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Robust and straightforward chemo-enzymatic enantiopure dipeptide syntheses and diketopiperazines thereof

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ABSTRACT

We have explored the scope of the synthetic route towards D-phenylglycyl diketopiperazines, involving a penicillin acylase catalysed formation of D-phenylglycyl dipeptides of L-amino acids with functional groups in the side chain. The synthesis of dipeptides from serine, threonine, glutamic acid, glutamine and methionine was successful. In contrast, aspartic acid, asparagine and cysteine only afforded trace amounts of dipeptides while no dipeptide was detected with arginine, lysine and tyrosine. Isolated dipeptide yields varied from 10% to 76%. The dipeptides were successfully converted into their corresponding enantiopure diketopiperazines by chemical esterification and cyclization under alkaline conditions, in 35–43% yield. In the case of glutamic acid, the procedure yielded the diketopiperazine with an esterified side chain. Remarkably with glutamine, the amide function in the side chain was transformed into an ester moiety, resulting in the same diketopiperazine as with glutamic acid.

1. Introduction

Diketopiperazines are cyclic dipeptides and constitute an important family of biologically active compounds that have attracted growing interest in several areas of research.[1,2] They are regarded as useful synthons for active pharmaceutical ingredients while histidine containing diketopiperazines are studied in the development of therapeutic agents.[3–6] Moreover, these dipeptides have been shown to possess a wide range of biological properties (antitumor, antiviral, antifungal and antibacterial), and have been linked with prebiotic activity while displaying enantioselective catalytic activity (Fig. 1).[7–12]

The goal of mimicking enzymatic activity with amino acids and peptides is not recent and important progress has been made in the development of asymmetric reactions with amino acid and peptide-based catalysts.[12–14] Another relevant example of both the growing interest in diketopiperazines as well as in developing biomimetic peptides is the peptide-catalysed synthesis of diketopiperazines and dipeptides. This makes diketopiperazines interesting for research, as well as showing the need for the development of new synthetic methods and strategies to enable access to a larger variety of such compounds.[15,16]

The use of biocatalysts in organic synthesis is widespread, mainly due to their main features: high selectivity and mild conditions. Unsurprisingly, steps towards the environmentally benign syntheses of peptides have been reported. Penicillin G acylase, for example, is an efficient biocatalyst used in the synthesis of β-lactam antibiotics and has also been shown to be effective in the synthesis of chiral dipeptides.[17–19]

Chemo-enzymatic routes to diketopiperazines containing the D-phenylglycine moiety have been described, either starting from enantiopure natural amino acids, or from inexpensive racemic mixtures in the case of unnatural amino acids.[20,21] In the latter case it was shown that when using a racemate, there was no interference with either the formation or isolation of the desired pure compound. In addition, expanding the scope of the procedure resulted in a green, efficient and inexpensive way of obtaining diketopiperazines containing unnatural amino acids with known anti-viral activity on a preparative scale.[20,22]

The coupling of D-phenylglycine amide with the L-enantiomers of glycine, alanine, valine, leucine, isoleucine, phenylalanine, tryptophan and histidine has already been successfully performed.[20] The route using penicillin G acylase provides a green and straightforward approach to the synthesis of dipeptides comprising D-phenylglycine as well as their cyclic diketopiperazine derivatives. The yields described are within the range of 34–70%, which combined with room temperature, normal pressure and an aqueous system, make it a powerful tool in the synthesis of enantiopure dipeptides. In order to obtain enantiopure diketopiperazines, the linear dipeptide undergoes esterification by treatment with thionyl chloride in methanol, followed by alkaline cyclization at room

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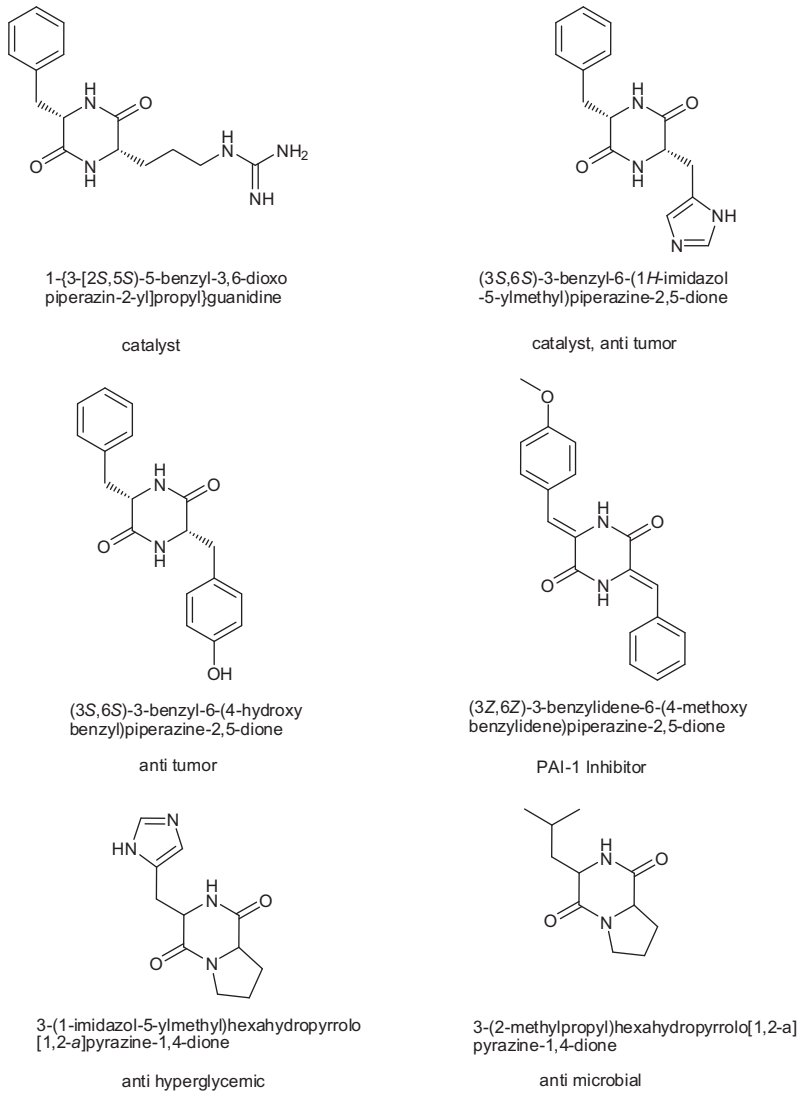


Figure 1. Examples of diketopiperazines and their functions.

temperature, after which the product formed precipitates from solution.

To explore the full potential of the penicillin G route towards the synthesis of enantiopure diketopiperazines, we decided to screen all natural amino acids that were not yet reported (Fig. 2). Herein we report the results obtained with the remaining amino acids, which comprise the interesting remainder of the polar and the charged amino acids. Proline was not tested, because secondary amines are not accepted as a nucleophile by penicillin G acylase. Bearing in mind the known broad selectivity of the enzyme towards nucleophiles and the reactivity of tryptophan and histidine in previous work, neither the volume nor the presence of a basic group in the residue was expected to constitute as a hindrance for the procedure.[20,23]

2. Results and discussion

2.1. Enzymatic coupling reactions

The proposed penicillin G acylase coupling method was successful in the coupling of the L-amino acids serine, threonine, glutamic acid, glutamine and methionine (Table 1). The variation in isolated yields is greater than that observed with amino acids with non-functionalized side-chains.[20] This suggests that the presence of a functional group in the side chain of one of the reactants can interfere with the reaction.

We observed that an increase in the initial amino acid concentration, starting from 200 mM, led to an improvement in purity with serine, threonine, glutamine and methionine. In these cases, this leads to a decreasing amount of the side product, D-phenylglycine. This was confirmed through NMR analysis of the obtained crude products. We concluded that, in these cases, hydrolysis of phenylglycine amide to phenylglycine decreases with increasing concentrations of the starting amino acid.

This was most notable in the case of the glutamine containing dipeptide, as we could only obtain pure dipeptide when performing the reaction with an initial concentration of 500 mM. On the other hand, we also observed that in the cases of L-serine, L-threonine and L-methionine, an increase in the initial concentration to 500 mM afforded an impure product. Further studies and optimization were not carried out.

The amino acids aspartic acid, asparagine and cysteine were tested without success. It was however possible to detect trace amounts of the formed dipeptide through mass spectrometry in

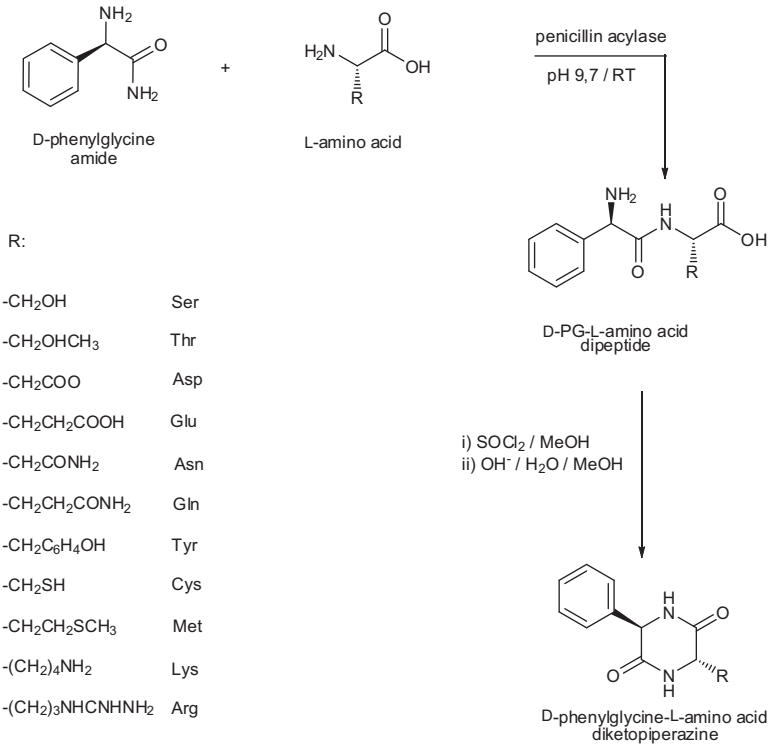


Figure 2. Proposed penicillin G acylase catalysed peptide synthesis with natural amino acids possessing chemical functionalities.

Table 1
Coupling reaction isolated yields and amino acid initial concentrations used (in the successful cases)

Dipeptide	Isolated yield (%)	Initial concentration (mM)
D-PG-L-Ser	10	260
D-PG-L-Thr	41	300
D-PG-L-Glu	73	200
D-PG-L-Gln	33	500
D-PG-L-Met	53	300
D-PG-L-Asp	Traces	—
D-PG-L-Asn	Traces	—
D-PG-L-Cys	Traces	—
D-PG-L-Arg	—	—
D-PG-L-Lys	—	—
D-PG-L-Tyr	—	—

these three cases. This indicates that the coupling reaction did occur to some extent, although it did not make it feasible for synthetic purposes without further studies.

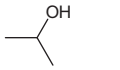
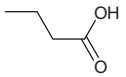
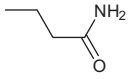
In the case of cysteine, trace amounts of phenylglycine were found with mass spectrometry, together with data fitting cystine, the corresponding disulfide. However, this could not be fully confirmed by the NMR data. The NMR profile obtained matched that described but the chemical shifts did not.[24]

Previous work indicated the affinity of L-aspartic acid, L-glutamic acid and L-asparagine with the enzyme to be comparable, in acyl transfer reactions where these species act as acyl acceptors.[23] However, we were able to obtain a dipeptide with both L-glutamic acid and L-glutamine but not with L-aspartic acid and L-asparagine.

In the trials with L-arginine, L-lysine and L-tyrosine, no evidence of the desired dipeptide was found, although the specificity of the enzyme to L-arginine was reported to be higher than the one of L-glutamic acid.[23] We did detect the side product D-phenylglycine, which indicates that there was no enzyme inactivation. The high solubility of L-lysine and L-arginine might have made it impossible to determine if there was product formation, because no precipitation occurred at the dipeptide's isoelectric point. D-Phenylglycine was obtained when lowering the pH.

The yields obtained varied considerably according to the side chain of the starting amino acid, not necessarily showing a dependency on the chemical function (Table 2). Previously, the nature of the side chain was found to influence the enzyme selectivity equally, that is, non-branched alkyl side-chains produced nucleophiles that were more effective.[20,21,23]

Table 2
Comparative apparent residue preference

Side-chain	Yield (%)		Side-chain	Yield (%)
	42	>		10
	73	>		Traces
	33	>		Traces
	53	>		Traces

We successfully reproduced the results obtained by Khimiuk et al.[20] in the case of phenylalanine and tryptophan, where the residue is bulky. This suggests that the volume of the residue should not be an influential variable. We were, however, unable to convert L-tyrosine into a dipeptide. This might have been due to tyrosine's low solubility.

The data regarding the pair aspartic or glutamic acid and the pair asparagine or glutamine, suggest amino acids with two

methylene groups in the residue are preferred (Table 2), whereas the equivalent amino acid with one methylene group less is not accepted, or only formed in very small amounts.

The isolated yield strongly depends on the dipeptide's solubility, not necessarily reflecting the real yield, as already observed.[20] However, due to the chemical similarity between this pair, one can consider aspartic acid and glutamic acid dipeptides to have comparable solubilities and the same applies for L-glutamine and L-asparagine. This difference in results could then be exclusively attributed to the enzyme's substrate preference.

The obtained yield for L-threonine was four fold higher than the one obtained with L-serine. This also suggests that the decisive factor is again not the solubility but the compatibility with the catalyst, since the solubility of both amino acids is very high.

The data regarding the sulfur containing amino acids L-methionine and L-cysteine, although suggestive, cannot be used to draw conclusions in this matter because both compounds differ not only in side-chain length but also in chemical functionality.

Dipeptides were obtained within a period of 2 days, either by single or sequential precipitation, with the exception of the L-serine containing dipeptide, which was obtained after 4 days. In this, and in the D-phenylglycyl-L-glutamic acid cases, significant amounts of product could still be obtained by precipitation after 50 and 10 days, respectively. These steps may be optimized by reducing the crystallization temperature, combined with either an earlier second isolation step or with a single isolation step, as a first isolation will necessarily translate into a larger second crystallization period. In these reactions only fresh enzyme was used. However, due to the known stability of this industrial catalyst, we think the effective re-use should be feasible.[25]

2.2. Esterification and condensation reactions

The subsequent steps in the chemo-enzymatic diketopiperazine syntheses are esterification with thionyl chloride in methanol, followed by in situ aqueous alkaline cyclization. The resulting dipeptide was isolated by precipitation from solution. This procedure allowed the diketopiperazine to be obtained pure and was successful in the cases of L-serine, L-threonine and L-methionine (Table 3).

Table 3
Esterification and condensation reactions of isolated yields

Diketopiperazine	Isolated yield (%)
Cyclo (D-PG, L-Ser)	43
Cyclo (D-PG, L-Thr)	48
Cyclo (D-PG, L-Met)	35
Cyclo (D-PG, L-Glu/Gln-OMe)	68

Both L-glutamic acid and L-glutamine containing dipeptide esters, afforded the same product. This was confirmed by infrared analysis and was consistently obtained in several trials (Fig. 3). This compound was obtained pure and was positively identified by NMR and mass spectrometry as the corresponding diketopiperazine methyl ester. Rather than having the acid or amide functional group, corresponding to L-glutamic acid and L-glutamine, the compound obtained had the same ester moiety in the side chain. Isolated yields varied between 66% and 69%.

With L-glutamic acid, a possible explanation for this result is that the ring closes before hydrolysis of the side chain's carboxylate ester, which then precipitates thus preventing any further reaction. An amine intra-molecular attack on the residue's ester group and not at the C terminus of the dipeptide could occur, which would yield an eight membered ring compound. However, we found only one of these two possible products (Fig. 4).

The explanation is still not clear in the case of glutamine because one must account for the loss of an amide group. One possible explanation is that the amide group is subjected to alcoholysis during esterification (Fig. 5). Although amides are stable bonds, the low pH at which esterification occurs might cause substitution of the amide group by the ester. However, the yields of diketopiperazine methyl ester found in both L-glutamic acid and L-glutamine are similar, although one equivalent of thionyl chloride is used over the course of the esterification of the latter and two equivalents in the former. This would presumably lead to a lower yield in the case of L-glutamine, thus suggesting amide alcoholysis was not the cause for our results.

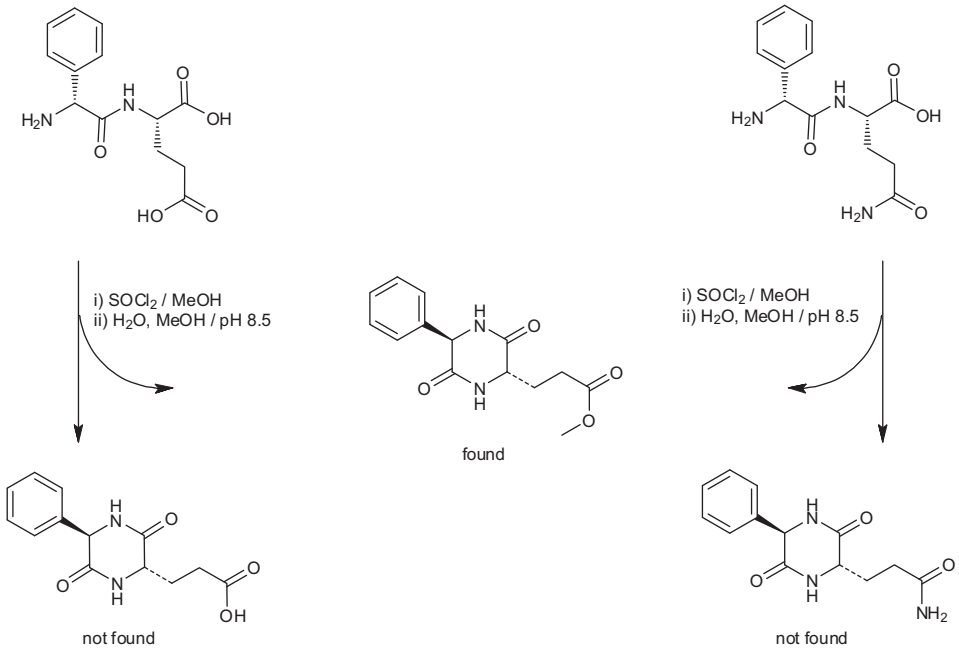


Figure 3. Same final product obtained in both cases is the corresponding diketopiperazine methyl ester and not the two expected corresponding diketopiperazines.

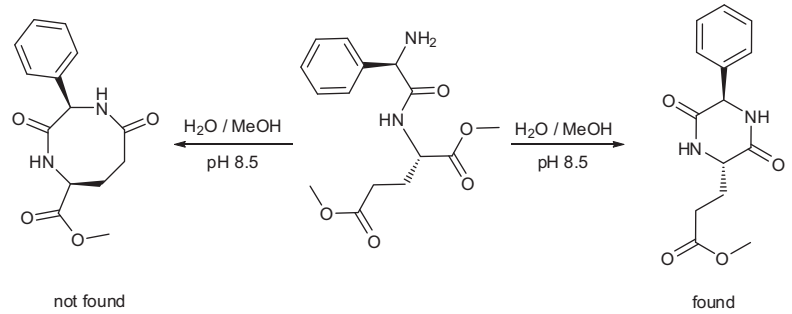


Figure 4. Amine intramolecular attack affords a cyclic dipeptide methyl ester and not eight membered ring product.

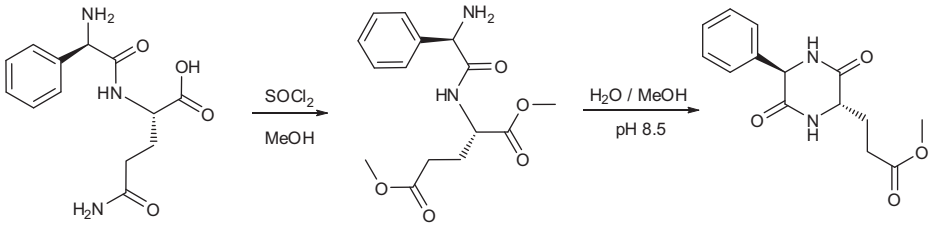


Figure 5. Putative formation of D-PG-L-Gln methyl ester via amide esterification.

An alternative, perhaps more likely explanation, involves a neighbouring group participation mechanism to account for the conversion of the amide into an ester moiety. In this hypothesis, this transformation takes place after ring-closure and not during the esterification (Fig. 6).

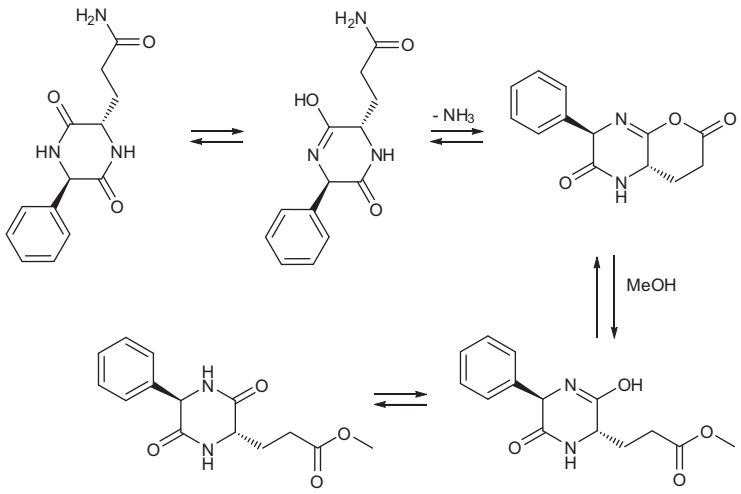


Figure 6. Proposed mechanism for the diketopiperazine ester formation with an enol intramolecular attack.

The hydroxyl group adjacent to the imine attacks the terminal amide, affording a six membered lactone with a loss of ammonia. Subsequent attack of methanol on the lactone carbonyl regenerates the open ring configuration, affording a methyl ester as indeed we have obtained.

Attack of a water molecule and not of a methanol molecule is unlikely, since no evidence of the formation of the diketopiperazine acid was found. In accordance with our results, and as observed in the case of the glutamic acid containing dipeptide, the ester presumably precipitates before hydrolysis of the side chain ester can take place. In addition to this, we observed that the time required for the precipitation during the ring-closure step was faster in the case of the L-glutamine containing dipeptide than in the case of the L-glutamic acid containing dipeptide, thus suggesting a different mechanism in the two cases.

3. Conclusion

The coupling procedure is applicable in the case of L-serine, L-threonine, L-methionine and L-glutamine, thus leading to a pure product without further purification. Data suggest there is room for optimization. In some cases it was possible to improve the product purity, simply by altering the initial concentration of amino acid.

The complete procedure is valid in the synthesis of the corresponding diketopiperazines, except in the case of L-glutamine and L-glutamic acid. In these cases, the same compound was

obtained pure and it was identified as the corresponding ester of the desired products. Two mechanisms have been proposed in order to explain this result.

A green and straightforward procedure was therefore established for the synthesis of dipeptides and diketopiperazines, containing both the D-phenylglycyl and chemically functionalized amino acid moieties, which are compounds of growing interest due to their catalytic and biological activity.

4. Experimental

4.1. Materials and methods

L-Amino acids and D-phenylglycine amide were obtained from commercial suppliers. Immobilized penicillin acylase from *Escherichia coli*, Assemblase 7500®, was kindly donated by DSM Anti-Infectives (Delft, The Netherlands). Mass spectrometry analysis gave coherent results, M_w or M_w+1, according to the technique used. The melting points were measured with a Büchi 510 and are not corrected. Optical rotations were measured with either a Perkin Elmer 241 Polarimeter or a Perkin Elmer 343 Polarimeter and concentrations were converted to the standard value of *c* 1.

4.2. Synthesis of D-phenylglycyl-L-amino acid dipeptides

4.2.1. Synthesis of D-phenylglycyl-L-serine

At first, 6.30 g (60.0 mmol) of L-serine and 2.25 g (15.0 mmol) of D-PGA were suspended in water and the pH was adjusted to 9.70 with NH_4OH 25%, making an overall volume of 230 mL. Next, 6.01 g of immobilized penicillin acylase was added at room temperature. Three further portions of D-PGA (2.25 g, 15.0 mmol) were added, with a 1.5-hour interval each, at which the pH was readjusted to 9.8 with the same base. After 6 hours, the enzyme was filtered and a turbid mixture was obtained. The pH was adjusted to 5.6 with H_2SO_4 (6 M). The mixture was left stirring for 4 days, after which precipitation occurred. The solid formed was filtered, washed with petroleum ether 40–60, dried, and 200 mg was obtained. The mixture was kept stirring for a period of 50 days and more precipitate was formed. This was collected, following the same procedure, after which 1.17 g was obtained in the second fraction, affording a total yield of 10%. The product was isolated pure. Mp: 224–226 °C (d). $[\alpha]_D^{20} = -68.9$ (*c* 1, 2.5 M HCl). ^{1}H NMR (300 MHz, D_2O+DCl): $\delta = 7.49–7.51$ (m, aromatic protons), $\delta = 5.22$ (s, C*H*NH_2), $\delta = 4.54$ (t, *J* = 4.5 Hz, C*H*CH_2OH), $\delta = 3.861$, $\delta = 3.86$, 3.73 (m, m, $J_1 = 3.9$ Hz, $J_2 = 11.7$ Hz, CHC*H*$_2$OH); ^{13}C NMR (75 MHz, D_2O+DCl): $\delta = 174.5$, 170.3 (*C*=O), $\delta = 133.3$, 132.0, 131.3, 129.6 (Ar); $\delta = 62.26$ *C*H_2OH, $\delta = 58.08$, 56.55 (C_α).

4.2.2. Synthesis of D-phenylglycyl-L-threonine

At first, 7.16 g (60.4 mmol) of L-threonine and 2.25 g (15.0 mmol) of D-PGA were suspended in water and the pH was adjusted to 9.80 with NH_4OH 25%, making an overall volume of 200 mL. Next, 6.27 g of immobilized penicillin acylase was added at room temperature. Three further portions of D-PGA (2.25 g, 15 mmol) were added, with a 1.5-hour interval each, at which the pH was readjusted to 9.8 with the same base. After 6 hours, the enzyme was filtered and a turbid mixture was obtained. The pH was then lowered to 6.4 with H_2SO_4 (6 M). The mixture was left stirring overnight, after which precipitation occurred. The solid was filtered, washed with petroleum ether 40–60 and dried. The yield was 41% (6.25 g) and the product was isolated NMR pure. Mp: 250–253 °C (d). $[\alpha]_D^{24} = -27.8$ (*c* 1, 2.5 M HCl). ^{1}H NMR (300 MHz, D_2O+DCl): $\delta = 7.54$ (m, aromatic protons), $\delta = 5.26$ (s, C*H*NH_2), $\delta = 4.515$ (*CH*CHOHCH$_3$), $\delta = 4.312$ (m, CHC*H*OHCH$_3$), $\delta = 0.852$ (d, $J = 6.3$ Hz, CHOHC*H*$_3$); ^{13}C NMR (75 MHz, D_2O+DCl): $\delta = 174.8$, 170.7 (C=O), $\delta = 133.5$, 132.0, 131.2, 129.5 (Ar); $\delta = 68.51$ *C*H_2OH, $\delta = 59.57$, 58.10 (C_α), $\delta = 20.05$ (CH*C*H$_3$CH$_2$OH).

4.2.3. Synthesis of D-phenylglycyl-L-glutamic acid

At first, 14.7 g (100 mmol) of L-glutamic acid and 5.52 g (36.2 mmol) of D-PGA were suspended in water and the pH was adjusted to 9.7, making an overall volume of 500 mL. To this, 12.2 g of immobilized penicillin acylase was added at room temperature. Two further portions of D-PGA (4.74 g, 31.6 mmol) were added, with a 2-hour interval each, at which the pH was adjusted to 9.9 with the NH_4OH. After 6 hours, the enzyme was filtered and a turbid mixture was obtained. The pH was then lowered to 3.3 with approximately 30 mL of H_2SO_4 (6 M). The mixture was left stirring for a period of two days, after which the formed precipitate was filtered, washed with petroleum ether 40–60, dried and 13.2 g was obtained. The mixture was kept stirring for an extra period of 10 days. More precipitate was formed and collected, following the same procedure, affording 7.28 g, which corresponds to an overall yield of 73%. The product was isolated NMR pure. Mp: 217–219 °C. $[\alpha]_D^{24} = -87.5$ (*c* 1, 2.5 M HCl). ^{1}H NMR (300 MHz, D_2O+DCl): $\delta = 7.487$ (m, aromatic protons), $\delta = 5.173$ (s, C*H*NH_2), $\delta = 4.485$ (dd, $J_1 = 3.9$ Hz, $J_2 = 9.6$ Hz, C*H*CH_2CH_2COOH), $\delta = 2.15$, 1.81 (mm, CHC*H*$_2$C*H*$_2$COOH); ^{13}C NMR (75 MHz, D_2O+DCl): $\delta = 178.1$, 176.0, 170.3 (*C*=O), $\delta = 133.4$, 132.0, 131.3, 129.5 (Ar); $\delta = 58.14$, 53.44 (C_α), $\delta = 30.99$, 26.91 (CH*C*H$_2$*C*H$_2$COOH).

4.2.4. Synthesis of D-phenylglycyl-L-glutamine

At first, 15.1 g (104 mmol) of L-glutamine and 5.21 g (34.7 mmol) of D-PGA were suspended in 200 mL of water and the pH was adjusted to 9.7 with NH_4OH 25%. To this, 4.12 g of immobilized penicillin G acylase was added at room temperature. Two further portions of D-PGA (5.20 g, 34.7 mmol) were added, with a 2-hour interval each, at which the pH was readjusted to 9.7 with the same base. After 6 hours of reaction time, the enzyme was filtered and the pH was then lowered to 5 with H_2SO_4 (6 M); precipitation was immediate. The mixture was left stirring overnight, after which the precipitate was filtered, washed with petroleum ether 40–60, dried and 8.59 g (29.7%) was obtained. A second precipitate fraction was filtered from the mother liquor the day after and a third fraction 2 days after, affording 840 mg and 1.68 g, respectively. The first fraction was NMR pure while the second had minor impurities. Mp: 238–240 °C. $[\alpha]_D^{24} = -89.5$ (*c* 1, 2.5 M HCl). ^{1}H NMR (300 MHz, D_2O+DCl): $\delta = 7.376$ (m, aromatic protons), $\delta = 5.061$ (s, C*H*NH_2), $\delta = 4.287$ (dd, $J_1 = 4.5$ Hz, $J_2 = 9.3$ Hz, C*H*$CH_2CH_2CONH_2$), $\delta = 2.08$, 1.944 (mm, CHC*H*$_2$C*H*$_2$-COOH); ^{13}C NMR (75 MHz, D_2O+DCl): $\delta = 177.6$, 174.5 , 168.9 (*C*=O), $\delta = 132.0$, 130.7, 129.9, 128.2 (Ar), $\delta = 56.74$, 52.47 (C_α), $\delta = 30.89$, 26.1 (CH*C*H$_2$*C*H$_2$CONH$_2$).

4.2.5. Synthesis of D-phenylglycyl-L-methionine

At first, 8.98 g (60.2 mmol) of L-methionine and 2.25 g (15 mmol) of D-PGA were suspended in water and the pH was adjusted to 9.80 with NH_4OH 25%, making an overall volume of 200 mL. To this, 6.05 g of immobilized penicillin G acylase was added at room temperature. Three further portions of D-PGA (2.25 g, 15 mmol) were added, with a 1.5-hour interval each, at which point the pH was readjusted to 9.8 with the same base. After 6 hours, the enzyme was filtered and a turbid mixture was obtained. The pH was then lowered to 5.8 with H_2SO_4 (6 M). The mixture was left stirring overnight, after which precipitation occurred and the formed solid was filtered, washed with petroleum ether 40–60 and dried under vacuum and P_2O_5 for a period of 9 days, affording 9.05 g of product corresponding to a yield of 53%. The product was isolated NMR pure. Mp: 235–237 °C. $[\alpha]_D^{24} = -97.9$ (*c* 1, 2.5 M HCl). ^{1}H NMR (300 MHz, D_2O+DCl):

δ = 7.485 (m, aromatic protons), δ = 5.157 (s, C*H*NH_2), δ = 4.6 (m, J_1 = 3.9 Hz, J_2 = 10.2 Hz, C*H*$CH_2CH_2SCH_3$), δ = 2.246, 2.061 (mm, $CHCH_2CH_2SCH_3$), δ = 1.856 (mm, $CHCH_2CH_2SCH_3$); ^{13}C NMR (75 MHz, D_2O+DCl): δ = 176.4, 170.3 (*C*=O), δ = 133.5, 132.0, 131.3, 129.5 (Ar), δ = 58.14, 52.86 (C_α), δ = 30.97, 30.71 ($CHCH_2CH_2SCH_3$), δ = 15.48 ($CHCH_2CH_2SCH_3$).

4.2.6. Synthesis of D-phenylglycyl-L-serine diketopiperazine

At first, 1.00 g (4.20 mmol) of D-phenylglycyl-L-serine was suspended in 44 mL of dry methanol. A white suspension was obtained which was cooled. To this suspension 370 μL of $SOCl_2$ (5.09 mmol, 1.21 equiv) was added dropwise. The mixture was allowed to reflux for 4 hours and after cooling to room temperature, 168 mL of water was added. The pH was adjusted to 8.6 with a saturated solution of sodium hydroxide. The mixture was left stirring and after 7 days, the formed precipitate was filtered. This was washed with petroleum ether 40–60 and dried. The product was isolated and 400 mg was obtained, which corresponds to a yield of 43%. Mp: 263–265 °C. $[\alpha]_D^{24}$ = −59.9 (*c* 1, DMSO). ^{1}H NMR (300 MHz, DMSO-d_6): δ = 8.47 (s, CHN*H*CO), δ = 7.99 (s, CHN*H*CO), δ = 7.35 (m, aromatic protons), δ = 5.08 (t, *J* = 4.9 Hz, C*H*CH_2OH), δ = 4.90 (d, *J* = 1.2 Hz, C*H*Ph), δ = 3.90 (s, $CHCH_2OH$), δ = 3.81 (dd, $CHCH_2OH$), δ = 3.63 (dd, $CHCH_2OH$); ^{13}C NMR (75 MHz, DMSO-d_6): δ = 167.6, 167.5 (*C*=O), δ = 140.0, 129.0, 128.5, 128.2 (Ar), δ = 63.27 (CH_2OH), δ = 59.45 (*C*HPh), δ = 57.62 (*C*CH_2OH).

4.2.7. Synthesis of D-phenylglycyl-L-threonine diketopiperazine

1.00 g (3.97 mmol) of D-phenylglycyl-L-threonine was suspended in 20 mL of dry methanol. A white suspension was obtained which was cooled. To this suspension 350 μL of $SOCl_2$ (4.82 mmol, 1.2 equiv) was added drop-wise. The mixture was allowed to reflux for 4 hours and after cooling to room temperature, 40 mL of water was added. The pH was adjusted to 8.6 with a saturated solution of sodium hydroxide. The mixture was left stirring and after 7 days, the formed precipitate was filtered. This was washed with petroleum ether 40–60 and dried. The product was isolated and 450 mg was obtained, which corresponds to a yield of 43%. Mp: 274–276 °C. $[\alpha]_D^{24}$ = −71.4 (*c* 1, DMSO). ^{1}H NMR (300 MHz, DMSO-d_6): δ = 8.39 (s, CHN*H*CO), δ = 8.13 (d, *J* = 1.6 Hz, CHN*H*CO), δ = 7.33 (m, aromatic protons), δ = 5.10 (d, *J* = 5.6 Hz, C*H*CHOHCH_3), δ = 4.94 (s, C*H*Ph), δ = 4.12 (t, CHC*H*OHCH_2), δ = 3.64 (s, $CHCH_2OH$), δ = 1.14 (d, *J* = 6.4 Hz, $CHCHOHCH_3$); ^{13}C NMR (75 MHz, DMSO-d_6): δ = 167.8, 167.7 (*C*=O), δ = 138.9, 128.0, 127.9, 127.6 (Ar), δ = 67.95 (*C*HOH), δ = 60.56 (*C*HPh), δ = 58.28 ($CHCHOHCH_3$), δ = 19.79 ($CHCH_2OHCH_3$).

4.2.8. Synthesis of D-phenylglycyl-L-methionine diketopiperazine

At first, 1.04 g (3.69 mmol) of D-phenylglycyl-L-methionine was suspended in 15 mL of dry methanol. A white suspension was obtained which was cooled. To this suspension 325 μL of $SOCl_2$ (4.48 mmol, 1.2 equiv) was added dropwise. The mixture was allowed to reflux for 4 hours and after cooling to room temperature, 30 mL of water was added. The pH was adjusted to 8.6 with a saturated solution of sodium hydroxide. The mixture was left stirring and after 10 days, the formed precipitate was filtered. This was washed with petroleum ether 40–60 and dried. The product was isolated and 590 mg was obtained, which corresponds to a yield of 35%. Mp: 253–255 °C. $[\alpha]_D^{24}$ = −47.6 (*c* 1, DMSO). ^{1}H NMR (300 MHz, DMSO-d_6): δ = 8.62 (d, *J* = 2.7 Hz, CHN*H*CO), δ = 8.29 (s, CHN*H*CO), δ = 7.35 (m, aromatic protons), δ = 4.93 (d, *J* = 2.4 Hz, C*H*Ph), δ = 4.08 (t, *J* = 5.0 Hz, C*H*$CH_2CH_2SCH_3$), δ = 2.905 (m, $CH_2CH_2SCH_3$); δ = 2.024 (m, $CH_2CH_2SCH_3$) ^{13}C NMR (75 MHz, DMSO-d_6): δ = 168.3, 167.4 (*C*=O), δ = 139.4, 129.2, 128.6, 127.7 (Ar), δ = 59.40 (*C*HPh), δ = 53.32 ($CHCH_2CH_2SCH_3$), δ = 32.21 ($CHCH_2CH_2SCH_3$), δ = 29.14 ($CHCH_2CH_2SCH_3$), δ = 15.21 ($CHCH_2CH_2SCH_3$).

4.2.9. Attempted synthesis of D-phenylglycyl-L-glutamic acid diketopiperazine

At first, 1.05 g (3.75 mmol) of D-phenylglycyl-L-glutamic acid was suspended in 20 mL of dry methanol. A white suspension was obtained which was cooled to 0–5 °C. To this suspension 570 μL of $SOCl_2$ (7.86 mmol, 2.1 equiv) was added dropwise. The mixture was allowed to reflux for 4 hours and after cooling to room temperature, 40 mL of water was added. The pH was adjusted to 8.7 with a saturated solution of sodium hydroxide. The mixture was left stirring and after 7 days, the formed precipitate was filtered. This was washed with petroleum ether 40–60 and dried. The product was isolated and 680 mg was obtained, which corresponds to a yield of 66%. The product was identified as the corresponding methyl ester. Mp: 236–238 °C. $[\alpha]_D^{24}$ = −43.8 (*c* 1, DMSO). ^{1}H NMR (300 MHz, DMSO-d_6): δ = 8.64 (d, *J* = 2.4 Hz, CHN*H*CO), δ = 8.26 (s, CHN*H*CO), δ = 7.35 (m, aromatic protons), δ = 4.93 (d, *J* = 2.7 Hz, C*H*Ph), δ = 4.06 (t, C*H*$CH_2CH_2CO_2CH_3$), δ = 3.32 (s, $CH_2CH_2CO_2CH_3$), δ = 2.905, δ = 2.905 (m, m, $CH_2CH_2CO_2CH_3$); ^{13}C NMR (75 MHz, DMSO-d_6): δ = 173.6, 168.2, 167.5 (*C*=O), δ = 139.4, 129.2, 128.6, 127.7 (Ar), δ = 59.40 ($CHCO_2CH_3$), δ = 53.3 (*C*HPh), δ = 52.1 ($CHCH_2CH_2CO_2CH_3$), δ = 29.5 ($CHCH_2CH_2CO_2CH_3$), δ = 27.9 ($CHCH_2CH_2CO_2CH_3$).

4.2.10. Attempted synthesis of D-phenylglycyl-L-glutamine diketopiperazine

At first, 1.02 g (3.66 mmol) of D-phenylglycyl-L-glutamine was suspended in 20 mL of dry methanol. A white suspension was obtained which was cooled to 0–5 °C. To this suspension 320 μL of $SOCl_2$ (4.41 mmol, 1.2 equiv) was added dropwise. The mixture was allowed to reflux for 4 hours and after cooling to room temperature, 40 mL of water was added. The pH was adjusted to 8.6 with a saturated solution of sodium hydroxide. The mixture was left stirring and after 7 days, the formed precipitate was filtered. This was washed with petroleum ether 40–60 and dried. The product was isolated and 700 mg was obtained, which corresponds to a yield of 69%. This was identified as the same methyl ester that was obtained from the phenyl glycine glutamic acid dipeptide (see above). Mp: 236–238 °C. $[\alpha]_D^{24}$ = −44.9 (*c* 1, DMSO). ^{1}H NMR (300 MHz, DMSO-d_6): δ = 8.63 (d, *J* = 2.7 Hz, CHN*H*CO), δ = 8.24 (s, CHN*H*CO), δ = 7.35 (m, aromatic protons), δ = 4.92 (d, *J* = 2.7 Hz, C*H*Ph), δ = 4.05 (t, C*H*$CH_2CH_2CO_2CH_3$), δ = 3.60 (s, $CH_2CH_2CO_2CH_3$), δ = 2.45, δ = 2.03 (m, m, $CH_2CH_2CO_2CH_3$); ^{13}C NMR (75 MHz, DMSO-d_6): δ = 173.6, 168.2, 167.5 (*C*=O), δ = 139.3, 129.2, 128.6, 127.7 (Ar), δ = 59.4 (CO_2CH_3), δ = 53.3 (*C*HPh), δ = 52.1 ($CHCH_2CH_2CO_2CH_3$), δ = 32.21 ($CHCH_2CH_2CO_2CH_3$), δ = 29.5 ($CHCH_2CH_2CO_2CH_3$), δ = 27.9 ($CHCH_2CH_2CO_2CH_3$).

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