Frakefamide, an Analgesic Tetrapeptide: Development of a Pilot-Plant-Scale Process

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Abstract:

A pilot-plant-process is described where frakefamide × HCl (L-tyrosyl-D-alanyl-*p*-fluoro-L-phenylalanyl-L-phenylalaninamide hydrochloride) was synthesised from its amino acid monomers in seven steps. The synthesis was performed in 70-L equipment, and the final product was obtained in 70% overall yield and in 99.5% purity. Only two intermediates were isolated, and the process required no chromatography. Peptide bond formation was promoted by isobutyl chloroformate-mediated mixed anhydride coupling reactions. The formed mixed anhydrides proved to be surprisingly stable, in most cases for several hours at −10 °C, and therefore suitable for large-scale peptide synthesis. Only traces, if any, of racemised coupling products were obtained. Benzyloxycarbonyl was used as amino protecting group throughout the synthesis, and its removal by hydrogenolysis proved to be fast and convenient on a large scale.

Introduction

Frakefamide (**1a**) is a tetrapeptide with an amide function at the C-terminus (Figure 1). The amino acid sequence of frakefamide is L-tyrosyl-D-alanyl-*p*-fluoro-L-phenylalanyl-L-phenylalaninamide. Hence, it contains both natural and unnatural α-amino acids. Frakefamide (**1a**) has shown interesting properties for use in acute and chronic pain treatment.[1] It acts as a selective agonist on μ-opioid receptors[2] and belongs to the dermorphin peptide family.[3] These peptides show analgesic properties and were discovered in the early 1980s. Dermorphin itself (Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH$_2$) and other naturally occurring peptides in the dermorphin family have a conserved *N*-terminal sequence of Tyr-D-Ala-Phe. The D-Ala residue is essential for activity; corresponding L-Ala peptides are practically inactive.[4] Furthermore, the amide function at the C-terminus is also important for activity; it increases the affinity to the μ-opioid receptor about 30−100 times compared to carboxyl group analogues. Suppression of the negative charge from a carboxyl group and better protection against carboxypeptidase
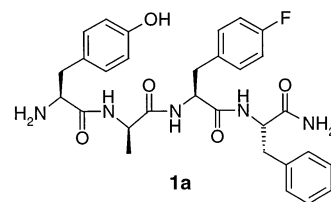

Figure 1. **Frakefamide (1a).**

cleavage have been suggested as reasons for the better activity.[3] For drug development the hydrochloride salt, frakefamide × HCl (**1b**), would be more suitable as drug substance rather than the neutral peptide. Any efforts for large-scale production would therefore be directed towards this compound.

For pre-clinical studies, investigational product in amounts up to 100 g was provided by solid-phase synthesis. A clinical development program would require multikilogram amounts, and future commercial production for market needs could be predicted in the range of tonne quantities per annum. A solid-phase approach was not considered realistic for this, mainly due to suspected problems with capacity. Costs would also be a problem for a solid-phase method even if enough capacity could be reached. The need for a large-scale solution synthesis was obvious, and the results from the development of an advanced pilot-plant process are described below.

Results and Discussion

Both a linear and a convergent solution synthesis of frakefamide × HCl (**1b**) can easily be designed. For a peptide of this size it is not obvious that one approach would be superior to the other. The number of synthetic steps is similar so other factors are important. These include crystallinity of intermediates, possibility to outsource the synthesis of intermediates, design of process logistics, and the obtained quality and yield of the final substance. From early on, energy was devoted to a linear synthesis that was utilised in the pilot plant process presented here. However, a convergent block synthesis was also developed to challenge the linear route. For both approaches there was initially a need to define some basic chemistry, a peptide coupling method, and a protecting group strategy suitable for large scale.

Coupling Methodology. Ideally, all peptide bonds in frakefamide (**1a**) should be formed by identical reactions to allow an efficient process flow with the best operability. Finding a suitable coupling reagent was therefore a key issue, and many aspects had to be considered and examined. The reagent should first of all be efficient (high-yielding, fast) and possible to handle on a large scale (relatively inert,

(1) Preclinical development has been made in collaboration with Biochem Pharma, Montreal, Canada, now part of Shire Pharmaceutical Group Inc.
(2) For a report on μ-opioid receptor binding with relevant related compounds, see: Schiller, P. W.; Nguyen, T. M.-D.; Chung, N. N.; Lemieux, C. *J. Med. Chem.* **1989**, *32*, 698−703.
(3) Melchiorri, P.; Negri, L. *Gen. Pharmacol.* **1996**, *27*, 1099−1107.
(4) Erspamer, V. *Int. J. Dev. Neurosci.* **1992**, *10*, 3−30.
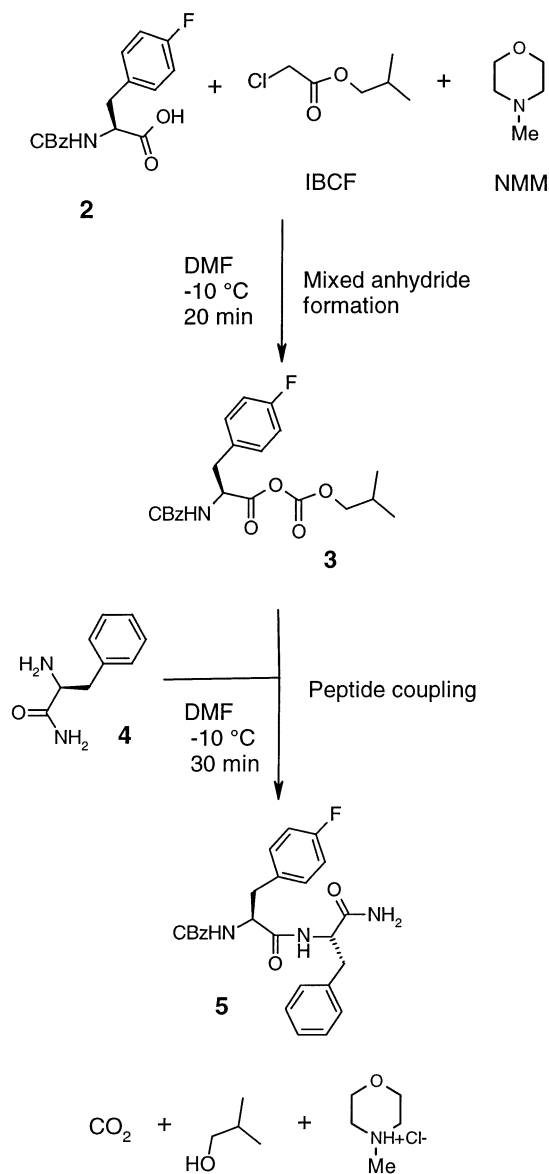
Figure 2. Mixed anhydride-promoted peptide coupling.

nontoxic). It should also be possible to remove residues of the reagent conveniently post-coupling (e.g., by extraction or filtration). A number of reagents fulfill these first important requirements, but if a future production process is planned, factors such as cost and availability, stability on storage, environmental profile, and safety in use also have to be considered.

After initial experiments with a few possible reagents, further development work was focused around isobutyl chloroformate (IBCF)-mediated mixed anhydride coupling reactions.[5-9] An example from the frakefamide × HCl (**1b**) synthesis is shown in Figure 2. IBCF is a cheap and readily available bulk reagent that together with the base *N*-methylmorpholine (NMM) quickly (within minutes) activates

the acid at low temperature (typically −10 to −25 °C). We found that the mixed anhydrides formed in our process were stable at −10 °C for 30 min up to several hours, an important feature adding operability to the process. Following activation, the peptide coupling reaction is also fast. By addition of the dissolved free-amino-block the bulk of the peptide bond formation is done within a few minutes, and the reaction is completed within 1 h. Yields of the coupling product in the range of 95−98% were achieved. The byproducts formed by this technique, carbon dioxide, isobutanol, and NMM-hydrochloride, are easy to remove and suitable for large-scale manufacture.

The mixed anhydride approach has been studied in detail by several investigators,[9-11] and our results follow most of the findings described. However, DMF is suggested to be a solvent where some racemisation can be expected, and the stability of the mixed anhydride might not be the best. To our benefit, DMF was found to be an excellent solvent when used in the couplings described here, with long-lasting stability of the mixed anhydrides and only trace amount of racemisation in the peptide products. No other base than NMM was evaluated for use in the frakefamide process. From literature it is clear that NMM is a very suitable base in combination with IBCF, especially with regards to low racemisation.[9] The mixed anhydride approach has earlier been used for large-scale peptide synthesis,[12] and examples show that it is suitable for many peptide couplings;[13] however, in some cases side reactions can be problematic.[14] The nature of the amino acids/peptides to be coupled, and careful optimisation of the conditions (temperature, solvent, and stoichiometry) will determine the outcome. Clearly, the mixed anhydride methodology suits the amino acid components to be coupled in the frakefamide synthesis. That together with thorough investigation of the conditions for each coupling has provided good results.

Protecting-Group Strategy. Since activation with IBCF and NMM occurs on the COOH function of the amino acid, the amino group needs protection[15,16] to prevent coupling with itself. Any protecting group, and several can be envisioned, therefore needs to be stable during activation and the coupling reaction. Furthermore, dependable, selective, and complete deprotection was a necessity to obtain clean material for the next coupling and low overall impurity formation. At the start of the development, the benzyloxycarbonyl group[17] (CBz group) was chosen for amino protection. It can easily be removed by catalytic hydrogenolysis,

(5) Vaughan, J. R. *J. Am. Chem. Soc.* **1951**, *73*, 3547.

(6) Wieland, T.; Bernhard H. *Ann.* **1951**, *572*, 190.

(7) Boissonnas, R. A. *Helv. Chim. Acta* **1951**, *34*, 874.

(8) Meienhofer, J. In *The Peptides*; Gross, E., Meienhofer, J., Eds.; Academic Press: London, 1979; Vol. 1, pp 263−314.

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(11) Chen, F. M. F.; Benoiton N. L. *Can. J. Chem.* **1987**, *65*, 619−625.

(12) For some general considerations of large-scale peptide synthesis in solution, see: Gorup, B. *Biochem. Soc. Trans.* **1990**, *18*, 1299−1306.
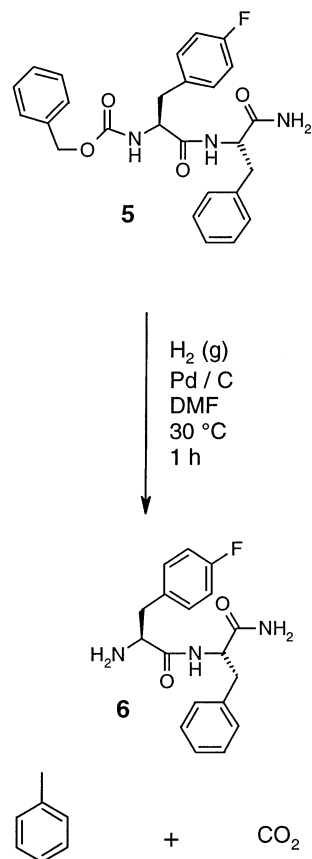
(13) Brady, S. F.; Freidinger, R. M.; Paleveda, W. J.; Colton, C. D.; Homnick, C. F.; Witter, W. L.; Curley, P.; Nutt, R. F.; Veber, D. F. *J. Org. Chem.* **1987**, *52*, 764−769.

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(16) Geiger, R.; König, W. In *The Peptides*; Gross, E., Meienhofer, J., Eds.; Academic Press: London, 1981; Vol. 3, pp 1−99.
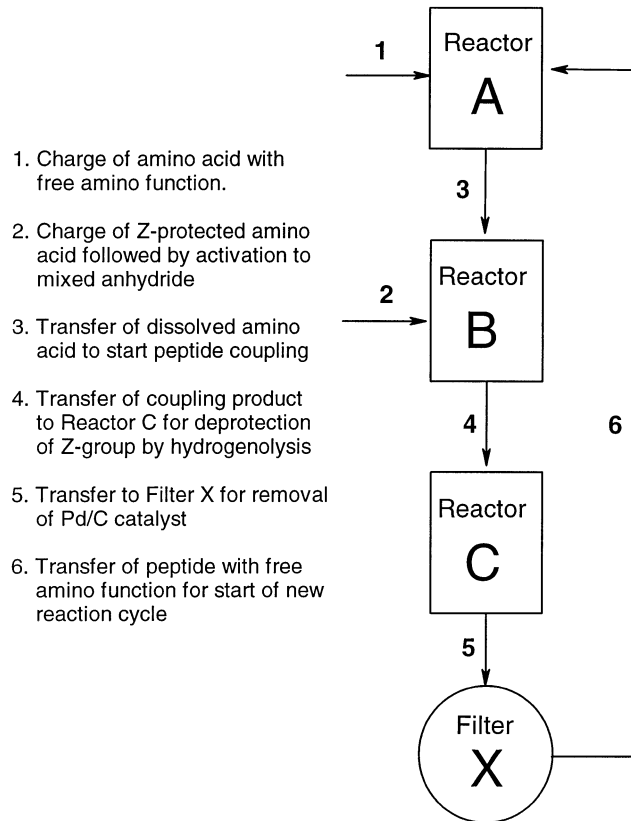
(17) Bodansky, M.; Bodansky, A. In *The Practice of Peptide Synthesis*, 2nd ed.; Springer-Verlag: Berlin, 1994; pp 11−14.

Figure 3. Detrotection of CBz group by hydrogenolysis.

1. Charge of amino acid with free amino function.

2. Charge of Z-protected amino acid followed by activation to mixed anhydride

3. Transfer of dissolved amino acid to start peptide coupling

4. Transfer of coupling product to Reactor C for deprotection of Z-group by hydrogenolysis

5. Transfer to Filter X for removal of Pd/C catalyst

6. Transfer of peptide with free amino function for start of new reaction cycle

Figure 4. Process flow in a three-reactor unit.

fulfilling all demands described above. The CBz group is also a standard item in the art of peptide synthesis, making sourcing of the desired CBz acids uncomplicated. In our typical procedure (see Figure 3) the CBz group is removed completely within 1−2 h by hydrogenolysis catalysed by 5% Pd/C at 3 atm. Similar to the coupling step, the byproducts carbon dioxide and toluene are easy to remove on large scale. Initially the Boc-group (*tert*-butyloxycarbonyl) was also considered. It worked well as a protecting group but its removal by TFA or HCl caused other problems. TFA turned out to be difficult to remove from the final substance without chromatography. If HCl was used instead, solvolysis of the peptide amide started to occur, and the corresponding ester was partly obtained. The CBz group had no side reactions that caused similar problems. To our benefit, CBz protection is also more economical due to lower reagent and raw material costs.

Process Logistics. The overall process for a stepwise synthesis of frakefamide hydrochloride (**1b**) (see Scheme 1) consists of three couplings, three deprotections and a final salt formation. Since the same procedures could be used consecutively for each coupling and each deprotection step, an attractive process design could be developed (see Figure 4). In a three-reactor unit, each reactor (A, B, and C respectively) was utilised for a specific purpose. The amino acid/peptide with free amino function to be coupled was dissolved in reactor A, and the CBz-protected amino acid to be added was charged to and dissolved in reactor B where it was activated by IBCF and NMM. The content of reactor

A was then transferred to reactor B where coupling occurred. After completion of the coupling reaction, the newly formed CBz-protected peptide was transferred to reactor C where deprotection by hydrogenolysis was performed. The solid Pd/C catalyst was removed by filtration with Filter X (a pressure filter), and the solution with the amino free peptide was transferred to reactor A. This reaction cycle was then repeated for the introduction of the next amino acid. In principle, the whole process is possible to run without any isolation of intermediates. However, isolation is possible by crystallisation after each reaction step by addition of an antisolvent. To keep control of impurities and stoichiometry, crystallisation and isolation of intermediates **9** and **1a** by use of Filter X was necessary.[18] These intermediates, isolated as solvent-moist crystals (only dry blown, not dried), were taken out and weighed before recharging. By gaining more experience of the process and better predictions of the yields, it should not be necessary to empty the filter after these isolations. The crystals can simply be redissolved in the filter and transferred to the appropriate reactor for the next reaction.

Step-by-Step Analysis of the Synthesis (Scheme 1): Step 1. Preparation of Dipeptide 5. In this first step, CBz-protected L-*p*-fluorophenylalanine (**2**) is activated in DMF by IBCF and NMM. Activation is performed at −10 °C, whereupon the mixed anhydride **3** is formed. Under these conditions the anhydride is stable for 3 h with negligible racemisation. Activation can be performed in *N*-methyl-2-

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(18) An additional filter was used for isolation of intermediates and final product if efficient cleaning of Filter X from Pd/C catalyst could not be ensured.

Scheme 1. Synthesis of frakefamide hydrochloride (1b)
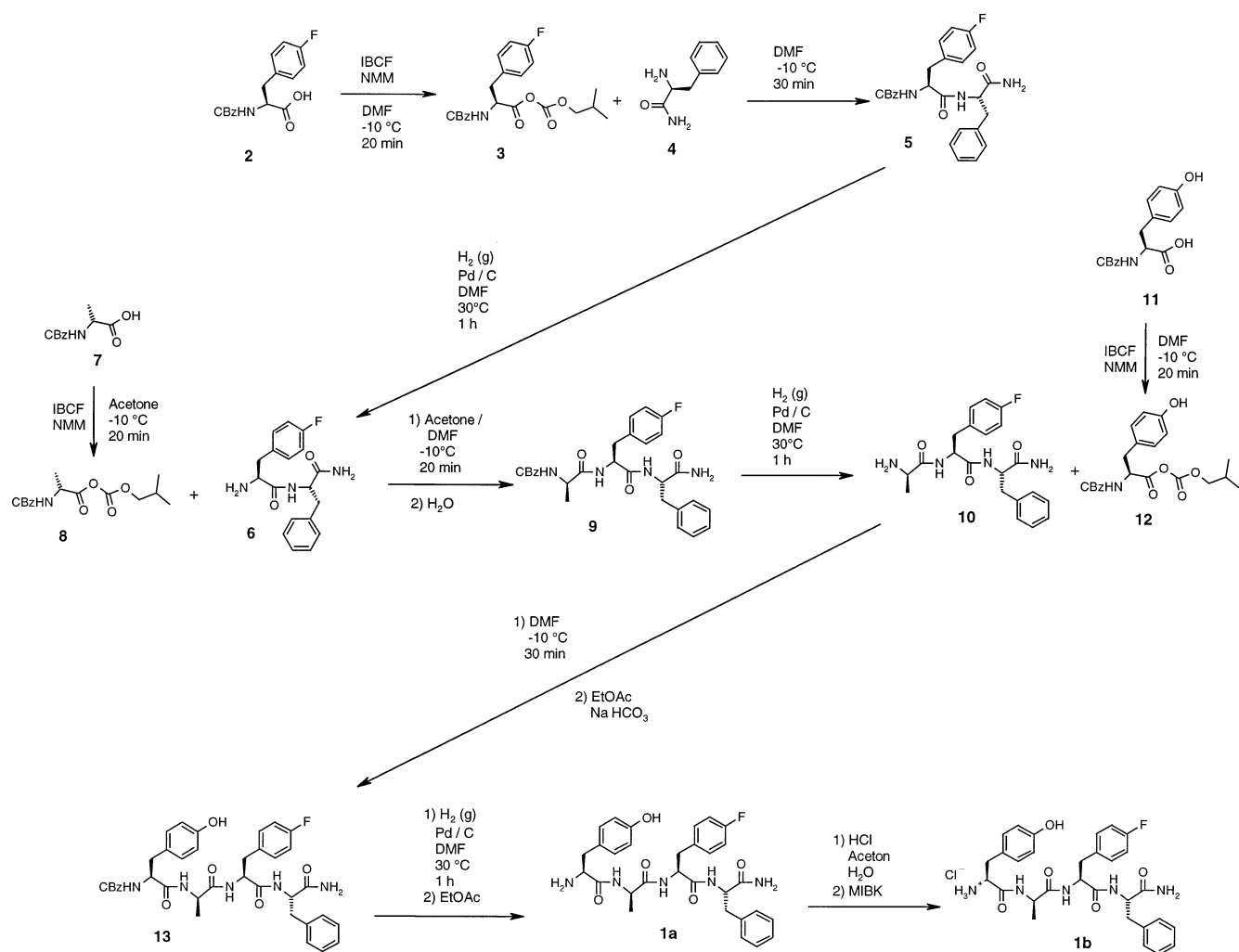
pyrrolidone (NMP) and EtOAc with similar results, but DMF was the best choice for the overall process. The peptide-coupling reaction is performed at the same temperature by addition of precooled phenylalanine amide (**4**) in DMF. Coupling is fast, complete within a few minutes, and a nonisolated yield of **5** of 98% is typically achieved. The DMF slurry of **5** is used directly in the next step.

This step has been modified and enhanced considerably during development. In the first pilot plant campaigns performed, H-Phe-OMe (**15**) was used in place of H-Phe-NH$_2$ (**4**) (see Figure 5). The coupling product thus obtained, dipeptide **16**, was transformed by ammonolysis to the desired dipeptide amide **5**. Ammonolysis was, however, very time-consuming, inconvenient, and volume-inefficient. Replacement of H-Phe-OMe (**15**) by H-Phe-NH$_2$ (**4**) gives an overall performance benefit that compensates well for the higher cost. In addition, intramolecular diketopiperazine formation (see Figure 6) occurs much slower with a dipeptide amide than with the corresponding ester. As a consequence, the process becomes more robust, and the product contains less of the piperazine impurity. Early on, dipeptide amide **5** was isolated by crystallisation from DMF and water at 35−40 °C. The procedure is rather sensitive to the addition rate of H$_2$O, relative volumes of DMF−water and gives thin fibrous

crystals that are best isolated by use of a pressure filter. Better control of the reaction conditions rendered this isolation unnecessary.

Step 2. Hydrogenolysis To Obtain Dipeptide 6. The second step, the first CBz group removal by hydrogenolysis, is straightforward. The incoming slurry of CBz-protected dipeptide amide **5** is warmed to 30 °C to improve solubility and, as a result, the reaction rate. Catalytic Pd/C is added and hydrogenolysis for 1−2 h at 3 atm removes the CBz-group cleanly and completely. The catalyst is removed by filtration and the obtained solution of **6** is used directly in the next step. It can be noted that compound **6** is stable for several days under the reaction conditions. For example, only trace amounts of diketopiperazine is formed. The fact that the incoming material for this step is not isolated has a positive effect on the filtration of the catalyst. Most of the *N*-methylmorpholine hydrochloride (NMM × HCl) formed in the first step precipitates in the DMF phase and is filtered off together with the catalyst. This makes the filter cake less dense, and the filtration time is considerably shortened.

Different grades of Pd/C catalyst have been examined for this reaction. For most pilot batches dry 5% Pd/C was used and added to the reaction as a pre-made DMF slurry. However, handling of dry Pd/C catalyst in regular manu-
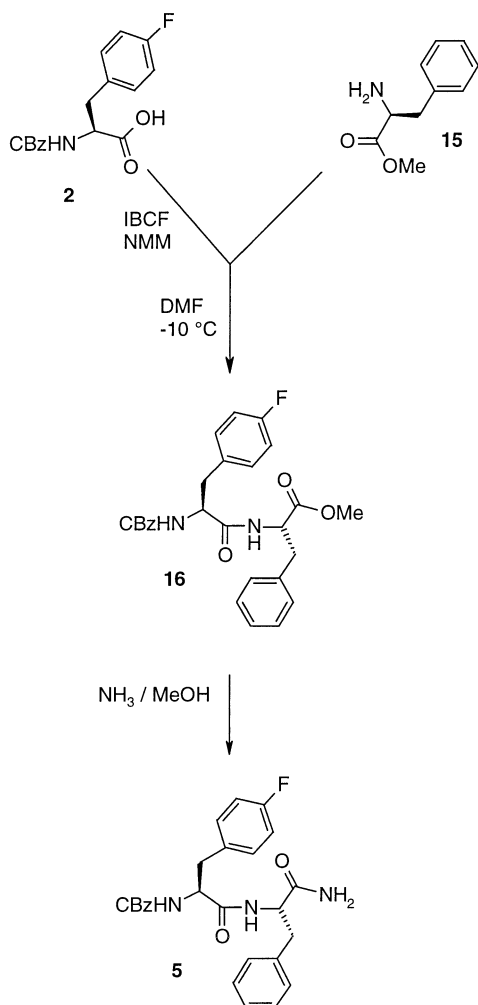
Figure 5. Previous synthesis of dipeptide 5.
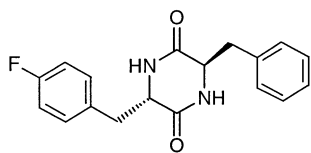
Figure 6. Diketopiperazine formed at dipeptide stage.

facture is not desirable from a safety point of view. Therefore water-wet catalyst (10% Pd/C, 50% H_2O content) was examined both in the laboratory and the pilot plant. The results were equivalent with those obtained with a dry catalyst with perhaps a small increase of the reaction rate. How much water that can be tolerated in the overall process remains to be investigated. Initial studies on the presence of water in the peptide couplings as well as literature precedence[10,19] are promising.

Step 3. Preparation of Tripeptide Amide 9. Activation of CBz-D-Ala-OH (**7**) with IBCF and NMM is best performed in acetone or acetonitrile. Both offer good stability (6 h at -10 °C) of the mixed anhydride **8** with very low rate of racemisation. Acetone was used in the pilot plant as the cheaper and more environmentally friendly choice. Control of temperatures, especially during the IBCF charge

(19) Chen, F. M. F.; Steinauer, R.; Benoiton, N. L. *J. Org. Chem.* **1983**, *48*, 2939−2941.

(should be kept ≤ -10 °C), is essential to avoid breakdown and byproduct formation. However, if the temperature is too low, a build-up of reagent will occur that may lead to a runaway reaction. The coupling reaction is fast, as in step 1, when dipeptide amide **6** is added to mixed anhydride **8**. It is finished within a few minutes with a nonisolated yield of 98%. The product **9** is crystallised by careful addition of H_2O, and the isolation is best performed with a pressure filter. Centrifugation works less well due to the small particle size of the crystals and high compressibility. Isolation is then completed by acetone washes of the filter cake followed by dry blowing with nitrogen. Dry blowing is efficient, leaving only 2−3% of the solvent within the crystals, and no further drying is needed prior to the next step. The isolated material typically has a purity of 99% at this stage, and the overall yield over the first three steps from **2** is about 93%.

Step 4. Hydrogenolysis To Obtain Tripeptide 10. Hydrogenolysis of CBz-protected tripeptide amide **9** is performed in the same way as for compound **5** in step 2. In this case no NMM × HCl is present so that filtration of the catalyst is much more time-consuming. The Pd/C-filter cake becomes very dense and almost impenetrable. The reaction time is about 1−2 h, and the resulting DMF solution of tripeptide **10** is used directly in the next step. Both dry and water-wet Pd/C catalysts have been utilised in this step with very similar results.

Step 5. Preparation of Tetrapeptide Amide 13. This step, the final peptide coupling where CBz-tyrosine **11** is added to the tripeptide **10**, is the most delicate in the synthesis. A small excess of CBz-tyrosine (**11**) is used and activated by IBCF/NMM in DMF in the same manner as described in steps 1 and 3. DMF and NMP work fine for the activation, but stability of the mixed anhydride **12** was poor in EtOAc. The outcome of the reaction is sensitive to the relative amounts of IBCF and NMM charged, and equimolar amounts of the reagents and CBz-tyrosine should be used. Too little reagents charged means, of course, incomplete formation of the mixed anhydride **12**, but only slight overcharge will promote isobutyl carbonate acylation of the tyrosine phenolic hydroxyl group (see Figure 7). If formation of the acylated byproduct **17** occurs, peptide coupling will still be possible, and a tyrosine acylated tetrapeptide **18** will be formed as a coupling byproduct. This byproduct can be converted to the desired product **13** by ammonolysis. In essence, if a significant amount of byproduct **18** is formed, ammonia treatment is included in the workup procedure. Crystallisations later in the synthesis remove this byproduct poorly so that the development of the optional ammonia treatment made the overall process more robust.

In early development, compound **13** was crystallised by addition of H_2O and isolated. This crystallisation was very sensitive and gave crystals of poor quality that were very difficult to filter due to partial gel formation. Experience gave better control of the reaction, and isolation at this stage could be omitted. However, the obtained solution of **13** was not suitable for the next step since the presence of NMM × HCl would greatly disturb the crystallisation of frakefamide (**1a**)
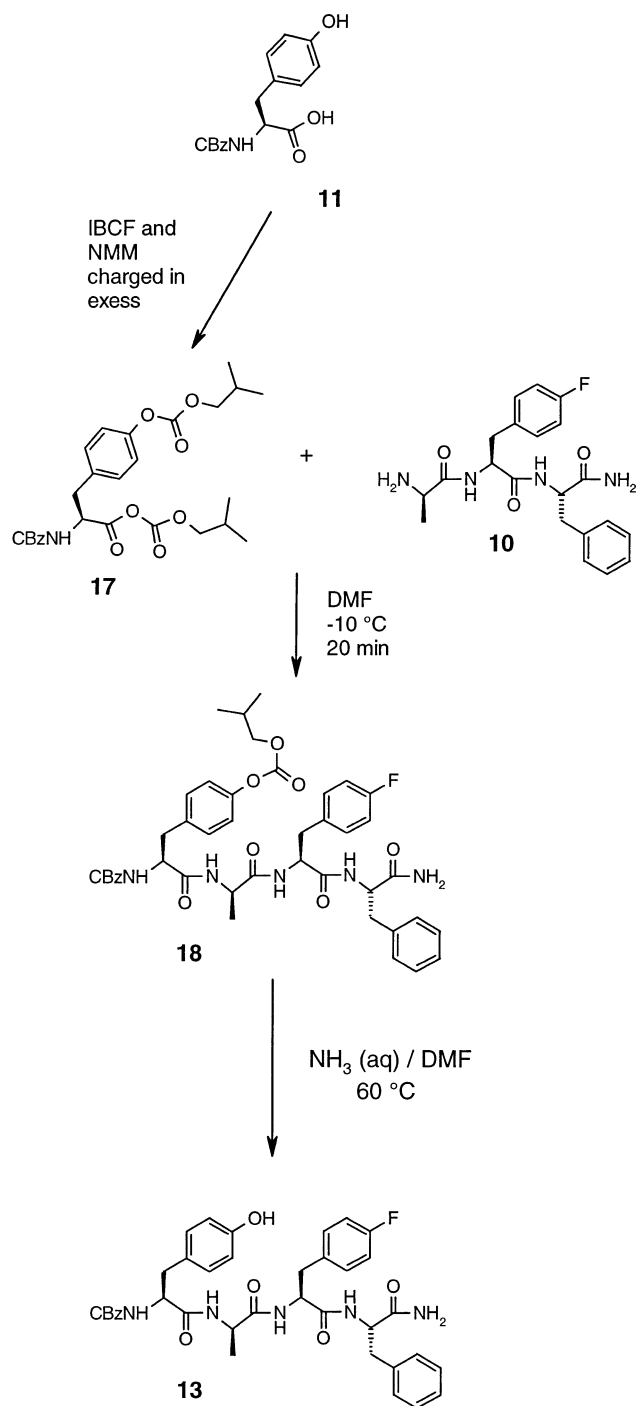
Figure 7. Formation and removal of byproduct 18.

to be produced in the next step. To circumvent this problem, one-third of the DMF is replaced with EtOAc after which NaHCO$_3$ is added. This converts NMM × HCl into neutral NMM and NaCl. The NaCl precipitates in the EtOAc−DMF mixture and can easily be filtered off. The NMM is distilled off at a later stage.

Step 6. Hydrogenolysis To Obtain Frakefamide (1a). CBz-protected tetrapeptide **13**, incoming as a DMF−ETOAC solution, is hydrogenolysed as described for steps 2 and 4 with formation of the unmasked product **1a**. The reaction time is about 1−2 h. Filtration of the Pd/C-catalyst is best performed with a heated filter (30−40 °C) so that unwanted

precipitation of product can be avoided. This will also facilitate efficient DMF-wash of the filter cake that is necessary to achieve a good yield. After Pd/C filtration, frakefamide (**1a**) is crystallised by addition of EtOAc and isolated by filtration. The crystals are blow-dried and are used EtOAc-wet in the next step. A solvent content up to 25% (w/w) can be tolerated. Removal of NMM × HCl in the previous step, as well as ammonia treatment to remove any tyrosine-*O*-acylated product, together with the hot Pd/C filtration have made this step more robust and better yielding. The isolated yield of frakefamide **1a** is about 80% over two steps starting from tripeptide **9**, with majority of the loss occurring in the crystallisation of product **1a**.

Step 7. Formation of Frakefamide × HCl (1b). The free base **1a** is charged EtOAc-wet and is dissolved in a H$_2$O−acetone mixture. HCl is charged (1 equiv), and the solution is clear filtered. When more acetone and seed crystals are added, the crystallisation starts, and addition of MIBK then completes crystallisation. The temperature is kept at 20 °C throughout the procedure, and the crystals are preferably isolated with a pressure filter. The yield is good, about 95%, and a purity of 99.5% of the final product **1b** can be expected, a purity improvement of 3% over the step. If this procedure is followed, frakefamide × HCl (**1b**) is isolated in a stable crystal form. However, if the crystals are left MIBK-wet for more than a week, other forms and amorphous material will start to appear. The dried product is, on the other hand, stable, only weakly hygroscopic, and has a shelf life exceeding 2 years.

Investigation of a Convergent Alternative. An alternative route was developed where two dipeptides were coupled to yield tetrapeptide **13** as an alternative to the step-by-step synthesis described above (Scheme 2). The synthesis was performed up to 2-L scale. The first fragment, dipeptide **6**, was synthesised as described earlier. The next building block CBz-Tyr-D-Ala-OH (**20**) was also synthesised using the IBCF/NMM coupling technique. CBz-Tyr-OH (**11**) and H-D-Ala-OMe × HCl (**19**) were starting monomers, and the coupling could be performed entirely in acetone. NaOH was subsequently used to remove the methyl ester, and the dipeptide fragment **20** was ready for coupling. Dipeptide fragment **20** could also be prepared from free D-alanine to avoid the acid deprotection step. A poly(ethylene glycol)−EtOAc mixture was found to be efficient, and K$_2$CO$_3$ was added to ensure desired deprotonation of the D-alanine. Formation of dipeptide **20** by this route was promising, but isolation turned out to be more problematic.

The two peptide fragments were coupled using the same IBCF-promoted mixed anhydride approach. A slight variation of the procedure was found to be efficient.[20] Both components (**6** and **20**) were charged to a reactor, and IBCF was added at −20 °C. Peptide-bond formation was then promoted by controlled addition of NMM. Also in this case, the whole procedure could be performed in acetone, and tetrapeptide **13** was isolated in 75−80% yield.

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(20) A similar activation procedure has been described by: Shieh, W.-C.; Carlson, J. A.; Shore M. E.; *Tetrahedron Lett.* **1999**, *40*, 7167−7170.

Scheme 2. Synthesis of tetrapetide 13 using dipeptide building blocks
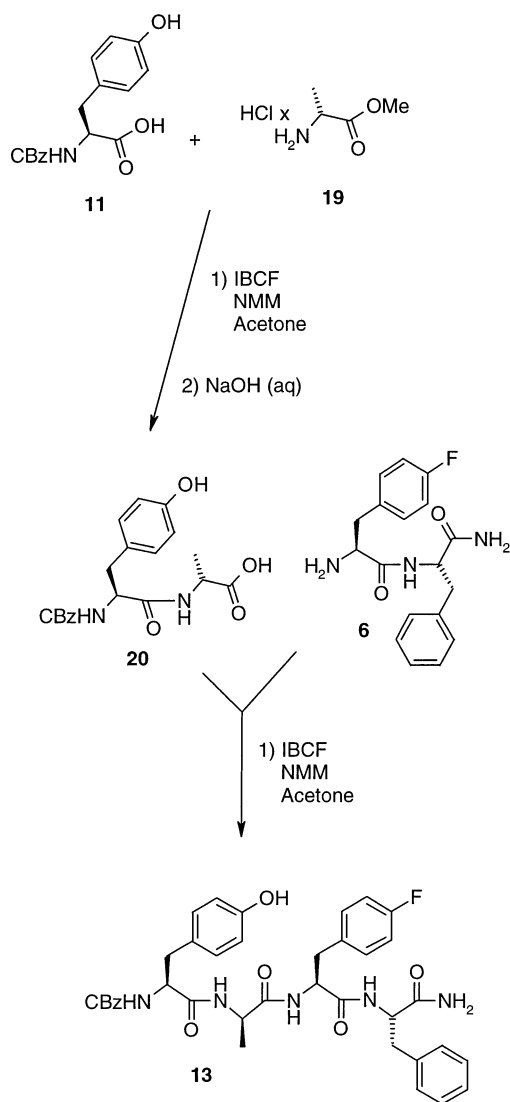
Although the convergent route was not developed to the same extent as the linear synthesis, its potential could be estimated and the two alternatives compared. Yield, quality, and cost of final substance were comparable from both routes, but batch size could be larger using the convergent approach. On the other hand, process logistics suggest more efficient use, and shorter batch cycle times, for the linear synthesis. The potential for outsourcing of the dipeptide blocks **6** and **20** and less use of DMF were other benefits with the convergent approach. In light of the relatively small differences between the routes and more expertise with the linear one, further development of a convergent approach was not carried out.

Resources and Costs. This process was developed during a period of 4 years during which seven pilot-plant campaigns were carried out. A total of about 20 man-years of effective development time was used, including all aspects of process development. Estimation of the cost of frakefamide × HCl (**1b**) by the described process is $2500/kg for production of less than 1000 kg/year. Higher annual production would

decrease the cost due to lower raw material prices. Of the raw materials, CBz-Phe(F)-OH (**2**) is the most costly followed by H-Phe-NH₂ × HCl (**4**). These two components are about 5−10 times more expensive than CBz-D-Ala-OH (**7**) and CBz-Tyr-OH (**11**). Reagents and solvents will only add negligible sums to the raw material cost. In an overall estimate, raw materials contribute 60−70% of the cost of goods, and the remaining part is used for operation of the process.

Conclusions

Starting from amino acid monomers, the overall yield in the tetrapeptide synthesis over seven steps of frakefamide × HCl (**1b**) is just over 70%. That together with the high purity (99.5%) of the final substance is in itself a good measure of the standard of this process. The chemistry that is used, IBCF/NMM-promoted couplings and CBz-group deprotection by hydrogenolysis is straightforward and simple. It is also robust, high-yielding, and provides very little formation of undesired byproducts. For example, there is very little racemisation (only traces detected) of the amino acid components occurring during the peptide-coupling steps. Crystallisations of intermediates and final substance are somewhat sensitive but provide good purification and yields.

Experimental Section

Structure-Contributing Components. All amino acid components were obtained from commercial sources, H-Phe-NH₂ × HCl (**4**) from Senn Chemicals AG, CBz-Phe(F)-OH (**2**) from Synthetech Inc., CBz-D-Ala-OH (**7**) and CBz-Tyr-OH (**11**) from Rexim Degussa.

Reagents. IBCF (SNPE Chemie), NMM (BASF), and Pd/C (5% Pd/C type 5R87L, from Johnson Matthey) were purchased from the listed commercial sources in reagent grade. Other chemicals were standard bulk chemicals.

Solvents. DMF was purchased in drums (p.a. quality from Merck). Acetone, water, MIBK, and EtOAc were of bulk quality taken from the local plant solvent supply system.

Equipment. Reactor A and reactor B were both 70-L glass-lined reactors, each equipped with a three-bladed retreat curve impeller. Reactor C was a 70-L stainless steel reactor equipped with a 12-bladed turbine. Filter X was a pressure filter-nutsche equipped with a K200-filterplate.[18]

Analytical Methods. A generic LC-purity method was developed that was used for all steps. The method was used both for in-process control and for determination of the purity of isolated substance. The degree of purity was determined by comparison with reference standards of known purity. Correct identity of intermediates and isolated material were controlled by comparison with reference standards, which were characterised by NMR and MS.

The Generic LC-Purity Method. Column: Kromasil C8, 3.5 μm, 100 mm × 4.6 mm or equivalent; flow rate: 1.0 mL/min; injection volume: 10 μL; wavelength: 210 and 254 nm; run time: 18.5 min; mobile phase A: 0.1% (v/v) TFA in H₂O; mobile phase B: 0.09% (v/v) TFA in 80/20 (v/v) CH₃CN/H₂O.

Gradient

time	%A	%B
0	75	25
2.5	75	25
12.5	10	90
16	10	90
16.5	75	25
18.5	75	25

^{1}H NMR spectra were recorded on a Bruker Avance 400 (400 MHz) or a Varian Gemini 300 (300 MHz) at 25 °C. The samples were dissolved in DMSO-d_6, and the DMSO peak at δ 2.50 ppm was used as internal standard.

CBz-Phe(F)-Phe-NH$_2$ (5). H-Phe-NH$_2$ × HCl (**4**) (1.27 kg, 6.33 mol) and DMF (6.3 kg, 6.7 L) were charged to reactor A at 20 °C. After complete dissolution, the temperature was adjusted to −10 °C. NMM (0.66 kg, 719 mL, 6.53 mol) was then charged to the solution to neutralise the hydrochloride salt. CBz-Phe(F)-OH (**2**) (1.94 kg, 6.10 mol) and DMF (6.3 kg, 6.7 L) were charged to reactor B at 20 °C. After almost complete dissolution, the temperature in reactor B was adjusted to −10 °C, and isobutyl chloroformate (IBCF) (0.95 kg, 902 mL, 6.95 mol) was charged over 2 min. This charge was directly followed by a charge of NMM (0.71 kg, 774 mL, 7.02 mol) over 15 min, and reaction temperature was kept at −5 to −15 °C. (CAUTION: Addition of NMM causes an exothermic reaction, and temperature needs to be carefully monitored.) Without delay, the entire contents of reactor A were transferred to reactor B over 10 min; the reaction temperature was kept at −5 to −10 °C. (CAUTION: The peptide-coupling reaction is exothermic, and temperature needs to be carefully monitored.) Reactor A was washed with DMF (3 L) which then was transferred to reactor B. The reaction mixture was stirred at −10 °C and sampled after 20 min. Analysis showed LC-purities of 97.2 and 0.9 area % of **5** and **2**, respectively. (If the level of **2** had been over 4 area %, reaction time would have been prolonged or additional IBCF charged or both). The thin DMF slurry of **5** in reactor B was used directly in the next step.

Crystallisation of dipeptide **5** is possible by slow addition of water at 20−30 °C. The formed crystals can then be isolated by filtration and dried. Dipeptide **5** isolated by this procedure gave NMR data: ^{1}H NMR (DMSO-d_6) δ 2.62−3.07 (m, 4H), 4.22 (m, 1H), 4.48 (m, 1H), 4.94 (m, 2H), 7.0−7.54 (m, 17H), 8.05 (d, J = 8.2 Hz, 1H).

H-Phe(F)-Phe-NH$_2$ (6). The DMF slurry of **5**, obtained in the previous step, was transferred from reactor B to reactor C. Reactor B was washed with DMF (4 L) which then was transferred to reactor C, and the inner temperature was adjusted to 30 °C. Pd/C (143 g, 5% Pd content, dry) was mixed with DMF (420 mL) in a steel vessel, and the slurry was charged to reactor C via the manhole. H$_2$ (g) was charged to reactor C until pressure reached 3 atm (0.3 Mpa), and the agitation was adjusted to strong. Within 45 min, the hydrogen pressure was released and new hydrogen gas charged twice to the same pressure. The mixture was kept for 2 h with strong agitation while temperature was allowed to fall to 25

°C. After the agitation was lowered and the hydrogen pressure was released, the mixture was sampled. LC-purity analysis showed that 99.7% of **5** (based on total area % calculations) had been consumed and that content of **6** was 96.3 area %. (If conversion of **5** had been lower than 98%, new H$_2$ would have been charged to 3 atm pressure and the reaction continued.) The content of reactor C was filtered by Filter X and the filtrate collected in reactor A. DMF (8 L) was used to rinse reactor C and wash Filter X; the wash liquid was collected in reactor A. The solution, containing **6**, was cooled to −10 °C and used directly in the next step.

Simple isolation of smaller amounts is possible by evaporation and drying of the residue. These operations gave **6** as a solid powder, not further purified but solvent-free, which had NMR data: ^{1}H NMR (DMSO-d_6) δ 1.65 (s (broad), 2H) 2.47−2.53 (m, 1H), 2.74−3.00 (m, 3H), 3.93 (m, 1H) 4.47 (m, 1H), 7.02−7.43 (m, 11H), 7.99 (d, J = 6.9 Hz, 1H)

CBz-D-Ala-Phe(F)-Phe-NH$_2$ (9). CBz-D-Ala-OH (**7**) (1.42 kg, 6.35 mol) and acetone (5.8 kg, 7.3 L) were charged to reactor B. The mixture was stirred at 25 °C, and after 15 min a thin opaque solution was obtained, and the temperature could be lowered to −10 °C. Isobutyl chlorofomate (IBCF) (0.87 kg, 826 mL, 6.35 mol) was then charged to reactor B over 2 min. This charge was directly followed by a charge of NMM (0.74 kg, 804 mL, 7.32 mol) over 20 min, while the reaction temperature was kept at −5 to −15 °C. (CAUTION: Addition of NMM causes an exothermic reaction, and temperature needs to be carefully monitored.) Without delay, the entire DMF solution of **6** in reactor A (obtained in previous step) was transferred to reactor B over 20 min; the reaction temperature was kept at −5 to −10 °C. (CAUTION: The peptide-coupling reaction is exothermic, and temperature needs to be carefully monitored.). Reactor A was washed with DMF (3 L) which then was transferred to reactor B. After 30 min the reaction mixture was sampled; the temperature of the reaction mixture was adjusted to 20 °C. LC-purity analysis of the sample showed that all **6** was consumed and that **9** was present in 92.8 area %. The reaction mixture was left overnight and then distilled at reduced pressure (100 mbar, temperature of jacket 50 °C). About half the volume was removed (∼20 L) by the distillation. Acetone (8 L) was charged to the remaining thin slurry and the temperature was adjusted to 40 °C. Over 1 h, water (41 L) was then charged to reactor B in portions; the temperature was kept between +30 to +40 °C. After completed H$_2$O charge the crystal slurry was gently stirred overnight and the temperature adjusted to 20 °C. The crystals were isolated by filtration with Filter X and then washed at 20 °C with a water−acetone mixture (1:1, 5 L) followed by two identical acetone washes (5 L each). After blow-drying with N$_2$ (100 min) the filter was emptied, and 3.34 kg of **9** was obtained. The solvent content was 2.5%, and the LC purity of **9** in the isolated material was 99.0%. A calculation of the yield would then give 3.03 kg of pure **9** that correlates to 5.7 mol and 93% yield over three steps starting from **2**. The crystals of **9** had NMR data: ^{1}H NMR (DMSO-d_6) δ 0.97 (d, J = 6.8 Hz, 3H), 2.66−3.08 (m, 4H), 4.02 (m, 1H), 4.45 (m, 2H),

5.01 (q, 2H), 7.00−7.46 (m, 17H), 8.04 (d, $J = 8.2$ Hz, 1H), 8.13 (d, $J = 8.3$ Hz, 1H)

H-D-Ala-Phe(F)-Phe-NH$_2$ (10). Tripeptide **9** (3.3 kg of material isolated in the previous step, correlates to 3.0 kg, 5.7 mol dry and pure material) and DMF (23 L, 17 kg) was charged to reactor B at 20 °C. The jacket temperature was then set to 45 °C and vacuum was applied. Under these conditions about 8 L of solvent was removed to ensure that all acetone was removed. After distillation the vacuum was released, and the thin slurry was transferred to reactor C; DMF (4 L) was used to rinse reactor B. The inner temperature was adjusted to 30 °C. Pd/C (153 g, 5% Pd content, dry) was mixed with DMF (610 mL) in a steel vessel, and the obtained slurry was charged to reactor C via the manhole. H$_2$ (g) was charged to reactor C until pressure reached 3 atm (0.3 Mpa), and the agitation was adjusted to strong. Within 45 min, the hydrogen pressure was released and new hydrogen gas charged twice to the same pressure. The mixture was kept for 1.5 h with strong agitation. After adjustment to lower agitation and releasing of the hydrogen pressure, the mixture was sampled. LC-purity analysis showed that 99.9% of **9** (based on total area % calculations) had been consumed and that the content of **10** was 95.3 area %. (If conversion of **9** had been lower than 98%, new H$_2$ would have been charged to 3 atm pressure and the reaction continued.) The content of reactor C was filtered by Filter X and the filtrate collected in reactor A. DMF (4 L) was used to rinse reactor C and wash Filter X, the wash liquid was collected in reactor A. The solution, containing **10**, was cooled to −10 °C and used directly in the next step.

Simple isolation of smaller amounts is possible by evaporation and drying of the residue. These operations gave **10** as a solid powder, not further purified but solvent-free, which had NMR data: ^{1}H NMR (DMSO-d_6) δ 0.96 (d, $J = 6.8$ Hz, 3H), 1.73 (s (broad), 2H) 2.73−3.05 (m, 4H), 3.18 (q, $J = 6.9$ Hz, 1H), 4.46 (m, 2H), 6.99−7.40 (m, 11H), 8.04 (s (broad), 1H), 8.11 (d, $J = 8.3$ Hz, 1H).

CBz-Tyr-D-Ala-Phe(F)-Phe-NH$_2$ (13). CBz-Tyr-OH (**11**) (1.87 kg, 5.92 mol) and DMF (7.7 kg, 10 L) were charged to reactor B. The mixture was stirred at 25 °C and after 15 min a clear gray solution was obtained, and the temperature could be lowered to −10 °C. Isobutyl chlorofomate (IBCF) (0.808 kg, 770 mL, 5.92 mol) was then charged to reactor B over 2 min. This charge was directly followed by a charge of NMM (0.604 kg, 659 mL, 5.97 mol) over 15 min; reaction temperature was kept at −5 to −15 °C. (CAUTION: Addition of NMM causes an exothermic reaction, and temperature needs to be carefully monitored.) Without delay, the entire DMF solution of **10** in reactor A (obtained in the previous step) was transferred to reactor B over 10 min; the reaction temperature was kept at −5 to −10 °C. (CAU-TION: The peptide-coupling reaction is exothermic, and temperature needs to be carefully monitored.) Reactor A was washed with DMF (3 L) which then was transferred to reactor B. After 30 min the reaction mixture was sampled. LC-purity analysis of the sample showed that 4.2 area % of **10** was unreacted. Since internal criteria for complete reaction was <1 area % of remaining **10**, extra charges of IBCF (31

mL, 0.04 mol) and NMM (29 mL, 0.05 mol) were made. After 10 min of continued stirring the temperature was adjusted to 20 °C, and the mixture was left for an additional 2 h. (If the sample had shown formation of byproduct **18** (>0.2 area % in LC-purity test) charge of 2.33 L NH$_4$OH (25% aqueous solution) would have been necessary. Heating of the obtained mixture at 60 °C for 4 h under strong agitation removes byproduct **18**, and the process can proceed with the next operation.) After 18 L of DMF was removed by distillation performed at jacket temperature of 50 °C and reduced pressure, EtOAc (20 L) was charged. NaHCO$_3$ (1.14 kg, 17.1 mol) was charged via the manhole, and the mixture was left with powerful stirring for 4 h at 30 °C. The temperature was then adjusted to 5 °C, and the mixture was filtered (precipitated NaCl isolated on filter) with filter X to reactor A. A DMF−EtOAC mixture (1:1, 4 L) was used to rinse reactor B and wash Filter X; the wash liquid was collected in reactor A. The DMF solution of **13** was used directly in the next step. LC-purity analysis of the solution showed a content of **13** of 91.2 area % (by calculation this correlates to 3.6 kg and 5.6 mol).

Crystallisation of tetrapeptide **13** is possible by slow addition of CH$_3$CN (0.7 L/ L DMF) at 45 °C followed by seeding with good quality crystals. The temperature is then slowly lowered to 20 °C before a slow addition of water (0.7 L/ L DMF). The crystals can then be isolated by filtration and dried. Tetrapeptide **13** isolated by this procedure has NMR data: ^{1}H NMR (DMSO-d_6) δ 0.90 (d, $J = 6.9$ Hz, 3H), 2.51−3.03 (m, 6H), 4.21 (m, 2H), 4.45 (m, 2H), 4.93 (q, 2H), 6.64 (d, $J = 8.2$ Hz, 2H), 7.00−7.45 (m, 19H), 8.04 (d, $J = 7.3$ Hz, 1H), 8.09 (d, $J = 8.2$ Hz, 1H), 8.17 (d, $J = 8.4$ Hz, 1H), 9.2 (s, 1H).

Frakefamide (H-Tyr-D-Ala-Phe(F)-Phe-NH$_2$) (1a). The DMF solution of **13** (3.91 kg, 5.6 mol) was transferred from reactor A to reactor C; DMF (4 L) was used to rinse reactor A. The inner temperature was adjusted to 30 °C. Pd/C (234 g, 5% Pd content, dry) was mixed with DMF (940 mL) in a steel vessel, and the obtained slurry was charged to reactor C via the manhole. Hydrogen (g) was charged to reactor C until pressure reached 3 atm (0.3 Mpa), and agitation was adjusted to strong. Within 45 min, the hydrogen pressure was released, and new hydrogen gas charged twice to the same pressure. The mixture was kept for 1.5 h with strong agitation. After adjustment to lower agitation and release of the hydrogen pressure, the mixture was sampled. LC-purity analysis showed that 99.8% of **13** (based on total area % calculations) had been consumed. (If conversion of **13** had been lower than 97%, new hydrogen would have been charged to 3 atm pressure and reaction continued at 40 °C for 30 min.) The content of reactor C was filtered at 35 °C by Filter X, and the filtrate was collected in reactor A. DMF (4 L) was used twice to rinse reactor C and wash Filter X; the wash liquids were collected in reactor A. Both washes were performed at 35 °C. Twenty-three liters of DMF was removed by distillation performed at jacket temperature of 50 °C and reduced pressure. EtOAc (7.8 + 15 + 28 L) was charged in portions at 30−40 °C over 2 h. Crystals forms spontaneously after the first portion. Once all EtOAc was

added, the slurry was cooled to 5 °C within 30 min and then left with stirring for 2 h. The crystals were isolated by filtration with Filter X, and the crystals were washed twice with EtOAc (5+5 L) and blow-dried with N_2. The filter was emptied, and the crystals were dried in a drying cabinet at 50 °C and 20 mbar for 100 h; 3.20 kg frakefamide (**1a**) was finally isolated. The solvent content of **1a** was 22% (mainly EtOAc), and LC-purity was 97.6 area %. This correlates to a dry and pure weight of 2.48 kg (4.41 mmol) and a yield of 80% from **9**. ^{1}H NMR data correlates to those obtained for compound **1b**, except that the signal from aminoprotons will vary in δ value, depending on pH.

Frakefamide Hydrochloride (H-Tyr-D-Ala-Phe(F)-Phe-NH$_2$ × HCl) (1b). Aqueous HCl (37% w/w, 510 g, 5.17 mmol), water (249 g, 249 mL), and acetone (7.9 kg, 10 L) were added to a charge vessel and mixed at 20 °C. Frakefamide (H-Tyr-D-Ala-(F)Phe-Phe-NH$_2$) (**1a**) (3.166 kg EtOAc moist, obtained in the previous step correlates to 4.38 mmol) was charged to reactor A followed by a rapid charge of the acetone−hydrochloride mix from the charge vessel. Stirring for 15 min at 20 °C gave an almost clear solution that was clear filtered, using a polish filter, to reactor B. Reactor A and the filter was washed with an acetone−water mixture (1.37 L acetone + 0.071 L water, 95:5 v/v) and the wash liquid added to the content in reactor B. Stirring was adjusted to slow and acetone (10.5 L) was charged while the temperature was kept at 20 °C. Good quality frakefamide hydrochloride crystals (H-Tyr-D-Ala-(F)Phe-Phe-NH$_2$ × HCl) (**1b**) (30 g, 0.05 mol) and acetone (535 mL) were mixed to a seed slurry in a flask. The seed slurry was carefully charged to reactor B via the manhole which initiated crystallisation. After 20 min, stirring was adjusted to strong,

and methyl isobutyl ketone (MIBK) was charged in four portions (4 × 14 L) to reactor B. Each portion was charged with a rate of about 0.4 L/min with a 10−15 min break and slow stirring between each portion, the temperature was kept at 20 °C. After the MIBK charge was completed, the crystal slurry was stirred for 1 h and then filtered using Filter X. The crystals were washed twice with MIBK (8 + 8 L) and then blow-dried with N_2. The moist crystals were emptied from the filter and dried in a drying cabinet for 150 h at 50 °C and <15 mbar. Finally, dry crystals (solvent + H_2O content was 0.3%) of **1b** (2.52 kg, 4.18 mmol) could be collected. LC purity of the material was 99.5%, and the yield from **1a**, 96%. The obtained crystals of **1a** had NMR data: ^{1}H NMR (DMSO-d_6) δ 0.74 (d, J = 6.9 Hz, 3H), 2.60−3.06 (m, 6H), 3.95 (m, 1H), 4.27 (m, 1H), 4.41 (m, 2H), 6.68 (d, J = 8.2 Hz, 2H), 6.95−7.32 (m, 12H), 7.51 (s, 1H) 8.23 (s (broad), 3H), 8.36 (m, 2H), 8.48 (d, J = 8.2 Hz, 1H), 9.39 (s, 1H).