptors and since eutectic formation in these systems is under strong kinetic control, equilibration and complete formation of eutectics were not achieved. This resulted in the presence of both substrates in the solid phase and lower than expected liquid volume fractions. However, eutectic formation between the substrate pairs was independently confirmed by DSC and microscopic analysis.

Preliminary experiments indicated that chymopapain and subtilisin stably tolerated temperatures of 35−45 °C when the water content was 0−10%, and that the reaction rates were optimal in this temperature range. Thus, the screening was conducted at 40 °C, and at this temperature the liquid-phase fractions of the mixtures amounted to 0, 26, and 33% respectively, and the addition of proteases resulted in the production of the di- tri-, and tetrapeptides with low yields of 7, 16, and 23%, respectively, and no detectable production of hydrolysis products. The inclusion of 4, 6, and 4% w/w water in the respective reaction mixtures increased the rates of peptide synthesis from 3.6, 9.8, and 9.4 mol kg$^{-1}$ h$^{-1}$ (mol of product per kg of biocatalyst per h) to 37.2, 83.0, and 79.2 mol kg$^{-1}$ h$^{-1}$ respectively, and elevated the corresponding product yields to 56, 53, and 46%, whilst incurring 8, 11, and 14% hydrolysis of acyl donors. This reflected direct activation of the enzymes by water, combined with the depression of the melting temperature of the reaction mixture and accompanying increase in liquid-phase fraction ($X_L$) and liquid-phase mobility.[26−29] Higher water contents of 6, 8, and 6%, although increasing the synthesis rates to 51.7, 92.3, and 91.5 mol kg$^{-1}$ h$^{-1}$ respectively, resulted in 11, 23, and 28% decreases in product yields due to the excessive hydrolysis of the acyl donors.

In view of the above results, the co-addition of ethanol and water, with the aim of reducing $T_e$ further, and increasing $X_L$ and mobility while suppressing acyl donor hydrolysis was investigated.[26−29] Indeed, the inclusion of 12−16% w/w of ethanol together with 2−4% w/w of water gave the best results, furnishing **1**, **2**, and **3** in optimal yields of 73, 74, and 67% respectively, and with 4, 6, and 9% of hydrolysis.

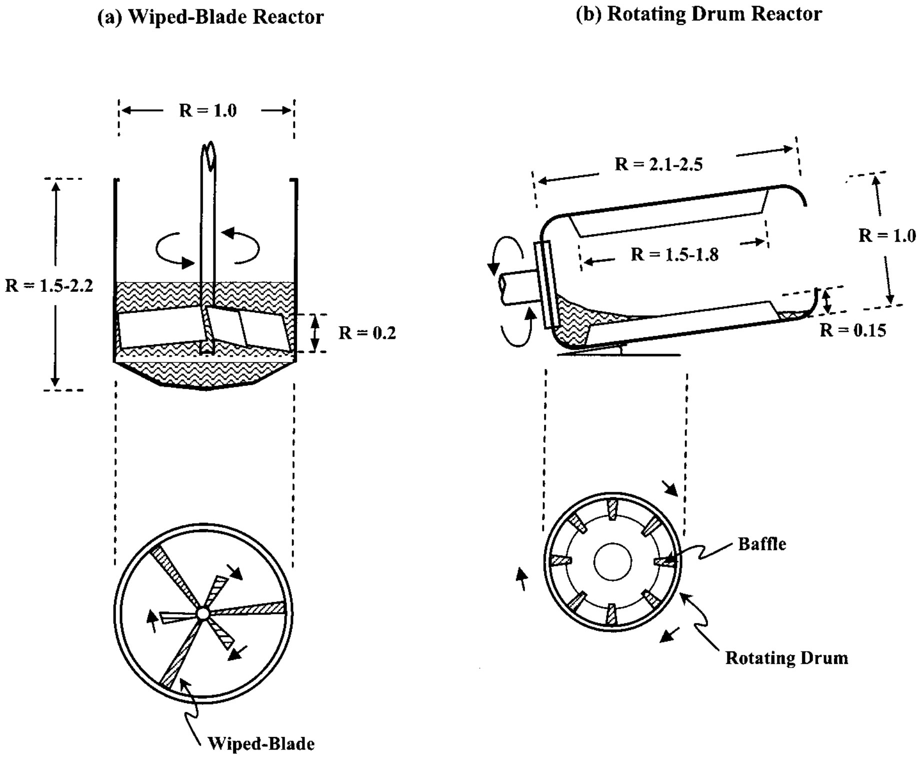

***Figure 2.*** **Schematics of the wiped-blade and rotating-drum reactors used for scale-up.**

This compares with the yields of 82, 84, and 76% obtained for the synthesis of the respective peptides in homogeneous solution.[22] This was accomplished with some reduction in the kinetics, with overall rate enhancements of 8.2-, 4.9-, and 7.4-fold observed as compared to the solvent (adjuvant)-free reactions. It should be noted that the course of peptide formation was observed to occur with a decrease in the amount of solid phase, in other words dissolution of suspended substrates and liquefaction of the reaction mixture, followed by the gradual precipitation of crystalline product and resulting partial solidification.[26−29]

The addition of water, and especially water plus ethanol, was observed to increase the extent of liquefaction observed during the progress of the reactions (Table 1). Thus, solvent (adjuvant)-free syntheses of **1**, **2**, and **3**, resulted in ca. 8, 29, and 44% (net) liquefaction of the reaction mixtures, while 94, 100, and 95% liquefaction was attained under optimal conditions with ethanol−water. In addition the solvents (adjuvants) also generally increased the time period during which liquefaction was observed and delayed the onset of solidification, especially in the case of **1**. It can be postulated that the speed and extent of liquefaction and subsequent precipitation should control the rate of synthesis and accumulation of and final yield of product. Thus, reduced liquefaction or rapid and extensive solidification or both were often found to result in low product yields, presumably due to only partial dissolution of substrates due to a low $X_L$ in the early stages, and premature termination of the coupling reaction due to a sharp increase in viscosity and reduction in $X_L$ in the final stages. It should be noted that the implementation of reactions in open vessels resulted in the evaporative loss of ca. 43−61% of the added solvents during the course of the reactions. The importance of the evaporation process to the progress of the reactions was indicated with the reduction in yields (ca. 13−26, 9−16, and 11−21% lowering in yields of **1**, **2**, and **3**, respectively) resulting from the use of closed reaction vessels or the application of reduced pressure. These deleterious effects presumably arose from the build-up of alcohols adversely affecting biocatalyst function in the former case, and the reduced liquefaction and premature solidification of the reaction mixtures in the latter.

It is important to note a critical appeal of eutectic-based media, namely the high substrate concentrations and correspondingly elevated product concentrations gained (Table 1). Thus, under the optimal conditions wherein 14−20% w/w of solvent (adjuvant) were employed, substrate (acyl donor) concentrations of 1.01, 0.98, and 0.81 M were achieved, and provided 0.37, 0.48, and 0.45 g of product per gram of reaction mixture. These productivities are ca. 9−25 times higher than those attainable when the syntheses are performed in homogeneous (saturated) solutions of the substrates in ethanol−water mixtures.[22]

Once optimal conditions had been determined for the syntheses, the reactions were scaled up to the multikilogram scale (Table 2). Scale-up studies were implemented in three types of reactor: (i) shaken beakers, where the reaction was conducted in an open beaker placed in an orbital shaker; (ii) wiped-blade reactors where the reaction mixture was actively mixed with a turbine fitted with flexible blades capable of efficiently blending the slurry (Figure 2a); (iii) rotating-drum reactors, where the reaction mixture was mixed by placement in baffled drums rotating at a slight inclination to the horizontal (Figure 2b). Rather poor scale-up was observed for the first configuration, with both synthesis rates and final yields dropping significantly above the 50-g scale, probably due to poor mixing of the high viscosity mixtures at this

***Table 2.*** **Scale-up of protease-mediated synthesis of peptides 1, 2, and 3 in heterogeneous solid−liquid mixtures**

| product | adjuvant[a] $H_2O$:EtOH (% w/w) | reaction scale (kg) | mol | reactor type | initial rate[b] (mol $kg^{-1}$ $h^{-1}$) | [prod.][c] (g $g^{-1}$) | yield (%) (40 h) | activity[d] recovered biocatalyst (%, 40 h) |
|---|---|---|---|---|---|---|---|---|
| **1** | 4:12 | 0.01 | 0.01 | 50 mL vial, orbital shaker, 200 rpm | 31.8 | 0.35 | 69 | 70 |
| | | 0.05 | 0.05 | 0.2-L beaker, orbital shaker, 200 rpm | 30.6 | 0.33 | 65 | 73 |
| | | 0.11 | 0.11 | 0.5-L beaker, orbital shaker, 150 rpm | 28.8 | 0.32 | 64 | 69 |
| | | 0.50 | 0.51 | 2-L beaker, orbital shaker, 150 rpm | 26.1 | 0.31 | 62 | 74 |
| | | 0.17 | 0.17 | 0.5-L wiped-blade reactor, 150 rpm | 33.5 | 0.38 | 75 | 68 |
| | | 0.54 | 0.55 | 2-L wiped-blade reactor, 150 rpm | 34.7 | 0.37 | 73 | 70 |
| | | 1.21 | 1.22 | 10-L wiped-blade reactor, 150 rpm | 36.0 | 0.39 | 77 | 67 |
| | | 2.90 | 2.93 | 10-L wiped-blade reactor, 150 rpm | 36.5 | 0.39 | 78 | 69 |
| | | 5.12 | 5.17 | 20-L wiped-blade reactor, 150 rpm | 34.8 | 0.38 | 74 | 74 |
| | | 11.43 | 11.54 | 50-L wiped-blade reactor, 150 rpm | 33.5 | 0.36 | 71 | 73 |
| | | 0.53 | 0.54 | 4-L inclined rotating-drum, 150 rpm | 33.0 | 0.35 | 69 | 76 |
| | | 1.35 | 1.52 | 12-L inclined rotating-drum, 150 rpm | 31.6 | 0.37 | 72 | 77 |
| | | 3.08 | 3.11 | 12-L inclined rotating-drum, 150 rpm | 30.2 | 0.36 | 70 | 74 |
| | | 5.03 | 5.08 | 46-L inclined rotating-drum, 120 rpm | 28.1 | 0.34 | 66 | 68 |
| **2** | 4:16 | 0.01 | 0.01 | 50 mL vial, orbital shaker, 200 rpm | 59.0 | 0.46 | 71 | 69 |
| | | 0.06 | 0.06 | 0.2-L beaker, orbital shaker, 200 rpm | 56.1 | 0.47 | 72 | 64 |
| | | 0.13 | 0.13 | 0.5-L beaker, orbital shaker, 150 rpm | 53.8 | 0.42 | 65 | 66 |
| | | 0.48 | 0.47 | 2-L beaker, orbital shaker, 150 rpm | 51.9 | 0.40 | 61 | 67 |
| | | 0.15 | 0.15 | 0.5-L wiped-blade reactor, 150 rpm | 55.6 | 0.47 | 73 | 62 |
| | | 0.50 | 0.49 | 2-L wiped-blade reactor, 150 rpm | 56.0 | 0.49 | 76 | 69 |
| | | 1.93 | 0.89 | 10-L wiped-blade reactor, 150 rpm | 55.7 | 0.49 | 75 | 66 |
| | | 6.30 | 6.17 | 20-L wiped-blade reactor, 150 rpm | 55.3 | 0.48 | 73 | 61 |
| | | 0.53 | 0.52 | 4-L inclined rotating-drum, 150 rpm | 54.4 | 0.45 | 70 | 62 |
| | | 2.04 | 2.00 | 12-L inclined rotating-drum, 150 rpm | 52.2 | 0.43 | 67 | 63 |
| **3** | 2:12 | 0.01 | 0.008 | 50 mL vial, orbital shaker, 200 rpm | 77.5 | 0.46 | 68 | 88 |
| | | 0.05 | 0.04 | 0.2-L beaker, orbital shaker, 200 rpm | 74.6 | 0.42 | 63 | 87 |
| | | 0.10 | 0.09 | 0.5-L beaker, orbital shaker, 150 rpm | 72.0 | 0.42 | 63 | 83 |
| | | 0.51 | 0.43 | 2-L beaker, orbital shaker, 150 rpm | 69.8 | 0.39 | 58 | 81 |
| | | 0.10 | 0.09 | 0.5-L wiped-blade reactor, 150 rpm | 78.3 | 0.48 | 71 | 86 |
| | | 0.60 | 0.51 | 2-L wiped-blade reactor, 150 rpm | 79.2 | 0.50 | 74 | 85 |
| | | 1.29 | 1.10 | 5-L wiped-blade reactor, 150 rpm | 77.4 | 0.49 | 72 | 84 |
| | | 5.83 | 4.96 | 20-L wiped-blade reactor, 150 rpm | 75.9 | 0.48 | 71 | 82 |
| | | 0.56 | 0.48 | 4-L inclined rotating-drum, 150 rpm | 76.0 | 0.46 | 69 | 84 |
| | | 2.15 | 1.83 | 12-L inclined rotating-drum, 150 rpm | 74.3 | 0.48 | 72 | 87 |

[a] Ethanol and water were used as solvents (adjuvants). [b] Initial rate of product formation, determined at 10 min, and quoted as mol per kg of Celite-deposited biocatalyst per h. [c] Product concentration attained at the end of the reaction. [d] Activity of the biocatalyst relative to that of the fresh material, after extraction of the reaction mixture with ethanol upon completion of the reaction. The substrates and solvents (adjuvants) were mixed at room temperature in a closed glass or stainless steel vessel, heated at ca. 5−10 °C $min^{-1}$ to 70 °C, held at this temperature for 5 min, cooled at ca. 5−10 °C $min^{-1}$ to 40 °C, and maintained at this temperature for 10−20 min prior to addition of the biocatalyst. Chymopapain and subtilisin deposited onto Celite (at 10% w/w) were used as catalysts at 10% w/w of the reaction mixtures. DTT (0.1% w/w) was added as activator when chymopapain was used as a catalyst. Weighed samples were quenched with methanol and analyzed by HPLC.

scale and the formation of a dry skin of substrate at the surface of the reaction mixtures. Rotating-drum reactors fared better, although inadequate mixing and the formation of agglomerates at reaction scales above ca. 1 kg significantly reduced kinetics and yields, probably due to excessive evaporation of solvents. In contrast, the wiped-blade reactors performed much better and provided efficient mixing and good dispersions throughout the course of reactions, and syntheses were readily scaled up to 5−11 kg without significant deterioration in rates or yields. Thus, at reaction scales of 11.43, 6.30, and 5.83 kg (11.54, 6.17, and 4.96 mol scale respectively), **1**, **2**, and **3** were synthesized with yields of 71, 73, and 71%, and with product concentrations of 0.36, 0.48, and 0.48 kg $kg^{-1}$. These correspond to space−time yields of 0.30, 0.64, and 0.30 kg $kg^{-1}$ $d^{-1}$, well above the acceptable margin of 0.06 kg $kg^{-1}$ $d^{-1}$ that was required for industrial scale production of the peptides. An important concern for scale-up economics, namely physical deterioration of the biocatalysts and deterioration in catalyst activity was also evaluated (Table 2). The shaken reactor and rotating-drum configurations produced little discernible mechanical damage to the catalysts, while the wiped-blade format did result in significant abrasion of catalyst particles and generated 6−13% of fines. However, good activity retentions were observed, with the Celite-deposited catalysts from the syntheses of **1**, **2**, and **3** being recovered with remaining activities of 67−77, 61−69, and 81−88%, respectively, after extraction of the reaction mixtures with ethanol. Indeed, the biocatalysts were reused 6−9 times before the activities dropped below the economically acceptable level of 30%, and this reusability furnished net productivities of 166, 235, and 312 kg of product per kg of enzyme, respectively.

To evaluate the flexibility of the methodology with regard to multistep protocols, we attempted to carry out the N-to-C synthesis of **3** in one-pot and three steps, using the sequential addition of the requisite substrates and enzymes. Indeed, providing that water and ethanol were added to replace the solvents (adjuvants) lost due to evaporation and re-liquefy the mixtures upon the completion of each step, the coupling reactions could be readily integrated in sequence without undue loss of yield. Thus, **3** was synthesized with a yield of 34% (isolated yield 30%), a product concentration of 0.25 kg $kg^{-1}$, and a space−time yield of 0.07 kg $kg^{-1}$ $d^{-1}$, at a (net) reaction scale of 5.98 kg (4.83 mol) in a 20-L wiped-blade reactor. The corresponding coupling yields of 82, 66, and 63% for the individual steps, and the overall performance compares very well with the net yield of 36−38% obtained when the reactions are carried out separately in heterogeneous media.

## Conclusions

Protease-mediated peptide synthesis in heterogeneous solid−liquid mixtures composed of eutectics plus excess solid substrate together with small amounts of ethanol and water can be readily scaled up in wiped-blade and rotating-drum reactors to produce kilogram amounts of di-, tri-, and tetrapeptides in high yields and productivities. Although these reactor configurations are not appropriate for multiton batches, the high productivity of the reaction systems should enable the use of relatively compact reactors (a 1 $m^3$ reactor with a 30% v/v working volume could deliver ca. 110−150 kg of product per cycle) which should be effective for performing the syntheses on the hundreds-of-kilogram scale, which is sufficient for the commercial production of such oligopeptides. The results suggest that the methodology provides facile, highly efficient, and economic protocols suitable for the industrial production of short peptides. We are further exploring the synthesis of peptides containing multifunctional amino acids in heterogeneous media and are studying the development of reactor configurations suitable for performing the syntheses on the hundreds-of-kilogram scale.

## Experimental Section

**Chemicals and Enzymes.** Subtilisin, chymopapain, dithiothreitol (DTT), $N_\alpha$-Cbz-L-Lys($N_\zeta$-Cbz)OH, GlyOEt•HCl, L-Asp$(OAll)_2$•TosOH, L-Glu$(OAll)_2$•TosOH, L-Ser(OH)OEt•HCl, L-LeuOEt•HCl, and L-Ala$NH_2$•HBr were purchased from Sigma Chemical Co. Celite, thionyl chloride, and all solvents were obtained from Aldrich Chemical Co.

**Analytical Methods.** Reactions were monitored by reverse phase HPLC using an Agilent 1100 LC system equipped with a diode array detector and HP Chemstation data collection and processing software. Samples were dissolved/quenched in pure methanol, centrifuged (10000*g*, 10 min), then analyzed on a Hichrom RPB column, 5 $\mu$m, 0.46 cm × 15 cm, maintained at 45 °C, and eluted at 1 mL $min^{-1}$ with 9:1 water−methanol (A) and methanol (B), each containing 0.05% v/v of phosphoric acid, with detection at 220 and 257 nm. The following elution conditions were used: 90:10 (A:B) to 40:60 (A:B) over 20 min; 70:30 (A:B) to 10:90 (A:B) over 20 min, with a 5 min hold at 10:90 (A:B).

$^1$H NMR spectra were recorded on a JEOL EX270 FT spectrometer at 270 MHz, 40 °C, with $d_6$-DMSO as solvent, using broad-band proton decoupling and a solvent field lock. FAB-MS spectra were recorded on a Kratos MS9 instrument using a xenon gun operating at 7 kV. Samples were dispersed in glycerol and applied to a copper probe, and spectra were recorded in positive ionization mode using polyglycerol ions as reference. Optical rotations were measured on an AA-100 polarimeter at 589.3 nm, 20 °C, using 0.25- or 0.5-cm path length cells, with methanol as solvent, and are given in units of $10^{-1}$ deg $cm^2$ $g^{-1}$. Melting points were determined on a Buchi 540 melting point apparatus using sealed capillaries, and are uncorrected.

The formation of eutectics and the determine determination of eutectic melting points was established by DSC. This was performed with a Perkin-Elmer DSC-7 instrument with a TAC controller and with nitrogen purging. Substrate mixtures (ca. 10−20 mg) were sealed under nitrogen in 50−100-$\mu$L aluminium pans, heated at 5 °C $min^{-1}$ to 70 °C, held at this temperature for 5 min, cooled at 5 °C $min^{-1}$ to −20 °C, and then equilibrated at this temperature for 24−48 h. The frozen samples were then analysed at a ramp of 1 °C $min^{-1}$ and melting and freezing peaks determined. Calibration was performed with hexane, distilled water, octan-1-ol, and acetamide. In addition the eutectic temperatures were also determined from contact slides (ca. 100−300 $\mu$m thickness, ca. 100−500 $\mu$m contact zone) by polarizing light microscopy analysis with a Zeiss Axiophot photomicroscope fitted with a heating stage and sealed chamber with nitrogen purging.

The volume fractions of the solid and liquid phases of heterogeneous mixtures were determined by centrifugation analysis. The substrates (ca. 5−10 g) were mixed together, solvent (adjuvant) was added as required, and the mixture was heated in a centrifugal microfilter tube (10 or 20 mL, 0.5 $\mu$m nylon filter) at 5 °C $min^{-1}$ to 70 °C in a heating block, cooled at 5 °C $min^{-1}$ to 40 °C, and maintained at this temperature for 10 min. A weighed amount of biocatalyst was added as required, and the sample was incubated for the required time and then centrifuged (5000*g*, 20 min). The expelled liquid phase was collected and weighed, and its density and composition were determined by microdensity bottle and HPLC methods. The retained solid phase was separately analysed by HPLC. These results were used to calculate the fractions of the solid and liquid phases.

**Preparative Liquid Chromatography.** Preparative column chromatography was performed using a jacketed 12 cm × 120 cm Buchi glass chromatography column connected to Buchi 688 LC or Ismatec Gear pumps and a Buchi UV−vis detector fitted with a preparative flow cell. The column was slurry packed with 40−63 $\mu$m Vydac 218TP, thermostated at 40 °C, and step-eluted with mixtures of pure methanol and 9:1 water−methanol.

**Preparation of Celite-Deposited Enzymes.** The proteases were deposited onto Celite at a loading of 10% w/w as follows: An ice-cold solution of the protease (4.5 L, protease dissolved at 40 g $L^{-1}$ in 100 mM potassium phosphate buffer, preadjusted to pH 9, containing 10 mM each of calcium and magnesium acetates and 5 mM EDTA) was added slowly to Celite powder (5 kg) in an inclined rotating-drum mixer (20 L, operated at ca. 70−100 rpm) and the wet adsorbate thoroughly blended for 10 min. The wet powder was then dried at room temperature in a Buchi fluidized bed dryer until the moisture content was below 2% w/w, and the obtained Celite-deposited biocatalyst was stored at 5 °C until used. Immobilization efficiencies (100 × fraction of applied protein immobilized × relative specific activity of immobilized protein in an aqueous activity assay) of 93 and 89% were obtained for subtilisin and chymopapain, respectively.

**Preparation of Substrates.** $N_\alpha$-Cbz-L-Lys($N_\zeta$-Cbz)OEt was prepared from the corresponding acid using the thionyl chloride method.[39] L-Asp(OAll)OAll and L-Glu(OAll)OAll were prepared from the corresponding tosylate salts by neutralizing ice-cold concentrated aqueous solutions with aqueous sodium hydroxide, followed by extraction with ethyl acetate or ether and rotary evaporation. The obtained free bases were stored at −20 °C until use.

**Reactors for Scale-Up of Heterogeneous Reactions.** Two types of reactor configurations were employed for scale-up trials, as illustrated in Figure 2: (a) wiped-blade reactors, consisting of jacketed, open-top cylindrical glass vessels with volumes of 0.5, 1, 2, 5, 10, 20, and 50 L, with aspect ratios of ca. 1.5:1−2.2:1 (height: diameter). Each reactor was fitted with an overhead stirrer carrying a turbine consisting of six 40° inclined polyethylene blades, each with a width ca. 0.2 × reactor vessel height and a thickness of 1−6 mm. Three of the blades were of length ca. 0.25 × vessel diameter, while the remaining blades were of length ca. 0.52 × vessel diameter, and these blades wiped the reactor walls. (b) inclined-rotating-drum reactors, consisting of cylindrical polypropylene or stainless steel vessels with nominal volumes of 4, 12, and 46 L, with aspect ratios of ca. 2.1:1−2.5:1 (height: diameter). The reactors were fitted with six or eight stainless steel or polyethylene baffles (triangular cross section) of length ca. 0.7 × reactor length and of width ca. 0.15 × reactor diameter, and were inclined at ca. 10−20° (to the horizontal) during operation.

**Preparation of $N_\alpha$-Cbz-L-Lys($N_\zeta$-Cbz)-GlyOEt (1).** $N_\alpha$-Cbz-L-Lys($N_\zeta$-Cbz)OEt (1.30 kg, 3 mol) was mixed with water (121 g) and ethanol (353 g) in an open 10-L wiped-blade reactor, and the slurry was heated with stirring (200 rpm) to 70 °C to form a viscous liquid. This was cooled to 40 °C, and to this were added GlyOEt·HCl (558 g, 4 mol), diisopropylethylamine (505 g, 3.92 mol, 98 mol %), DTT (10 g) and a 1:1 mix of finely powdered potassium carbonate and potassium hydrogen carbonate (50 g), and stirring (ca. 160 rpm) continued at 40 °C to furnish a semiliquid heterogeneous multicomponent mixture. The final water and ethanol contents were ca. 4.2 and 12.1% w/w, respectively. Celite-deposited chymopapain (500 g) was added to the mixture, and stirring continued (200 rpm) at 40 °C for 28 h. The formation of dipeptide was accompanied by partial liquefaction of the reaction mixture, with the mixture transforming to a mobile liquid at 4−7 h, and this was followed by an increase in viscosity as the reaction progressed further and the dipeptide partially crystallized out. After 28 h, a semisolid mixture was obtained, and the yield of dipeptide had reached 76%. Ethanol (ca. 4 L) was added, and the suspension was stirred for 20 min and filtered, and the filtrate containing the product was collected and rotary-evaporated to dryness at room temperature. The recovered biocatalyst retained 73% of its initial activity. The sticky solid (ca. 2.3 kg) was dissolved in methanol (ca. 2.4 L) and purified by column chromatography (step elution with 7:3, then 5:5, then 4:6 water−methanol) to give the dipeptide as a white solid: 1.031 kg; 69% yield; 98−99% pure (by HPLC); mp 90−92 °C; $[\alpha]_D^{20}$ −12.3° (*c* 0.10, MeOH); $^1$H NMR (DMSO-$d_6$, 40 °C): d 1.18 (t, 3H, *J* = 7.2 Hz), 1.25−1.47 (m, 4H), 1.57 (m, 1H), 1.65 (m, 1H), 2.97 (m, 2H), 3.74 (dd, 1H, *J* = 6.0 Hz, 16.7 Hz), 3.88 (dd, 1H, *J* = 5.7 Hz, 16.9 Hz), 4.02 (dt, 1H, *J* = 7.0 Hz, 7.2 Hz), 4.08 (q, 2H, *J* = 6.9 Hz), 4.98 (s, 2H), 5.03 (s, 2H), 7.15 (m, 1H), 7.26 (m, 1H), 7.31−7.46 (m, 10H), 8.26 (dd, 1H, *J* = 5.6 Hz, 5.8 Hz); FAB-MS (M + H) calculated for $C_{26}H_{34}N_3O_7$ 500.2396, observed 500.2438.

**Preparation of $N_\alpha$-Cbz-L-Lys($N_\zeta$-Cbz)-Gly-L-Asp(OAll)OAll (2).** $N_\alpha$-Cbz-L-Lys($N_\zeta$-Cbz)-GlyOEt (**1**, 936 g, 1.87 mol) was mixed with L-Asp(OAll)OAll (600 g, 2.80 mol), water (81 g), ethanol (310 g), and DTT (5 g) in an open 10-L wiped-blade reactor, and the slurry was heated with stirring (200 rpm) to 50 °C to form a viscous suspension. The final water and ethanol contents were ca. 4.1% w/w and 15.4% w/w, respectively. The mixture was allowed to cool to 40 °C, Celite-deposited chymopapain (200 g) was added, and stirring was continued (ca. 190 rpm) at 40 °C for 18 h. Tripeptide formation was accompanied by near complete liquefaction of the reaction mixture, with a homogeneous liquid being formed at ca. 3−7 h, followed by an increase in viscosity and gradual precipitation of the tripeptide thereafter. After 18 h, a viscous semisolid mixture was obtained, and the yield of tripeptide had reached 71%. Ethanol (ca. 1.7 L) was added, the suspension was stirred for 20 min and filtered, and the filtrate containing the product was collected and rotary evaporated at room temperature to a pale yellow syrup (ca. 1.2 kg). The recovered biocatalyst retained 69% of its initial activity. The crude product was dissolved in methanol (ca. 1.5 L) and purified by column chromatography (step elution with 8:2, then 6:4, then 5:5, then 4:6 water−methanol) to furnish the tripeptide as a white solid: 0.799 kg; 64% yield; >99% pure (by HPLC); mp 115−116 °C; $[\alpha]_D^{20}$ −7.6° (*c* 0.10, MeOH); $^1$H NMR (DMSO-$d_6$, 40 °C): 1.26−1.43 (m, 4H), 1.53 (m, 1H), 1.60 (m, 1H), 2.75 (dd, 1H, *J* = 6.3 Hz, 16.0 Hz), 2.88 (dd, 1H, *J* = 6.7 Hz, 16.1 Hz), 2.98 (m, 2H), 3.72 (dd, 1H, *J* = 6.1 Hz, 15.7 Hz), 3.80 (dd, 1H, *J* = 5.5 Hz, 15.3 Hz), 4.00 (dt, 1H, *J* = 6.5 Hz, 7.4 Hz), 4.51−4.62 (m, 4H), 4.75 (ddd, 1H, *J* = 6.1, 6.8, 8.2 Hz), 4.99 (s, 2H), 5.03 (s, 2H), 5.15−5.30 (m, 4H), 5.82−6.01 (m, 2H), 7.17 (t, 1H, *J* = 6.4 Hz), 7.25−7.40 (m, 11H), 8.15 (dd, 1H, *J* = 5.7 Hz, 6.3 Hz), 8.28 (dd, 1H, *J* = 5.7 Hz, 5.9 Hz); FAB-MS (M + H) calculated for $C_{34}H_{43}N_4O_{10}$ 667.2979, observed 667.2983.

**Preparation of $N_\alpha$-Cbz-L-Lys($N_\zeta$-Cbz)-Gly-L-Asp(OAll)-L-Glu(OAll)OEt (3).** $N_\alpha$-Cbz-L-Lys($N_\zeta$-Cbz)-Gly-L-Asp(OAll)OAll (**2**, 720 g, 1.08 mol) was mixed with L-Glu-

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(39) Bodanzsky, M. *Peptide Chemistry*, 2nd ed.; Springer-Verlag: Berlin, 1993.

(OAll)OAll (392 g, 1.73 mol), water (9 g), and ethanol (167 g) in an open 5-L wiped-blade reactor, and the slurry was heated with stirring (200 rpm) to 50 °C to form a viscous suspension. The final water and ethanol contents were ca. 1.9% w/w and 12.6% w/w, respectively. The mixture was cooled to 40 °C, Celite-deposited subtilisin (200 g) was added, and stirring was continued (ca. 140 rpm) at 40 °C for 38 h. The reaction was accompanied by near complete liquefaction of the reaction mixture, with minimum viscosity occurring at ca. 6−9 h, followed by a gradual increase in viscosity and increasing precipitation of the tetrapeptide thereafter. After 38 h, the reaction mixture had partially solidified, and the yield of tetrapeptide had reached 73%. Ethanol (ca. 2.1 L) was added, the suspension was stirred for 20 min and filtered, and the filtrate containing the product was collected and rotary evaporated at room temperature to a pale yellow syrup (ca. 1.0 kg). The recovered biocatalyst retained 78% of its initial activity. The crude product was dissolved in methanol (ca. 0.8 L) and purified by column chromatography (step elution with 8:2, then 6:4, then 5:5, then 4:6, then 2:8 water−methanol) to give the tetrapeptide as a white solid: 0.551 kg; 62% yield; > 99% pure (by HPLC); mp 112−115 °C; $[\alpha]_D^{20}$ −16.2° (*c* 0.10, MeOH); $^1$H NMR (DMSO-$d_6$, 40 °C): 1.25 (t, 3H, $J = 7.3$ Hz), 1.30−1.54 (m, 4H), 1.58 (m, 1H), 1.67 (m, 1H), 1.96 (m, 1H), 2.08 (m, 1H), 2.50 (dd, 2H, $J = 7.4$ Hz, 7.6 Hz), 2.68 (dd, 1H, $J = 8.0$ Hz, 15.9 Hz), 2.89 (dd, 1H, $J = 5.7$ Hz, 15.5 Hz), 3.01 (dt, 2H, $J = 6.2$ Hz, 6.5 Hz), 3.75 (d, 2H, $J = 5.0$ Hz), 4.05 (m, 1H), 4.11 (q, 2H, $J = 7.5$ Hz), 4.30 (m, 1H), 4.58−4.63 (m, 4H), 4.75 (m, 1H), 5.07 (d, 1H, $J = 12.8$ Hz), 5.09 (s, 2H), 5.13 (d, 1H, $J = 12.7$ Hz), 5.17−5.35 (m, 4H), 5.85−6.07 (m, 2H), 7.20 (t, 1H, $J = 6.3$ Hz), 7.29−7.48 (m, 11H), 8.15−8.26 (m, 3H); FAB-MS (M + H) calculated for $C_{41}H_{54}N_5O_{13}$ 824.3718, observed 824.3741.

**One-Pot Synthesis of $N_\alpha$-Cbz-L-Lys($N_\zeta$-Cbz)-Gly-L-Asp(OAll)-L-Glu(OAll)OEt (3).** $N_\alpha$-Cbz-L-Lys($N_\zeta$-Cbz)OEt (2.10 kg, 4.83 mol) was mixed with water (0.19 kg) and ethanol (0.56 kg) in an open 20-L wiped-blade reactor, and the slurry was heated with stirring (200 rpm) to 70 °C to form a viscous liquid. This was cooled to 40 °C, and to this were added GlyOEt·HCl (0.898 kg, 6.44 mol), diisopropylethylamine (0.81 kg, 6.31 mol, 98 mol %), DTT (10 g), and a 1:1 mix of finely powdered potassium carbonate and potassium hydrogen carbonate (70 g). Stirring (ca. 160 rpm) continued at 40 °C to furnish a semiliquid heterogeneous mixture. Celite-deposited chymopapain (0.8 kg) was added to the mixture, and stirring continued (200 rpm) at 40 °C for 28 h, when 82% of dipeptide had been formed. Water (0.10 kg) and ethanol (0.41 kg) were added to liquefy the mixture, followed by L-Asp(OAll)OAll (0.56 kg, 2.60 mol) and DTT (7 g), and the mixture was stirred (ca. 170 rpm) for 20 min to give a viscous heterogeneous mixture. A further portion of Celite-deposited chymopapain (0.26 kg) was added, and the mixture was stirred at 40 °C for a further 23 h, by which time the yield of tripeptide had reached 54% (66% based upon dipeptide), and a thick crystalline suspension had formed. Once again, this was liquefied by adding water (0.06 kg) and ethanol (0.60 kg) and stirring, followed by the addition of L-Glu(OAll)OAll (0.78 kg, 3.44 mol). Stirring was continued (ca. 150 rpm) for 30 min to provide a viscous suspension. Celite-deposited subtilisin (0.35 kg) was added, and stirring was continued (ca. 130 rpm) at 40 °C for 32 h, when the tetrapeptide yield had reached 34% (63% based upon tripeptide) and the mixture had partially solidified. Ethanol (ca. 7.0 L) was added, the suspension was stirred for ca. 1 h min. It was then filtered through Celite, and the filtrate containing the product was collected and rotary-evaporated at room temperature to a yellow syrup (ca. 5.3 kg). This was dissolved in methanol (ca. 4.8 L) and purified by column chromatography (as above) in six batches to furnish the tetrapeptide as a white solid: 1.22 kg; 30% yield; 98−99% pure (by HPLC); mp 111−113 °C; $[\alpha]_D^{20}$ −15.8° (*c* 0.10, MeOH); $^1$H NMR spectrum identical to that above.

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