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SYNTHESIS AND CYTOSTATIC ACTIVITY OF N-(CHLOROETHYLAMINOETHYL)-4-CHLORO-L-PROLINE ETHYL ESTER

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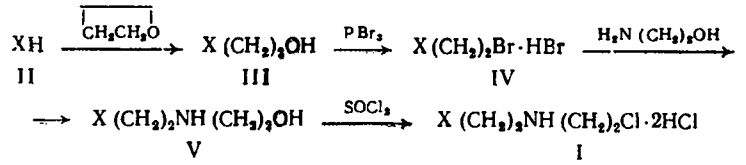
We describe here the preparation of N-(2-chloroethylaminoethyl)-4-chloro-L-proline ethyl ester (I), and the results of experimental studies of its cytostatic and antitumor activity.

We have developed a four-stage route for the preparation of I, based on the introduction of the β-hydroxyethyl substituent into the 4-chloro-L-proline ethyl ester molecule (II), followed by attachment of the alkyl chain by treatment of the hydroxyester (III) with PBr_3 and of the N-β-bromethylproline derivative (IV) with ethanolamine, followed by reaction of the hydroxyaminoester (V) with thionyl chloride.

SCHEME 1

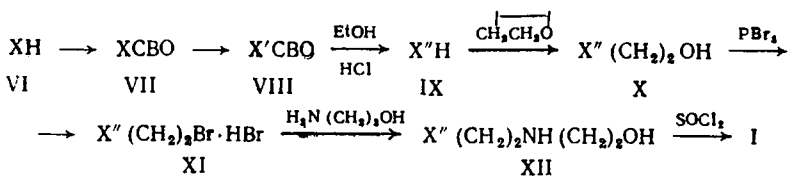


X = 2-ethoxycarbonyl-4-chloropyrrolidin-1-yl

The yields obtained using this method did not however exceed 15–20%. It was therefore used only for analytical purposes, to obtain a chemically pure sample of I.

In a search for the best method for the synthesis of I, two new methods were examined in turn:

SCHEME 2



VI, VII; X = 2-carboxy-4-hydroxypyrrolidiny-1-yl; VIII: X' = 2-carboxy-4-tosyloxypyrrolidin-1-yl; IX, XII: X" = 2-ethoxycarbonyl-4-tosyloxypyrrolidin-1-yl; CBO is carbobenzoxy.

*Deceased.

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SCHEME 3

$$VI \xrightarrow[SOCl_2]{EtOH} II \xrightarrow{ClSiMe_3} [XSiMe_3] \xrightarrow{XIII} I$$

X = 2-Ethoxycarbonyl-4-chloropyrrolidin-1-yl

These methods required the use of different protecting groups. In the second route (Scheme 2), carbobenzoxy- and tosyl protecting groups are introduced into L-hydroxyproline (compounds VII-IX) asdescribed in [5, 6], and the subsequent conversion of the hydroxyester (X) into I was affected by the methods used previously in the first route. Yields of I were 45-50%.

The third method of synthesis (Scheme 3), namely reaction of di-(2-chloroethyl)amine (XIII) with the silyl derivative of II, involves only two steps, and affords high yields of the required product (65-70%). The purity of I was checked by chromatography .

Examination of the cytostatic activity of I in suspended cultures of tumor cells, and of antitumor activity *in vitro*, showed this compound to possess moderatecytostatic activity, but no antitumor activity in transplanted tumors in animals.

EXPERIMENTAL (CHEMISTRY)

The compounds prepared were identified by TLC on Silufol UV-254 plates in three solvent systems: 1) butanol—acetic acid—water, 4:0.3:1 (R_f), 2) propanol—0.2 N ammonia, 3:1 (R_f'), and 3) chloroform—methanol, 8:1 (R_f''). The mass spectrum of I was obtained on an MAT-311A instrument (West Germany) under standard conditions [2]. Fragment ions were identified in the mass sepectrum corresponding to structural fragments in I: (m/z): 246 $[M - HCl]^+$, 233$[M - CH_2Cl]^+$, 209 $[M - COOC_2H_5]^+$, 182 $[M - CH_2{=}CHCOOC_2H_5]^+$, 168 $[ClCH_2CH_2)_2N{=}C{=}CH_2]^+$ 141$[CH_2CH_2NHCH_2CH_2Cl]^+$, 115 $[C_5H_9NO_2]$, 68 $[C_4H_6N]^+$, 49 $[CH_2Cl]^+$, 38 $[H^{37}Cl]$, 36$[^{35}ClH]$.

N-(Chloroethylaminoethyl)-4-chloro-L-proline Ethyl Ester Dihydrochloride (I). Method 1.

N-(Hydroxyethyl)-4-chloro-L-proline Ethyl Ester (III). To 0.8 g (4.5 mmoles) of II, obtained as described in [3], in 2 ml of methanol and 0.5 ml of water was added 0.23 ml (4.5 mmoles) of ethylene oxide. The mixture was kept at room temperature for 72 h. When the reaction was complete, the solvent was distilled off under reduced pressure at 50-60°C. The residue, a mobile yellow oil, was dried *in vacuo* over P_2O_5. Yield of III, 0.8 g (80%). Found, %: C 48.98; H 7.21; Cl 15.78; N 6.25. $C_9H_{16}ClNO_3$. Calculated, %: C 48.70; H 7.20; Cl 16.0; N 6.32.

N-(Bromoethyl)-4-chloro-L-proline Ethyl Ester Hydrobromide (IV). To 0.77 g (2.8 mmoles) of PBr_3 in 7 ml of dry benzene was added slowly with stirring 0.06 ml of dry pyridine, followed by the dropwise addition over 1 h at 0-5°C with stirring of 1.76 g (7.8 mmoles) of III in 6 ml of benzene. The mixture was stirred at this temperature for 2.5 h, and kept overnight at ~20°C. The hygroscopic, oil precipitate was filtered off, washed with dry ether, and dried over P_2O_5 *in vacuo* for several days. The dried solid was purified by twice reprecipitating it from dry chloroform with dry ether. The yield of IV was 7.68 g (58%). Found, %: C 29.92; H 4.67; N 4.11. $C_9H_{16}Br_2ClNO_2$. Calculated %: C 29.60; H 4.40; N 3.84.

N-(hydroxyethylaminoethyl)-4-chloro-L-proline Ethyl Ester (V). To 0.94 g (2.5 mmoles) of IV in 20 ml of 70% methanol was added 0.346 g (5.6 mmoles) of monoethanolamine, and the mixture boiled for 3 h. When the reaction was complete, the solvent was removed under reduced pressure after first filtering off the solid which separated from the reaction mixture. The yellow oil obtained, which crystallized on drying over P_2O_5, was purified on a column of silica gel grade L 40/100 μ in the system chloroform—methanol (8:1). The solvent was removed under reduced pressure at 40-50°C, and the resulting deep yellow oil dried *in vacuo* over P_2O_5. The yield of V was 0.17 g (25%). Found, %: C 49.67; H 8.07; Cl 13.72; N 10.58. $C_{11}H_{21}ClN_2O_3$. Calculated, %: C 49.91; H 7.93; Cl 13.50; N 10.60.

Hydrochloride of I. To 0.0645 g (0.5 mmole) of thionyl chloride was added dropwise with water cooling and stirring 0.12 g (0.5 mmole) of V in 5 ml of dry chloroform. The mixture was kept for 1 h with stirring at room temperature, and the solvent and excess $SOCl_2$ removed under reduced pressure at 40-50°C. The product was treated successively with dry benzene

and dry ether, and dried *in vacuo* over P_2O_5. I was obtained as a gray hygroscopic powder, R_f 0.89 Found, %: C 37.68, H 5.87; Cl 39.53; N 8.14, $C_{11}H_{22}Cl_4N_2O_2$. Calculated %: C 37.20; H 6.18; Cl 39.80; N 7.85.

Method 2.

The synethesis of I commenced with 4-hydroxy-L-proline VI. Compounds VII and VIII were obtained by literature methods [6], and IX-XII were obtained as for II and III-V.

4-Tosyloxy-L-proline ethyl ester hydrochloride (IX) was obtained by heating VIII in boiling absolute ethanol saturated with HCl. The oily product was treated with dry ether, and dried over P_2O_5. Yield of IX, 78%, mp 53-54°C (fromalcohol), R_f' 0.77. Found, %: C 47.79; H 5.53; Cl 9.92; N 3.91; S 9.43; $C_{14}H_{20}ClNO_5$. Calculated %: C 48.10; H 5.72; Cl 10.15; N 4.03; S 9.15.

N-(hydroxyethyl)-4-tosyloxy-L-proline ethyl ester (X) was obtained by reacting (IX) with ethylene oxide in aqueous methanol. Yield of X, 95%. The product was hygroscopic, R_f 0.55. Found, %: C 53.92; H 6.50; N 3.84; S 9.27. $C_{16}H_{23}NO_6S$. Calculated, %: C 53.70; H 6.44; N 3.92; S 8.99.

N-(Bromoethyl)-4-tosyloxyl-L-proline Ethyl Ester Hydrobromide (XI). Compound X was treated with phosphorus tribromide in benzene in the presence of pyridine, with cooling. The oily, hygroscopic XI obtained was washed with dry ether, dried, and crystallized from dry chlorofrom. Yield of XI, 83%, R_f' 0.48. Found, %: C 39.29; H 4.53; N 2.90; S 6.50. $C_{16}H_{23}Br_2NO_5S$. Calculated, %: C 38.41; H 4.59; N 2.78; S 6.39.

N-(hydroxyethylaminoethyl)-4-tosyloxy-L-proline Ethyl Ester (XII). Reaction of XI with an excess of monoethanolamine in aqueous methanol afforded XII as a hygroscopic, crystalline solid which was purified by reprecipitation from dry chloroform with dry ether. Yield of XII, 68%, R_f' 0.65. Found, %: C 53.88; H 6.90; N 7.20; S 7.73. $C_{18}H_{28}N_2O_6$. Calculated %: C 54.0; H 7.0; N 7.0; S 8.0.

Hydrochloride of I was obtained as described above as a hygroscopic powder, yield 79%, R_f 0.89, R_f' 0.62.

Method 3.

To a solution of 3.75 g (20 mmoles) of II (free base) in 10 ml of dry chloroform was added dropwise 2.66 ml (20 mmoles) of $ClSiMe_3$. Triethylamine (2.71 ml; 20 mmoles) was than added with ice-water cooling, and the triethylamine hydrochloride which separated was filtered off. To the chloroform solution was added a solution of 5.54 g (40 mmoles) of XIII [7] (free base) in dry ether, and the mixture boiled for 15 h. Volatile materials were than removed to dryness under reduced pressure. Tne residue was dissolved in 15 ml of dry chloroform, the solution saturated with gaseous HCl with cooling, and 250 ml of strongly cooled dry ether added dropwise. The oil which separated crystallized on chilling, The ether was decanted off, and the residual (I) dried *in vacuo* over P_2O_5. Purification of I from traces of XIII was effected by chromatography on a column of length 50 cm and diameter 2.8 cm. The stationary phase was 40/100 μ silica gel, and the mobile phase chloroform–methanol, with gradient elution. The ratio of absorbent to be separated was 100:1.5. The composition of the fractions obtained was followed by TLC on Silufol UV-254 plates in the system chloroform–methanol (8:1), previously treated with ammonia, the compounds being visualized on the chromatograms with ninhydrin. The fraction with R_f'' 0.34-0.4 was taken, filtered, and the eluent distilled off. The pure (I), as an oil, was dried *in vacuo* over P_2O_5. Yield 4.9 g (68%), hygroscopic, $[\alpha]_D^{20} = -11.0°$ (in water), R_f 0.89, R_f' 0.62.

EXPERIMENTAL (BIOLOGICAL)

The ability of I to inhibit nucleic acid synthesis in suspended cultures of sarcoma 37 and NK/Ly, and Ehrlich's tumor cells was examined by the method described in [1]. It was found that (I) is cytostatic *in vitro* in marginal doses (50-100 μg/ml).

Antitumor activity was examined by the method described in [4], in C57Bl/6 hybrid mice and mongrel mice with model transplanted strains of experimental tumors sarcoma 37 (S-37), sarcoma 180 (S-180), adenocarcinoma 755 (Ca-755), Lewis tumor (LLC), and La leukemia. The

original weight of the animals was 16-18 g. One hundred six animals were used in the experiments, six animals being used for each dose. Administration of the drug was commenced 48 h after transplantation of the solid tumors, and 24 h after transplantation of leukemia, five doses being given intraperitoneally at 24 h intervals. Observations were continued for seven days following termination of treatment. Antitumor activity was measured by the percentage inhibition of tumor growth for solid tumors, and increased lifespan in leukemia (T, %), calculated from the formula:

$$T\,\% = [(P_c - P_e)/P_c] \cdot 100\%,$$

where P_c and P_e are the mean weights of the tumors in the controls and the experimental group respectively (in the case of leukemia, the mean increase in lifespan in the controls and the experimental group). The numerical data were subjected to statistical treatment by the Belen'kii method.

The studies showed that the extent of inhibition of the above tumor strains by (I) at a therapeutic dose of 70-100 μg/kg was 17-39%. The compound was found to be of low toxicity, its LD_{50} in mongrel female mice by the Kerber method and the intraperitoneal route being 620 mg/kg.

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