Pergamon

Tetrahedron: Asymmetry, Vol. 8, No. 3, pp. 425–434, 1997

Printed in Great Britain
PII: S0957-4166(96)00535-6
0957-4166/97 $17.00 + 0.00

Pig liver esterase catalyzed hydrolysis of dimethyl and diethyl 2-methyl-2-(*o*-nitrophenoxy)malonates †

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Abstract: Pig liver esterase (PLE) catalyzed hydrolysis of dimethyl and diethyl 2-methyl-2-(*o*-nitrophenoxy)malonates affords (*R*)-monoethyl/monomethyl 2-methyl-2-(*o*-nitrophenoxy)malonates in moderate to good enantiomeric excesses (69–81%). These data indicate that the nitro group may be accommodated in the large hydrophobic pocket of the Jones active-site model of PLE. © 1997 Elsevier Science Ltd. All rights reserved.

Introduction

Pig liver esterase (PLE) has been widely used for the creation of chiral synthons by asymmetric hydrolysis of *meso*- and prochiral diesters.[1] The binding of substrates in the active site of PLE and stereoselective hydrolysis thereof is readily rationalized by the Jones cubic active-site model of PLE for which specificity can be interpreted in terms of substrate interactions with two polar binding sites and two hydrophobic pockets.[2] Whereas the volumes of two polar binding sites and the small hydrophobic pocket (H_S) were confirmed as initially specified, the size of the large hydrophobic pocket (H_L) was amended several times when new specificity data became available.[3–5] The model predicts preferential binding of hydrophobic portions of the substrate into one of the two hydrophobic pockets. Bulkier groups such as aromatic residues bind in the larger H_L binding site. According to the model the H_L site can accommodate less polar heteroatom functions such as halogen, ether or ketal oxygen and silicon atoms, whereas polar groups such as hydroxy, amino, carbonyl, nitro, etc. should be excluded from this area.[2] The stereoselectivity of PLE-catalyzed hydrolysis of a large number of malonate substrates, mainly dimethyl esters, has been well documented.[1a,c] Among dialkyl malonates diethyl esters generally provide worse substrates and afford products of lower enantiomeric purity as compared to the corresponding methyl analogs.[1a,c,6]

We wish to report herein that hitherto unknown (*R*)-monoalkyl 2-methyl-2-(*o*-nitrophenoxy)malonates **3**, which were needed as key intermediates in the course of our synthesis of diastereomerically pure immunomodulatory compounds,[7,8] can be obtained in moderate to good enantiomeric excesses (e.e.) by PLE-catalyzed stereoselective hydrolysis of prochiral dimethyl or diethyl 2-methyl-2-(*o*-nitrophenoxy)malonates **2** in a phosphate buffer (pH 7)/dimethyl sulfoxide (DMSO) (8:2) mixture (Scheme 1). Our results indicate that in the substrates **2** a polar nitro group is tolerated on a phenyl ring, which according to the Jones active-site model[2] is bound into the large hydrophobic pocket of PLE.

Results and discussion

Dialkyl 2-methyl-2-(*o*-nitrophenoxy)malonates **2** were prepared in excellent yields by alkylation of the corresponding *o*-nitrophenols **1** with dialkyl 2-bromo-2-methylmalonates in the presence of potassium fluoride in dimethylformamide (DMF). The PLE-catalyzed hydrolyses were carried out at pH 7.0 and ambient temperature using a high capacity buffer providing a constant pH. After initial experiments on the hydrolysis of diethyl 2-methyl-2-(*o*-nitrophenoxy)malonate **2ab** in a phosphate

† Dedicated to Professor Miha Tišler on the occasion of his 70th birthday.

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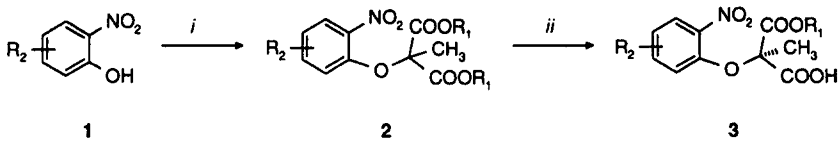


Scheme 1. *i*: $Br(CH_3)C(COOR_1)_2$, KF, DMF, 60°C, 6 h; *ii*: PLE, buffer (pH 7)/DMSO (8:2), room temp., 24 h.

Table 1. PLE-Catalyzed hydrolyses of **2aa–2ga**

Substrate	Product	R_1	R_2	Product Yield (%)	e.e. (%)
2aa	**3aa**	CH_3	H	80	77.2[a]
2ab	**3ab**	C_2H_5	H	66	69.3[a]
2ba	**3ba**	CH_3	4'-CH_3	85	80.6[a]
2bb	**3bb**	C_2H_5	4'-CH_3	69	76.7[a]
2ca	**3ca**	CH_3	5'-CH_3	79	72.4[a]
2cb	**3cb**	C_2H_5	5'-CH_3	59	72.1[a]
2da	**3da**	CH_3	4'-OCH_3	78	80.5[a]
2db	**3db**	C_2H_5	4'-OCH_3	60	72.7[a]
2ea	**3ea**	CH_3	4'-Cl	14	78.6[a]
2eb	**3eb**	C_2H_5	4'-Cl	66	77.8[a]
2fa	**3fa**	CH_3	5'-F	84	69.1[a]
2fb	**3fb**	C_2H_5	5'-F	63	68.5[a]
2ga	**3ga**	CH_3	5'-NO_2	77	79.1[b]

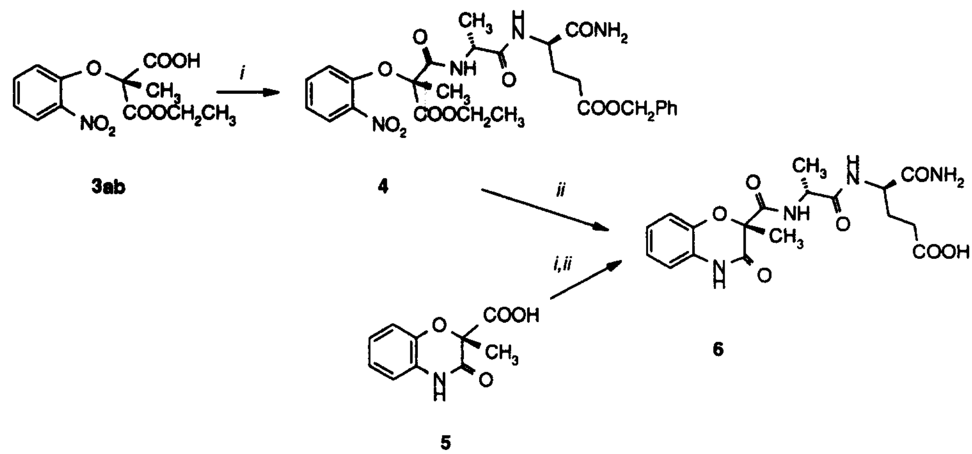
[a]Method A; Integration of signals of 2-CH_3 group at 1.53-1.66 ppm (R_1=CH_3) and 1.57-1.71 ppm (R_1=C_2H_5) in ^{1}H-NMR spectra in the presence of (+)-(*R*)-1-methylbenzylamine. Chemical shift difference between the major and the minor singlet was 0.02-0.03 ppm; [b]Method B; Integration of signals of 2-CH_3 group at 1.89 ppm in ^{1}H-NMR spectrum in the presence of (-)-cinchonidine.

buffer (pH 7) and in phosphate buffer (pH 7)/DMSO mixtures containing various proportions of DMSO, the phosphate buffer (pH 7)/DMSO (8:2) mixture was selected as the optimal system yielding the highest enantiomeric excess of the resulting monoester **3ab**. Monoalkyl 2-methyl-2-(*o*-nitrophenoxy)malonates **3** were obtained in 59–85% yield[9] (Table 1) which could not be raised with prolongation of the reaction time. Thin layer chromatography revealed that even after 7 days substrates **2** were still present in the reaction mixture, probably due to gradual denaturation of the enzyme in a medium containing a high proportion of DMSO.

Determination of enantiomeric excess and of the absolute configuration

The enantiomeric excesses of the acid–ester products **3aa–3fb** were determined by ^{1}H-NMR using (+)-(*R*)-1-methylbenzylamine[10] as a chiral solvating agent (Method A).[11,12] Since determination of enantiomeric excess of **3ga** was not possible by Method A due to overlapping signals, (−)-cinchonidine was used as a chiral solvating agent (Method B) to determine the enantiomeric excess of **3ga**. In both cases the identity of signals used for e.e. determination was unequivocally established using (−)-(*S*)-1-methylbenzylamine and (+)-cinchonine, respectively. The results of PLE-catalyzed hydrolyses of **2** are recorded in Table 1. Dimethyl esters proved to be better substrates of the enzyme as compared to diethyl esters and generally gave better enantiomeric excesses and yields.

The absolute configuration of monoethyl 2-methyl-2-(*o*-nitrophenoxy)malonate **3ab**, the parent product in the ethyl series, was determined (Scheme 2) by its conversion into the L-alanyl-D-



Scheme 2. *i*: HCl·L-Ala-D-iGln(OCH_2Ph), DPPA, Et_3N, DMF; *ii*: H_2, 10% Pd/C, EtOH.

isoglutamine derivative **4** followed by reduction and concomitant cyclization to give (+)-(2*R*)-N-(2-methyl-3-oxo-3,4-dihydro-2*H*-benzo[*b*][1,4]-oxazine-2-carbonyl)-L-alanyl-D-isoglutamine **6** which was obtained also independently[7,8] by the coupling of (+)-(*S*)-2-methyl-3-oxo-3,4-dihydro-2*H*-benzo[*b*][1,4]oxazine-2-carboxylic acid **5**, for which the absolute configuration had been determined previously by X-ray analysis,[13,14] with L-alanyl-D-isoglutamine benzyl ester[15] using diphenylphosphoryl azide[16] as a coupling agent. The absolute configuration of the other structures in the series was deduced from the same pattern of ^{1}H-NMR chemical shift anisochronicity.[17,18a] Thus, monoalkyl malonates **3** possess the *R* absolute configuration which is in accordance with the Jones active-site model predicting hydrolysis of the pro-*S* ester group affording (*R*)-monoalkyl malonates.[2]

Stereoselective hydrolysis of substrates **2** comprising a phenyl ring, which according to the Jones active-site model[2] is bound into the larger hydrophobic pocket of PLE, suggested that a polar nitro group bound to a phenyl ring might be accommodated into H_L of the PLE active-site. Taking into account the dimensions of the molecules of **2** and specification of the model, the nitro group could be accommodated in the hydrophobic pocket or could locate in the area above the model which is postulated to be open and accessible to any substrate moiety which needs to locate there. To investigate this possibility we attempted the PLE-catalyzed hydrolysis of dimethyl 2-methyl-2-(2′,5′-dinitrophenoxy)malonate **2ga** in which according to the Jones active-site model, orientation of one nitro group in the place above the model necessarily requires the second nitro group to be located in the hydrophobic pocket. Interestingly, the PLE-catalyzed hydrolysis of **2ga** under the same conditions afforded (+)-(*R*)-monomethyl 2-methyl-2-(2′,5′-dinitrophenoxy)malonate in ca. 80% e.e.

From these results we conclude that the nitro group, on a bulky hydrophobic portion such as phenyl substituent, can be accommodated in the large hydrophobic pocket of the PLE active-site; such results can give more information for the interpretation and the prediction of the Jones active-site model.

Experimental

Melting points were taken on a Reichert hot stage microscope and are uncorrected. IR spectra were recorded on a Perkin–Elmer 1600 Series FTIR spectrometer as KBr discs for solids and neat films for oils. Optical rotations were measured on a Perkin–Elmer 1241 MC polarimeter. The reported values for specific rotation are average values of ten successive measurements using an integration time of 10 seconds. Elemental analyses were performed at Faculty of Chemistry and Chemical Engineering, University of Ljubljana on a Perkin–Elmer C, H, N-Analyzer 240 C. Mass spectra were obtained on an Autospec Q, VG-Analytical mass spectrometer using EI or FAB ionization. NMR spectra were obtained on a Bruker Avance DPX 300 instrument operating at 300.13 MHz for protons and 75.47 MHz for ^{13}C nuclei with tetramethylsilane as an internal standard. Pig liver esterase suspension in 3.2 M ammonium sulfate solution (activity 130 U/mg protein) was obtained from Fluka. The enzyme used in all experiments was from the same Fluka lot, number 46063. Enantiomeric excess determinations were performed at 298 K in 0.06 M $CDCl_3$ solution using 10–40 molar equivalents of (+)-(*R*)-1-methylbenzylamine (Method A) or 1.5 molar equivalents of (−)-cinchonidine (Method B). The starting compounds *o*-nitrophenols and diethyl 2-bromo-2-methylmalonate were commercially available.

Dimethyl 2-bromo-2-methylmalonate

Bromine (11 g, 68.5 mmol) was added dropwise from a dropping funnel to stirred dimethyl 2-methylmalonate (10 g, 68.5 mmol) in a 500 mL two-neck flask equipped with an efficient condenser. Towards the end of addition a vigorous reaction took place with concomitant discoloration of the reaction mixture. After the addition of bromine was complete, the mixture was stirred for an additional 1 h and distilled *in vacuo* to give dimethyl 2-bromo-2-methylmalonate; yield: 13.71 g (89%), bp 115–117°C/20 Torr; IR (film): ν 2958, 2847, 1747, 1447, 1379, 1234, 1119, 1070, 975, 886 cm^{-1}; ^{1}H-NMR (300 MHz, DMSO-d_6): δ 2.01(s, 3H, CH_3), 3.77(s, 6H, 2×$COOCH_3$) ppm; MS (70 eV, EI): m/z=224 (M^+, 12%), 226 [$(M+2)^+$, 12%], 59 (100%); Anal. Calcd. for $C_6H_9BrO_4$: C 32.02%, H 4.03%. Found: C 32.33%, H 3.94%.

*Diethyl 2-methyl-2-(2′-nitrophenoxy)malonate (**2ab**). General procedure for the synthesis of substrates **2aa–2ga***

Diethyl 2-bromo-2-methylmalonate (22.78 g, 90 mmol) was added to a stirred suspension of potassium fluoride (13.07 g, 0.225 mol) in dry N,N-dimethylformamide (60 mL). After stirring for 15 minutes at room temperature 2-nitrophenol (12.51 g, 90 mmol) was added. The resulting mixture was stirred for 6 hours at 60°C, cooled to room temperature and poured into crushed ice/water (250 g). After dissolution of the ice the aqueous solution was extracted with ether (3×200 mL), the ethereal phase was washed successively with 0.1 N sodium hydroxide (3×50 mL) and 0.1 N hydrochloric acid (3×50 mL), dried over sodium sulfate, filtered and evaporated *in vacuo* to give 23.2 g (83%) of **2ab** as an orange oil; IR (film):ν 2985, 1748, 1605, 1532, 1483, 1361, 1278, 1243, 1116, 1017, 849, 764 cm^{-1}; ^{1}H-NMR (300 MHz, DMSO-d_6): δ 1.19 (t, 6H, J=7.1 Hz, 2×$COOCH_2CH_3$), 1.74 (s, 3H, CH_3), 4.25 (q, 4H, J=7.1 Hz, 2×$COOCH_2CH_3$), 7.23 (d, 1H, J=8.0 Hz, H-6′), 7.29 (dd, 1H, J=8.0 Hz, H-4′), 7.64 (ddd, 1H, J=8.0 Hz, J=1.7 Hz, H-5′), 7.91 (dd, 1H, J=8.0 Hz, J=1.7 Hz, H-3′) ppm; MS (70 eV, EI): m/z=311 (M^+, 15%), 265 (100%); Anal. Calcd. for $C_{14}H_{17}NO_7$: C 54.02%, H 5.50%, N 4.50%. Found: C 53.65%, H 5.80%, N 4.66%.

*Dimethyl 2-methyl-2-(2′-nitrophenoxy)malonate (**2aa**)*

Prepared from 2-nitrophenol (12.51 g, 90 mmol) and dimethyl 2-bromo-2-methylmalonate (20.3 g, 90 mmol); yield: 21.7 g (85%), brownish crystals; mp (°C): 63–66; IR (film): ν 3854, 3743, 1747, 1537, 1289, 1244, 1134 cm^{-1}; ^{1}H-NMR (300 MHz, DMSO-d_6): δ 1.72 (s, 3H, CH_3), 3.77 (s, 6H, 2×$COOCH_3$), 7.16 (dd, 1H, J=8.4 Hz, J=1.0 Hz, H-6′), 7.29 (m, 1H, H-4′), 7.63 (ddd, 1H, J=8.4 Hz, J=7.5 Hz, J=1.7 Hz, H-5′), 8.06 (dd, 1H, J=8.0 Hz, J=2.0 Hz, H-3′) ppm; MS (70 eV, EI): m/z=283 (M^+, 37%), 122 (100%); Anal. Calcd. for $C_{12}H_{13}NO_7$: C 50.89%, H 4.63%, N 4.95%. Found: C 50.88%, H 4.57%, N 4.83%.

*Dimethyl 2-methyl-2-(4′-methyl-2′-nitrophenoxy)malonate (**2ba**)*

Prepared from 4-methyl-2-nitrophenol (13.77 g, 90 mmol) and dimethyl 2-bromo-2-methylmalonate (20.3 g, 90 mmol); yield: 20.0 g (75%), yellow crystals; mp (°C): 85–87; IR (film): ν 3859, 2956, 1750, 1539, 1361, 1249, 1119 cm^{-1}; ^{1}H-NMR (300 MHz, DMSO-d_6): δ 1.67 (s, 3H, 2-CH_3), 2.32 (s, 3H, 4′-CH_3), 3.76 (s, 6H, 2×$COOCH_3$), 7.08 (d, 1H, J=8.5 Hz, H-6′), 7.43 (dd, 1H, J=8.5 Hz, J=1.7 Hz, H-5′), 7.72 (d, 1H, J=1.7Hz, H-3′) ppm; MS (70 eV, EI): m/z=297 (M^+, 15%), 153 (100%); Anal. Calcd. for $C_{13}H_{15}NO_7$: C 52.53%, H 5.09%, N 4.71%. Found: C 52.30%, H 5.04%, N 4.86%.

*Diethyl 2-methyl-2-(4′-methyl-2′-nitrophenoxy)malonate (**2bb**)*

Prepared from 4-methyl-2-nitrophenol (13.77 g, 90 mmol); yield: 25.7 g (88%), orange oil; IR (film): ν 2984, 1745, 1536, 1501, 1446, 1357, 1249, 1116, 1017, 858, 816 cm^{-1}; ^1H-NMR (300 MHz, DMSO-d_6): δ 1.16 (t, 6H, J=7.1 Hz, 2×$COOCH_2CH_3$), 1.65 (s, 3H, 2-CH_3), 2.31 (s, 3H, 4′-CH_3), 4.20 (q, 4 H, J=7.1 Hz, 2×$COOCH_2CH_3$), 7.09 (d, 1H, J=8.5 Hz, H-6′), 7.42 (dd, 1H, J=8.5 Hz, J=2.0 Hz, H-5′), 7.70 (dd, 1H, J=2.0 Hz, J=0.7 Hz, H-3′) ppm; MS (70 eV, EI): m/z=325 (M^+, 24%), 154 (100%); Calcd. for $C_{15}H_{19}NO_7$: 325.116152. Found: 325.117250.

*Dimethyl 2-methyl-2-(5′-methyl-2′-nitrophenoxy)malonate (**2ca**)*

Prepared from 5-methyl-2-nitrophenol (13.77 g, 90 mmol) and dimethyl 2-bromo-2-methylmalonate (20.3 g, 90 mmol); yield: 23.25 g (87%), yellow crystals; mp (°C): 46–48; IR (film): ν 2954, 1746, 1523, 1356, 1233, 1118 cm^{-1}; ^1H-NMR (300 MHz, DMSO-d_6): δ 1.77 (s, 3H, 2-CH_3), 2.35 (s, 3H, 5′-CH_3), 3.77 (s, 6H, 2×$COOCH_3$), 6.98 (m, 1H, H-6′), 7.08–7.12 (m, 1H, H-4′), 7.80 (d, 1H, J=8.3 Hz, H-3′) ppm; MS (70 eV, EI): m/z=297 (M^+, 4%), 251 (100%); Calcd. for $C_{13}H_{15}NO_7$: 297.085850. Found: 297.084852.

*Diethyl 2-methyl-2-(5′-methyl-2′-nitrophenoxy)malonate (**2cb**)*

Prepared from 5-methyl-2-nitrophenol (13.77 g, 90 mmol); yield: 24.9 g (85%), orange oil; IR (film): ν 2984, 1749, 1606, 1525, 1351, 1271, 1112, 1017, 847 cm^{-1}; ^1H-NMR (300 MHz, DMSO-d_6): δ 1.17 (t, 6H, J=7.1 Hz, 2×$COOCH_2CH_3$), 1.71 (s, 3H, 2-CH_3), 2.36 (s, 3H, 5′-CH_3), 4.23 (q, 4 H, J=7.1 Hz, 2×$COOCH_2CH_3$), 7.00 (dd, 1H, J=1.7 Hz, J=0.7 Hz, H-6′), 7.11 (ddd, 1H, J=8.3 Hz, J=1.7 Hz, J=0.7 Hz, H-4′), 7.81 (d, 1H, J=8.3 Hz, H-3′) ppm; MS (FAB): m/z=326 [$(M+1)^+$, 100%]; Calcd. for $C_{15}H_{19}NO_7$: 325.116152. Found: 325.117350.

*Dimethyl 2-methyl-2-(4′-methoxy-2′-nitrophenoxy)malonate (**2da**)*

Prepared from 4-methoxy-2-nitrophenol (15.21 g, 90 mmol) and dimethyl 2-bromo-2-methylmalonate (20.3 g, 90 mmol); yield: 23.9 g (85%), yellow crystals; mp (°C): 33–35; IR (film): ν 2957, 2847, 1750, 1534, 1446, 1355, 1282, 1221, 1119, 1035 cm^{-1}; ^1H-NMR (300 MHz, DMSO-d_6): δ 1.61 (s, 3H, CH_3), 3.75 (s, 6H, 2×$COOCH_3$), 3.80 (s, 3H, OCH_3), 7.17–7.20 (m, 2H, H-6′, H-5′), 7.45–7.46 (m, 1H, H-3′) ppm; MS (70 eV, EI): m/z=313 (M^+, 72%), 169 (100%); Anal. Calcd. for $C_{13}H_{15}NO_8$: C 49.84%, H 4.83%, N 4.47%. Found: C 49.58%, H 4.77%, N 4.31%.

*Diethyl 2-methyl-2-(4′-methoxy-2′-nitrophenoxy)malonate (**2db**)*

Prepared from 4-methoxy-2-nitrophenol (15.21 g, 90 mmol); yield: 26.1 g (85%), orange oil; IR (film): ν 3477, 2986, 1741, 1529, 1458, 1360, 1152, 1022, 844, 799 cm^{-1}; ^1H-NMR (300 MHz, DMSO-d_6): δ 1.18 (t, 6H, J=7.1 Hz, 2×$COOCH_2CH_3$), 1.61 (s, 3H, CH_3), 3.80 (s, 3H, OCH_3), 4.21 (q, 4 H, J=7.1 Hz, 2×$COOCH_2CH_3$), 7.22 (m, 2H, H-5′, H-6′), 7.46–7.48 (m, 1H, H-3′) ppm; MS (70 eV, EI): m/z=341 (M^+, 36%), 169 (100%); Anal. Calcd. for $C_{15}H_{19}NO_8$: C 52.79%, H 5.61%, N 4.10%. Found: C 52.42%, H 5.56%, N 4.47%.

*Dimethyl 2-methyl-2-(4′-chloro-2′-nitrophenoxy)malonate (**2ea**)*

Prepared from 4-chloro-2-nitrophenol (15.57 g, 90 mmol) and dimethyl 2-bromo-2-methylmalonate (20.3 g, 90 mmol); yield: 22.25 g (78%), yellow crystals; mp (°C): 68–71; IR (film): ν 3105, 2955, 1749, 1532, 1360, 1238, 1118, 977, 882 cm^{-1}; ^1H-NMR (300 MHz, DMSO-d_6): 1.71(s, 3H, CH_3), 3.76 (s, 6H, 2×$COOCH_3$), 7.19 (d, 1H, J=9.0 Hz, H-6′), 7.68 (dd, 1H, J=9.0 Hz, J=2.7 Hz, H-5′), 8.08 (d, 1H, J=2.7 Hz, H-3′) ppm; MS (70 eV, EI): m/z=317 (M^+, 5%), 319 [$(M+2)^+$, 2%], 113 (100%); Anal. Calcd. for $C_{12}H_{12}ClNO_7$: C 45.37%, H 3.81%, N 4.41%. Found: C 45.41%, H 3.83%, N 4.16%.

*Diethyl 2-methyl-2-(4′-chloro-2′-nitrophenoxy)malonate (**2eb**)*

Prepared from 4-chloro-2-nitrophenol (15.57 g, 90 mmol); yield: 27.9 g (90%), orange oil; IR (film): ν 2985, 1746, 1604, 1536, 1480, 1446, 1357, 1274, 1116, 1016, 857 cm^{-1}; ^1H-NMR (300 MHz, DMSO-d_6): δ 1.16 (t, 6H, J=7.1 Hz, 2×$COOCH_2CH_3$), 1.70 (s, 3H, CH_3), 4.21 (q, 4 H, J=7.1 Hz, 2×$COOCH_2CH_3$), 7.22 (d, 1H, J=9.0 Hz, H-6′), 7.68 (dd, 1H, J=9.0 Hz, J=2.5 Hz, H-5′), 8.06 (d, 1H, J=2.5 Hz, H-3′) ppm; MS (70 eV, EI): m/z=345 (M^+, 27%), 347 [$(M+2)^+$, 9%], 43 (100%); Anal. Calcd. for $C_{14}H_{16}ClNO_7$: C 48.64%, H 4.66%, N 4.05%. Found: C 48.41%, H 4.69%, N 4.33%.

Dimethyl 2-methyl-2-(5′-fluoro-2′-nitrophenoxy)malonate (2fa)

Prepared from 5-fluoro-2-nitrophenol (14.13 g, 90 mmol) and dimethyl 2-bromo-2-methylmalonate (20.3 g, 90 mmol); yield: 21.6 g (80%), brown crystals; mp (°C): 45–50; IR (film): ν 3102, 2960, 1764, 1743, 1531, 1286, 1148, 1004 cm^{-1}; ^{1}H-NMR (300 MHz, DMSO-d_6): 1.77(s, 3H, CH_3), 3.79 (s, 6H, 2×$COOCH_3$), 7.05 (dd, 1H, J=10.2Hz, J=2.6Hz, H-6′), 7.20 (ddd, 1H, J=9.1 Hz, J=7.8 Hz, J=2.6 Hz, H-4′), 8.06 (dd, 1H, J=9.1 Hz, J=6.0 Hz, H-3′) ppm; MS (70 eV, EI): m/z=301 (M^+, 0.6%), 140 (100%); Anal. Calcd. for $C_{12}H_{12}FNO_7$: C 47.85%, H 4.02%, N 4.65%. Found: C 47.93%, H 4.01%, N 4.53%.

Diethyl 2-methyl-2-(5′-fluoro-2′-nitrophenoxy)malonate (2fb)

Prepared from 5-fluoro-2-nitrophenol (14.13 g, 90 mmol); yield: 21.6 g (73%), orange oil; IR (film): ν 2986, 1748, 1618, 1533, 1448, 1353, 1281, 1115, 1016, 858 cm^{-1}; ^{1}H-NMR (300 MHz, DMSO-d_6): δ 1.20 (t, 6H, J=7.1 Hz, 2×$COOCH_2CH_3$), 1.78 (s, 3H, CH_3), 4.26 (q, 4 H, J=7.1 Hz, 2×$COOCH_2CH_3$), 7.08 (dd, 1H, J=10.2 Hz, J=2.7 Hz, H-6′), 7.20 (dddd, 1H, J=9.0 Hz, J=7.8 Hz, J=2.7 Hz, J=0.7 Hz, H-4′), 8.07 (dd, 1H, J=9.0 Hz, J=5.9 Hz, H-3′) ppm; MS (70 eV, EI): m/z=330 [$(M+1)^+$, 2%], 283 (100%); Due to intensive decarboxylation of this product, elemental analysis could not be obtained.

Dimethyl 2-methyl-2-(2′,5′-dinitrophenoxy)malonate (2ga)

Prepared from 2,5-dinitrophenol (16.56 g, 90 mmol) and dimethyl 2-bromo-2-methylmalonate (20.3 g, 90 mmol); yield: 10.33 g (35%), brown crystals; mp (°C): 59–64; IR (film): ν 3118, 2958, 1750, 1551, 1293, 1267, 1122 cm^{-1}; ^{1}H-NMR (300 MHz, DMSO-d_6): δ 1.79 (s, 3H, CH_3), 3.80 (s, 6H, 2×$COOCH_3$), 7.92 (d, 1H, J=2.2 Hz, H-6′), 8.13 (dd, 1H, J=8.9 Hz, J=2.2 Hz, H-4′), 8.21 (d, 1H, J=8.9 Hz, H-3′) ppm; Anal. Calcd. for $C_{12}H_{12}N_2O_9$: C 43.62%, H 3.76%, N 8.46%. Found: C 43.90%, H 3.66%, N 8.53%.

*(R)-Monoethyl 2-methyl-2-(2′-nitrophenoxy)malonate (**3ab**). General procedure for PLE-catalyzed hydrolyses of **2aa–2ga***

Buffer solution (pH=7, 100 mL) containing 0.3 mL of PLE suspension was added to a solution of diethyl 2-methyl-2-(2′-nitrophenoxy)malonate (**2ab**) (0.50 g, 1.6 mmol)) in DMSO (25 mL). The mixture was stirred for 24 hours at room temperature. During this time the pH remained constant. Then, the reaction mixture was washed with ether (2×15 mL) and made alkaline by addition of saturated aqueous $NaHCO_3$ solution (15 mL). The aqueous phase was acidified with 1H hydrochloric acid, saturated with NaCl and extracted with ether (5×15 mL). The ethereal phase was washed with water (3×15 mL), dried over $MgSO_4$, filtered and evaporated *in vacuo* to afford 0.3 g (66%) of **3ab** as yellow oil; $[\alpha]_D^{20}$=+8.25° (c=0.6, methanol); IR (film): ν 3531, 2987, 1747, 1605, 1531, 1483, 1356, 1279, 1128, 1014, 746 cm^{-1}; ^{1}H-NMR (300 MHz, DMSO-d_6): δ 1.14 (t, 3H, J=7.1 Hz, $COOCH_2CH_3$), 1.69 (s, 3H, CH_3), 4.19 (q, 2H, J=7.1 Hz, $COOCH_2CH_3$), 7.18 (dd, 1H, J=8.5 Hz, J=1.0 Hz, H-6′), 7.22 (ddd, 1H, J=8.0 Hz, J=7.5 Hz, J=1.0 Hz, H-4′), 7.59 (ddd, 1H, J=8.5 Hz, J=7.5 Hz, J=1.7 Hz, H-5′), 7.85 (dd, 1H, J=8.0 Hz, J=1.7 Hz, H-3′) ppm; MS (FAB): m/z=284 [$(M+1)^+$, 100%)]; Calcd. for $C_{12}H_{13}NO_7$: 284.07800. Found: 284.077027.

(R)-Monomethyl 2-methyl-2-(2′-nitrophenoxy)malonate (3aa)

Prepared from dimethyl 2-methyl-2-(2′-nitrophenoxy)malonate (**2aa**) (0.74 g, 2.6 mmol); yield: 0.56 g (80%), brown oil; $[\alpha]_D^{20}$=−2.24° (c=0.7, methanol); IR (film): ν 3539, 2958, 1747, 1529, 1280, 1126 cm^{-1}; ^{1}H-NMR (300 MHz, $CDCl_3$): δ 1.89 (s, 3H, CH_3), 3.83 (s, 3H, $COOCH_3$), 7.09 (dd, 1H, J=8.3 Hz, J=1.1Hz, H-6′), 7.23 (ddd, 1H, J=8.2 Hz, J=7.5 Hz, J=1.1 Hz, H-4′), 7.55 (ddd, 1H, J=8.3 Hz, J=7.5 Hz, J=1.7 Hz, H-5′), 7.99 (dd, 1H, J=8.2 Hz, J=1.7 Hz, H-3′), 7.70 (s, 1H, COOH) ppm; MS (70eV, EI): m/z=269 (M^+, 2%), 123 (100%); Anal. Calcd. for $C_{11}H_{11}NO_7$x0.5H_2O: C 47.48%, H 4.32%, N 5.03%. Found: C 47.72%, H 4.78%, N 4.95%.

(R)-Monomethyl 2-methyl-2-(4′-methyl-2′-nitrophenoxy)malonate (3ba)

Prepared from dimethyl 2-methyl-2-(4′-methyl-2′-nitrophenoxy)malonate (**2ba**) (0.77 g, 2.6 mmol); yield: 0.63 g (85%), brown oil; $[\alpha]_D^{20}$=+1.70° (c=0.66, methanol); IR (film): ν 3299, 2957, 1749, 1533, 1355, 1251, 1125 cm^{-1}; ^{1}H-NMR (300 MHz, $CDCl_3$): δ 1.82 (s, 3H, 2-CH_3), 2.38 (s, 3H, 4′-CH_3), 3.83 (s, 3H, COOCH_3), 7.05 (d, 1H, J=8.5 Hz, H-6′), 7.34 (dd, 1H, J=8.5 Hz, J=1.7 Hz, H-5′), 7.74 (d, 1H, J=1.7 Hz, H-3′), 8.71 (s broad, 1H, COO*H*) ppm; MS (70 eV, EI): m/z=283 (M^+, 65%), 136 (100%); MS (FAB): m/z=284 [$(M+1)^+$, 20%], 163 (100%); Anal. Calcd. for $C_{12}H_{13}NO_7$x0.5H_2O: C 49.31%, H 4.79%, N 4.79%. Found: C 48.96%, H 4.35%, N 4.91%.

(R)-Monoethyl 2-methyl-2-(4′-methyl-2′-nitrophenoxy)malonate (3bb)

Prepared from diethyl 2-methyl-2-(4′-methyl-2′-nitrophenoxy)malonate (**2bb**) (0.52 g, 1.6 mmol); yield: 0.33 g (69%), yellow oil; $[\alpha]_D^{20}$=+8.24° (c=0.65, methanol); IR (film): ν 2986, 1746, 1532, 1355, 1251, 1122, 1014 cm^{-1}; ^{1}H-NMR (300 MHz, DMSO-d_6): δ 1.16 (t, 3H, J=7.1 Hz, $COOCH_2CH_3$), 1.65 (s, 3H, 2-CH_3), 2.32 (s, 3H, 4′-CH_3), 4.20 (q, 2H, J=7.1 Hz, $COOCH_2CH_3$), 7.10 (d, 1H, J=8.6 Hz, H-6′), 7.42 (dd, 1H, J=8.6 Hz, J=1.9 Hz, H-5′), 7.69 (m, 1H, H-3′), 13.87 (s broad, 1H, COOH) ppm; MS (70 eV, EI): m/z=297 (M^+, 21%), 163 (100%); MS (FAB): m/z=298 [$(M+1)^+$, 45%)], 136 (100%); Calcd. for $C_{13}H_{15}NO_7$: 297.084852. Found: 297.085920; Anal. Calcd. for $C_{13}H_{15}NO_7$xH_2O: C 49.53%, H 5.43%, N 4.44%. Found: C 49.34%, H 4.98%, N 4.40%.

(R)-Monomethyl 2-methyl-2-(5′-methyl-2′-nitrophenoxy)malonate (3ca)

Prepared from dimethyl 2-methyl-2-(5′-methyl-2′-nitrophenoxy)malonate (**2ca**) (0.77 g, 2.6 mmol); yield: 0.58 g (79%), yellow oil; $[\alpha]_D^{20}$=−1.38° (c=0.68, methanol); IR (film): ν 3542, 2956, 1747, 1606, 1522, 1350 cm^{-1}; ^{1}H-NMR (300 MHz, $CDCl_3$): δ 1.88 (s, 3H, CH_3), 2.40 (s, 3H, 5′-CH_3), 3.82 (s, 3H, COOCH_3), 6.82 (m, 1H, H-6′), 7.02 (dq, 1H, J=8.4 Hz, J=0.8 Hz, H-4′), 7.95 (d, 1H, J=8.4 Hz, H-3′), 6.44 (s, 1H, COO*H*) ppm; MS (70 eV, EI): m/z=283 (M^+, 0.03%), 73 (100%). Due to intensive decarboxylation of this product, elemental analysis could not be obtained.

(R)-Monoethyl 2-methyl-2-(5′-methyl-2′-nitrophenoxy)malonate (3cb)

Prepared from diethyl 2-methyl-2-(5′-methyl-2′-nitrophenoxy)malonate (**2cb**) (0.52 g, 1.6 mmol); yield: 0.28 g (59%), yellow oil; $[\alpha]_D^{20}$=+7.93° (c=0.64, methanol); IR (film): ν 2986, 1747, 1606, 1524, 1351, 1270, 1125, 1014, 843 cm^{-1}; ^{1}H-NMR (300 MHz, DMSO-d_6): δ 1.16 (t, 3H, J=7.1 Hz, $COOCH_2CH_3$), 1.69 (s, 3H, CH_3), 2.35 (s, 3H, 5′-CH_3), 4.21 (q, 2H, J=7.1 Hz, $COOCH_2CH_3$), 6.99 (s, 1H, H-6′), 7.07 (dd, 1H, J=8.3 Hz, J=0.7 Hz, H-4′), 7.79 (d, 1H, J=8.3 Hz, H-3′), 13.7 (s broad, 1H, COOH) ppm; MS (70 eV, EI): m/z=297 (M^+, 5%), 43 (100%); MS (FAB): m/z=298 [$(M+1)^+$, 41%)], 136 (100%); Calcd. for $C_{13}H_{15}NO_7$: 297.084852. Found: 297.085850.

(R)-Monomethyl 2-methyl-2-(4′-methoxy-2′-nitrophenoxy)malonate (3da)

Prepared from dimethyl 2-methyl-2-(4′-methoxy-2′-nitrophenoxy)malonate (**2da**) (0.81 g, 2.6 mmol); yield: 0.60 g (78%), yellow oil; $[\alpha]_D^{20}$=+3.67° (c=0.71, methanol); IR (film): ν 3532, 2956, 1746, 1535, 1220, 1124 cm^{-1}; ^{1}H-NMR (300 MHz, $CDCl_3$): δ 1.97 (s, 3H, CH_3), 3.78 (s, 3H, COOCH_3), 3.85 (s, 3H, 4′-OCH_3), 7.96 (d, 1H, J=2.2 Hz, H-3′), 8.10 (dd, 1H, J=8.9 Hz, J=2.2 Hz, H-5′), 8.19 (d, 1H, J=8.9 Hz, H-6′), 5.09 (s, 1H, COO*H*) ppm; MS (70 eV, EI): m/z=299 (M^+, 23%), 169 (100%); Anal. Calcd. for $C_{12}H_{13}NO_8$x0.5H_2O: C 46.75%, H 4.54%, N 3.90%. Found: C 46.35%, H 4.62%, N 4.43%.

*(R)-Monoethyl 2-methyl-2-(4′-methoxy-2′-nitrophenoxy)malonate (**3db**)*

Prepared from diethyl 2-methyl-2-(4′-methoxy-2′-nitrophenoxy)malonate (**2db**) (0.54 g, 1.6 mmol); yield: 0.30 g (60%), yellow oil; $[\alpha]_D^{20}$=+13.30° (c=0.52, methanol); IR (film): ν 2984, 1746, 1539, 1497, 1355, 1275, 1120, 1034, 856 cm^{-1}; ^{1}H-NMR (300 MHz, DMSO-d_6): δ 1.17 (t, 3H, J=7.1 Hz, $COOCH_2CH_3$), 1.59 (s, 3H, CH_3), 3.79 (s, 3H, 4′-OCH_3), 4.19 (q, 2H, J=7.1 Hz, $COOCH_2CH_3$), 7.21–7.22 (m, 2H, H-5′, H-6′), 7.44 (dd, 1H, J=2.2 Hz, J=1.1 Hz, H-3′), 13.62 (s broad, 1H, COOH) ppm; MS (70 eV, EI): m/z=313 (M^+, 7%), 169 (100%); MS (FAB): m/z=314 [$(M+1)^+$, 35%)], 152 (100%); Calcd. for $C_{13}H_{15}NO_8$: 313.079767. Found: 313.080760.

*(R)-Monomethyl 2-methyl-2-(4′-chloro-2′-nitrophenoxy)malonate (**3ea**)*

Prepared from dimethyl 2-methyl-2-(4′-chloro-2′-nitrophenoxy)malonate (**2ea**) (0.77 g, 2.4 mmol); yield: 0.11 g (14%), brown oil; IR (film): ν 3512, 2957, 1747, 1535, 1482, 1275, 1124 cm^{-1}; ^{1}H-NMR (300 MHz, $CDCl_3$): 1.86(s, 3H, CH_3), 3.84 (s, 3H, $COOCH_3$), 7.09 (d, 1H, J=9.0 Hz, H-6′), 7.49 (dd, 1H, J=9.0 Hz, J=2.7 Hz, H-5′), 7.94 (d, 1H, J=2.7 Hz, H-3′) ppm; MS (70 eV, EI): m/z=M^+, not present, 63 (100%); Anal. Calcd. for $C_{11}H_{10}ClNO_7$: C 43.51%, H 3.32%, N 4.61%. Found: C 43.21%, H 3.51%, N 4.20%.

*(R)-Monoethyl 2-methyl-2-(4′-chloro-2′-nitrophenoxy)malonate (**3eb**)*

Prepared from diethyl 2-methyl-2-(4′-chloro-2′-nitrophenoxy)malonate (**2eb**) (0.55 g, 1.6 mmol); yield: 0.33 g (66%), yellow oil; $[\alpha]_D^{20}$=+3.44° (c=0.6, methanol); IR (film): ν 2986, 1747, 1538, 1481, 1355, 1247, 1121 cm^{-1}; ^{1}H-NMR (300 MHz, DMSO-d_6): δ 1.17 (t, 3H, J=7.1 Hz, $COOCH_2CH_3$), 1.70 (s, 3H, CH_3), 4.21 (q, 2H, J=7.1 Hz, $COOCH_2CH_3$), 7.23 (d, 1H, J=9.0 Hz, H-6′), 7.69 (dd, 1H, J=9.0 Hz, J=2.7 Hz, H-5′), 8.06 (d, 1H, J=2.7 Hz, H-3′), 13.87 (s broad, 1H, COOH) ppm; MS (70 eV, EI): m/z=317 (M^+, 13%), 319 [$(M+2)^+$, 4%)], 183 (100%); MS (FAB): m/z=318 [$(M+1)^+$, 77%)], 183 (100%); Calcd. for $C_{12}H_{12}ClNO_7$: 317.030230. Found: 317.031230.

*(R)-Monomethyl 2-methyl-2-(5′-fluoro-2′-nitrophenoxy)malonate (**3fa**)*

Prepared from dimethyl 2-methyl-2-(5′-fluoro-2′-nitrophenoxy)malonate (**2fa**) (0.78 g, 2.6 mmol); yield: 0.63 g (84%), brown oil; $[\alpha]_D^{20}$=−9.52° (c=0.56, methanol); IR (film): ν 3542, 3120, 2959, 1748, 1618, 1593, 1530, 1283 cm^{-1}; ^{1}H-NMR (300 MHz, $CDCl_3$): δ 1.90 (s, 3H, CH_3), 3.84 (s, 3H, $COOCH_3$), 6.85–6.94 (m, 1H, H-4′, H-6′), 8.03 (dd, 1H, J=9.1 Hz, J=5.8 Hz, H-3′) ppm; MS (FAB): $(M+1)^+$, not present, 127 (100%); Anal. Calcd. for $C_{11}H_{10}FNO_7x0.25H_2O$: C 45.28%, H 3.43%, N 4.80%. Found: C 45.15%, H 3.90%, N 4.61%.

*(R)-Monoethyl 2-methyl-2-(5′-fluoro-2′-nitrophenoxy)malonate (**3fb**)*

Prepared from diethyl 2-methyl-2-(5′-fluoro-2′-nitrophenoxy)malonate (**2fb**) (0.52 g, 1.6 mmol); yield: 0.30 g (63%), yellow oil; $[\alpha]_D^{20}$=+4.00° (c=0.58, methanol); IR (film): ν 3118, 2987, 1746, 1619, 1593, 1531, 1353, 1283, 1128, 1001, 845 cm^{-1}; ^{1}H-NMR (300 MHz, DMSO-d_6): δ 1.16 (t, 3H, J=7.1 Hz, $COOCH_2CH_3$), 1.74 (s, 3H, CH_3), 4.22 (q, 2H, J=7.1 Hz, $COOCH_2CH_3$), 7.02 (dd, 1H, J=10.4 Hz, J=2.6 Hz, H-6′), 7.16 (ddd, 1H, J=9.0 Hz, J=7.9 Hz, J=2.6 Hz, H-4′), 8.03 (dd, 1H, J=9.0 Hz, J=6.0 Hz, H-3′); 13.50 (s broad, 1H, COOH) ppm; MS (FAB): m/z=302 [$(M+1)^+$, 77%)], 140 (100%); Calcd. for $C_{12}H_{12}FNO_7$: 301.05883. Found: 301.05978.

*(R)-Monomethyl 2-methyl-2-(2′,5′-dinitrophenoxy)malonate (**3ga**)*

Prepared from dimethyl 2-methyl-2-(2′,5′-dinitrophenoxy)malonate (**2ga**) (0.85 g, 2.6 mmol); yield: 0.63 g (77%), brown oil; $[\alpha]_D^{20}$=−14.34° (c=0.67, methanol); IR (film): ν 3539, 2958, 1747, 1529, 1280, 1126 cm^{-1}; ^{1}H-NMR (300 MHz, $CDCl_3$): δ 1.97 (s, 3H, CH_3), 3.78 (s, 3H, $COOCH_3$), 7.96 (d, 1H, J=2.2 Hz, H-6′), 8.10 (dd, 1H, J=8.9 Hz, J=2.2 Hz, H-4′), 8.19 (d, 1H, J=8.9 Hz, H-3′) ppm; MS (70 eV, EI): m/z=M^+, no signal, 59 (100%); Anal. Calcd. for $C_{11}H_{10}N_2O_9xH_2O$: C 39.76%, H 3.61%, N 8.43%. Found: C 40.21%, H 3.32%, N 7.89%.

*(2R)-Benzyl-N-[2-ethoxycarbonyl-2-(2′-nitrophenoxy)propionyl]-L-alanyl-D-isoglutaminate (**4**)*

To a stirred solution of benzyl L-alanyl-D-isoglutaminate hydrochloride (687 mg, 2 mmol) and (*R*)-monoethyl 2-methyl-2-(2′-nitrophenoxy)malonate (**3ab**) (566 mg, 2 mmol) in dry N,N-dimethylformamide (9 mL) diphenylphosphoryl azide (550 mg, 2 mmol) was added at 0–5°C, followed by the addition of triethylamine (0.56 mL, 4 mmol). Stirring was continued for 1 h on ice bath and then for 60 h at room temperature. Ethyl acetate (40 mL) was added and the mixture was extracted with 10% citric acid (3×15 mL). The combined citric acid phases were reextracted with ethyl acetate (5×25 mL). The combined ethyl acetate phases were successively washed with water (3×20 mL), brine (3×20 mL), saturated $NaHCO_3$ solution (3×20 mL), water (3×20 mL) and brine (3×20 mL). The solution was dried over $MgSO_4$, filtered and evaporated *in vacuo* to give 0.51 g (50%) of **4** as oil; IR (film): ν 3382, 2983, 2940, 1738, 1665, 1605, 1529, 1451, 1348, 1244, 1168, 1124, 1048, 748 cm^{-1}; ^{1}H-NMR (300 MHz, DMSO-d_6): δ 1.09 (t, 3H, J=7.1 Hz, $COOCH_2$*CH_3*), 1.39 (d, 3H, J=6.6 Hz, CH_3-Ala), 1.72 (s, 3H, CH_3), 1.77–1.88 (m, 1H, CH_2-βiGln), 1.99–2.11 (m, 1H, CH_2-βiGln), 2.39 (t, 2H, J=7.8 Hz, CH_2-γiGln), 4.17 (q, 2H, J=7.1 Hz, COO*CH_2*CH_3), 4.22–4.30 (m, 1H, CH-iGln), 4.35–4.45 (m, 1H, CH-Ala), 5.10 (s, 2H, *CH_2*Ph), 7.11 (d, 1H, J=8.3 Hz, H-6′), 7.17 (s, 1H, $CONH_2$), 7.26–7.41 (m, 7H, 5H-Ar, H-4′, $CONH_2$), 7.67 (dd, 1H, J=8.3 Hz, J=7.2 Hz, H-5′) 8.02 (d, J=8.1 Hz, iGln-CONH), 8.17 (d, 1H, J=7.3 Hz, 3′-H), 8.23 (d, 1H, J=8.1 Hz, Ala-CONH); MS (FAB): m/z=573 [$(M+1)^+$, 18%)], 91 (100%); Calcd. for $C_{27}H_{32}N_4O_{10}xH_2O$: C 54.91%, H 5.80%, N 9.49%. Found: C 55.22%, H 5.76%, N 9.07%.

*(2R)-(+)-N-[2-Methyl-3-oxo-3,4-dihydro-2H-benzo[b][1,4]oxazine-2-carbonyl]-L-alanyl-D-isoglutamine (**6**)*

A solution of **4** (0.3 g, 0.52 mmol) in ethanol (15 mL) was hydrogenated over 10% palladium on charcoal (30 mg) at normal pressure for 1 hour. After removal of the catalyst, evaporation of solvent and trituration with ether, the title product was obtained in the form of white foam; yield: 153 mg (72%); $[\alpha]_D^{20}$=+21.1° (c=1.15, tetrahydrofuran); IR (film): ν 3334, 2979, 1669, 1501, 1449, 1379, 1308, 1229, 1171, 1133, 757 cm^{-1}; ^{1}H-NMR (300 MHz, DMSO-d_6): δ 1.05 (d, 3H, J=7.1 Hz, CH_3-Ala), 1.65 (s, 3H, CH_3), 1.68–1.75 (m, 1H, CH_2-βiGln), 1.89–2.00 (m, 1H, CH_2-βiGln), 2.17 (t, 2H, J=7.8 Hz, CH_2-γiGln), 4.10–4.21 (m, 2H, CH-iGln, CH-Ala), 6.86–7.10 (m, 4H, 4H-Ar), 7.13 (s, 1H, $CONH_2$), 7.33 (s, 1H, $CONH_2$), 7.98 (d, 1H, J=8.3 Hz, iGln-CONH), 8.12 (d, 1H, J=7.3 Hz, Ala-CONH), 10.76 (s, 1H, NH); MS (FAB): m/z=407 [$(M+1)^+$, 87%)], 162 (100%); Calcd. for $C_{18}H_{22}N_4O_7x0.4\ H_2O$: C 52.27%, H 5.56%, N 13.55%. Found: C 52.76%, H 5.68%, N 13.06%.

Acknowledgements

This work was supported financially by the Ministry of Science and Technology of the Republic of Slovenia. The authors wish to thank the Faculty of Chemistry and Chemical Engineering at the University of Ljubljana for microanalyses and J. Stefan Institute for mass spectra.

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(Received in UK 18 November 1996)