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Penicillin acylase-catalyzed peptide synthesis in aqueous medium: a chemo-enzymatic route to stereoisomerically pure diketopiperazines

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Abstract—A range of non-natural dipeptides of the general formula D-(−)-phenylglycyl-L-X, where X is a natural α-amino acid, have been prepared by penicillin acylase-catalyzed synthesis in aqueous medium from D-(−)-phenylglycine amide and the corresponding amino acids. The conversion of the dipeptides to the corresponding dipeptide esters, followed by their subsequent spontaneous cyclization afforded the corresponding stereoisomerically pure diketopiperazines.
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1. Introduction

The chemical synthesis of dipeptides generally requires protection and activation steps, which result in lengthy and wasteful procedures. Enzymatic peptide synthesis, which is, for example, practiced on an industrial scale in the synthesis of the sweetener aspartame, is much more efficient.[1] Proteases have been widely applied in the synthesis of peptides, but they are restricted to L-amino acid derivatives and do not convert non-natural amino acids, such as phenylglycine.

We recently have shown that penicillin acylase (penicillin amidohydrolase, EC 3.5.1.11) from *Escherichia coli* can be used for the enzymatic synthesis of non-natural phenylglycine dipeptides including those containing the D-(−)-phenylglycyl residue.[2] The acyl-binding subsite in penicillin acylase, which is highly selective for phenylacetic acid and derivatives,[3] can also accept D-(−)-phenylglycine derivatives as the acyl donor. In contrast, the nucleophile binding subsite is extremely selective for the L-enantiomers of a wide range of amino acids.[3–5] Hence, penicillin acylase has been used, in combination with proteases, in peptide synthesis as a selective protection and deprotection catalyst.[6,7] Additionally, penicillin acylase provides the unique possibility to synthesize a wide range of D-(−)-phenylglycine dipeptides with different amino acids. These can easily be converted into the dipeptide esters, which, in turn, can be cyclized to give the corresponding diketopiperazines. The latter are interesting compounds in their own right; their application as food additives,[8] chitinase inhibitors,[9] anti-allergic agents[10] and building block for anti-viral compounds[11] has been claimed. Diketopiperazines also catalyze the enantioselective addition of hydrogen cyanide to aldehydes.[12–14]

We have recently reported a chemoenzymatic route to stereoisomerically pure 3,6-diphenyldiketopiperazines.[2] Herein we demonstrate the extension of our procedure to different D-phenylglycine-derived dipeptides and a range of the corresponding diketopiperazines.

2. Results and discussion

The chemoenzymatic procedure is outlined in Scheme 1. The initial step involves the penicillin acylase-catalyzed acyl transfer from D-phenylglycine amide **1** (acyl donor) to the appropriate amino acid **2a**–**h** as a

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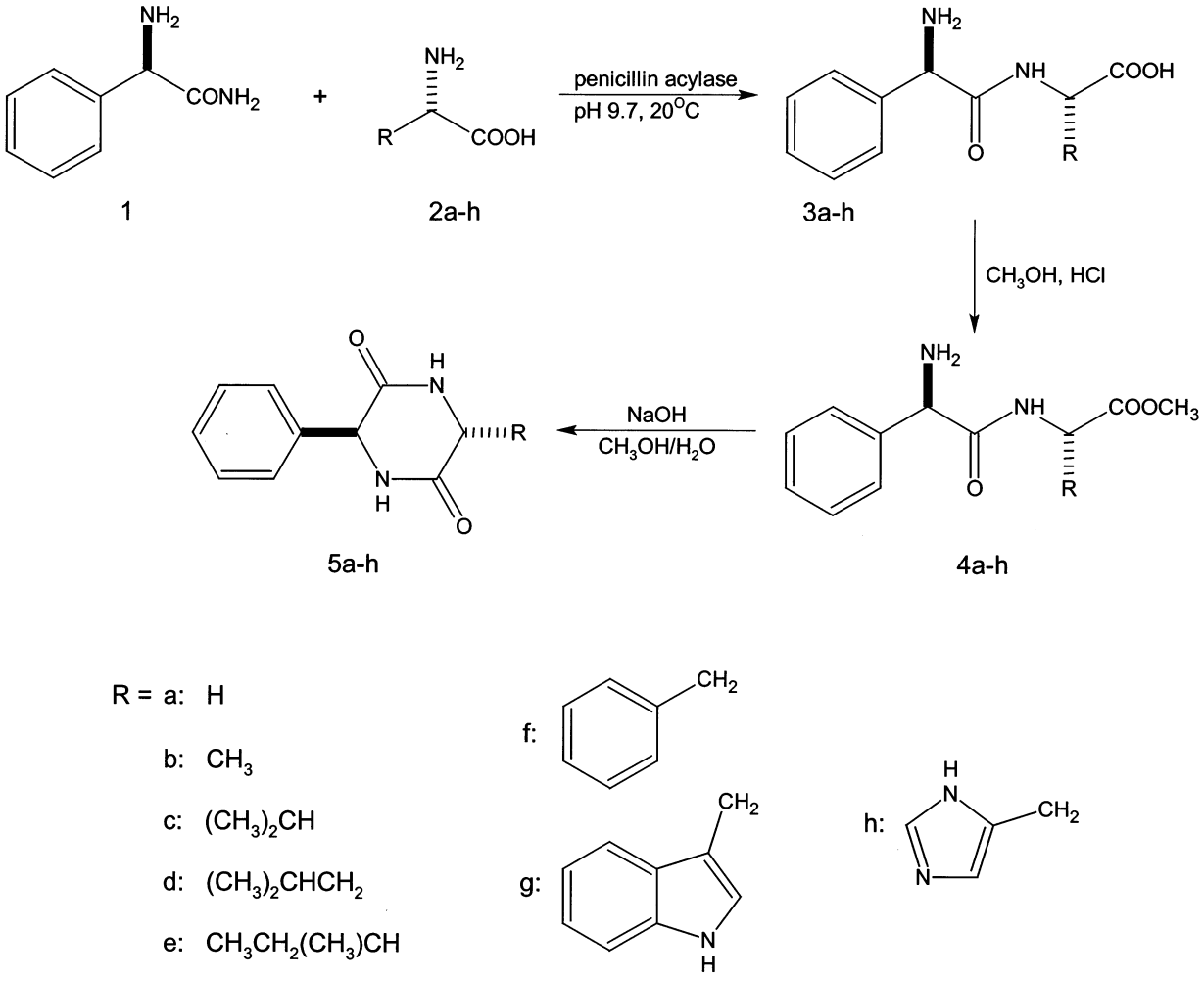

Scheme 1. Synthesis of diketopiperazines.

nucleophile. The reaction is kinetically controlled and proceeds via an acyl-enzyme intermediate, in which the D-phenylglycyl moiety is covalently bound to the catalytic B1 serine residue. A single product is obtained due to the unique substrate and stereospecificity of penicillin acylase, combined with a judicious choice of the reaction conditions. Any tendency of the nucleophile **2** to act as the acyl donor was suppressed by supplying the former as a free amino acid, which is unable to acylate penicillin acylase for thermodynamic reasons and also due to the strict specificity of its acyl group binding site.[3] The reactions were performed at high pH (9.7) to deprotonate the amino group in **2**. The nucleophile subsite is highly specific for L-amino acids;[3–5] hence, **1** does not act as an acyl acceptor and no D-phenylglycine dimers are formed.[2]

The penicillin acylase-catalyzed conversion of the reactants into the dipeptides **3a–h** was high (>80% on average) according to HPLC monitoring (see Table 1). Portionwise addition of **1** avoided the inhibition of the biocatalyst by high concentrations of **1** as well as the

Table 1. Synthesis of D-phenylglycine dipeptides according to Scheme 1

Nucleophile **2**		R	HPLC yield (%)	Isolated yield (%)		
			3	**3**	**4**	**5**
Glycine	**a**	H	85	61	n.d.	31
L-Alanine	**b**	CH_3	88	34	66	42
L-Valine	**c**	i-C_3H_7	72	41	82	n.d.
L-Leucine	**d**	i-C_4H_9	76	70	81	19
L-Isoleucine	**e**	s-C_4H_9	85	67	67	n.d.
L-Phenylalanine	**f**	$CH_2C_6H_5$	87	53	67	58
L-Tryptophan	**g**	3-Indolylmethyl	94	34	n.d.	25
L-Histidine	**h**	3-Imidazolylmethyl	85	n.d.	n.d.	26

irreversible inactivation of the former that takes place at high pH and is exacerbated at high substrate concentrations.[15] The isolated yields (Table 1) generally were much lower but the procedure was performed at a relatively small scale and was not optimized.

The dipeptide acids were converted into the corresponding methyl esters **4a**–**h** via a standard procedure and then incubated in alkaline aqueous solution in order to give the diketopiperazines **5a**–**h**. All tested dipeptide esters formed diketopiperazines sluggishly compared to D-phenylglycyl-L-phenylglycine methyl ester and L-phenylglycyl-L-phenylglycine methyl ester. D-Phenylglycyl-L-isoleucine methyl ester proved to be an extreme case and hardly any formation of diketopiperazine was observed after nearly 3 months. However, useful yields of diketopiperazines were obtained from most of the other dipeptide esters.

3. Conclusion

Penicillin acylase efficiently catalyzes the synthesis non-natural dipeptides from D-phenylglycine amide and L-amino acids. The ring closure of the corresponding dipeptide esters affords the stereoisomerically pure diketopiperazine derivatives.

4. Experimental

4.1. Materials and analysis

4.1.1. Materials. Immobilised penicillin acylase from *E. coli*, Assemblase® (315 U/g), was kindly donated by DSM Anti-Infectives (Delft, The Netherlands). D-(−)-Phenylglycine amide was received from DSM (Geleen, The Netherlands). The L-amino acids were from common suppliers.

4.1.2. Analytical methods. The synthesis of all compounds was monitored by HPLC using a Waters 6000A pump, a Phenomenex Luna C18(2) column (250×4.6 mm, 5 μm) and a Waters M481 LC detector at 208 nm with 7 mM phosphate pH 3.0, containing acetonitrile (from 30 to 45%, v/v) and a 0.7 g/L of sodium dodecylsulphate, as the eluent. The flow rate was 0.5 mL/min. Retention times (in min) for the eluent with 40% CH_3CN: D-phenylglycine amide (8.8), D-phenylglycine (5.34), L-phenylalanine (6.7), L-tryptophan (8.74), L-histidine (6.33), D-phenylglycyl-glycine (8.12), D-phenylglycyl-L-alanine (9.46), D-phenylglycyl-L-valine (15.8), D-phenylglycyl-L-leucine (21.9), D-phenylglycyl-L-isoleucine (21.3), D-phenylglycyl-L-phenylalanine (22.3), D-phenylglycyl-L-tryptophan (22.9), D-phenylglycyl-glycine methyl ester (11.7), D-phenylglycyl-L-alanine methyl ester (15.2), D-phenylglycyl-L-valine methyl ester (28.2), D-phenylglycyl-L-leucine methyl ester (42.0), D-phenylglycyl-L-isoleucine methyl ester (42.5), D-phenylglycyl-L-phenylalanine methyl ester (43.9), (*R*)-3-phenyl-2,5-piperazinedione (4.31), (3*S*,6*R*)-3-methyl-6-phenylpiperazine-2,5-dione (4.96), (3*S*,6*R*)-3-isopropyl-6-phenylpiperazine-2,5-dione (5.6), (3*S*,6*R*)-3-(2-methylpropyl)-6-phenylpiperazine-2,5-dione (7.67), (3*R*,6*S*)-3-phenyl-6-(phenylmethyl)piperazine-2,5-dione (7.61), (3*S*,6*R*)-3-(1*H*-indol-3-ylmethyl)-6-phenylpiperazine-2,5-dione (7.80), (3*S*,6*R*)-3-(1*H*-imidazol-4-ylmethyl)-6-phenylpiperazine-2,5-dione (8.80). Optical rotations were determined using a Jasco (Japan) DIP-360 Digital polarimeter.

4.1.3. General method A: enzymatic synthesis of dipeptides. **1** (4.4 g, 29.3 mmol) and amino acid **2a**–**e** (80 mmol) were suspended in 200 mL water at 20°C. The pH was adjusted to 9.7 (25% NH_4OH) and immobilized *E. coli* penicillin acylase (3.8–5 g) was added. The pH was maintained at 9.7 while stirring. Two further portions of **1** (4.4 g, 29.3 mmol) were added when the concentration of **1** was 5–15% from initial. When conversion of the by-product (D-phenylglycine) was about 10%, the enzyme was removed by filtration. The pH was adjusted to 5.5 (3 M H_2SO_4); the precipitate was collected and purified several times by dissolution at pH ~10 followed by precipitation at pH ~5.5

4.1.4. General method B: synthesis of diketopiperazines. The hydrochloride of the dipeptide methyl ester **4a**–**e**, **3g** was dissolved in 75 mL methanol–water (1:1, v/v), the pH was raised to 8.0 using KOH and the reaction mixture was stirred at room temperature for several days. The precipitate was collected and recrystallized from 1,2-dimethoxyethane.

4.2. Synthesis of (*R*)-3-phenyl-2,5-piperazinedione 5a

4.2.1. D-Phenylglycyl-glycine 3a. **3a** was synthesized using the general method A with a reaction time of 310 min using 4.2 g of immobilized *E. coli* penicillin acylase. Yield 10.2 g (49 mmol, 61%): dec. 245°C; $[\alpha]_D^{20} = -123.2$ (*c* 1, 2.5 M HCl). ^{1}H NMR (300 MHz) (D_2O+DCl): 3.96 (s, 2H, CH_2NHCO), 5.20 (s, 1H, CHNH_3^+), 7.49 (s, 5H, aromatic protons); ^{13}C NMR (75 MHz) (D_2O+DCl): 31.2, 42.7, 58.1, 129.8, 131.2, 132.0, 133.2, 170.7, 174.3. Anal. calcd for $C_{10}H_{12}N_2O_3$: C, 57.69; H, 5.81; N, 13.45. Found: C, 57.63; H, 5.93; N, 13.31.

4.2.2. (*R*)-3-Phenylpiperazine-2,5-dione 5a. **3a** (6 g, 28.8 mmol) was refluxed in 600 mL methanol/HCl for 3 h and subsequently concentrated in vacuo. The residue was dissolved in 150 mL methanol–water (1:1, v/v); the pH was raised to 8.0 (KOH) and the reaction mixture was stirred at 20°C for 10 days. The precipitate was collected and recrystallized from 1,2-dimethoxyethane, yielding **5a** (1.7 g, 8.9 mmol, 31%): mp 235°C, $[\alpha]_D^{20} = -74.2$ (*c* 1, DMSO). ^{1}H NMR (300 MHz) (DMSO-d_6): 3.73 (dd, 1H, CH_2, $J=3.2$, $J=17.8$), 3.94 (d, 1H, CH_2, $J=17.8$), 4.87 (d, 1H, CHC$_6$H$_5$, $J=2.9$), 7.29–7.43 (m, 5H, aromatic protons), 8.14 (s, 1H, CHNHCO), 8.61 (d, 1H, CHNHCO, $J=2.2$); ^{13}C NMR (75 MHz) (DMSO-d_6): 44.1, 58.4, 126.7, 127.8, 128.4, 138.7, 165.7, 166.1. Anal. calcd for $C_{10}H_{10}N_2O_2$: C, 63.15; H, 5.30; N, 14.73. Found: C, 60.81; H, 5.14; N, 13.91. MS *m*/*z*: 190 (26), 169 (5), 162 (8), 147 (100), 132 (9), 118 (46), 104 (72), 91 (24), 77 (42), 69 (17), 63 (8), 56 (6), 51 (29), 44 (7).

4.3. Synthesis (3*S*,6*R*)-3-methyl-6-phenylpiperazine-2,5-dione 5b

4.3.1. D-Phenylglycyl-L-alanine 3b. **3b** was synthesized using the general method A with a reaction time of 240 min using 3.8 g of immobilized *E. coli* penicillin acylase. Yield 6.0 g (27 mmol, 34%): mp 251–252°C, $[\alpha]_D^{20} = -119.8$ (*c* 1, 2.5 M HCl). ^{1}H NMR (300 MHz) (D_2O+DCl): 1.28 (d, 3H, CHCH_3, $J=7.3$), 4.35 (q, 1H, CHCH$_3$, $J=7.3$, $J=14.6$), 5.14 (s, 1H, CHNH_3^+), 7.42–7.55 (m, 5H, aromatic protons); ^{13}C NMR (75 MHz) (D_2O+DCl): 17.4, 31.2, 50.5, 58.0, 129.6, 131.3, 132.0, 133.4, 169.9, 177.6. Anal. calcd for $C_{11}H_{14}N_2O_3$: C, 59.45; H, 6.35; N, 12.61. Found: C, 58.38; H, 6.37; N, 13.07.

4.3.2. D-Phenylglycyl-L-alanine methyl ester 4b. **3b** (5 g, 22.5 mmol) was refluxed for 3 h in 600 mL methanol/HCl and subsequently concentrated in vacuo. The residue was crystallized from dioxane, yielding **4b**·HCl (3.5 g, 12.8 mmol, 57%): dec. 194°C, $[\alpha]_D^{20} = -118.4$ (*c* 1, EtOH). ^{1}H NMR (300 MHz) (DMSO-d_6): 1.21 (d, 3H, CHCH_3, $J=6.9$), 3.64 (s, 3H, OCH_3), 4.26 (m, 1H, CHCH$_3$, $J=7.2$), 5.05 (s, 1H, CHNH_3^+), 7.38–7.60 (m, 5H, aromatic protons), 8.86 (s, 3H, NH_3^+), 9.28 (d, 1H, CHNHCO, $J=7.2$); ^{13}C NMR (75 MHz) (DMSO-d_6): 16.7, 47.9, 51.9, 55.0, 127.7, 128.6, 128.9, 134.0, 167.1, 172.2. Anal. calcd for $C_{12}H_{17}ClN_2O_3$: C, 52.85; H, 6.28; N, 10.27. Found: C, 51.59; H, 6.24; N, 10.50.

4.3.3. (3*S*,6*R*)-3-Methyl-6-phenylpiperazine-2,5-dione 5b. **5b** was synthesized using the general method B with a reaction time of 5 days. Yield 1.1 g (5.3 mmol, 48%): mp 262–263°C, $[\alpha]_D^{20} = -57.2$ (*c* 1, DMSO). ^{1}H NMR (300 MHz) (DMSO-d_6): 1.29 (d, 3H, CHCH_3, $J=6.6$), 4.00 (m, 1H, CHCH$_3$, $J=6.9$), 4.90 (d, 1H, CHC$_6$H$_5$, $J=3.3$), 7.30–7.42 (m, 5H, aromatic protons), 8.26 (s, CHNHCO), 8.56 (d, 1H, CHNHCO, $J=2.7$); ^{13}C NMR (75 MHz) (DMSO-d_6): 18.1, 49.0, 58.8, 126.6, 127.8, 128.4, 138.4, 166.4, 168.7. Anal calcd. for $C_{11}H_{12}N_2O_2$: C, 64.69; H, 5.92; N, 13.72. Found: C, 65.00; H, 5.89; N, 14.00. MS *m*/*z*: 204 (21), 176 (14), 161 (100), 133 (32), 118 (26), 106 (85), 91 (28), 77 (34), 63 (6), 56 (12), 51 (21), 44 (80).

4.4. Synthesis of (3*S*,6*R*)-3-isopropyl-6-phenylpiperazine-2,5-dione 5c

4.4.1. D-Phenylglycyl-L-valine 3c. **3c** was synthesized using the general method A with a reaction time of 240 min using 5 g of immobilized *E. coli* penicillin acylase. Yield 8.1 g (32.4 mmol, 41%): mp 264–265°C, $[\alpha]_D^{20} = -75.6$ (*c* 1, 2.5 M HCl). ^{1}H NMR (300 MHz) (D_2O+DCl): 0.66 (dd, 6H, CH(CH_3)$_2$, $J=6.7$, $J=8.2$), 2.07 (m, 1H, CH(CH$_3$)$_2$), 4.32 (d, 1H, CHNHCO, $J=5.7$), 5.22 (s, 1H, CHNH_3^+), 7.49 (m, 5H, aromatic protons); ^{13}C NMR (75 MHz) (D_2O+DCl): 18.3, 19.9, 31.2, 31.6, 58.0, 59.8, 129.4, 129.6, 131.2, 131.9, 133.6, 170.3, 176.3. Anal. calcd for $C_{13}H_{18}N_2O_3$: C, 62.38; H, 7.25; N, 11.19. Found: C, 61.16; H, 7.23; N, 11.22.

4.4.2. D-Phenylglycyl-L-valine methyl ester 4c. **3c** (7 g, 28 mmol) was refluxed in 500 mL methanol/HCl for 3 h and subsequently concentrated in vacuo. The residue was crystallized from a mixture of hexane, dioxane and 2-propanol, yielding **4c**·HCl (6.9 g, 22.9 mmol, 82%): dec. 123–124°C, $[\alpha]_D^{20} = -77.0$ (*c* 1, EtOH). ^{1}H NMR (300 MHz) (DMSO-d_6): 0.63 (dd, 6H, CH(CH_3)$_2$, $J=6.9$, $J=14.1$), 1.96 (m, 1H, CH(CH$_3$)$_2$), 3.57 (s, 3H, OCH_3), 4.21 (dd, 1H, CHNHCO, $J=6.3$, $J=8.7$), 5.14 (s, 1H, CHNH_3^+), 7.33–7.65 (m, 5H, aromatic protons), 8.8 (s, 3H, NH_3^+), 9.03 (d, 1H, CHNHCO, $J=8.6$); ^{13}C NMR (75 MHz) (DMSO-d_6): 17.5, 18.5, 30.2, 51.8, 54.9, 57.2, 127.5, 128.5, 128.9, 134.4, 167.8, 171.3. Anal. calcd for $C_{14}H_{21}ClN_2O_3$: C, 55.90; H, 7.04; N, 9.31. Found: C, 55.94; H, 7.15; N, 8.90.

4.4.3. (3*S*,6*R*)-3-Isopropyl-6-phenylpiperazine-2,5-dione 5c. **5c** was synthesized using the general method B with a reaction time of 73 days. Yield 0.23 g (1 mmol, 10%): mp 264°C, $[\alpha]_D^{20} = -61.4$ (*c* 1, DMSO). ^{1}H NMR (400 MHz) (DMSO-d_6): 0.7–1.02 (m, 6H, CH(CH_3)$_2$), 1.95–2.32 (m, 1H, CH(CH$_3$)$_2$), 3.8 (m, 1H, CH′Pr), 4.95 (s, 1H, CHC$_6$H$_5$), 7.18–7.44 (m, 5H, aromatic protons), 8.14 (s, 1H, CHNHCO), 8.41 (s, 1H, CHNHCO); ^{13}C NMR (100 MHz) (DMSO-d_6): 16.6, 18.1, 31.4, 58.2, 59.1, 126.6, 127.3, 127.7, 128.2, 139.0, 166.8, 167.0.

4.5. Synthesis of (3*S*,6*R*)-3-(2-methylpropyl)-6-phenylpiperazine-2,5-dione 5d

4.5.1. D-Phenylglycyl-L-leucine 3d. **3d** was synthesized using the general method A with a reaction time of 470 min using 5 g of immobilized *E. coli* penicillin acylase. Yield 14.8 g (56 mmol, 70%): mp 262°C, $[\alpha]_D^{20} = -95.2$ (*c* 1, 2.5 M HCl). ^{1}H NMR (300 MHz) (D_2O+DCl): 0.64 (dd, 6H, CH(CH_3)$_2$, $J=6.6$, $J=11.5$), 1.11 (m, 1H, CH$_2$CH(CH$_3$)$_2$), 1.54 (m, 2H, CHCH_2CH(CH$_3$)$_2$, $J=7.4$), 4.38 (m, 1H, CHNHCO), 5.17 (s, 1H, CHNH_3^+), 7.47 (s, 5H, aromatic protons); ^{13}C NMR (75 MHz) (D_2O+DCl): 21.6, 23.6, 25.7, 31.2, 40.5, 52.9, 58.1, 71.6, 129.4, 131.2, 132.0, 133.3, 170.1, 177.5. Anal. calcd for $C_{14}H_{20}N_2O_3$: C, 63.62; H, 7.63; N, 10.6. Found: C, 62.83; H, 7.97; N, 9.48.

4.5.2. D-Phenylglycyl-L-leucine methyl ester 4d. **3d** (7 g, 26.5 mmol) was refluxed in 500 mL methanol/HCl over 3 h and subsequently concentrated in vacuo. The residue was recrystallized from a mixture of hexane and dioxane, yielding **4d**·HCl (6.8 g, 21.6 mmol, 81%): mp 107–110°C, $[\alpha]_D^{20} = -94.0$ (*c* 1, EtOH). ^{1}H NMR (300 MHz) (DMSO-d_6): 0.6 (d, 3H, (CH_3)CH(CH$_3$), $J=6.6$), 0.7 (d, 3H, (CH$_3$)CH(CH_3), $J=6.6$), 1.19 (m, 1H, CH$_2$CH(CH$_3$)$_2$), 1.46 (m, 2H, CHCH_2CH(CH$_3$)$_2$), 3.36 (s, 3H, OCH_3) 4.26 (m, 1H, CHCH$_2$CH(CH$_3$)$_2$), 5.02 (s, 1H, CHNH_3^+), 7.34–7.62 (m, 5H, aromatic protons), 8.77 (s, 3H, NH_3^+), 9.17 (d, 1H, CHNHCO, $J=8.1$); ^{13}C NMR (75 MHz) (DMSO-d_6): 20.7, 22.5, 23.9, 39.5, 50.3, 51.9, 55.1, 127.6, 128.4, 128.9, 134.2, 167.5, 172.2. Anal. calcd for $C_{15}H_{23}ClN_2O_3$: C, 57.23; H, 7.36; N, 8.90. Found: C, 57.61; H, 7.60; N, 9.33.

4.5.3. (3*S*,6*R*)-3-(2-Methylpropyl)-6-phenylpiperazine-2,5-dione 5d. **5d** was synthesized using the general method B with a reaction time of 21 days. Yield 0.432 g (1.8 mmol, 19%): dec. 262°C, $[\alpha]_D^{20}=-47.0$ (*c* 1, DMSO). ^{1}H NMR (400 MHz) (DMSO-d_6): 0.82–0.93 (m, 6H, CH(CH_3)$_2$), 1.65 (m, 2H, CHCH_2CH(CH$_3$)$_2$), 1.87 (m, 1H, CH$_2$CH(CH$_3$)$_2$), 3.89 (m, 1H, CHCH$_2$CH(CH$_3$)$_2$), 4.97 (s, 1H, CHC$_6$H$_5$), 7.3–7.43 (m, 5H, aromatic protons), 8.25 (s, 1H, CHNHCO), 8.52 (s, 1H, CHNHCO); ^{13}C NMR (100 MHz) (DMSO-d_6): 22.0, 22.7, 23.4, 41.4, 52.4, 58.4, 127.2, 127.7, 128.3, 138.6, 166.6, 168.3. Anal. calcd for $C_{14}H_{18}N_2O_2$: C, 68.27; H, 7.37; N, 11.37. Found: C, 70.26; H, 7.57; N, 10.94.

4.6. Synthesis of (3*S*,6*R*)-3-((*S*)-1-methylpropyl)-6-phenylpiperazine-2,5-dione 5e

4.6.1. D-Phenylglycyl-L-isoleucine 3e. **3e** was synthesized using the general method A with a reaction time of 315 min using 4.8 g of immobilized *E. coli* penicillin acylase. Yield 14.3 g (54.1 mmol, 67%): mp 253°C, $[\alpha]_D^{20}=-68.8$ (*c* 0.5, 2.5 M HCl). ^{1}H NMR (300 MHz) (D$_2$O+DCl): 0.67 (dd, 6H, CH(CH_3)CH$_2$CH_3, $J=7.1$, $J=13.2$), 0.81–1.20 (m, 2H, CH(CH$_3$)CH_2CH$_3$), 1.83 (m, 1H, CH(CH$_3$)CH$_2$CH$_3$), 4.34 (d, 1H, CHNHCO, $J=6.0$), 5.18 (s, 1H, CHNH$_3^+$), 7.48 (s, 5H, aromatic protons); ^{13}C NMR (75 MHz) (D$_2$O+DCl): 11.8, 16.5, 25.9, 31.2, 37.9, 58.0, 59.1, 129.1, 131.2, 132.0, 133.6, 170.3, 176.6. Anal calcd. for $C_{14}H_{20}N_2O_3$: C, 63.62; H, 7.63; N, 10.6. Found: C, 63.13; H, 7.62; N, 10.85.

4.6.2. D-Phenylglycyl-L-isoleucine methyl ester 4e. **3e** (7 g, 26.5 mmol) was refluxed esterified in 600 mL methanol/HCl for 3 h and concentrated in vacuo. The residue was crystallized from a mixture of hexane and 2-propanol, yielding **4e**·HCl (4.9 g, 15.6 mmol, 59%); mp 175–177°C, $[\alpha]_D^{20}=-76.8$ (*c* 1, EtOH). ^{1}H NMR (300 MHz) (DMSO-d_6): 0.63 (m, 6H, CH(CH_3)CH$_2$CH_3, $J=7.5$), 0.86–1.19 (m, 2H, CH(CH$_3$)CH_2CH$_3$), 1.72 (m, 1H, CH(CH$_3$)CH$_2$CH$_3$), 3.67 (s, 3H, OCH$_3$), 4.23 (dd, 1H, CHNHCO, $J=6.7$, $J=8.6$), 5.15 (s, 1H, CHNH$_3^+$), 7.37–7.64 (m, 5H, aromatic protons), 8.90 (s, 3H, NH$_3^+$), 9.10 (d, 1H, CHNHCO, $J=8.7$); ^{13}C NMR (75 MHz) (DMSO-d_6): 10.5, 15.1, 24.1, 36.4, 51.8, 54.8, 56.2, 127.5, 128.4, 128.9, 134.2, 167.6, 171.4. Anal. calcd for $C_{15}H_{23}ClN_2O_3$: C, 57.23; H, 7.36; N, 8.90. Found: C, 57.03; H, 7.42; N, 9.22.

4.6.3. (3*S*,6*R*)-3-((*S*)-1-Methylpropyl)-6-phenylpiperazine-2,5-dione 5e. An attempt to synthesize **5e** using the general method B did not succeed during 3 months of incubation. At higher temperatures and/or pH 8.5 there was increased hydrolysis of the dipeptide ester but cyclization was not observed.

4.7. Synthesis (3*R*,6*S*)-3-phenyl-6-(phenylmethyl)-piperazine-2,5-dione 5f

4.7.1. D-Phenylglycyl-L-phenylalanine 2f. **1** (6.75 g, 45 mmol) and L-(+)-phenylalanine (6.69 g, 40.5 mmol) were suspended in 90 mL water at 25°C. The pH was adjusted to 9.7 (25% NH$_3$) and immobilized *E. coli* penicillin acylase (10 g) was added; the pH was maintained at 9.7 while stirring. After 280 min the enzyme was removed by filtration. The pH was adjusted to 5.5 (3 M H$_2$SO$_4$); the precipitate was collected and twice purified by dissolution at pH ~10 followed by precipitation at pH ~5.5, yielding **3f** (6.36 g, 21.3 mmol, 53%): mp 238°C, $[\alpha]_D^{20}=-25.4$ (*c* 1, 2.5 M HCl). ^{1}H NMR (400 MHz) (D$_2$O+DCl): 2.8 (dd, 1H, CH_2C$_6$H$_5$, $J=9.9$, $J=14.1$), 3.16 (dd, 1H, CH_2C$_6$H$_5$, $J=4.5$, $J=14.1$), 4.8 (m, 1H, CHNHCO), 5.02 (s, 1H, CHNH$_3^+$) 6.83–7.5 (m, 10H, aromatic protons); ^{13}C NMR (100 MHz) (D$_2$O+DCl): 31.2, 37.9, 55.2, 58.1, 71.3, 128.5, 129.3, 130.2, 130.4, 131.3, 131.8, 133.1, 137.6, 169.7, 175.9. Anal. calcd for $C_{17}H_{18}N_2O_3$: C, 68.44; H, 6.08; N, 9.39. Found: C, 67.64; H, 6.02; N, 9.27. MS *m*/*z*: 280 (10), 189 (8), 161 (13), 118 (7), 106 (100), 91 (25), 79 (21), 51 (5).

4.7.2. D-Phenylglycyl-L-phenylalanine methyl ester 4f. **3f** (3 g, 10 mmol) was refluxed in 450 mL methanol/HCl for 3 h and concentrated in vacuo. The residue was crystallized from isopropylalcohol, yielding **4f**·HCl (1.93 g, 5.5 mmol, 55%): dec. 228–229°C, $[\alpha]_D^{20}=-40.2$ (*c* 1, EtOH). ^{1}H NMR (400 MHz) (DMSO-d_6): 2.76–3.20 (m, 2H, CH_2C$_6$H$_5$), 3.62 (s, 3H, OCH_3), 4.52 (m, 1H, CHNHCO), 5.02 (s, 1H, CHNH$_3^+$), 6.83–7.5 (m, 10H, aromatic protons), 8.75 (s, 3H, NH_3^+), 9.2 (d, 1H, CHNHCO); ^{13}C NMR (100 MHz) (DMSO-d_6): 36.1, 52.0, 53.5, 55.0, 126.3, 127.7, 128.0, 128.4, 128.7, 128.9, 133.9, 136.4, 167.3, 171.1. Anal. calcd for $C_{18}H_{21}ClN_2O_3$: C, 61.98; H, 6.07; N, 8.03. Found: C, 61.63; H, 6.02; N, 7.86. MS *m*/*z*: 161 (6), 117 (17), 106 (100), 91 (54), 77 (31), 65 (14), 51 (23), 45 (70).

4.7.3. (3*R*,6*S*)-3-Phenyl-6-(phenylmethyl)piperazine-2,5-dione 5f. **4f**·HCl (1.5 g, 4.3 mmol) was dissolved in 40 mL methanol–water (1:1 v/v), the pH was raised to 8.0 (KOH) and the reaction mixture was stirred at room temperature. After 14 days the precipitate was collected and crystallized from isopropyl alcohol, yielding **5f** (0.7 g, 2.5 mmol, 58%); mp 243–244°C, $[\alpha]_D^{20}=-17.4$ (*c* 1, DMSO). ^{1}H NMR (400 MHz) (DMSO-d_6): 2.9–3.3 (m, 2H, CH$_2$C$_6$H$_5$), 4.23 (s, 1H, CHCH$_2$), 4.35 (s, 1H, CHC$_6$H$_5$), 7.15–7.38 (m, 10H, aromatic protons), 8.24 (s, 1H, CHNHCO), 8.43 (s, 1H, CHNHCO); ^{13}C NMR (100 MHz) (DMSO-d_6): 37.8, 54.9, 58.2, 126.6, 127.7, 127.7, 128.0, 128.2, 130.1, 136.0, 138.9, 166.0, 166.7. Anal. calcd for $C_{17}H_{16}N_2O_2$: C, 72.84; H, 5.75; N, 9.99. Found: C, 73.00; H, 5.61; N, 10.02. MS *m*/*z*: 280 (62), 263 (6), 237 (9), 189 (51), 161 (87), 149 (10), 132 (11), 117 (46), 106 (60), 91 (100), 77 (34), 62 (22), 51 (19), 45 (46).

4.8. Synthesis of (3*S*,6*R*)-3-(1*H*-indol-3-ylmethyl)-6-phenylpiperazine-2,5-dione 5g

4.8.1. D-Phenylglycyl-L-tryptophan 3g. **1** (1.76 g, 11.7 mmol) and L-(+)-tryptophan (6.54 g, 32 mmol) were suspended in 80 mL water at 20°C. The pH was adjusted to 9.7 (25% NH$_3$) and immobilized *E. coli* penicillin acylase (8.5 g) was added. The pH was maintained at 9.7 while stirring and after 10 and 30 min two

subsequent portions of **1** (1.76 g, 11.7 mmol) were added. After 140 min the product started to precipitate. The enzyme and precipitated product were separated by filtration, resuspended in 150 mL of water to dissolve the product and the enzyme was removed by filtration. The filtrates were combined; approx. 100 mL of water was evaporated and ammonia was added, yielding **3g**·NH_3 (4.07 g, 11.5 mmol, 36%); mp 261–263°C, $[\alpha]_D^{20} = -72.8$ (*c* 1, 2.5 M HCl). ^{1}H NMR (400 MHz) (D_2O+DCl): 2.96 (dd, 1H, CH_2C, $J=10.0$, $J=14.8$), 3.27 (dd, 1H, CH_2C, $J=4.4$, $J=14.8$), 4.87 (dd, 1H, C*H*NHCO, $J=4.4$, $J=10.0$), 4.97 (s, 1H, C*H*NH_3^+), 6.5 (s, 1H, N*H*-aromatic ring Trp), 6.98–7.47 (m, 10H, aromatic protons); ^{13}C NMR (100 MHz) (D_2O+DCl): 28.3, 31.2, 54.2, 58.0, 109.9, 113.4, 119.6, 120.7, 123.2, 125.9, 127.8, 129.0, 131.0, 131.7, 133.1, 137.6, 169.7, 176.4. Anal. calcd for $C_{19}H_{22}N_4O_3$: C, 64.39; H, 6.26; N, 15.81. Found: C, 64.40; H, 6.12, N, 15.78.

4.8.2. (3*S*,6*R*)-3-(1*H*-indol-3-ylmethyl)-6-phenylpiperazine-2,5-dione 5g. **3g** (3 g, 8.9 mmol) was refluxed in 450 mL methanol/HCl for 5 h and subsequently concentrated in vacuo. The residue was dissolved in 100 mL methanol–water (80:20, v/v); the pH was raised to 8.0 (KOH) and the reaction mixture was stirred at 20°C for 28 days. The precipitate was collected and recrystallized from 2-propanol, yielding **5g** (0.71 g, 2.23 mmol, 25%): mp 271–272°C, $[\alpha]_D^{20} = -34.8$ (*c* 1, DMSO). ^{1}H NMR (300 MHz) (DMSO-d_6): 3.04–3.4 (m, 2H, CH_2), 4.3 (s, 2H, C*H*NHCO), 6.92–7.68 (m, 10H, aromatic protons), 8.14 (s, 1H, CHN*H*CO), 8.34 (s, 1H, CHN*H*CO), 10.92 (s, 1H, N*H*-aromatic ring Trp); ^{13}C NMR (75 MHz) (DMSO-d_6): 28.3, 54.8, 58.4, 108.3, 111.1, 118.3, 118.8, 120.7, 124.5, 127.1, 127.6, 127.7, 128.2, 135.8, 139.0, 166.0, 167.4. Anal. calcd for $C_{19}H_{17}N_3O_2$: C, 71.46; H, 5.37; N, 13.16. Found: C, 68.86; H, 5.18; N 12.82. MS *m*/*z*: 319 (7), 130 (100), 104 (7), 77 (11), 57 (10), 45 (24).

4.9. Synthesis of (3*S*,6*R*)-3-(1*H*-imidazol-4-ylmethyl)-6-phenylpiperazine-2,5-dione 5h

1 (4.4 g, 29.3 mmol) and L-(+)-histidine (12.41 g, 80 mmol) were suspended in 200 mL water at 20°C. The pH was adjusted to 9.7 (25% NH_3) and 5 g immobilized *E. coli* penicillin acylase was added. The pH was maintained at 9.7 while stirring. After 65 and 125 min two subsequent portions of **1** (4.4 g, 29.3 mmol) were added. After 200 min the enzyme was removed by filtration. The filtrate was evaporated to dryness, refluxed in 600 mL of methanol/HCl for 5 h and concentrated in vacuo. The crude ester was dissolved in 200 mL methanol–water (1:1, v/v), the pH was raised to 8.0 (KOH) and the resulting solution was stirred at 20°C for 18 days. The precipitate was collected and purified from ethanol/water by changing pH of the solution, yielding **5h** (5.6 g, 20.7 mmol, 26%): mp 222°C, $[\alpha]_D^{20} = -40.6$ (*c* 1, DMSO). ^{1}H NMR (300 MHz) (DMSO-d_6): 2.95 (dd, 1H, CH_2, $J=6.6$, $J=14.8$), 3.07 (dd, 1H, CH_2, $J=4.1$, $J=14.8$), 4.17 (t, 1H, C*H*CH_2, $J=5.1$), 4.70 (d, 1H, C*H*C_6H_5, $J=2.4$), 6.83–7.60 (m, 7H, aromatic protons), 8.19 (s, 1H, CHN*H*CO), 8.57 (d, 1H, CHN*H*CO, $J=2.2$); ^{13}C NMR (75 MHz) (DMSO-d_6): 29.5, 53.9, 58.5, 116.9, 126.9, 127.7, 128.3, 133.1, 134.8, 138.6, 166.2, 167.3. Anal. calcd for $C_{14}H_{14}N_4O_2$: C, 62.21; H, 5.22; N, 20.73. Found: C, 59.32; H, 5.19; N, 20.95. MS *m*/*z*: 270 (57), 190 (26), 179 (6), 169 (14), 149 (11), 136 (20), 118 (20), 106 (72), 94 (24), 82 (100), 72 (29), 59 (52), 44 (29). HRMS calcd. for $C_{14}H_{14}N_4O_2$: 270.1117. Found: 270.1123.

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