SYNTHESIS OF PROLINE ANALOGUES OF ANTINEOPLASTIC COMPOUNDS AS PRODRUGS FOR ACTIVATION BY PROLIDASE

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Abstract: The synthesis of proline analogues of chlorambucil [**II**], melphalan [**VI**] and anthraquinone–2–carboxylic acid [**VIII**] have been performed. The structures of the novel compounds were confirmed by elemental and spectral analyses. One of the compounds, proline analogue of melphalan [**VI**], was found as very good prolidase substrate with susceptibility over 2 fold higher compared to standard, endogenous its substrate – Gly–L–Pro.

Keywords: synthesis, prolidase, proline analogues.

Prolidase [E.C. 3.4.13.9] is a cytosolic exopeptidase widely distributed in man and animals (4,7). The enzyme cleaves imidodi– and imidotripeptides with C–terminal proline or hydroxyproline (5). Prolidase from various sources hydrolyze dipeptides in which the C–terminal aminoacid proline or hydroxyproline is linked through its tertiary nitrogen to the carbonyl of an amino acid residue bearing a free α-amino (5). However, when the amino group in imidodipeptides is replaced by methionyl group or haloacetylprolines, good substrates result, suggesting that α-amino group is not an absolute specificity requirement for prolidase (6). It creates possibility to conjugate drug with proline as a prodrug susceptible to the action of prolidase. The presence of prolidase in the cytoplasm allow to suspect that it may be targeted as a prodrug converting enzyme. This strategy should be of benefit in case of antineoplastic prodrugs, since at least some neoplastic tissues evoke increased prolidase activity in comparison to control tisssues (2,3). In such a case the release of drug from prodrug would be more efficient in neoplastic tissues than in normal tissues.

RESULTS AND DISCUSSION

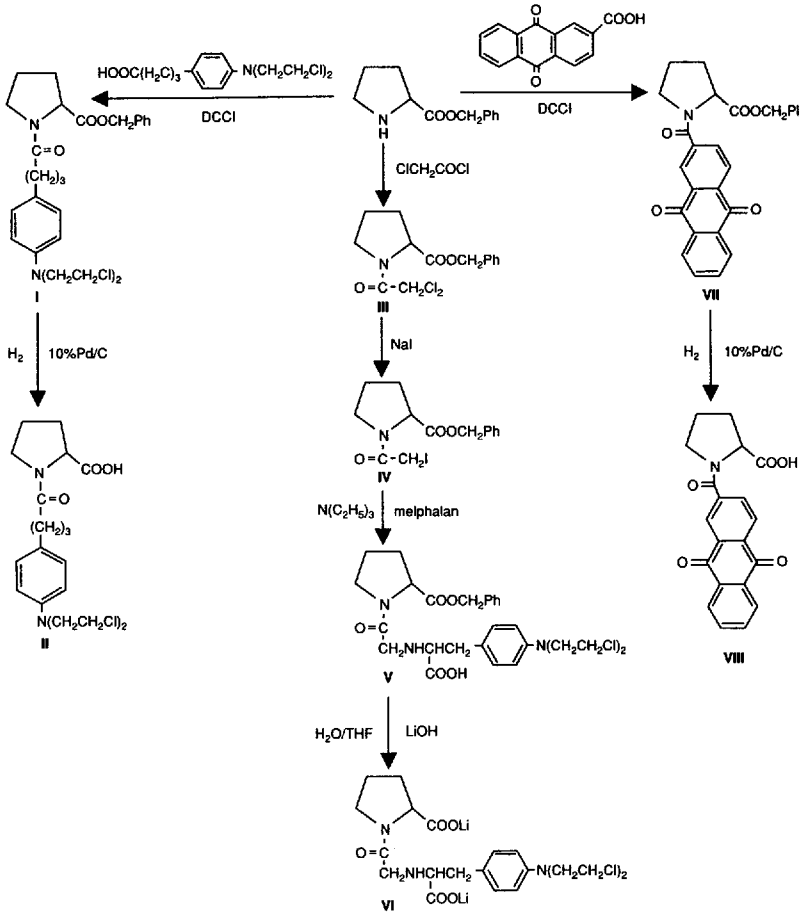
Results of chemical experiments are shown in Scheme 1. Compounds **II** and **VIII** were synthesized by using the carbodiimide coupling method. The protecting benzyl group was removed by catalytic hydrogenation at room temperature and atmospheric pressure gave the desired compounds. The compound **VI** was obtained in four steps from starting material L–proline benzyl ester. The reaction of L–proline benzyl ester and chloroacetyl chloride in methylene chloride at room temperature afforded (2S)–1–(Chloroacetyl)–2–(benzyloxycarbonyl)pyrrolidine [**III**]. The N–(chloroacetyl)proline derivative [**III**] was converted to its α-iodo derivative [**IV**]. This material was immediately reacted with melphalan to give N–[[[[(S)–benzyloxycarbonyl]pyrrolidin–1–yl]carbonyl]methyl]–4–[bis(2–chloroethyl)amino]–2–phenylalanine [**V**]. The mild experimental saponification afforded compound **VI**. The compound **VI** was isolated as a dilithium salt. The structures of the novel compounds were confirmed by elemental and spectral analyses.

The compounds **II**, **V**, **VI** and **VII** were used as a substrate for prolidase activity assays. The activity of prolidase was determined according to the method of Myara et al. (8) which is based on measurement of proline by Chinard's reagent (1). One of the compounds, proline analogue of melphalan [**VI**], was a very good prolidase substrate with susceptibility over 2 fold higher compared to standard, endogenous its substrate – Gly–L–Pro. In such a case proline analogue of melphalan could serve as a donor of proline for collagen synthesis and melphalan as antimitotic agent. The finding that proline analogue of melphalan evokes susceptibility to the action of prolidase creates possibility for its application in pharmacotherapy of neoplastic diseases. Simultaneously, the neoplastic tissue evokes high collagenolytic activity and decreased collagen biosynthesis (2,3). In such a case proline analogue of melphalan could serve as a donor of proline for collagen synthesis and melphalan as antimitotic agent. Compounds **II**, **VI** and **VIII** will be submitted to a pharmacological investigation and its results will be published elsewhere.

EXPERIMENTAL

Melting points were determined on Buchi 535 melting–point apparatus and are uncorrected. ^{1}H NMR (200 MHz) and 13C NMR (150 MHz) spectra were recorded on a Bruker AC 200F spectrometer, using TMS as an internal standard. Thin–layer chromatography was performed on Silica Gel 60 F_{254} (Merck) and visualized with UV or with iodine vapour. Chromatographic purification on silica gel (Merck, grade 60, 240–400 mesh) was done by flash or gravity methods. N–[4–[4–(N,N–bis(2–chloroethyl)amino)phenyl]butyryl]–L–proline benzyl ester [**I**].

L–proline benzyl ester hydrochloride (1.5 g, 6.2 mmol) was dissolved in 20 ml of chloroform. To this stirred solution triethylamine (0.86 cm^3, 6.2 mmol) was added. The solution was cooled to 0°C before adding chlorambucil (1.89 g, 6.2 mmol). This provided a clear



Scheme 1. Synthesis of proline analogues of antineoplastic compounds as prodrugs for activition by prolidase.

solution into which N,N'–dicyclohexylcarbodiimide (1.28 g, 6.2 mmol) was added all at once. It was stirred at 0°C for 2 hours, and then warmed to room temperature where it was kept for 24 hours. The precipitate of dicyclohexylurea was removed by filtration. Concentration under vacuum gave a colorless solid which crystallized from methanol to give **I** (2.1 g, 76%). M.p. 85°C.

^{1}H NMR ($CDCl_3$): 7.28–7.18 (m, 5H, Ph), 7.06 (m, 2H, Ar), 6.61 (m, 2H, Ar), 5.22 (m, 2H, $PhCH_2$), 4.38 (m, 1H, α-CH), 3.48 (m, 2H, δ-CH), 3.64 (t, 4H, NCH_2), 3.56 (t, 4H, CH_2Cl), 2.97 (m, 2H, CH_2CO), 2.64 (t, 2H, CH_2), 2.21 (q, 2H, CH_2), 1.82–1.68 (m, 4H, β- and γ- CH_2).

^{13}C NMR ($CDCl_3$): 172.0 (NCO), 169.0 (COO), 139.6, 139.2, 136.9, 136.7, 131.1, 130.7, 128.6, 126.1 (Ar), 67.3 ($PhCH_2$), 50.4 (α-C proline), 48.6 (NCH_2), 47.1 (δ-CH proline), 42.4 (CH_2Cl), 34.9 (CH_2), 28.9 (CH_2), 27.8 (β-C proline), 27.4 (CH_2), 23.5 (γ-CH proline).

N–[4–[4–(N,N–bis(2–chloroethyl)amino)phenyl] butyryl]–L–proline [**II**].

Compound **I** (1.7 g, 3.8 mmol) was dissolved in 21 ml of anhydrous ethanol, and 0.1g of 10%Pd/C was added under a blanket of N_2. The mixture was stirred vigorously at room temperature for 6 hours, at which time tlc analysis indicated complete removal of benzyl group. The catalyst was filtered through a bed of Celite and washed with ethanol (4x15 cm^3). Concentration under vacuum gave an oil which was chromatographed on a silica gel column using methanol/methylene chloride (8:2) as the eluant. The desired fractions were combined and concentrated to give **II** (1.1 g, 74%). M.p. 230.8°C.

^{1}H NMR ($CDCl_3$): 9.58 (s, 1H, COOH), 7.12 (m, 2H, Ar), 6.65 (m, 2H, Ar), 4.36 (m, 1H, α-CH), 3.48 (m, 2H, δ-CH), 3.64 (t, 4H, NCH_2), 3.56 (t, 4H, CH_2Cl), 2.92 (m, 2H, CH_2), 2.64 (t, 2H, CH_2), 2.21 (q, 2H, CH_2), 1.82–1.69 (m, 4H, β- and γ- CH_2).

^{13}C NMR ($CDCl_3$): 176.6 (COOH), 172.0 (NCO), 136.9, 136.3, 129.3, 128.9 (Ar), 50.4 (α-C proline), 48.7 (NCH2), 47.2 (δ-CH proline), 41.9 (CH_2Cl), 35.0 (CH_2), 28.9 (CH_2), 27.7 (β-C proline), 27.4 (CH_2), 23.4 (γ-CH proline).

$C_{19}H_{26}N_2O_3Cl_2$ (401.3)	C%	H%	N%
Calculated:	56.82	6.48	6.98
Found:	56.93	6.29	6.85

(2S)–1–(Chloroacetyl)–2–(benzyloxycarbonyl)pyrrolidine [**III**].

L–proline benzyl ester hydrochloride (1.9 g, 8.0 mmol) was dissolved in methylene chloride (22 ml). The solution was cooled to –30°C, after which NEt (1.22 ml, 8.0 mmol) was added slowly followed by the dropwise addition of chloroacetyl chloride (0.8 ml, 10 mmol) as a solution in 3.6 ml of CH_2Cl_2. After 4 h at

room temperature, 25 ml of 10% citric acid was added, and the layers were separated. The organic layer was washed with 25 ml each of 1M $NaHCO_3$ and saturated NaCl. The organic layer was dried over $MgSO_4$ and then concentrated to an oil, which was purified by chromatography on silica gel with EtOAc/hexane (1:1) as the eluant. The purified oil was obtained in a yield of 1.8 g (82%).

^{1}H NMR (DMSO–d_6): 7.26–7.18 (m, 5H, Ph), 5.22 (m, 2H, $PhCH_2$), 4.28 (m, 1H, α-CH), 3.86–3.94 (m, 2H, CH_2Cl), 3.50–3.40 (m, 2H, δ-CH), 1.82–1.68 (m, 4H, β- and γ- CH_2).

^{13}C NMR (DMSO–d_6): 169.2 (COO), 165.1 (CO), 139.2, 128.6, 128.3, 127.9 (Ar), 67.2 ($PhCH_2$), 50.4 (α-C proline), 47.1 (δ-CH proline), 42.4 (CH_2Cl), 27.7 (β-C proline), 23.2 (γ-CH proline).

N–[[[[(S)–carboxy]pyrrolidin–1–yl]carbonyl]methyl]–4–[bis(2–chloroethyl)amino]–2–phenyl–alanine [**VI**].

Compound **III** (1.65 g, 5.86 mmol) was dissolved in 10 ml of acetone, and NaI (0.87 g, 5.86 mmol) was added all at once to this solution. This solution was refluxed for 15 min after which the precipitate was removed by filtration and the golden–colored filtrate was concentrated under vacuum to give (2S)–1–(iodoacetyl)–2–(benzyloxcarbonyl)pyrrolidine [**IV**] as an oil (2.0 g, 94%). This material was dissolved in 8 ml DMF, and the solution was added dropwise to a solution of melphalan (1.64g, 5.4 mmol) in 8 ml DMF. The reaction mixture was stirred for 18 h at room temperature before the solution was concentrated under vacuum to a golden solid. The crude solid was purified by silica gel chromatography with CH_2Cl_2/MeOH (10:1) as the eluant. This provided 0.4 g (28%) of compound **V**. The compound **V** (0.4 g, 1.50 mmol) was dissolved in 3 ml of THF and 3 ml 1M LiOH. The reaction mixture was stirred for 12 h at room temperature before the solution was concentrated under vacuum to a white solid. The solid was washed with acetone twice and crystallized from 80% ethanol to give **VI** (0.56 g, 80%). The compound **VI** was isolated as a dilithium salt. M.p. 220°–221°C.

^{1}H NMR (DMSO–d_6): 7.08 (m, 2H, Ar), 6.60 (m, 2H, Ar), 5.32 (m, 2H, CH_2Ph), 4.45 (m, 1H, α-CH), 4.38 (m, 1H, α-CH), 3.70 (m, 4H, NCH_2), 3.64 (m, 4H, CH_2Cl), 3.49 (m, 2H, δ-CH_2), 3.05 (m, CH_2), 1.82–1.68 (m, 4H, β- and γ- CH_2).

^{13}C NMR (DMSO–d_6): 175.0 (COO), 172.1 (COO), 165.0 (NCO), 139.6, 136.9, 136.7, 131.1 (Ar), 62.5 ($PhCH_2$), 57.3 (CH), 50.5 (α-C proline), 48.6 (NCH_2), 47.1 (δ-CH proline), 42.4 (CH_2Cl), 34.97 (CH_2), 27.7 (β-C proline), 23.1 (γ-CH proline).

$C_{20}H_{25}N_3O_5Cl_2Li_2$ (472.2)	C%	H%	N%
Calculated:	50.83	5.29	8.89
Found:	50.73	5.2	18.75

Preparation of N–(anthraquinone–2–carbonyl)–L–proline benzyl ester [**VII**].

L–proline benzyl ester hydrochloride (0.75 g, 3.1 mmol) was dissolved in 10 ml of chloroform. To this stirred solution triethylamine (0.43 ml, 3.1 mmol) was added. The solution was cooled to 0°C before adding anthraquinone–2–carboxylic acid (0.78 g, 3.1 mmol). This provided a clear solution into which N,N'–dicyclohexylcarbodiimide (0.64 g, 3.1 mmol) was added all at once. It was stirred at 0°C for 2.5 hours, and then warmed to room temperature where it was kept for 24 hours. The precipitate of dicyclohexylurea was removed by filtration. Concentration under vacuum gave a colorless solid which crystallized from methanol to give **VII** (1.26 g, 95 %). M.p. 125°C.

^{1}H NMR (DMSO–d_6): 7.95–8.65 (m, 7H, anthraquinone), 7.38–7.18 (m, 5H, Ph), 5.22 (m, 2H, $PhCH_2$), 4.41 (m, 1H, α-CH), 3.50 (m, 2H, δ-CH2), 1.82–1.64 (m, 4H, β- and - CH_2)

^{13}C NMR (DMSO–d_6): 181.9, 181.8 (CO anthraquinone), 169.0 (COO), 159.3 (NCO), 135.4, 134.7, 134.6, 134.3, 133.2, 132.9 (anthraquinone), 130.3, 130.1, 129.6, 129.1 (Ph), 127.3, 127.3, 126.8 (anthraquinone), 67.2 ($PhCH_2$), 50.4 (α-C proline), 47.1 (δ-CH proline), 27.8 (β-C proline), 23.5 (γ-CH proline).

Preparation of N–(anthraquinone–2–carbonyl)–L–proline [**VIII**].

Compound **VII** (1.26 g, 2.87 mmol) was dissolved in 20 ml of chloroform, and 0,1 g of 10%Pd/C was added under a blanket of N_2. The mixture was stirred vigorously at room temperature for 5 hours, at which time tlc analysis indicated complete removal of the benzyl group. The catalyst was filtered through a bed of Celite and washed with ethanol (4x15 ml). Concentration under vacuum gave an oil which was chromatographed on a silica gel column using methanol/methylene chloride (7:3) as a eluant. The desired fractions were combined and concentrated to give **VIII** (0.82 g, 80 %). M.p. 152°C.

^{1}H NMR (DMSO – d_6): 9.58 (s, 1H, COOH), 7.95–8.65 (m, 7H, anthraquinone), 4.41 (m, 1H, α-CH), 3.48 (m, 2H, δ-CH_2), 1.82–1.68 (m, 4H, β- and γ- CH_2)

^{13}C NMR (DMSO–d_6): 182.0, 181.8 (CO anthraquinone), 169.0 (COO), 159.3 (NCO), 135.7, 134.7, 134.6, 134.3, 133.1, 132.9, 127.3, 127.3, 126.8 (anthraquinone), 50.4 (α-C proline), 47.1 (δ-CH proline), 27.7 (β-C proline), 23.3 (γ-CH proline).

$C_{20}H_{15}NO_5$ (349.34)	C%	H%	N%
Calculated:	68.70	4.29	4.01
Found:	68.45	4.18	3.95

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