Methotrexate analogs. 16. Importance of the side-chain amide carbonyl group as a structural determinant of biological activity

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Hydrolysis of 6 (R = 6-Cl; X = O). A solution of 88 mg (0.7 mmol) of $Na_2CO_3 \cdot H_2O$ in 1 mL of H_2O was added to a solution 100 mg (0.36 mmol) of 6 (R = 6-Cl; X = O) in 1 mL of THF. After stirring at 20 °C for 16 h, the mixture was diluted with 10 mL each of H_2O and EtOAc. The aqueous phase was washed with 10 mL of EtOAc and then acidified to pH 1 and extracted with 2 × 15 mL of EtOAc. These latter-combined EtOAc phases were dried ($MgSO_4$) and filtered, the filtrate was and vacuum evaporated to give 11: yield 63 mg (70%); mp 192–195 °C.

Preparation of 11 Using Ethyl Chloroformate and Imidazole. A mixture of 0.500 g (1.96 mmol) of the imidate 5 (R = 6-Cl; X = O) and 0.228 g (3.35 mmol) of imidazole was combined with 0.254 g (2.35 mmol) of ethyl chloroformate and heated at 120 °C for 15 min. After removal of the heat, the mixture was diluted with 100 mL of EtOAc, washed with 2 × 25 mL of 1 N HCl and 25 mL of brine, dried ($MgSO_4$), and filtered, and the filtrate was evaporated in vacuo to a residue, 11, which was crystallized from toluene: yield 360 mg (72%). The acidic washes were basified and extracted with Et_2O. A mixture of imidazole and *N*-ethylimidazole (*m/e* 96) was obtained from the concentrated Et_2O extracts.

Preparation of α,β-Unsaturated Amide 9 (R = 6-F; X = S). The Stroughton[6] procedure was used. Ice-cold concentrated HCl (1 mL) was added to a solution of 1.00 g (3.60 mmol) of 4-cyano-6-fluoro-4-hydroxy-4*H*-2,3-dihydrobenzothiopyran in 1.0 mL of Et_2O at 0 °C. The mixture was perfused with HCl gas for 2 min and then stoppered and kept 16 h at 20 °C. The amide was obtained after quenching the reaction with 60 mL of ice/H_2O and filtering. The solid was washed with H_2O and dried: yield 0.63 g (83%); mp 221–223 °C dec ($MeOH/H_2O$); IR (KBr) cm^{-1}; NMR ($CDCl_3$) δ 3.50 (d, *J* = 6 Hz, 2 H, CH_2), 6.60 (t, *J* = 6 Hz, 1 H, = CH), 6.9–7.6 (m, 3 H, arom); mass spectrum, *m/e* 209 (P). Anal. ($C_{10}H_8FNOS$) C, H, N.

Preparation of α-Chloro Amide (R = 6-Cl; X = O). A solution of 1.40 g (5.00 mmol) of 6-chloro-4-cyano-4-[(trimethylsilyl)oxy]-4*H*-2,3-dihydrobenzopyran in 1.5 mL of Et_2O at 0 °C was combined with 1.5 mL of concentrated HCl at 0 °C, perfused with HCl gas for 10 min, and then allowed to stand at room temperature for 16 h. The oil obtained from the concentrated extract of the quenched reaction mixture was column chromatographed on silica gel with Et_2O and gave four fractions, the first (R_f 0.82) was 6-chloro-4-chromanone, the second was α-chloroamide [R_f 0.44; yield 0.44 g (36%); mp 118–120 °C dec; IR (KBr) 1692 cm^{-1}; NMR ($CDCl_3$) δ 2.3–2.6 (m, 1 H, CH_2), 2.9–3.3 (m, 1 H, CH_2), 4.3–4.6 (m, 2 H, CH_2), 6.6–7.4 (m, 3 H, arom, and 2 H NH_2), mass spectrum *m/e* calcd, 246.9981; found, 246.9981], the third was α-hydroxy amide [R_f 0.144; yield 0.071 g (6%); mp 168–169 °C; IR (KBr) 1672 cm^{-1}; NMR [$(CD_3)_2SO$] δ 1.6–2.5 (m, 2 H, CH_3), 3.8–4.7 (m, 2 H, CH_2), 6.7–7.7 (m, 3 arom); mass spectrum *m/e* 227], and the fourth was a mixed fraction [R_f 0.15; mass spectrum, *m/e* 227 and 209].[13]

Acknowledgment. The authors are indebted to H. R. Howard, D. MacDonald, and C. aldinger for their technical assistance.

(13) The material with *m/e* 209 peak was shown to be the α,β-unsaturated amide by NMR, see above.

Methotrexate Analogues. 16. Importance of the Side-Chain Amide Carbonyl Group as a Structural Determinant of Biological Activity[1]

A. Rosowsky* and R. Forsch

The Sidney Farber Cancer Institute and Department of Pharmacology, Harvard Medical School, Boston, Massachusetts 02115.
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N-[[[(2,4-Diaminopteridin-6-yl)methyl]amino]benzyl]-L-glutamic acid ("deoxoaminopterin", 1), a new aminopterin analogue containing a CH_2 group in the side chain in place of the amide C=O, was synthesized by condensation of 2,4-diamino-6-(bromomethyl)pteridine with diethyl *N*-(*p*-aminobenzyl)-L-glutamate, followed by saponification with a stoichiometric amount of barium hydroxide in 50% ethanol. The apparent importance of the amide C=O group as a structural determinant of biological activity was indicated by the finding that 1 has 10- to 20-fold lower affinity for bacterial and mammalian dihydrofolate reductase than aminopterin, is not toxic to L1210 murine leukemia cells in culture at a concentration of up to 1.0 μM, and shows no antitumor effect in L1210 leukemic mice at doses as high as 240 mg/kg (q3d × 3).

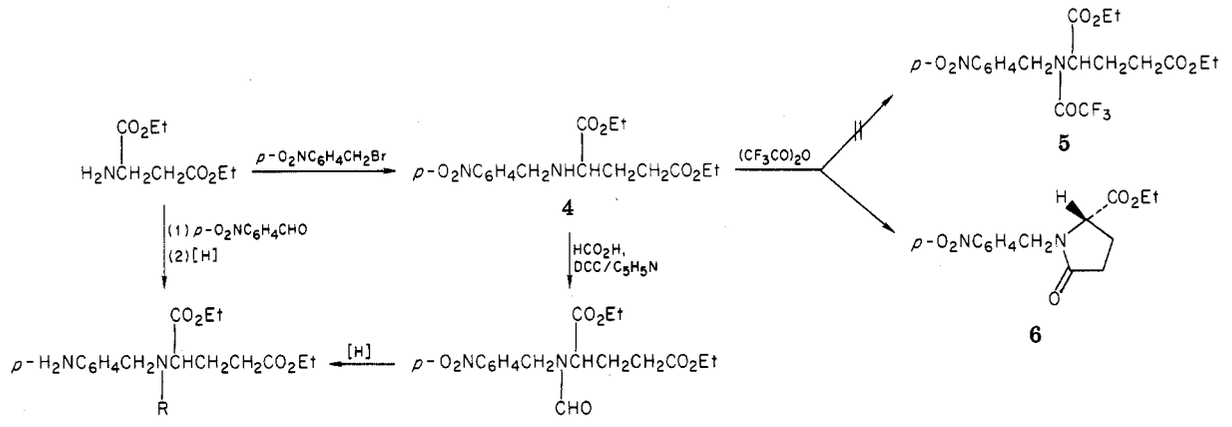
As part of an ongoing research effort aimed at correlating structure and biological activity in folate antagonists,[2-8] it was of interest to assess the possible importance of the C=O group in the amide bond separating the pteroyl and glutamate moieties of classical antifols such as aminopterin and methotrexate. The presence of the amide C=O in these compounds might be expected a priori to have several possible consequences. First, the extent of dissociation of the neighboring α-COOH might be affected, since *N*-acylamino acids are known to be less acidic than amino acids themselves. Secondly, the amide resonance of the conjugated CONH bond should restrict rotation, which might confer on the side chain of the molecule a certain amount of conformational rigidity. Thirdly, the C=O oxygen is potentially a hydrogen bond acceptor, and this might contribute to binding of the molecule to the active site of dihydrofolate reductase. Apart from these effects, there is also the possibility that the amide C=O could influence binding to the membrane-associated proteins that mediate active transport of folates and folate analogues into cells and that activity might be affected at the pharmacological level as a result of differences in plasma protein binding, tissue distribution, or renal/hepatobiliary excretion.

With these considerations in mind, we prepared and investigated some biological properties of *N*-[[[(2,4-diaminopteridin-6-yl)methyl]amino]benzyl]-L-glutamic acid

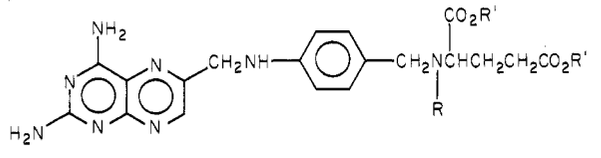
(1) For paper 15 in this series, see Rosowsky, A.; Wright, J. E.; Ginty, C. E.; Uren, J. *J. Med. Chem.* **1982**, *25*, 960.
(2) Rosowsky, A.; Chen, K. K. N. *J. Med. Chem.* **1974**, *17*, 1308 (and earlier papers cited therein).
(3) Rosowsky, A.; Chen, K. K. N.; Papathanasopoulos, N. *J. Heterocycl. Chem.* **1976**, *13*, 727.
(4) Rosowsky, A.; Ensminger, W. D.; Lazarus, H.; Yu, C.-S. *J. Med. Chem.* **1977**, *20*, 925.
(5) Rosowsky, A.; Yu, C.-S. *J. Med. Chem.* **1978**, *21*, 170.
(6) Rosowsky, A.; Beardsley, G. P.; Ensminger, W. D.; Lazarus, H.; Yu, C.-S. *J. Med. Chem.* **1978**, *21*, 380.
(7) Rosowsky, A.; Yu, C.-S.; Uren, J.; Lazarus, H.; Wick, M. *J. Med. Chem.* **1981**, *24*, 559 (paper 13 in this series).
(8) Rosowsky, A.; Forsch, R.; Uren, J.; Wick, M. *J. Med. Chem.* **1981**, *24*, 1450 (paper 14 in this series).

0022-2623/82/1825-1454$01.25/0

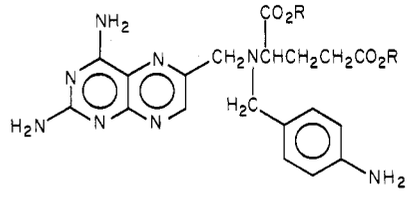
Scheme I



(1), a heretofore unknown aminopterin analogue in which the amide C=O is replaced by CH_2.[9] For simplicity the compound is referred to, in our laboratory, as "deoxoaminopterin". The chemical synthesis of 1 and the results of its initial in vitro and in vivo biological evaluation are the subject of this report.



1, R = R′ = H
8, R = CHO; R′ = Et
9, R = CHO; R′ = H
10, R = H; R′ = Et



11, R = Et
12, R = H

Two related approaches to the chemical synthesis of 1 were investigated (Scheme I). In one of them, 2,4-diamino-6-(bromomethyl)pteridine hydrobromide[10] was condensed with diethyl *N*-(*p*-aminobenzyl)-*N*-formyl-L-glutamate (**2**) with the intent of removing the *N*-formyl group after coupling. In the other approach, the pteridine was coupled directly to diethyl *N*-(*p*-aminobenzyl)-L-glutamate (**3**) without prior protection of the aliphatic nitrogen atom.

Reaction of the HCl salt of diethyl L-glutamate with 1 molar equiv of *p*-nitrobenzyl bromide in DMF containing a stoichiometric proportion of potassium carbonate gave, after 3 days at room temperature, a 92% yield of the oily monosubstituted adduct **4**, which was characterized as a crystalline hydrochloride derivative. Lack of formation of a disubstitued product in this reaction was presumably a consequence of the inductive deactivating effect of the *p*-nitrobenzyl group in **4**. Attempted reaction of **4** with a small excess of trifluoroacetic anhydride in $CHCl_3$ at room temperature unexpectedly failed to produce the desired N-protected derivative **5** but afforded instead the *N*-(*p*-nitrobenzyl)-L-pyroglutamate ester **6** (65% yield). The five-membered lactam structure of **6** was apparent from its infrared spectrum, which contained a peak of 1695 cm^{-1}, and from its NMR spectrum, which showed one ethyl group and an A_2B_2 pattern more characteristic of CH_2CH_2 protons in cyclic than open-chained compounds. The mechanism of the cyclization of **4** to **6** is unclear but apparently is not the result of trifluoroacetic acid being present adventitiously in the reaction, since **4** could be recovered unchanged on deliberate treatment with either trifluoroacetic acid or ethyl trifluoroacetate under similar conditions. Protection of the aliphatic nitrogen in **4** was achieved successfully, on the other hand, by acylation with formic acid and *N*,*N*-dicyclohexylcarbodiimide in a 2:1 mixture of $CHCl_3$ and pyridine, which gave the crystalline amide diester **7** (81% yield). Reduction of the nitro group in **7** by catalytic hydrogenation over 5% Pd/C yielded the noncrystalline aniline **2**, which was added directly to 2,4-diamino-6-(bromomethyl)pteridine hydrobromide[10] in *N*,*N*-dimethylacetamide and left to stir at room temperature for 7 days. The still esterified product (**8**; 59% crude yield) was thereupon saponified by overnight treatment with a stoichiometric ratio of barium hydroxide in 50% aqueous ethanol. Purification at the stage of the free acid **9** was accomplished by elution from a DEAE-cellulose ion-exchange column with 3% ammonium bicarbonate, followed by freeze-drying of pooled TLC-homogeneous fractions. The overall yield for the coupling and saponification steps was 37%.

A model experiment with compound **7** was conducted to determine how readily an *N*-alkyl-*N*-formyl amino acid ester would be deprotected under acidic conditions. Treatment of **7** with aqueous ethanolic HCl at room temperature for 3 days afforded a product whose infrared spectrum showed the expected disappearance of the *N*-formyl C=O peak at 1675 cm^{-1} and retention of the ester C=O peak at 1735 cm^{-1}. These typical deformylation conditions were thus judged to be adequate for our needs, and we attempted the same reaction with compound **8** with the expectation that the deformylated diester **10** would be formed. Unfortunately, however, when **8** was stirred in aqueous ethanolic HCl there was rapid precipitation of an insoluble hydrochloride salt, which on subsequent saponification yielded a product with an elemental analysis indicating it to be still N-formylated; i.e., it was **9**. It appears that formation of an insoluble HCl salt of **8** pre-

(9) Aminopterin analogues with CH_2 groups inserted between the phenyl ring and CONH group have been described: see Piper, J. R.; Montgomery, J. A. *J. Heterocycl. Chem.* **1974**, *11*, 279.
(10) Piper, J. R.; Montgomery, J. A. *J. Org. Chem.* **1977**, *42*, 208.

vented the desired deformylation from taking place. An attempt was also made to remove the *N*-formyl group in **9** under alkaline conditions. Not surprisingly, however, treatment of **9** with 0.1 M NaOH on the steam bath for 15 min promptly brought about the ca. 20-nm downward shift in the ultraviolet absorbance maximum that is characteristic of the conversion of 2,4-diaminopteridines to 2-aminopteridin-4(3*H*)-ones.[11]

In the second approach to the chemical synthesis of **1**, diethyl L-glutamate hydrochloride was condensed with *p*-nitrobenzaldehyde in refluxing benzene, with azeotropic removal of water in a Soxhlet apparatus containing molecular sieves. The resultant *p*-nitrobenzylidene derivative was subjected directly to catalytic hydrogenation (5% Pd/C), followed by treatment with $NaBH_4$ in ethanol. While we had anticipated that catalytic hydrogenation alone would convert the *p*-nitrobenzylidene derivative to **3**, there consistently remained an orange-colored trace impurity that could be monitored by NMR and was difficult to separate from the main product. However, the additional $NaBH_4$ step effectively removed all traces of this impurity and gave **3** as a colorless oil whose NMR spectrum was consistent with the assigned structure and which on treatment with ethanolic HCl afforded a crystalline product analyzing correctly for the dihydrochloride salt **3**·2HCl. Condensation of **3** with 2,4-diamino-6-(bromomethyl)pteridine hydrobromide[10] in *N*,*N*-dimethylacetamide at room temperature for 42 h gave a 56% yield of impure diester (**10**), which could be purified to homogeneity by silica gel column chromatography with $CHCl_3$–MeOH (95:5) as the eluent. Saponification with barium hydroxide in the same manner as with **8** yielded, after elution from a DEAE-cellulose column with 3% ammonium bicarbonate and freeze–drying, a 60% yield of a TLC-homogeneous product analyzing correctly for the dihydrate of the desired diacid **1**.

Since **3** contained two potentially reactive nitrogen atoms, alkylation of 2,4-diamino-6-(bromomethyl)pteridine hydrobromide could conceivably occur on either the aromatic NH_2 or the α-NH of the glutamate moiety. While coupling on α-NH was regarded as unlikely because this would involve alkylation of a secondary nitrogen in preference to the less sterically hindered primary NH_2, we nonetheless deemed it necessary to obtain chemical evidence that the reaction had in fact occurred at the desired site, giving **10** and **1** as opposed to the alternative structures **11** and **12**, respectively. This was done by demonstrating that both the diester formed on coupling and the diacid obtained after saponification lacked a diazotizable NH_2 group as determined by the classical Bratton–Marshall color test.[12] When the *p*-aminobenzyl compound **3** was diazotized in acid solution and treated with *N*-(1-naphthyl)ethylenediamine, an intense purple color formed rapidly at room temperature, whereas with the products assigned structures **10** and **1** there was only a very light violet tinge, which was comparable to that observed with aminopterin itself. This faint coloration in aminopterin samples is assumed to be due to the presence of a trace of *p*-aminobenzoyl-L-glutamic acid, carried along as a contaminant during synthesis and purification or generated on storage via oxidative cleavage of the C^9–N^{10} bond. A similar, essentially negative Bratton–Marshall test was observed with the saponified *N*-formyl adduct **9**, which presumably had formed via unequivocal alkylation of the nonprotected aromatic NH_2 group.

Since the condensation of **3** and 2,4-diamino-6-(bromomethyl)pteridine appeared to yield deoxoaminopterin (**1**) directly, efforts to synthesize this compound via the *N*-formyl intermediates **2**, **8**, and **9** were not pursued further.

Only limited in vitro and in vivo data on the biological activity of **1** could be obtained due to the small quantity of material available for testing, but the results clearly indicated this compound to be at least tenfold less potent than aminopterin, *attesting to the importance of the amide C=O group*. Binding assays were performed with dihydrofolate reductase from MTX-resistant *Lactobacillus casei* and with partially purified enzyme from L1210 mouse leukemia cells as described previously.[8] Functional inhibition of the bacterial reductase was measured spectrophotometrically at 340 nm, whereas binding to the mammalian enzyme was determined by a competitive radioligand assay with [^{3}H]methotrexate. In each instance, aminopterin was used as the positive control. Against the *Lactobacillus casei* enzyme, the ID_{50} concentrations for **1** and aminopterin were 0.47 and 0.027 μM (a 17-fold difference), and against the L1210 enzyme the doses for 50% inhibition of [^{3}H]methotrexate binding were 0.024 and 0.0022 μM (an 11-fold difference). Cytotoxicity assay against L1210 cells in culture (48-h incubation)[8] revealed **1** to be completely devoid of growth-inhibitory activity at up to 1 μM. This contrasted sharply with the activity of aminopterin, for which ID_{50} values of 0.002–0.02 μM are typically observed in this system. Thus, there is at least a 50-fold loss of cytotoxicity when the C=O group in the side chain of the aminopterin molecule is replaced by CH_2. In vivo, the activity of **1** was similarly low. When $B6D2F_1J$ mice were treated with **1** at doses of 30, 60, 120, and 240 (mg/kg)/day (q3d × 3) starting 24 h after ip implantation of 10^5 L1210 leukemia cells, there was a statistically nonsignificant +22% increase in life span (ILS) at the highest dose and no activity at the lower doses. This slight ILS was accompanied by a 5% loss in mean body weight on day 7, as compared with a 13% weight gain in untreated controls, suggesting that this amount of drug was probably near the toxic limit. In contrast to these results, aminopterin administered on a comparable intermittent schedule (q3d × 3) has been observed in this laboratory to produce a +27% ILS at 1 (mg/kg)/day, while at 4 (mg/kg)/day there was a +45% ILS, with a small weight loss indicating near-toxicity.[13] It thus appears that **1** is at least 100-fold less toxic to mice than aminopterin and that on the single schedule tested, at least, this compound has no significant antitumor activity.

The lack of in vivo activity of deoxoaminopterin (**1**) was consistent with both its low affinity for dihydrofolate reductase and its low toxicity to cells. However, it may be noted that while there was a 10- to 20-fold difference in enzyme affinity between **1** and aminopterin, the difference in activity in culture and in vivo was even greater (i.e., 100- to 200-fold). This suggests that low affinity for dihydrofolate reductase is probably not the sole cause for the observed absence of antitumor effect.

The decreased affinity of **1** for dihydrofolate reductase relative to aminopterin is most probably due to the fact that the side-chain NH in the deoxo compound is protonated at the pH of the enzyme active site. The proximity of the resultant positive center would be expected to diminish electrostatic interaction between the ionized COOH

(11) Seeger, D. R.; Cosulich, D. B.; Smith, J. M., Jr.; Hultquist, M. E. *J. Am. Chem. Soc.* **1949**, *71*, 1753.
(12) Bratton, A. C.; Marshall, E. K., Jr. *J. Biol. Chem.* **1939**, *128*, 537.
(13) Comparable in vivo results have been reported for aminopterin by others; see, for example, Goldin, A.; Venditti, J. M.; Humphreys, S. R.; Dennis, D.; Mantel, N.; Greenhouse, S. W. *J. Natl. Cancer Inst.* **1955**, *15*, 1657.

and the protonated arginine-57 residue of the enzyme, which is highly conserved in all known dihydrofolate reductases and is believed to be a major contributing factor to binding.[14] A further consequence of replacing the amide C=O by CH_2 in aminopterin ought to be a decrease in pK_a for the α-COOH group and, to a lesser extent, the γ-COOH group as well. It is well-known that the pK_a for carboxy proton dissociation is 1.0–1.5 log units lower in *N*-acylamino acids than in the corresponding nonacylated amino acids, which can form zwitterions. For example, the pK_a for the COOH in glycine is 2.3, whereas for *N*-acetylglycine the pK_a is 3.6.[15] The α- and γ-COOH groups in glutamic acid have pK_a's of 2.19 and 4.25, whereas the corresponding values for *N*-(*p*-aminobenzoyl)glutamic acid are 3.76 and 4.83, respectively.[16] Similarly the α- and γ-COOH groups in methotrexate are reported to have pK_a's of 3.36 and 4.70,[17] and it is likely that aminopterin would have similar values. Under typical cell culture conditions approximating the physiological pH of 7.4, all these compounds would therefore be >99% in the dianion form. The "average" pK_a for the two COOH groups in glutamic acid is 3.2, whereas for *N*-(*p*-aminobenzoyl)glutamic acid this value is 4.3, representing roughly a 10-fold difference in ease of ionization to a dianion. Using the same approximation, the "average" pK_a for aminopterin and the deoxo analogue 1 would presumably differ by the same factor of ca. 10. It has been pointed out that in the uptake of folates and 4-amino analogues there is an electrostatically unfavorable interaction between the ionized glutamate carboxys and the negatively charged cell membrane, such that the species actually penetrating the cell membrane may be in the undissociated (i.e., noncharged) form even though this represents only a minute fraction of the total extracellular drug.[18,19] Thus, the very weak cytotoxicity observed with 1 in culture is consistent not only with low dihydrofolate reductase binding but also with inefficient cell membrane penetration.[20] An additional possibility that cannot be ruled out at present is that 1 owes its lack of cytotoxicity to failure to form polyglutamates, a biochemical process which is increasingly recognized as being significant in the molecular pharmacology of methotrexate and related compounds.[21]

In summary, the studies reported in this paper indicate that deoxoaminopterin (1) has an affinity for dihydrofolate reductase that is about one order of magnitude lower than that of aminopterin and that its in vitro and in vivo antitumor activity relative to aminopterin is diminished at least another tenfold. While these results have discouraged us from further investigating the therapeutic potential of this new side-chain-altered analogue, we belive they are of interest insofar as they suggest a previously not recognized role for the amide C=O group as a structural determinant of biological activity in classical antifols.

Experimental Section

Infrared (IR) spectra were obtained on a Perkin-Elmer Model 137B double-beam recording spectrophotometer, and NMR spectra were measured with a Varian T60A instrument with Me_4Si as the internal reference. TLC was performed on Eastman 13181 silica gel or Eastman 13254 cellulose sheets containing a fluorescent indicator, and spots were visualized in an ultraviolet viewing chamber at 254 nm or with the aid of iodine staining. Column chromatography was carried out on Baker 3405 silica gel (60–200 mesh) or Whatman DEAE-cellulose. Melting points were measured in Pyrex capillary tubes in a Mel-Temp apparatus (Laboratory Devices, Cambridge, MA) and are not corrected. Microanalyses were performed by Galbraith Laboratories, Knoxville, TN, and were within ±0.4% of theory unless otherwise specified.

Diethyl *N*-(*p*-Nitrobenzyl)-L-glutamate (4). A mixture of diethyl L-glutamate hydrochloride (12 g, 0.05 mol), *p*-nitrobenzyl bromide (11 g, 0.05 mol), and K_2CO_3 (14 g, 0.1 mol) in dry DMF (250 mL) was stirred at room temperature for 3 days. The solvent was removed by rotary evaporation (vacuum pump, dry ice/acetone), and the residue was taken up in benzene. After being washed repeatedly with water, the benzene solution was evaporated to an oil, which was partially purified by dissolving it in $CHCl_3$ (150 mL), adding silica gel (20 g), stirring briefly, filtering, and evaporating to dryness: yield 16.2 g (92%); IR (neat) ν 3350, 2990, 1730 (ester C=O), 1630, 1520, 1345 cm^{-1}; NMR ($CDCl_3$) δ 1.17 (2 overlapping t, 6, CH_3CH_2O), 1.6–2.2 and 2.2–2.7 (2 m, 5, $CH_2CH_2CO_2$ and NH), 3.0–3.4 (m, 1, CH_2NHCH), 3.7–4.4 (m, 6, CH_3CH_2O and benzylic CH_2), 7.45 (d, 2, J = 9 Hz, aromatic protons), 8.14 (d, 2, J = 9 Hz, aromatic protons).

The **hydrochloride salt**, mp 116–117 °C, was prepared by treatment of an ether solution of the free base with dry HCl gas. Anal. ($C_{16}H_{22}H_2O_6$·HCl) C, H, Cl, N.

Ethyl *N*-(*p*-Nitrobenzyl)-L-pyroglutamate (6). Trifluoroacetic anhydride (0.63 g, 3 mmol) was added dropwise with stirring at room temperature to a solution of the nitro compound 4 (0.75 g, 2.2 mmol) in $CHCl_3$ (15 mL). After being left to stand for 15 min, the solution was washed with 5% $NaHCO_3$ and evaporated to dryness under reduced pressure. Column chromatography on silica gel ($CHCl_3$) gave two products. The faster-moving compound (0.027 g) was discarded. The slower-moving major product (0.42 g, 65%) was an oil whose spectral properties indicated that cyclization had occurred to give a lactam: IR (neat) ν 2985, 1745 (ester C=O), 1695 (five-membered lactam C=O), 1605, 1500, 1350 cm^{-1}; NMR ($CDCl_3$) δ 1.25 (t, 3, J = 7 Hz, CH_3CH_2O), 1.9–2.7 (m, 4, ring methylenes), 3.9–4.4 (m, 4, CH_3CH_2O and benzylic CH_2), 4.9–5.2 (m, 1, CHNCO), 7.43 (d, 2, J = 8 Hz, aromatic protons), 8.20 (d, 2, J = 8 Hz, aromatic protons). Anal. ($C_{14}H_{16}N_2O_5$) C, H, N.

Diethyl *N*-Formyl-*N*-(*p*-nitrobenzyl)-L-glutamate (7). A mixture of 98% formic acid (0.18 g, 4 mmol) and $CHCl_3$ (20 mL) was added dropwise with cooling to a stirred solution of *N,N'*-dicyclohexylcarbodiimide (0.41 g, 2 mmol) in $CHCl_3$ (25 mL). After 5 min, the reaction mixture was added over 0.5 h to a stirred ice-cold mixture of the diester hydrochloride 4·HCl (3.7 g, 0.01 mol) and Et_3N (1.0 g, 0.01 mol) in dry pyridine (25 mL). The mixture was kept in the ice bath for 4 h and then left at room temperature overnight. Following evaporation of the solvent under reduced pressure, ether was added, the precipitated *N,N'*-dicyclohexylurea was filtered and washed with ether, and the combined filtrates were evaporated to an oil. Column chromatography on silica gel with $CHCl_3$ as the eluent gave an oil (3.0 g, 81%), which crystallized after several days: mp 60–61 °C; IR (neat) ν 2980, 1735 (ester C=O), 1675 (amide C=O), 1600, 1520,

(14) For recent discussions on this subject and references to the literature, see Kumar, A. A.; Mangum, J. H.; Blankenship, D. T.; Freisheim, J. H. *J. Biol. Chem.* **1981**, *256*, 8970. Volz, K. W.; Matthews, D. A.; Alden, R. A.; Freer, S. T.; Hansch, C.; Kaufman, B. T.; Kraut, J. *Ibid.* **1982**, *257*, 2528.

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1345 cm^{-1}; NMR ($CDCl_3$) δ 1.16 (2 overlapping t, 6, CH_3CH_2O), 2.1–2.4 (m, 4, $CH_2CH_2CO_2$), 3.8–4.4 (m, 5, CH_3CH_2O and NHCH), 4.62 (s, 2, benzylic CH_2), 7.48 (d, 2, J = 8 Hz, aromatic protons), 8.18 (d, 2, J = 8 Hz, aromatic protons), 8.25 (s, 1, CH=O). Anal. ($C_{17}H_{22}N_2O_7$) C, H, N.

Diethyl *N*-(*p*-Aminobenzyl)-*N*-formyl-L-glutamate (2). A solution of the foregoing nitro compound **7** (0.68 g, 1.9 mmol) was catalytically reduced overnight in a Parr apparatus in EtOH solution (25 mL) in the presence of 5% Pd/C (100 mg). The catalyst was removed by filtration through Celite, and the alcohol was evaporated under reduced pressure to form a waxy material, which failed to crystallize on standing: yield 0.48 g (78%); IR (neat) ν 3360, 2970, 1725 (ester C=O), 1665 (amide C=O), 1615, 1515 cm^{-1}. Anal. ($C_{17}H_{24}N_2O_5 \cdot 1.25H_2O$) C, H, N.

Diethyl *N*-(*p*-Aminobenzyl)-L-glutamate (3). Diethyl L-glutamate hydrochloride (2.4 g, 0.01 mol) was added to a solution of K_2CO_3 (1.4 g, 0.01 mol) in water (10 mL), the mixture was immediately extracted with benzene (50 mL), and to the extract was added a solution of *p*-nitrobenzaldehyde (1.5 g, 0.01 mol) in benzene (50 mL). After being refluxed for 3 days in a Soxhlet apparatus (Linde 4A molecular sieves), the reaction mixture was evaporated, and the crude product (3.2 g), whose TLC still showed some unreacted aldehyde, was taken up in absolute EtOH (30 mL) and reduced catalytically (5% Pd/C, 0.6 g) overnight in a Parr bottle. The catalyst was removed by filtration through Celite, dry HCl gas was bubbled through the filtrate, the orange solution was concentrated to dryness by rotary evaporation, and the residue was triturated with ether. The solid obtained after decantation of the ether was stirred in *i*-PrOH (125 mL) at room temperature for 30 min, a small amount of undissolved material was filtered off, the filtrate was treated with decolorizing carbon and filtered (Celite) again, and the filtrate was diluted with a large volume of ether: yield 1.5 g. Since it appeared to still be incompletely reduced, as evidenced by UV absorption at 332 nm (0.1 N HCl), a portion of this product (0.34 g, 0.001 mol) was dissolved in absolute EtOH (10 mL), and $NaBH_4$ (0.34 g, 0.003 mol) was added gradually while the solution was cooled below 20 °C. After being stirred for 15 min at room temperature, the reaction mixture was partitioned between water and CH_2Cl_2, and the organic layer was separated, dried (Na_2SO_4), and evaporated to a light-yellow oil (0.25 g, 35% yield); IR (neat) ν 3350, 2960, 1725 (C=O), 1615, 1510 cm^{-1}; NMR ($CDCl_3$) δ 1.25 (2 t, 6, J = 8 Hz, CH_3CH_2O), 1.8–2.1 and 2.3–2.6 (2 m, 4, $CH_2CH_2CO_2$), 3.6 (d, 2, J = 4 Hz, benzylic CH_2), 4.15 (2 q, 4, J = 8 Hz, CH_3CH_2O), 6.6 (d, 2, J = 9 Hz, aromatic protons), 7.1 (d, 2, J = 9 Hz, aromatic protons). The remainder of the incompletely reduced product was treated with $NaBH_4$ in the same manner, and the oily diamino diester was used directly for coupling to 2,4-diamino-6-(bromomethyl)-pteridine (vide infra). The diamino diester was characterized as a **dihydrochloride salt**, mp ~190 °C dec, prepared by passing dry HCl gas through a solution of the free base in ethanol and diluting the solution with ether. Anal. ($C_{16}H_{24}N_2O_4 \cdot 2HCl \cdot 0.25H_2O$) C, H, Cl, N.

***N*-[[[(2,4-Diaminopteridin-6-yl)methyl]amino]benzyl]-*N*-formyl-L-glutamic Acid (9).** A mixture of the *N*-formyl derivative **2** (1.2 g, 3.2 mmol) and 2,4-diamino-6-(bromomethyl)-pteridine hydrobromide[10] (1.1 g, 3.2 mmol) in *N*,*N*-dimethylacetamide (50 mL) was stirred at room temperature for 7 days. Then K_2CO_3 (0.55 g, 4 mmol) in a small volume of water was added, and the mixture was stirred briefly before being concentrated to dryness by rotary evaporation (vacuum pump, dry ice/acetone cooled receiver). The residue was treated first with acetonitrile and then with ethanol (50 mL) with the aid of a sonicator. After removal of the ethanol-insoluble material, the filtrate was concentrated to a small volume, and ether was added to produce 0.28 g of yellow powder. The solid remaining after the initial ethanol trituration was extracted two more times with refluxing ethanol overnight, and the combined extracts were concentrated and added to ether to obtain an additional 0.7 g of the same yellow product (total yield 59%). A portion (0.1 g, 0.2 mmol) of the combined solids was suspended in 50% ethanol (4 mL). Then $Ba(OH)_2 \cdot 8H_2O$ (0.124 g, 0.4 mmol) was added, the mixture was stirred in an open flask overnight, and Na_2SO_4 (0.06 g, 0.4 mmol) in a minimum volume of water was added. After several minutes of stirring, the $BaSO_4$ precipitate was collected, and the filtrate was acidified with 10% acetic acid and freeze–dried. The residue was applied onto a DEAE-cellulose ion-exchange column, which was eluted first with water and then with 3% NH_4HCO_3. Fractions judged to be TLC-homogeneous (cellulose, pH 7.4 buffer) were pooled and freeze–dried to obtain a yellow solid (0.034 g). The overall yield for the coupling and saponification, based on consumption of 0.2 mmol of the starting material **4**, was 37%. Anal. ($C_{20}H_{22}N_2O_5 \cdot H_2O$) C, H, N.

Attempted Removal of the *N*-Formyl Blocking Group in 8 and 9. A. Model Experiment. A mixture of the *N*-formyl derivative **7** (0.32 g, 0.87 mmol) in absolute ethanol (20 mL) and concentrated HCl (1 mL) was stirred at room temperature for 3 days. The clear solution was then poured into a large volume of CH_2Cl_2, the acid was neutralized by washing with 5% $NaHCO_3$ (**caution**: CO_2 evolution), and the organic layer was evaporated to an oil, whose IR spectrum still contained an ester C=O peak at 1730 cm^{-1} but no amide C=O peak at 1675 cm^{-1}. These conditions were thus judged to be adequate for *N*-formyl group cleavage.

B. The unpurified *N*-formyl derivative **8** (0.7 g, 1.4 mmol) was left to stir at room temperature in a mixture of ethanol (20 mL) and concentrated HCl (1 mL), but instead of a clear solution being formed as in the model experiment, a tan-colored precipitate deposited in the flask. This product was collected and subjected directly to $Ba(OH)_2$ saponification in 50% ethanol in the anticipation that the product would be the desired diacid **1**. However the saponified material still contained an intact *N*-formyl group on the basis of elemental microanalysis (C, H, N), and it thus appeared that the formation of the insoluble HCl salt of **8** had blocked the deformylation reaction. An attempt was also made to deformylate **9** with 0.1 M NaOH (5 equiv, 90 °C, 15 min), but this gave only a product whose UV spectrum in pH 7.4 buffer (λ_{max} 239, 273, 349 nm) indicated loss of the 4-amino group. The UV spectrum of **9** at pH 7.4 showed λ_{max} 257, 282 (sh), and 372 nm, which was consistent with 2,4-diaminopteridine substitution.

Diethyl *N*-[[[(2,4-Diaminopteridin-6-yl)methyl]amino]-benzyl]-L-glutamate (10). A mixture of the diamino diester **3** (0.13 g, 0.43 mmol) and 2,4-diamino-6-(bromomethyl)pteridine hydrobromide (0.14 g, 0.43 mmol)[10] in *N*,*N*-dimethylacetamide (5 mL) was stirred at room temperature for 42 h. After solvent evaporation with the aid of a rotary evaporator connected to a vacuum pump and dry ice/acetone cooled receiver, the residue was partitioned between 5% $NaHCO_3$ and $CHCl_3$, and the $CHCl_3$ layer was separated and added dropwise to a large volume of ether. The crude product (0.12 g, 56% yield) was collected by filtration and purified by column chromatography on silica gel with $CHCl_3$–MeOH (95:5) as the eluent: yield 37 mg (20%); mp 192–196 °C dec; IR (KCl) ν 3400, 2985 (w), 1735 (ester C=O), 1635, 1565, 1525, 1460 cm^{-1}. Anal. $C_{23}H_{30}N_8O_4 \cdot 0.5CH_3OH \cdot 0.5H_2O$) C, H, N.

***N*-[[[(2,4-Diaminopteridin-6-yl)methyl]amino]benzyl]-L-glutamic Acid (1).** A suspension of the diester **11** (0.43 g, 0.9 mmol) and $Ba(OH)_2 \cdot 8H_2O$ (0.56 g, 1.8 mmol) in 50% ethanol (18 mL) was stirred in an open flask overnight. A small volume of water containing Na_2SO_4 (0.26 g, 1.8 mmol) was then added, and the mixture was sonicated briefly and suction filtered to remove the $BaSO_4$ that had formed. Acidification of the filtrate to pH 4.5–5.0 with 10% acetic acid, followed by refrigeration for several hours, gave a yellow solid, which was collected, washed with water, and passed through a DEAE-cellulose ion-exchange column with 3% NH_4HCO_3 as the eluent. Fractions found to be TLC-homogeneous were pooled and freeze–dried: yield 0.24 g (60%); mp >300 °C; R_f 0.4 (cellulose, 0.05 M potassium phosphate buffer, pH 7.4). Anal. ($C_{19}H_{22}N_8O_4 \cdot 2H_2O$) C, H, N.

Bratton-Marshall Reaction for Diazotizable Amino Groups. This color test was performed essentially as described by Mishra et al.[22] To a 1 mM stock solution of test compound in 1 mL of water were added with mixing 9 mL of phosphate buffer (pH 7.4), 2 mL of 5 N HCl, and 8 mL of acetone. To this mixture was then added 1 mL of 0.5% $NaNO_2$, followed after 3 min by 1 mL of 2.5% ammonium sulfamate, and 2 min later by 1 mL of 0.5% *N*-(1-naphthyl)ethylenediamine dihydrochloride. The solution was left to stand at room temperature for 15 min

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to allow color development. Diethyl *N*-(*p*-aminobenzyl)-L-glutamate (**3**) gave an intense purple color under these conditions and was used as the positive control, whereas with methotrexate and diethyl *N*-(*p*-nitrobenzyl)-L-glutamate (**4**) the solution remained practically colorless. With aminopterin a faint purple color was observed, which probably represented contamination by a trace of *N*-(*p*-aminobenzoyl)-L-glutamic acid. Compounds **1**, **9**, and **10** all gave only a faint violet tinge intermediate between methotrexate and aminopterin and were thus judged to lack a diazotizable amino group.

Biological Testing. Cytotoxicity assays in 48-h cultures were performed as previously described.[8] Dihydrofolate reductase inhibition was determined spectrophotometrically at 340 nm against *Lactobacillus casei* enzyme (New England Enzyme Center, Boston, MA) and by competitive [^{3}H]methotrexate binding against partially purified L1210 leukemic cell enzyme as reported earlier.[7,8] In vivo antitumor tests were conducted according to NCI protocols.[23]

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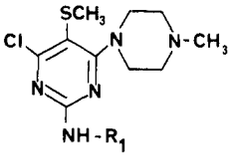
2-Amino-6-chloro-4-(*N*-methylpiperazino)pyrimidines, Inhibitors of Spiroperidol Binding

Claude Guérémy,*,† François Audiau,† André Uzan,‡ Gérard Le Fur,‡ Jean-Michel Léger,§ and Alain Carpy§

Département de Recherches Thérapeutiques, Pointet-Girard, Groupe Pharmuka, F 92390 Villeneuve-la-Garenne, France, Département de Recherches Biologiques, Pharmuka-Laboratoires, Groupe Pharmuka, F 92231 Gennevilliers, France, and ERA-890 CNRS, Faculté de Pharmacie, F 33000 Bordeaux, France. *Received December 1, 1981*

A series of 30 6-chloro-2,4-diaminopyrimidines was synthesized and tested in vitro as inhibitors of [^{3}H]spiroperidol binding. The affinity for the dopamine receptor was shown to be related to the 6-chloro-4-(*N*-methylpiperazine)pyrimidine structure bearing a NH_2 or NHR_1 group as a substituent in position 2, provided that R_1 was not an α branched alkyl group. The nature of the substituent in position 5 is also of importance for the affinity; 2-(benzylamino)-6-chloro-4-(*N*-methylpiperazino)-5-(methylthio)pyrimidine (**22**) is the most active member of the series. Molecular structures of three compounds were analyzed by X-ray diffraction and PCILO computation.

A prior communication[1] described the synthesis and the pharmacological activities of a number of 5-(methylthio)-4-piperazinopyrimidines. Two of them, mezilamine (**1**) and UK-177 (**22**), were shown to be dopamine (DA)

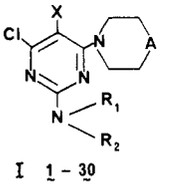


1, $R_1 = CH_3$, mezilamine

22, $R_1 = CH_2-C_6H_5$, UK-177

antagonists and potential antipsychotic drugs.[2-4] Although these compounds fall into the Janssen definition,[5] they do not seem to be related to any well-established class of neuroleptics.

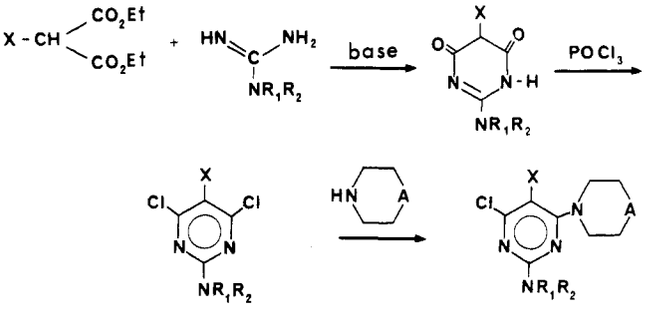
The atypical structure of 4-piperazinopyrimidines prompted us to synthesize a series of mezilamine analogues (I). In the present report we describe the synthesis of



I **1**–**30**

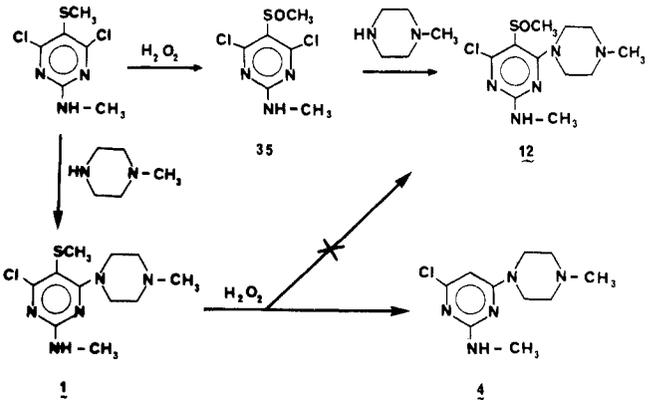
these derivatives (listed in Table I) and their effects on the binding of [^{3}H]spiroperidol. The molecular structures of two active compounds (**1** and **22**) and of one inactive

Scheme I



5, **6**, **8**–**11**, **21**, **30**

Scheme II



1 **4**

† Groupe Pharmuka, Département de Recherches Thérapeutiques.

‡ Groupe Pharmuka, Département de Recherches Biologiques.

§ ERA-890 CNRS, Faculté de Pharmacie.

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