Synthesis of the retro-inverso peptide analogues of *N*-acetylmuramyl-L-alanyl-D-isoglutamine (MDP)

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Dedicated to Professor Miha Tišler on the occasion of his 75th birthday

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Abstract

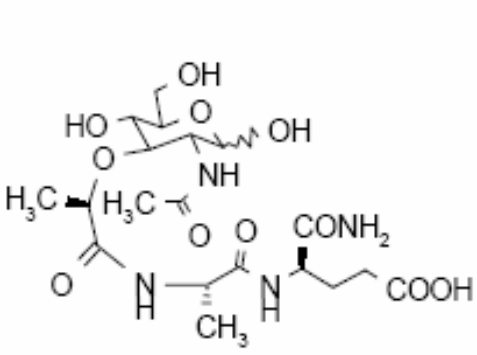
Two novel retro-inverso analogues of a conformationally restricted carbocyclic muramyl dipeptide (MDP) derivative N-(2-methyl-3-oxo-3,4-dihydro-2H-1,4-benzoxazine-2-carbonyl)-L-alanyl-D-isoglutamine were prepared and tested in a immunorestoration test in mice. Both retro-inverso MDP analogues did not enhance non-specific resistance to experimental fungal infection in immunosuppressed animals, suggesting that the intact amide bond is essential for the immunorestorating activity in this type of rigidified carbocyclic MDP analogues.

Keywords: Muramyl dipeptide, retro-inverso analogues, immunomodulators

Introduction

Muramyl dipeptide (**1**) (MDP) was identified in 1974 as the minimal immunologically active component of bacterial cell wall peptidoglycan.[1] Numerous derivatives of this natural lead were designed and synthesized with the aim of obtaining molecules with improved and more defined immunological profiles.[2-4] Recently we have shown that replacement of the polyhydroxy substituted pyranose ring of D-glucosamine in MDP by a

benzene ring and partial rigidification of the molecule involving incorporation of the lactoyl moiety and acetamido group into a benzo-fused 3-morpholinone ring results in a new series of potent immunostimulatory compounds of general structure **2** (R^1=H, Me).[5,6] However, modifications of the L-Ala-D-iGln bond of MDP and analogues have not been described so far although modification of the labile peptide bonds is one of the most frequently applied strategy in the design of peptidomimetics.[7] Among them, the retro-inverso modification of peptide bonds has evolved into one of the most widely used peptidomimetic approaches for the design of novel bioactive molecules which has been applied to many families of biologically active peptides[7,8] including immunostimulatory tuftsin analogues.[9]



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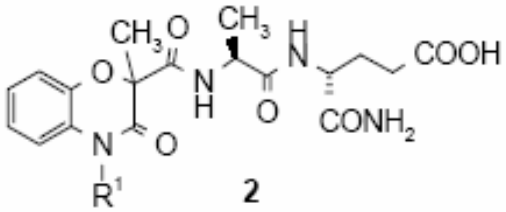

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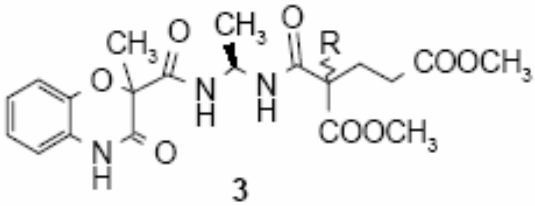

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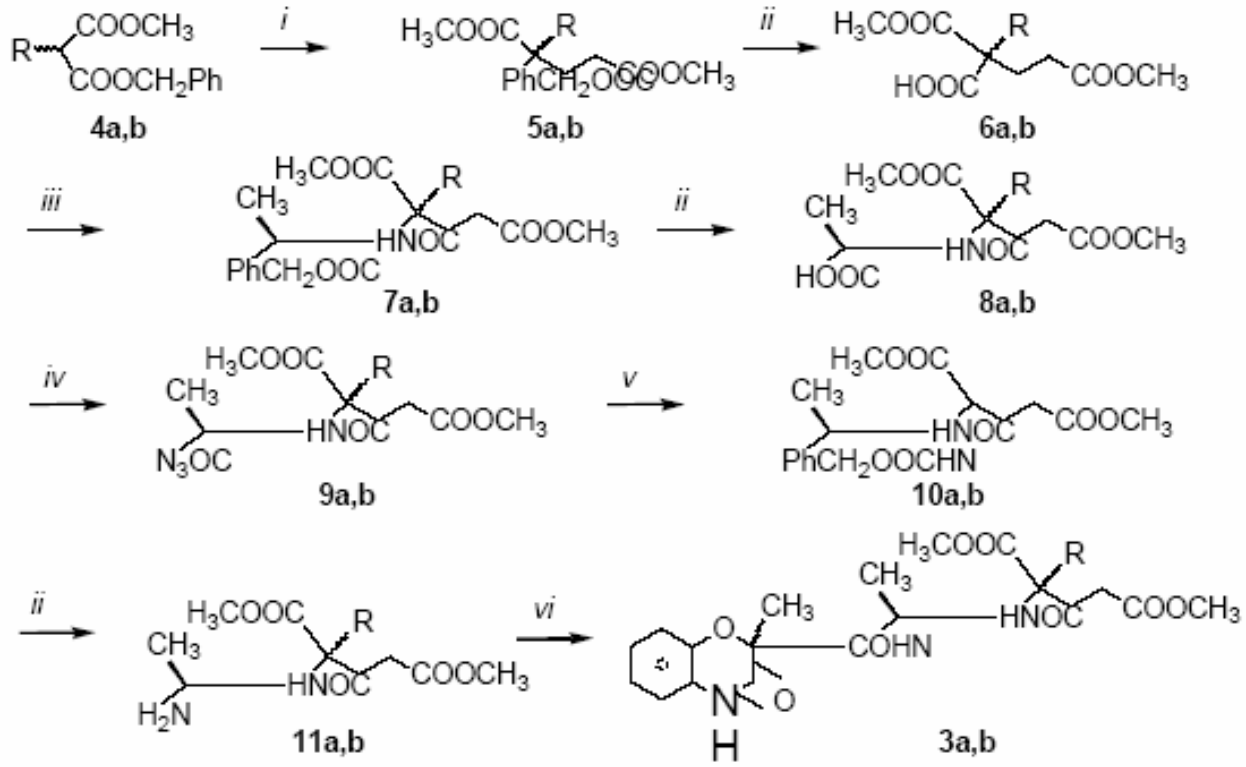
We report now on preparation and results of biological testing of two novel carbocyclic MDP analogues, represented by a general structure **3**, with a retro-inverso modified peptide bond between the L-alanyl and D-glutamate moieties. These compounds were prepared with the aim of obtaining information about the importance of the intact peptide bond for immunostimulating activity in this class of partially rigidifed carbocyclic MDP analogues.

Results and Discussion

Starting from commercially available benzyl methyl malonate **4a** (R = H) and **4b** (R = CH_3) prepared by methylation of **4a**, alkylation with methyl 3-bromopropionate in the presence of sodium hydride and subsequent hydrogenolysis afforded carboxylic acids **6a** and **6b**. The intermediary esters **5a** and **5b** were purified by a high-vacuum distillation to avoid decarboxylation. Coupling of carboxylic esters **6** with D-alanine benzyl ester[10] in the presence of diphenylphosphoryl azide (DPPA)[11-13] followed by hydrogenolytical removal of the benzyl group gave intermediates **8a** and **8b** which were converted to acyl azides **9a,b**. These acyl azides were then subjected to Curtius rearrangement in the presence of benzyl alcohol. The rearrangement of acyl azide to isocyanate could be very successfully followed by IR spectroscopy as a gradual disappearing of the azide absorption band at ca. 2150 cm^{-1} and appearance of the isocyanate band at ca. 2250 cm^{-1}. The yields of carbamates **10a**,**b** were generally low due to a well-known side product formation during addition of alcohols to isocyanates.[14] Deprotection of carbamates **10a**,**b** by hydrogenolysis afforded the *gem*-diamine derivatives **11a** and **11b** which were coupled immediately with pure enantiomers of 2-methyl-3-oxo-3,4-dihydro-2*H*-1,4-benzoxazine-2-carboxylic acid (**12**) prepared by us previously[15] to give **3a** and **3b** in the form of diastereomeric mixtures each containing four diastereomers as evidenced by proton NMR spectra, indicating racemization at C-2 of the morpholinone ring during the coupling reaction. Racemization at C-2 could be deduced from identical ^{1}H-NMR spectra of **3a** obtained either from (*R*)-**12** or (*S*)-**12**; similarly, also ^{1}H-NMR spectra of **3b** obtained either from (*R*)-**12** or (*S*)-**12** were identical and displayed the presence of 4 diastereomers. If racemization had occured at the *gem*-diamine carbon atom, the opposite enantiomers of **12** would have led with high probability to different ^{1}H-NMR spectra of **3a** and **3b**, respectively. Racemization at C-2 had been previously observed also in 2-methyl-3-oxo-3,4-dihydro-2*H*-1,4-benzoxazine-2-amines.[15] However, a mechanism of racenization at C-2 of the heterocyle has not yet been clarified. An attempt to separate the diastereomers of **3b** by HPLC gave two pure distereomers (**3bI** and **3bII**) as well as an equimolar mixture of enantiomers **3bIII** and **3bIV** which were inactive in the immunorestoration test in mice[16,] thus supporting a hypothesis that a retro-inverso modified peptide bond between L-alanine and D-glutamate moieties in this carbocyclic series of MDP analogues is not beneficial for immunostimulating activity.

D. Kikelj, unpublished results



Scheme 1. *i:* NaH, dioxane, 10 °C, 1 h, $BrCH_2CH_2COOMe$, 50 °C, 4 h then r.t., 20 h; *ii:* H_2, Pd/C, MeOH, r.t., 1 h; *iii:* D-Ala(OCH_2Ph)*p*-TsOH, DPPA, Et_3N, DMF, 0 °C, 1 h then r.t., 3 days; *iv:* ClCOOEt, Et_3N, THF, -15 °C, 1 h then NaN_3, H_2O, r.t., 1 h; *v:* toluene, 80 °C, 0.25 h then $PhCH_2OH$, toluene, 110 °C, 20 h; *vi*: (*R*)-**12** or (*S*)-**12**, DPPA, Et3N, DMF, 0 °C, 1 h then r.t., 3 day.

Experimental Section

General Procedures. All reagents and solvents were of commercial grade and were used as such unless specified. Melting points were determined using a Reichert hot-stage microscope and are uncorrected. IR spectra were recorded on Perkin-Elmer FTIR 1600 instrument. NMR spectra were recorded on a Varian VXR-300 spectrometer with tetramethylsilane as internal standard. Mass spectra were recorded on a VG-Analytical Autospec Q mass spectrometer. Microanalyses were carried out at the Department of Organic Chemistry, Faculty of Chemistry and Chemical Technology, Ljubljana, on a Perkin Elmer elemental analyzer 240 C. Optical rotations were determined on a Perkin-Elmer polarimeter 1241 MC using a 1 dm cell. HPLC analysis was performed on a Hewlet-Packard 1090 system with Licrospher 100 RP18 5μm (150 x 4.6 mm) column. The mobile phase was a linear gradient from 0.1 % trifluoroacetic acid in water to 60 % acetonitrile and 0.1 % trifluoroacetic acid in water. For preparative purification a semipreparative column Microsorb C18 3 μm (50 x 22 mm) was used.

General procedure for the synthesis of α-alkyl benzyl methyl malonates 4b, 5a and 5b

Sodium hydride (0.24 g, 10 mmol) was added gradually to a stirred solution of benzyl methyl malonate (2.08g, 10.0mmol) or benzyl methyl 2-methylmalonate (2.22g, 10.0mmol) in dry 1,3-dioxane (16 mL), cooled on an ice-bath. After the addition was completed, the mixture was allowed to warm to room temperature and alkylating agent (10.3 mmol) in dry 1,4-dioxane (10 mL) was added dropwise. The reaction mixture was stirred for 4 h at 50 ^{o}C and then overnight at room temperature. The solvent was removed under reduced pressure, the residual oil dissolved in ether (25 mL), washed successively with water (5×3 mL) and brine (3×3 mL), dried over Na_2SO_4, filtered and the solvent evaporated. The crude product was purified by vacuum distillation.

***(R,S)*-Benzyl methyl 2-methylmalonate (4b).**

Prepared from benzyl methyl malonate (27.28 g, (131 mmol) and methyl iodide (19.02 g , 134 mmol); yield: 13.58g (47 %); bp 105 ^{o}C / 2×10^{-4} mbar, colorless viscous liquid; R_f (silica gel; Et_2O/hexane = 1/2) 0.39; *IR (film, cm^{-1})*: 3655, 3574, 3458, 3051, 3010, 2981, 2946, 2888, 2854, 1763, 1602, 1565, 1492, 1451, 1434, 1381, 1329, 1271, 1219, 1161, 1132, 1091, 1074, 981, 906, 853, 743, 691; *^{1}H-NMR (300MHz, $CDCl_3$, TMS)*: δ(ppm):

1.45 (d, 3H, *J* = 7.24 Hz, CH_3), 3.52 (q, 1H, *J* = 7.29 Hz, CH), 3.70 (s, 3H, OCH_3), 5.17 (s, 2H, CH_2), 7.27 - 7.37 (m, 5H, 5Harom.); *MS (EI),* m/z (%): 222 (M^+, 67), 91 (100); CHN-analysis: calcd for $C_{12}H_{14}O_4$: C 64.85, H 6.35; found: C 64.57, H 6.53.

***(R,S)*-Benzyl methyl 2-[2-(methoxycarbonyl)ethyl]malonate (5a).**
Prepared from benzyl methyl malonate (33.31 g, 160 mmol) and methyl 3-bromopropionate (27.39 g, 164mmol); yield: 24,45 g (52%); bp 155 °C / 10^{-4} mbar, viscous colorless liquid; Rf (silica gel; Et2O/hexane = 1/2) 0.25; *IR (film, cm^{-1}):* 3636, 3463, 3019, 2954, 2847, 1733, 1498, 1437, 1378, 1240, 1153, 1082, 1022, 987, 910, 850, 795, 751, 699; *^{1}H-NMR (300MHz, $CDCl_3$, TMS)*: δ (ppm): 2.25 (m, 2H, CH_2-CH), 2.40 (m, 2H, CH_2-CH_2-CH), 3.53 (t, 1H, *J* = 7.32 Hz, CH), 3.66 (s, 3H, OCH_3), 3.71 (s, 3H, OCH_3), 5.18 (s, 2H, CH_2-Ph), 7.28 - 7.37 (m, 5H, 5H-arom.); *MS (EI),* m/z (%): 294 (M^+, 49), 91 (100); CHN-analysis: calcd for $C_{15}H_{18}O_6$ × 1/3 H_2O: C 59.99, H 6.27; found: C 60.37, H 6.21.

***(R,S)*-Benzyl methyl 2-methyl-2-[2-(methoxycarbonyl)ethyl]malonate (5b).** Prepared from benzyl methyl 2-methylmalonate (14.22 g, 64 mmol) and methyl 3-bromopropionate (11.02 g, 66mmol); yield: 13.59 g (69%), colorless viscous liquid; bp 145 °C, 2×10^{-4} mbar; R_f (silica gel; Et_2O/hexane = 1/2) 0.23; *IR (film, cm^{-1}):* 2953, 2846, 1732, 1587, 1498, 1455, 1436, 1380, 1260, 1174, 1110, 1060, 989, 912, 830, 751, 699; *^{1}H-NMR (300MHz, $CDCl_3$, TMS)*: δ(ppm): 1.43 (s, 3H, CH_3), 2.24 (m, 2H, CH_2), 2.32 (m, 2H, CH_2), 3.65 (s, 6H, (2 × OCH_3), 5.16 (s, 2H, CH_2-Ph), 7.33 (m, 5H, 5H-arom.); *MS (EI):*m/z (%): 308 (M^+, 9), 91 (100); CHN-analysis: calcd for $C_{16}H_{20}O_6$: C 62.33, H 6.54; found: C 62.35, H 6.68.

General procedure for preparation of carboxylic acids 6a and 6b
A solution of benzyl methyl 2-alkyl malonate **5a** or **5b** (10.0mmol) in methanol (30 mL) was purged with argon and hydrogenated over 10 weight % of 10% palladium on charcoal at room temperature and normal pressure until the reaction was complete (ca 1 h). The solvent was removed in vacuo to give the product as a colorless viscous oil.

***(R,S)*-2-[2-(Methoxycarbonyl)ethyl] monomethyl malonate (6a).**
Prepared from *(R,S)*-benzyl methyl 2-[2-(methoxycarbonyl)ethyl]malonate (**5a**) (11.77 g, 40 mmol); yield: 8.15 g (100%), colorless viscous liquid; *IR (film, cm^{-1}):* 3519, 3225, 2955, 2602, 1955, 1743, 1437, 1367, 1190, 1096, 1049, 1020, 991, 891, 797; *^{1}H-NMR*

(300MHz, $CDCl_3$, TMS): δ(ppm): 2.26 (q, 2H, *J* = 7.16 Hz, CH_2-CH), 2.46 (t, 2H, *J* = 7.20 Hz, CH_2-CO), 3.54 (t, 1H, *J* = 7.15 Hz, CH), 3.69 (s, 3H, OCH_3), 3.77 (s, 3H, OCH_3), 8.43 (s broad, 1H, COOH); *MS (EI):* m/z (%): 205 (MH^+, 22), 55(100); CHN-analysis: calcd for $C_8H_{12}O_6$: C 47.06, H 5.92; found: C 47.40, H 5.60.

***(R,S)*-2-Methyl-2-[2-(methoxycarbonyl)ethyl] monomethyl malonate (6b).**

Prepared from 20.97 g (68 mmol) *(R,S)*-benzyl methyl 2-methyl-2-[2-(methoxycarbonyl)ethyl]-malonate (**5b**); yield: 14.90 g (100%), colorless viscous oil; *IR (film, cm^{-1}):* 3223, 2999, 2956, 1737, 1439, 1383, 1256, 1118, 992, 940, 880, 785, 670, 688; ^{1}H-NMR (300MHz, $CDCl_3$, *TMS)*: δ(ppm): 1.64 (s, 3H CH_3), 2.22 (m, 2H, CH_2-CH), 2.42 (m, 2H, CH_2COOCH_3), 3.69 (s, 3H, OCH_3), 3.76 (s, 3H, OCH_3), 9.98 (s broad, 1H, COOH); *MS (FAB):* m/z (%): 219 (MH^+, 100); CHN-analysis: calcd for $C_9H_{14}O_6$: C 49.54, H 6.47; found: C 49.67, H 6.48.

***N*-{*(R,S)*-2-[2-(Methoxycarbonyl)ethyl]-monomethylmalonyl}-*D*-alanine benzyl ester (7a).**

Prepared from *(R,S)*-2-[2-(methoxycarbonyl)ethyl] monomethyl malonate (**6a**) (10.21 g, 50 mmol) and D-alanine benzyl ester *p*-toluenesulfonate (17.57 g, 50 mmol) as described for **7b**. A solid product obtained after evaporation of EtOAc was recrystallized from MeOH : Et2O (1/3); *yield*: 15.14 g (83%), white crystals; mp 77 – 79 °C; $[\alpha]_D^{20}$ = +0.03° (c = 0.629, MeOH); *IR (KBr, cm^{-1}):* 3304, 2954, 2252, 2146, 1738, 1651, 1542, 1455, 1197, 750, 698; *^{1}H-NMR (300MHz, $CDCl_3$, TMS)*: δ(ppm): 1.43 (d, 3H, *J* = 4.21 Hz, CH_3-CH), 2.21 (m, 2H, CH_2-CH), 2.40 (t, 2H, *J* = 7.32 Hz, CH_2-CO-), 3.38 (3.37) [t (t), 1H, *J* = 7.30Hz (*J* = 7.22Hz), CH_2-CH], 3.67 (s, 3H, OCH_3), 3.73 (3.72) [s (s), 3H, OCH_3], 4.61 (m, 1H, CH_3-CH), 5.17 (AB-system, 2H, J_{AB} = 12.16 Hz, CH2-Ph), 7.0 (m, 1H, NHCO), 7.33 (m, 5H, H-arom); *MS (FAB)*: m/z (%): 366 (MH^+, 98), 91(100); CHN-analysis: calcd for $C_{18}H_{23}NO_7$: C 59.17, H 6.34, N 3.83; found: C 59.29 H 6.33, N 3.60.

***N*-{*(R,S)*-2-Methyl-2-[2-(methoxycarbonyl)ethyl]-monomethylmalonyl}-*D*-alanine benzyl ester (7b).**

To a stirred solution of *(R,S)*-2-methyl-2-[2-(methoxycarbonyl)ethyl]monomethyl malonate (**6b**) (10.03 g, 46 mmol) and D-alanine benzyl ester *p*-toluene-sulfonate (16.10g, 46mmol) in dry DMF (150 mL) precooled to –15 °C was added on ice bath diphenylphosphoryl azide (DPPA) (9.9 mL, 46 mmol) and subsequently Et_3N (12.8mL, 92mmol). After stirring the reaction mixture for 1 h on an ice bath and then for 72 h at

room temperature, EtOAc (600 mL) was added and the solution was extracted wit 10% citric acid (3 x 75mL). The combined citric acid solutions were reextracted with EtOAc (5x300mL) and the combined organic phases were washed with water (3 x 300 mL), brine (3 x 300 mL), saturated $NaHCO_3$ solution (3 x 300 mL), water (3 x 300 mL) and again with brine (3 x 300mL). The organic solution was dried over Na_2SO_4, filtered and evaporated in vacuo. The resulting viscous yellow liquid was purified by column chromatography on silica gel using CH_2Cl_2 (50/1) as eluent: *yield*: 14.48 g (83%), colorless viscous liquid; $[\alpha]_D^{20}$= +25.57° (c = 0.472, MeOH); *IR (film, cm^{-1})*: 3373, 2953, 1739, 1668, 1525, 1456, 1385, 1248, 1202, 1121, 989, 751, 699; *^{1}H-NMR (300MHz, $CDCl_3$, TMS)*: δ(ppm): 1.41-1.45 (m, 6H, CH_3-CH, CH_3-C), 2.13-2.40 (m, 4H, CH_2-CH_2), 3.64 (3.65) (s, 3H, OCH_3), 3.73 (3.74) (s, 3H, OCH_3), 4.61 (m, 1H, CH_3-CH) 5.11-5.23 [AB-system, 2H, J_{AB} = 12.16 Hz, CH_2-Ph); 5.12 (dd, 1H, J_{AB} = 2.16 Hz, J = 3.54 Hz); 5.16 (d, 1H, J_{AB} = 12.16Hz)], 7.35 (m, 5H, H-arom), 7.36 (m, 1H, NHCO); *MS (FAB):* 380 (MH^+, 63), 91(100); *MS (EI)*: m/z (%): 379 (M^+, 54), 201 (100); *HRMS* calcd for $C_{19}H_{25}NO_7$: 379.163102; found: 379.162013.

_N_-{_(R,S)_-2-Methyl-2-[2-(methoxycarbonyl)ethyl]-monomethylmalonyl}-_D_-alanine (8b).

N-{*(R,S)*-2-Methyl-2-[2-(methoxycarbonyl)ethyl]-monomethylmalonyl}-*D*-alanine benzyl ester (**7b**) (4.0 g, 10.5 mmol) was dissolved in MeOH (100 mL), purged with argon and hydrogenated over 10% Pd/C (800 mg) at room temperature and normal pressure for 0.75 h. The catalyst was filtered off and MeOH was evaporated in vacuo; *yield*: 3.03 g (100%), colorless viscous liquid; $[\alpha]_D^{20}$ = +18.09° (c = 0.365, MeOH); *IR (film, cm^{-1})*: 3371, 2956, 2360, 1738, 1652, 1532, 1456, 1383, 1204, 1123, 988; *^{1}H-NMR (300MHz, $CDCl_3$, TMS)*: δ(ppm): 1.46-1.49 (m, 6H, CH_3-C, CH_3-CH), 2.15-2.27 (m, 2H, CH_2-CH), 2.30-2.39 (m, 2H, CH_2-$COOCH_3$), 3.66 (3.67) [s (s), 3H, OCH_3], 3.76 (3.77) [s (s), 3H, OCH_3], 4.55 [m, 1H, CH_3-CH], 7.56 (m, 1H, NHCO], 8.66 (s broad, 1H, COOH]; *MS (FAB):* m/z (%): 290 (MH^+, 100); *HRMS* calcd for $C_{12}H_{19}NO_7$: 289.116152, found: 289.116532; CHN-analysis: calcd for $C_{12}H_{19}NO_7 \times 0.125H_2O$: C 49.42, H 6.66, N 4.82; found: C 49.27, H 6.79 N 4.59.

_N_-{_(R,S)_-2-[2-(Methoxycarbonyl)ethyl]-monomethylmalonyl}-_D_-alanine (8a).

Prepared from *N*-{*(R,S)*-2-[2-(methoxycarbonyl)ethyl]-monomethylmalonyl}-*D*-alanine benzyl ester (**7a**) (6.02 g, 16.5 mmol) as described for **7b**; *yield*: 4.51g (100%), colorless

viscous liquid; $[\alpha]_D^{20}$=+11.12°(c = 0.666, MeOH); *IR (film, cm^{-1})*: 3344, 2957, 1736, 1659, 1547, 1439, 1376, 1206, 1175, 1052, 1018, 822; *^{1}H-NMR (300MHz, $CDCl_3$, TMS):* δ(ppm): 1.45 (1.46) [d (d), 3H, *J* = 11.37 Hz (*J* = 7.73Hz), CH_3-CH], 2.19 - 2.27 (m, 2H, CH_2-CH), 2.43 (t, 2H, *J* = 7.44 Hz, CH_2-CO-), 3.40 -3.46 (m, 1H, CH_2-CH), 3.68 (s, 3H, OCH_3), 3.75 (s, 3H, OCH_3), 4.57 (m, 1H, CH_3-CH), 5.73 (s broad, 1H, COOH), 7.20 [d (d), 1H, *J* = 7.17 Hz, (*J* = 7.02 Hz), NHCO]; *MS (FAB):* m/z (%): 276 (MH^+, 100); CHN analysis: calcd for $C_{11}H_{17}NO_7$: C 48.00, H 6.23, N 5.09; found: C 47.59, H 6.48, N5.24.

Dimethyl 2-{[((1*S*)-1-{[(benzyloxy)carbonyl]amino}ethyl)amino]carbonyl}pentane-dioate [Z-D-Ala-Ψ (NHCO)-(*R,S*)-Glu(OMe)$_2$] (10a).

To a stirred solution of *N-{(R,S)*-2-[2-(methoxycarbonyl)ethyl]-monomethylmalonyl}-*D*-alanine (**8a**) (10.0 g, 36.4 mmol) in dry THF (110 mL) precooled to –15 °C was added on an ice bath Et_3N (3.68 g, 36.4 mmol) and ethyl chloroformate (3.95 g, 36.4 mmol). After stirring for 1 h at -15°C a solution of NaN_3 (4.73 g, 72.8mmol) in water (36 mL) was added. The mixture was allowed to reach room temperature and after being stirred for 1 h diluted with EtOAc (110 mL) and extracted with saturated $NaHCO_3$ solution (3 × 110 mL), water (3 × 110 mL), and brine (3 × 110 mL). The organic solution was dried over Na_2SO_4, filtered and evaporated in vacuo. The obtained crude azide **9b** was dissolved in toluene (120 mL) and the solution heated until the rearrangement was complete (determined by disappearence of the azide band at 2140cm^{-1} and appearance of the isocyanate band at 2252cm^{-1} in IR spectra), whereupon benzyl alcohol (3.90 mL, 36.4 mmol) was added and the mixture was refluxed for further 20 h. The volatiles were removed in vacuo and the crude product triturated with ether to give a white solid which was recrystallized from EtOH; *yield*: 3.15g (23%); mp 109 – 111 °C; $[\alpha]_D^{20}$=+0.44° (c = 0.585, MeOH); *IR (KBr, cm^{-1})*: 3345, 3027, 2920, 1944, 1741, 1604, 1496, 1454, 1379, 1241, 1178, 1082, 1036, 829, 695; *^{1}H-NMR (300MHz, $CDCl_3$, TMS)*: δ(ppm): 1.49 (d, 3H, *J* = 6.54 Hz, CH_3-CH), 2.17 (m, 2H, CH_2-CH), 2.36 (t, 2H, *J* = 6.98 Hz, CH_2-CH_2 - CO), 3.30 (t, 1H, *J* =7.28 Hz, CH_2-CH- CO), 3.65 (s, 3H, OCH_3), 3.68 (3.69) [s (s), 3H, *J* =3.16 Hz, OCH_3], 5.08 (s, 2H, CH_2-Ph), 5.33 (m, 1H, CH_3-CH), 5.83 (d, 1H, *J* = 5.30 Hz, NHCO), 7.26-7.37 (m, 6H, 5H-arom., NHCO); *MS (FAB):* m/z (%): 381(MH^+, 67), 230 (100): CHN analysis: calcd for $C_{18}H_{24}N_2O_7$: C 56.82, H 6.36, N 7.37; found: C 56.55, H 6.41, N 7.47.

Dimethyl2-{[((1*S*)-1-{[(Benzyloxy)carbonyl]amino}ethyl)amino]carbonyl}-2-methyl pentanedioate [Z-D-Ala- Ψ (NHCO)-(*R,S*)-α-Me-Glu(OMe)$_2$] (10b).

Prepared from *N*-{*(R,S)*-2-methyl-2-[2-(methoxycarbonyl)ethyl]-monomethylmalonyl}-*D*-alanine (8b) (3.00 g, 10.4 mmol) according to the procedure described for the synthesis of 10a; *yield*: 1.47g (36%), colorless viscous oil; $[\alpha]_D^{20} = +1.55°$ (c = 0.510, MeOH); *IR (film, cm^{-1}):* 3027, 2920, 2360, 2253, 1741, 1604, 1496, 1458, 1379, 1081, 1030, 728, 694; *^{1}H-NMR (300MHz, CDCl$_3$, TMS)*: δ(ppm): 1.39 (1.40) [s (s), 3H, CH_3-C], 1.50 (d, 3H, *J* = 5.86 Hz, CH_3-CH), 2.03-2.28 (m, 4H, CH_2-CH_2-CH), 3.64 (3.65) [s (s), 3H, OCH_3], 3.70 (3.71) [s (s), 3H, OCH_3], 5.09 (s, 2H, CH_2-Ph), 5.30 (m, 1H, CH_3-CH), 5.69 (s broad, 1H, NHCO), 7.27-7.35 (m, 5H, H-arom.) 7.51 (s broad, 1H, NHCO); *MS (FAB):* m/z (%): 395 (MH^+, 62), 91 (100); CHN analysis: calcd for $C_{19}H_{26}N_2O_7$: C 57.86, H 6.64, N 7.10; found: C 57.87, H 6.71, N7.50.

Dimethyl 2-({[(1*R*)-1-Aminoethyl]amino}carbonyl)pentanedioate [D-Ala-Ψ(NHCO)- (R,S)-Glu(OMe)$_2$] (11a): Z-D-Ala-Ψ(NHCO)-(*R,S*)-Glu(OMe)$_2$ (10a). (380 mg, 1.00 mmol) was dissolved in MeOH (40 mL) and hydrogenated for 1 h over 10% palladium on charcoal at room temperature and ambient pressure. The solvent was removed in vacuo and the crude product was used immediately for the next reaction step; *yield*: 246 mg (100%); *IR (film, cm^{-1}):* 3268, 2955, 1738, 1654, 1540, 1438, 1377, 1260, 1202, 1163, 991; *^{1}H-NMR (300 MHz, CDCl$_3$, TMS)*: δ(ppm): 1.31 (1.32) [d (d), 3H, *J* = 6.31 Hz (*J* = 6.31 Hz), CH_3-CH], 2.07 (s broad, 2H, NH_2), 2.21 (m, 2H, CH_2-CH_2), 2.40 (m, 2H, CH_2-CH_2), 3.29 (m, 1H, CH-CH_2), 3.67 (s, 3H, OCH_3), 3.74 (s, 3H, OCH_3), 4.87 (m, 1H, CH_3-CH), 6.82 (s broad, 1H, NHCO).

Dimethyl 2-({[(1*R*)-1-aminoethyl]amino}carbonyl)-2-methylpentanedioate [D-ala-Ψ(NHCO)-(*R,S*)-α-Me-Glu(OMe)$_2$] (11b).

Prepared from Z-D-Ala-Ψ(NHCO)-(*R,S*)-α-Me-Glu(OMe)$_2$ (**10b**) (394 mg, 1.00 mmol) following the procedure for the synthesis of **11a**. The crude product was used immediately for the next synthetic step; *yield*: 260mg (100%); *IR (film, cm^{-1}):* 3369, 2954, 2059, 1736, 1654, 1522, 1438, 1379, 1248, 1120, 1031, 990, 907, 871, 832, 800, 760; *^{1}H-NMR (300 MHz, CDCl$_3$, TMS)*: δ (ppm): 1.37 (1.39) [d (d), 3H, *J* = 6.10 Hz (*J* = 6.35 Hz), CH_3-CH], 1.43 (1.44) [s (s), 3H, CH_3-C], 2.10 - 2.38 (m, 4H, CH_2-CH_2), 3.13 (s broad, 2H, NH_2), 3.67 (s, 3H, OCH_3), 3.77 (s, 3H, OCH_3), 4.92 (m, 1H, CH_3-CH), 7.32 (d, 1H, *J* = 5.00 Hz, NHCO).

Dimethyl 2-{[((1*S*,2*R*,*S*)-1-{[(2-methyl-3-oxo-3,4-dihydro-2*H*-1,4-benzoxazin-2-yl)-carbon-yl]amino}ethyl)amino]carbonyl}pentanedioate [(2*R*,*S*)-3,4-dihydro-2-ethyl-3-oxo-2*H*-1,4-benzoxazin-2-carbonyl-*D*-Ala- Ψ *(*NHCO)-*(R,S)*Glu(OMe)$_2$] (3a).

To a stirred solution of *D*-Ala-Ψ(NHCO)-*(R,S)*-Glu(OMe)$_2$ (11a) (1.29 g, 5.26 mmol) and (*S*)-(+)-3,4-dihydro-2-methyl-3-oxo-2*H*-1,4-benzoxazin-2-carboxylic acid [(*S*)-12] (1.09 g, 5.26 mmol) in DMF (50 mL) precooled to -15°C was successively added on an ice bath DPPA (1.45 g, 5.26 mmol) and Et_3N (1.06 g, 10.52 mmol). The mixture was stirred for 1 h on an ice bath and then for 5 days at room temperature whereupon the reaction mixture was diluted with EtOAc (200mL) and extracted with 10% citric acid (3 × 14 mL). The combined citric acid solutions were reextracted with EtOAc (5 x 50 mL) and the combined organic phases were washed with water (3 x 50 mL), brine (3 x 50 mL), saturated $NaHCO_3$ solution (3 x 50 mL), water (3 x 50 mL) and again with brine (3 x 50 mL). The organic solution was dried over Na_2SO_4, filtered and evaporated in vacuo. The crude product was purified by column chromatography on silica gel using EtOAc/hexane (3/1) as eluent; *yield*: 495mg (22%); mp 68 – 73 °C, pale yellow crystals; *IR (KBr, cm^{-1}):* 3317, 2989, 2954, 1741, 1707, 1614, 1502, 1438, 1378, 1228, 1156, 756; *^{1}H-NMR (300 MHz, DMSO-d_6, TMS)*: δ (ppm): 1.057 (1.062) (1.187) (1.192) [d (d) (d) (d), 3H, *J* = 6.51 Hz (*J* =6.56 Hz) (*J* = 6.54 Hz) (*J* =6.56 Hz), CH_3-CH], 1.597 (1.608) (1.614) (1.617) [s (s) (s) (s), 3H, CH_3-C], 1.88 (m, 2H CH_2-CH_2-), 2.25 (m, 2H, CH_2-CH_2), 3.30 (m, 1H, CH-CH_2), 3.60 (m, 6H, 2 × OCH_3), 5.33 (m, 1H, CH_3-CH), 6.85 - 6.97 (m, 4H, 4H-arom.), 8.21 - 8.29 (m, 2H, NHCO), 10.70 (10.72) (10.74) [s, (s) (s), 1H, NH]; *MS (FAB):* m/z (%): 436, (43, MH^+), 230 (100); CHN analysis calcd. for $C_{21}H_{25}N_3O_8$×1/3H_2O: C 54.42, H 5.86, N 9.52; found: C 54.64, H 5.93, N 9.94.

The same product (containing 4 diastereomers as indicated by ^{1}H-NMR) was obtained when **11a** was coupled with (*R*)-(-)-3,4-dihydro-2-methyl-3-oxo-2*H*-1,4-benzoxazin-2-carboxylic acid.

Dimethyl 2-methyl-2-{[((1*S*,2*R*,*S*)-1-{[(2-methyl-3-oxo-3,4-dihydro-2*H*-1,4-benzoxazin-2-yl)carbonyl]amino}ethyl)amino]carbonyl}pentanedioate [(2*R*,*S*)-3,4-dihydro-2-methyl-3-oxo-2*H*-1,4-benzoxazin-2-carbonyl-*D*-Ala-Ψ(NHCO)-*(R,S)*-α-Me-Glu(OMe)$_2$] (3b).

Prepared from D-Ala-Ψ(NHCO)-(*R,S*)-α-Me-Glu(OMe)$_2$ (11b) (0.78 g, 3.0 mmol) and (*S*)-(+)-3,4-dihydro-2-methyl-3-oxo-2*H*-1,4-benzoxazin-2-carboxylic acid [(*S*)-12] (0.62 g, 3.0 mmol) as described for the synthesis of 3a; *yield*: 473mg (35%), pale yellow

crystals; mp 51 – 56 °C; *IR (KBr, cm^{-1}):* 3340, 2989, 2954, 1731, 1708, 1614, 1502, 1438, 1378, 1229, 1122, 755; *^{1}H-NMR (300 MHz, CDCl$_3$, TMS)*: δ (ppm): 0.99 (1.01) [d (d), 3H, *J* = 6.45 Hz (*J* = 6.45 Hz), CH_3], 1.15 - 1.27 (m, 3 H, CH_3), 1.597 (1.60) (1.61) [s (s) (s), 3H, CH_3-C], 1.97 [m, 2H, CH_2-CH_2), 2.19 (m, 2H, CH_2-CH_2), 3.46 (m, 6H, OCH_3), 5.41 (m, 1H, CH_3-CH), 6.95 (m, 4H, 4H-arom.), 7.7 (m, 1H, NHCO), 8.03 (m, 1H, NHCO), 10.7 (s broad, 1H, NH); *MS (FAB):* m/z (%): 450 (MH^+, 35), 73 (100); CHN analysis calcd. for $C_{21}H_{27}N_3O_8$: C 56.10, H 6.05, N 9.35; found: C 55.68, H 6.20, N 9.23.

The same product (containing 4 diastereomers as indicated by ^{1}H-NMR) was obtained when **11b** was coupled with (*R*)-(-)-3,4-dihydro-2-methyl-3-oxo-2*H*-1,4-benzoxazin-2-carboxylic acid.

Compound **3b** (a mixture of 4 diastereomers) was separated by HPLC to give two pure distereomers (**3bI** and **3bII**) as well as an equimolar mixture of enantiomers **3bIII** and **3bIV**. **3bI**: mp 104 – 109 °C; =$[\alpha]_D^{20}$ -31,12° (c = 0.50, MeOH); *^{1}H-NMR (300MHz, DMSOd$_6$, TMS):* δ (ppm): 1.16 (d, 3H, *J* = 6.42 Hz, CH_3-CH), 1.19 (s, 3H, CH_3-C), 1.62 (s, 3H, CH_3-C) 1.89 - 1.94 (m, 2H, CH_2-CH_2), 2.12 - 2.17 (m, 2H, CH_2-CH_2), 3.59 (s, 3H, OCH_3), 3.60 (s, 3H, OCH_3), 5.41 (m, 1H, CH_3-CH), 6.83 -7.04 (m, 4H, 4H-arom.), 7.64 (d, 1H, *J* = 8.14 Hz, NHCO), 8.04 (d, 1H, *J* = 8.24 Hz, NHCO), 10.75 (s broad, 1H, NH). 20**3bII**: mp 108 – 111 °C; $[\alpha]_D^{20}$ =-31,60° (c = 0.50, MeOH); *^{1}H-NMR (300MHz, DMSOd$_6$, TMS):* δ(ppm): 1.16 (d, 3H, *J* = 7.14 Hz, CH_3-CH), 1.17 (s, 3H, CH_3-C), 1.62 (s, 3H, CH_3-C), 1.85 - 2.05 (m, 2H, CH_2-CH_2), 2.07 - 2.25 (m, 2H, CH_2-CH_2), 3.60 (s, 6H, 2 × OCH_3), 5.40 (m, 1H, CH_3-CH), 6.84 - 7.04 (m, 4H, 4H-arom.), 7.67 (d, 1H, *J* = 8.13Hz, NHCO), 8.05 (d, 1H, *J* = 8.20 Hz, NHCO), 10.76 (s broad, 1H, NH). **3bIII** and **3bIV**: mp 54 – 57 °C; *^{1}H-NMR (300 MHz, DMSO-d$_6$, TMS):* δ(ppm): 0.99 (1.0) [d (d), 3H, *J* = 6.48 Hz (*J* = 6.38 Hz), CH_3-CH], 1.25 (1.26) [s (s), 3H, CH_3-C], 1.59 (1.60) [s (s), 3H, CH_3-C], 1.95 - 2.05 (m, 2H, CH_2-CH_2), 2.15 - 2.25 (m, 2H, CH_2CH_2), 3.59 (s, 3H, OCH_3), 3.61 (3.62) [s (s), 3H, OCH_3], 5.41 (m, 1H, CH_3-CH), 6.85 -7.07 (m, 4H, 4H-arom.), 7.75 [d, 1H, *J* = 7.91 Hz, NHCO], 8.00 (8.01) [d (d), 1H, *J* = 8.45 Hz (*J* = 8.35Hz), NHCO], 10.74 (s, broad, 1H, NH).

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