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Technical Notes

The Use of PeptiCLEC-TR in the Preparation of Dipeptides

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Abstract:

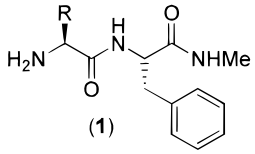
Dipeptides are important intermediates in many pharmaceutical products. To support a research programme on matrix metalloproteinases (MMP) we needed to prepare the dipeptide, Leu-Phe-NHMe (3). Chemical methods provided the material but not to the quality required, and thus an alternative preparation was sought. The use of enzymes in chemical synthesis was a tool that we had used before but not in the preparation of dipeptides. This contribution will show the utility of PeptiCLEC-TR in the preparation of dipeptide (3) and its application to other dipeptides. It will highlight the amenability of PeptiCLEC-TR to scale-up and demonstrate the recycling of this enzyme, which will make this a cost-effective route.

Introduction

The matrix metalloproteinases (MMPs) are a large and expanding family of zinc-dependent endopeptidases that have attracted much attention as targets for drug discovery over the past decade.[1] The discovery that the MMP enzymes are overexpressed in many pathological conditions has led to the belief that inhibitors of the MMPs could be useful for the treatment of a range of inflammatory disorders for which there is an unmet therapeutic need. From the literature it can be seen that the amino-carboxylate families of inhibitors all contain dipeptides of the form (**1**) as a common motif[2] (Scheme 1).

To support a research program dipeptides of the form (**1**) were prepared on a gram to kilogram scale[3−5] (Scheme 2).

Scheme 1



(**1**)

R= alkyl, phenyl

Initially dipeptide (**3**) was prepared by chemical coupling of CBZ-leucine and amino amide (**2**) in 10 vol of dichloromethane at 10−20 °C using 1.1 equiv of 1-[3-dimethylamino)propyl]-3-ethylcarbodiimine hydrochloride (EDAC). The dipeptide product was filtered off directly from the reaction mixture and a second crop isolated by stripping the liquors. In the chemically mediated coupling there is little discrimination of amino acid impurities present in the feedstock other than that obtained during isolation by crystallisation. A particular issue in the synthesis of Z-Leu-Phe-NHMe was the impurity Z-Ile-Phe-NHMe which results from the presence of isoleucine in the leucine feedstock. This gives rise to a significant impurity which is impossible to remove from the final product by crystallisation. In addition a small but significant amount of racemisation of the leucine occurs during the coupling giving rise to ∼0.1% of the *RS*,*SR* diastereoisomers after recrystallisation. In view of this, alternative methods were required.

Although the use of enzymes in organic synthesis is a well-established area,[6] application of lipases as amidation catalysts has received much less attention.[7] At the outset of this work the use of enzymes in the preparation of dipeptides was not widespread, particularly on a large scale. There were some notable exceptions to this, which included the industrial production of aspartame.[8] More recently the use of cross-linked enzyme crystals (CLECs) of thermolysin[9] and sub-

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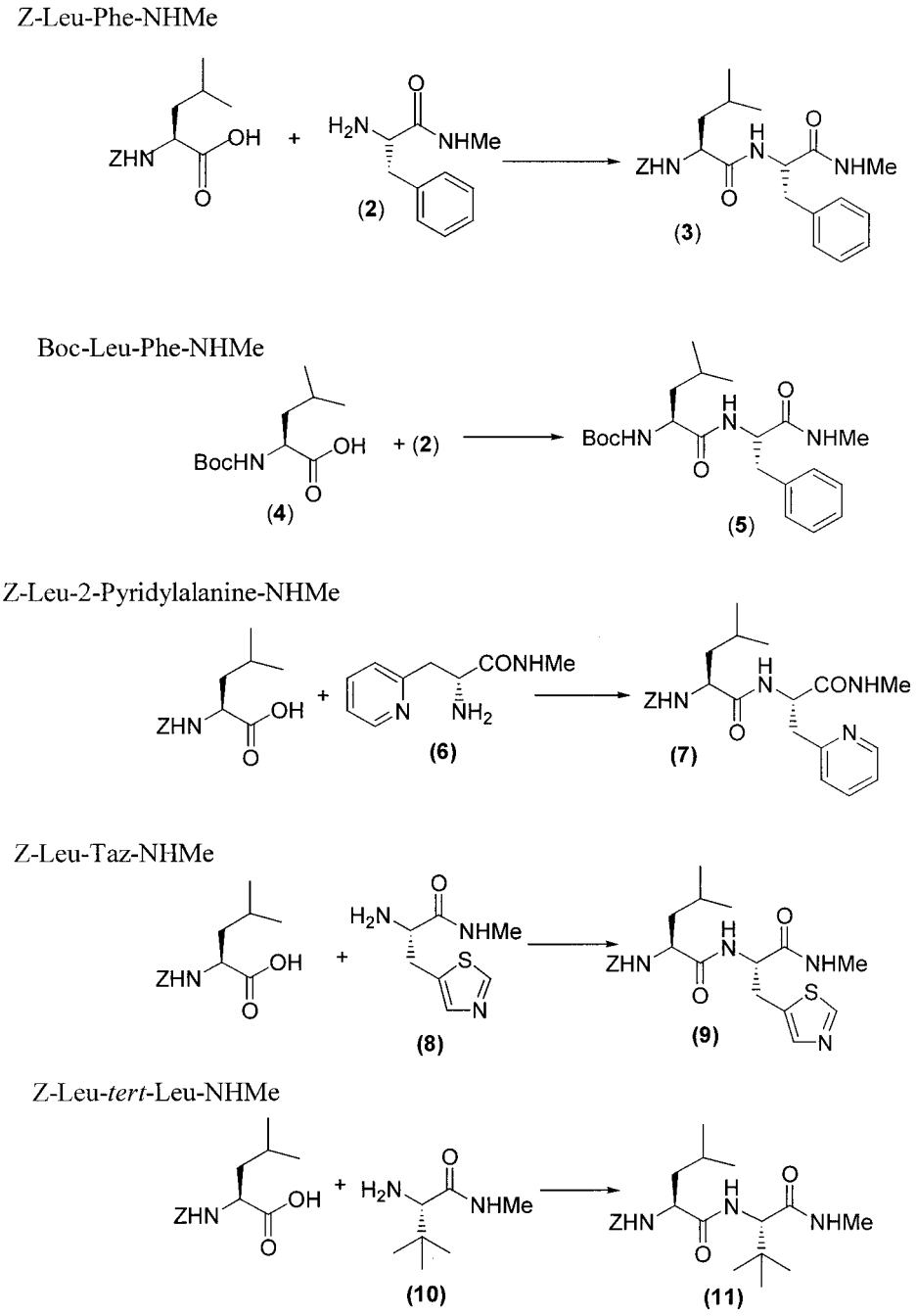
Scheme 2



tilisin[10] has been recommended for the formation of di- and polypeptides and peptide alkylamides. CLECs offer the advantages of increased product purity and potential for enzyme recycling reducing the cost of the process and minimising waste.

Enzyme Preparation of Z-Leu-Phe-NHMe (3). Thus, having reviewed the literature, we investigated the use of the thermolysin CLEC (PeptiCLEC-TR) for the synthesis of Z-Leu-Phe-NHMe (**3**). The initial conditions focused on the use of 50% aqueous ethanol as solvent with dry PeptiCLEC-TR, which furnished a 68% yield of isolated product **3** after 24 h. Disappointingly, recycle of the enzyme gave only a 34% yield of **3**. The use of the enzyme in the commercially available slurry form gave much better results; 87% yield both on first run and first recycle after a total of 24 h. Changing the solvent to 40% aqueous ethanol was even more encouraging and the results are summarised in the table. Although some deactivation of the enzyme was apparent a total of 255 g of **3** was produced using 3 g of PeptiCLEC-TR over five runs (Table 1).

The influence of solvent was then examined especially the use of ethyl acetate, the extraction solvent used in the preparation of **2**. Gratifyingly, it was found that there was no significant loss in the reaction rate. This had the benefit that the enzyme charge could be maintained at 1% w/w, could still achieve 95% conversion in 7.5 h, and could use (**2**) without the need to isolate the solid. On a larger scale

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Table 1. **Showing yield and % purity of product when recycling 3 g PeptiCLEC TR over five runs**

run	yield of (**3**)/%	L.C. purity/%	reaction time/h
1	83	94	6.5
2	88	94	14
3	88	92	19
4	91	88	20
5	91	94	20

[a] Conditions: 1wt %/wt PeptiCLEC-TR, 20 g/L Z-leu, 36 g/L amine, 40 °C.

with increased substrate loading a 91% yield of 96% pure **3** was obtained and demonstrated three recycles without significant loss of activity. The economic benefits of this are clearly apparent. The increased substrate loading was achieved by way of multiple additions of Z-leucine and *N*-methylamide to prevent the mixture becoming too thick to stir.

An added advantage of the enzyme-coupling procedure was that the product was essentially free of any isoleucine contaminant. It is presumed that the low levels result from the fact that isoleucine is more sterically encumbered than leucine and hence undergoes coupling at a slower rate than leucine.

A comparison of Boc-leucine with Z-leucine in the coupling reaction (Scheme 2) was examined. The former reagent had a number of potential advantages over the latter. First, Boc-leucine is a crystalline solid and may be purified by crystallisation, whereas the Z-leucine is a viscous oil. Second the resultant Boc dipeptide (**5**) can be converted to the desired dipeptide (**1**) by simple acidolysis. In the event a 77% yield of isolated **5** was obtained after 70 h using PeptiCLEC-TR. Thus, the reaction of Boc-leucine appears to be about 2−3 times slower when compared to that of Z-leucine.

A further advantage of using an enzyme for this coupling is the fact that the procedure is racemisation free without the need for further purification as seen for the chemical method. Indeed the highly stereoselective nature of the reaction would allow one to use racemic **4** in the coupling and only produce the desired (*S*,*S*)-dipeptide (**5**). In this way, particularly during early-stage research, where material supply to a specified quality standard may override cost issues, the need for developing chiral assays could be avoided and instead a de assay (HPLC or NMR) utilised to measure the quality of the dipeptide (**5**).

To investigate the generality of PeptiCLEC-TR for the synthesis of leucine dipeptides, a range of amino acids were tested in the coupling.

Z-Leu-2-Pyridylalanine-NHMe (7). A possible replacement for phenylalanine is 2-pyridylalanine. CLEC coupling of both racemic **6** and 82% ee **6** gave rise to a single new peak in the HPLC assay (Scheme 2). Furthermore, with the racemic material **6** an ee assay of the recovered starting material confirmed that it was being resolved (50% ee after 4 h). This example highlights the specificity of the enzyme to only couple in one enantiomer.

Z-Leu-Taz-NHMe (9). The TAZ-amide (**8**) was coupled to Z-leucine to give the dipeptide (**9**) in 66% isolated yield (Scheme 2). The HPLC conversion was 93%, and the modest isolated yield was attributed to the workup in which some of the water-soluble dipeptide was lost during the aqueous washes.

Z-Leu-*tert*-Leu-NHMe (11). To test the reaction out with respect to steric constraints the formation of the dipeptide (**10**) from *tert*-leucine *N*-methylamide (Scheme 2) was examined. Although product was formed only 10−20% conversion was reached after 3 days!

Conclusions

In summary, the utility of PeptiCLEC-TR in the preparation of diastereomerically enriched dipeptides has been shown. This method has limitations in that the more sterically hindered the components, the slower the reaction, but diastereomeric integrity is maintained. When compared with traditional chemical methods this is a simple means of ensuring chemical and diasteriomeric purity. This method also has the advantage of recycling of the enzyme, thus reducing costs and minimising waste.

Experimental Section

General Procedure for Dipeptide Formation. Protected leucine (1 mol equiv) is dissolved in 3:2 0.2 M sodium acetate, 50 mM calcium acetate solution, pH 6.5:ethyl alcohol at a concentration of 20 g/L. Amine (2 mol equiv) is added and the mixture warmed to 40 °C. Enzyme (3.5 mg/mL) is added and the reaction shaken vigorously at 40 °C for the required length of time with regular analysis by HPLC.

HPLC analysis method: C18 250 × 4.6 mm i.d. Hypersil Shandon HPLC column, 1 mL/min, 210 nm. 50:50 methanol:10 mM phosphate solution pH 3.1 hold for 5 min, gradient to 100% methanol at 20 min, then back to original conditions over 2 min.

Preparation of Boc-Leu-Phe-NHMe (5): as general procedure, 77% yield. ^{1}H NMR ($CDCl_3$, 200 MHz) δ 0.9 (6H, 2xCH_3), 1.4 (9H, m, tBu), 1.7 (3H, m, CH_2, CH), 2.75 (3H, d, NHMe), 3.1 (2H, dd, CH_2Ph), 4.0 (1H, m, CH), 4.7 (2H, m, NH, CH), 6.45 (1H, m, NH), 6.6 (1H, m, NH), 7.3 (5H, s, Ph).

Preparation of Z-Leu-2-Pyridylalanine-NHMe (7): as general procedure, new peak at 14.8 min using HPLC method above.

Preparation of Z-Leu-Taz-NHMe (9): as general procedure, 66% yield. ^{1}H NMR ($CDCl_3$, 200 MHz) δ 0.8 (6H, d, 2xCH_3), 1.5 (3H, m, CH_2, CH), 2.5 (3H, d, NHMe), 2.8 (1H, dq, CH_2), 4.1(1H, m, CH), 4.8 (1H, m, CH), 5.0 (2H, dd, CH_2Ph), 5.8 (2H, dd, CH_2Ph), 6.9 (1H, d, NH), 7.05 (1H, s, CH), 7.2 (5H, s, Ph).

Preparation of Z-Leu-*tert*-Leu-NHMe (11): as general procedure, new peak at 16.4 min using HPLC method above.

Preparation of Z-leucine-phenylalanine-*N*-methylamide (3). *Enzyme preparation*. PeptiCLEC TR (3.75 mL) in storage solution was filtered until no surface water could be seen and then re-filtered with 2 × 3.75 mL absolute ethanol until a paste.

A mixture of Z-leucine (37.5 g, 0.14 mol) and PheNHMe amide (27.4 g, 0.15 mol) in ethyl acetate (500 mL) and 10

mM calcium acetate solution (15 mL) was warmed to 50 °C; an insoluble salt formed, but this dissolved after about 45 min. The enzyme prepared as above was added in a small amount of reaction mixture. After 1 h a pre-warmed mixture of Z-leucine (20 g, 0.07 mol) and PheNHMe (14.77 g, 0.08 mol) in ethyl acetate (4 mL) was added to bring the concentration to 100 g/L. Again, the insoluble salt was present, but this had dissolved after 2 h, and thus a second addition of the above quantities of Z-leucine and PheNHMe was performed. The mixture was then stirred overnight at 50 °C.

At completion methanol (410 mL) was added and the mixture warmed to 50 °C, the enzyme filtered off (was stored in 10mM calcium acetate solution for future use). The reaction mixture was cooled to 15 °C, and the product was filtered off and then re-dissolved in methanol (1.25 L). The enzyme was then filtered off and discarded before cooling to recrystallise the product for isolation, giving a 63% yield first crop and a further 15% yield as second crop on concentration of the mother liquor. ^{1}H NMR (DMSO, 400 MHz) δ 0.8 (6H, dd, 2 $\times$ CH_3), 1.35 (2H, dq, CH_2), 1.5 (1H, m, CH), 2.5 (3H, d, NHMe), 2.9 (2H, dq, CH_2Ph), 4.0 (1H, m, CH), 4.4 (1H, m, CH), 5.0 (2H, dd, CH_2Ph), 7.25 (10H, m, 2 $\times$ Ph), 7.4 (1H, d, NH), 7.9 (2H, m, 2 $\times$ NH).

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