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The preparation of isonipecotinoyl analogues of aminopterin and methotrexate is described. Condensation of diethyl *N*-isonipecotinoyl-L-glutamate **4** with 2-amino-5-bromomethyl-3-cyanopyrazine **5** afforded diethyl *N*-(*N*-[(2-amino-3-cyanopyrazin-5-yl)methyl]isonipecotinoyl)-L-glutamate **6**. Cyclisation of **6** with guanidine followed by blocking group hydrolysis afforded *N*-([*N*-(2,4-diaminopteridin-6-yl)methyl]isonipecotinoyl)-L-glutamic acid **8**. Coupling of *N*-(2-amino-4(3*H*)-oxopteridin-6-yl]methylisonipecotinic acid **11** with diethyl L-glutamate gave diethyl *N*-[(*N*-[2-amino-4(3*H*)-oxopteridin-6-yl]methyl)isonipecotinoyl]-L-glutamate **12**. Blocking group hydrolysis afforded *N*-[(*N*-[2-amino-4(3*H*)-oxopteridin-6-yl]methyl)isonipecotinoyl]-L-glutamic acid **13**.

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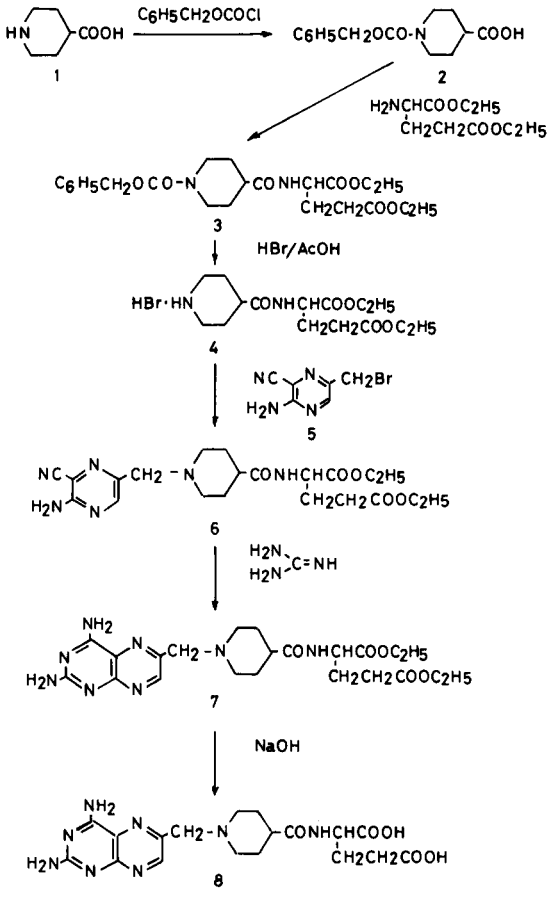
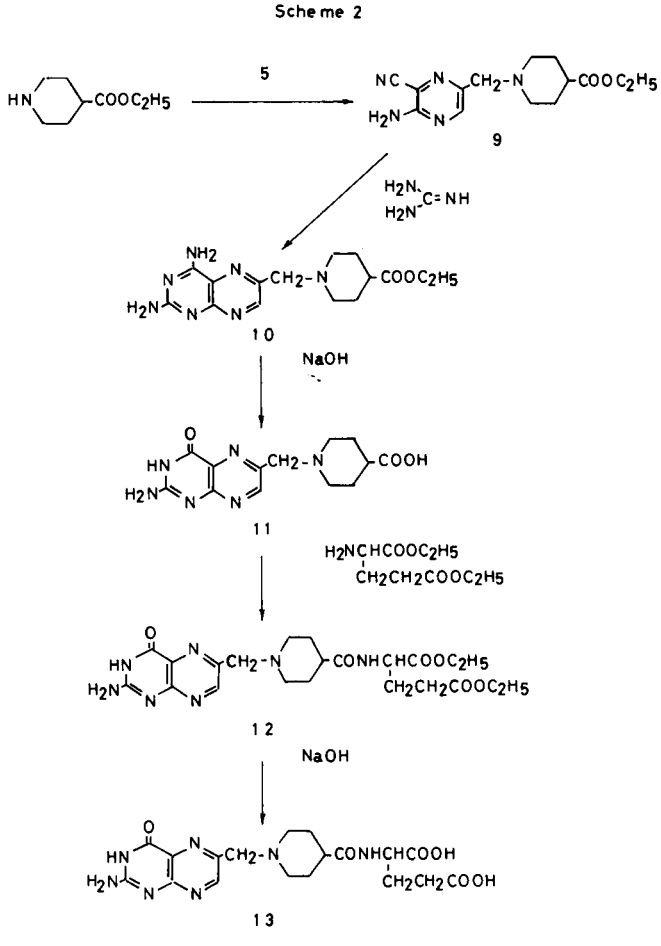
Since the first report that folic acid antagonists, aminopterin and its N^{10}-methyl derivative methotrexate, are useful in the treatment of human neoplastic disease, various modifications have been made in an attempt to increase the potency and lessen the toxicity of these compounds. Both compounds interact with dihydrofolate reductase to give complexes with low dissociation constants that inhibit the function of this enzyme. The interaction depletes the tetrahydrofolate pool, resulting in a decreased synthesis of thymidylate and, in turn, an inhibition of DNA synthesis (1,2,3,4). One of the major problems associated with methotrexate therapy is the development of resistance to drugs (5). The present report describes the synthesis of new analogs which had the isonipecotinoyl group substituted for the *p*-aminobenzoyl moiety of aminopterin and folic acid. The sequence of reaction is presented in Scheme 1. The *N*-protected amino acid **2** was readily prepared in

Scheme 1



Scheme 2



83% yield by treatment of **1** with an excess of benzyl chlorocarbonate in the presence of sodium hydrogen carbonate. Reaction of **2** with ethyl chloroformate gave a solution of the mixed anhydride, which was condensed with diethyl L-glutamate to give **3**. While removal of the *N*-benzyloxycarbonyl blocking group of **3** could be readily achieved by treatment with hydrogen bromide in acetic acid at room temperature, its removal by catalytic hydrogenolysis under milder conditions was not successful. Condensation of 2 equivalents of **4** in tetrahydrofuran with 1 equivalent of 2-amino-5-bromomethyl-3-cyanopyrazine **5** (6) followed by chromatographic purification gave the desired compound **6**. Reaction of **6** with guanidine in refluxing ethanol gave **7**. Saponification of **7** with sodium hydroxide in ethanol gave the desired diacid **8**. Next, the intermediate isonipecotinoyl derivative **11** was prepared as shown in Scheme 2. Under conditions that were used for the preparation of **6**, the condensation of ethyl isonipecotinate in chloroform with **5** gave **9**, which on cyclisation with guanidine afforded **10**. Hydrolysis of this compound with 5% sodium hydroxide gave the isonipecotinoyl analog of pteric acid **11**. The glutamic acid moiety was then introduced *via* the mixed anhydride method yielding **12**. Finally, hydrolysis of **12** with sodium hydroxide in aqueous ethanol yielded the desired compound **13**. The biological activities of compound **8** and **13** are now under investigation.

EXPERIMENTAL

Melting points were recorded with a capillary melting point apparatus and are uncorrected. The ^{13}C nmr spectra were obtained on a Jeol-FX-100 spectrometer using solutions in the indicated solvents and dioxan as internal standard.

N-Benzyloxycarbonylisonipecotinic Acid (**2**).

Isonipecotinic acid (**1**, 6.5 g, 0.05 mole), sodium bicarbonate (12.6 g, 0.125 mole), and benzyloxycarbonyl chloride (9.0 g, 0.052 mole) were combined in a mixture of water (120 ml) and ether (20 ml) at room temperature. The solution was stirred for 4 hours and then extracted with ether (3 × 20 ml). The aqueous layer was cooled to 0° and acidified to *p*H 4 with 6*N* hydrochloric acid. A thick white oil separated. The supernatant was removed and extracted with ethyl acetate (2 × 20 ml). The oily residue was dissolved in the combined ethyl acetate extracts and the ethyl acetate layer was washed with water, dried over sodium sulfate and evaporated to dryness to give a thick oily residue (12 g). The oily material was treated with petroleum ether (60-80°) at 0°. After the extraction the upper layer was discarded, and the inside surface of the vessel was scratched with a glass bar under fresh petroleum ether at 0°. Then the mixture was stored in a deep freeze for 2 days and **2** was isolated as white crystals, 11 g (83%), mp 66-68°. The analytical sample, mp 72-73°, was prepared by recrystallization from benzene-petroleum ether; nmr (deuteriochloroform): δ 27.6 (C-3), 40.6 (C-4), 43.2 (C-2), 67.3 (CH_2O), 127.8, 127.9, 128.4, 136.5 (aromatic), 155.2 (CON), 179.7 (CO_2).

Anal. Calcd. for $C_{14}H_{17}NO_4$: C, 63.86; H, 6.51; N, 5.32. Found: C, 63.87; H, 6.41; N, 5.37.

Diethyl *N*-(*N*-Benzyloxycarbonylisonipecotinoyl)-L-glutamate (**3**).

Ethyl chloroformate (2.7 g, 0.025 mole) was added to a mixture of *N*-benzoyloxycarbonylisonipecotinic acid (**2**, 6.5 g, 0.025 mole) and triethylamine (2.57 g, 0.025 mole) in dry ethyl acetate (60 ml). After stirring 1 hour at room temperature, a solution of diethyl L-glutamate (5 g, 0.025 mole) in dry ethyl acetate (10 ml) was added. Stirring was continued at room temperature for 2 hours, the triethylamine hydrochloride was filtered and the filtrate was washed successively with water (3 × 10 ml), cold 0.1*N* sodium bicarbonate (3 × 10 ml) and cold 0.1*N* hydrochloric acid (2 × 10 ml) and then the organic layer was dried over sodium sulfate. After removal of the ethyl acetate, the resulting residue was recrystallized from benzene-petroleum ether and **3** was isolated as white crystals, 8 g (72%), mp 61-62°; nmr (deuteriochloroform): δ 14.5 (CH_3), 27.4 (glu-β), 28.7, 28.4 (iso-β), 30.8 (glu-γ), 43.1 (iso-γ), 43.7 (iso-α), 52.0 (glu-α), 61.1, 61.9, (CH_2O, CH_2N), 128.2, 128.3, 128.8, 137.1 (aromatic), 155.5 (OCON), 172.2, 173.2, 174.5 (CO_2 × 2, CON).

Anal. Calcd. for $C_{23}H_{32}N_2O_7$: C, 61.59; H, 7.19; N, 6.25. Found: C, 61.55; H, 7.35; N, 6.10.

Diethyl *N*-Isonipecotinoyl-L-glutamate Hydrobromide (**4**).

Compound **3** (8.9 g, 0.02 mole) was treated with 25% hydrogen bromide in acetic acid (16 g) at room temperature. When carbon dioxide evolution stopped, the product was precipitated by adding ether (200 ml) to the reaction mixture. After standing overnight in the refrigerator, the clear ethereal solution was removed by decantation. After two reprecipitations from ethanol (30-50 ml) solution by addition of ether (300-500 ml), the gummy product was dried over sodium hydroxide *in vacuo*. To a solution of this compound in absolute ethanol (10 ml), ether was added until the solution became faintly cloudy. The solution was cleared by addition of a few drops of absolute ethanol, and kept 2 days in a refrigerator, **4** was isolated as white crystals, 6.8 g (89%), mp 162-163°.

Anal. Calcd. for $C_{15}H_{27}N_2O_5 \cdot H_2O$: C, 43.58; H, 7.07; N, 6.78. Found: C, 43.41; H, 6.73; N, 7.01.

Diethyl *N*-(*N*-[(2-Amino-3-cyanopyrazin-5-yl]methyl]isonipecotinoyl)-L-glutamate (**6**).

Compound **4** (6.0 g, 0.015 mole) was suspended in chloroform (200 ml) and anhydrous potassium carbonate (30 g) was added. After stirring at room temperature for 1 hour, the suspension was filtered through Celite. The filtrate was concentrated to approximately 20 ml and cooled to 0° and a solution of 2-amino-5-bromomethyl-3-cyanopyrazine **5** (6) (1.34 g, 0.006 mole) in tetrahydrofuran (20 ml) was gradually added dropwise with stirring over 20 minutes. The mixture was stirred at room temperature for an additional 3 hours. The mixture was filtered and the filtrate was concentrated *in vacuo* to give a brown gum which was chromatographed on silica gel (70-230 mesh). Elution with chloroform gave unreacted **5**. Elution with ethyl acetate gave pure **6**, 1535 mg (45%), mp 63-64°; nmr (deuteriochloroform): δ 14.1 (CH_3), 27.1 (glu-β), 28.6 (iso-β), 30.4 (glu-γ), 42.7 (iso-γ), 51.5 (glu-α), 53.0 (iso-α), 60.6, 61.1, 61.5 (CH_2 × 2, CH_2N), 111.4, 144.0, 146.8, 155.2 (heteroaromatic), 115.1 (CN), 171.6, 172.6, 174.5 (CO_2 × 2, CON).

Anal. Calcd. for $C_{21}H_{30}N_6O_5$: C, 56.49; H, 6.77; N, 18.82. Found: C, 56.77; H, 6.87; N, 18.70.

Diethyl *N*-([*N*-(2,4-Diaminopteridin-6-yl)methyl]isonipecotinoyl)-L-glutamate (**7**).

Guanidine hydrochloride (190 mg, 3 mmoles) was added to a stirred solution of sodium ethoxide prepared from sodium metal (46 mg, 2 mg-atom) and ethanol (10 ml). After 20 minutes the mixture was filtered to remove sodium chloride and the filtrate was refluxed with **6** (892 mg, 2 mmoles) for 4 hours. Evaporation under reduced pressure left a gummy solid, which was dissolved in ethanol, a small amount of silica gel was added, and the solvent was removed under reduced pressure. The powder was then added to the top of a silica gel column (70-230 mesh, 30 g) which was eluted with ethyl acetate-methanol (9:1). The main fraction yielded **7**, 130 mg (13%), mp 166-168° dec.

Anal. Calcd. for $C_{22}H_{32}N_8O_5 \cdot H_2O$: C, 52.16; H, 6.76; N, 22.12. Found: C, 52.29; H, 6.47; N, 22.35.

N-([*N*-(2,4-Diaminopteridin-6-yl)methyl]isonipecotinoyl)-L-glutamic Acid (**8**).

A mixture of **7** (244 mg, 0.5 mmole) and 1*N* sodium hydroxide (1 ml) in 50% ethanol (2 ml) was stirred for 48 hours at room temperature. After concentration, the residue was dissolved in water (5 ml) and the solution was neutralized by addition of Amberlite IRC-50 (H^+ form), then filtered. The filtrate was acidified to *p*H 4 with 1*N* hydrochloric acid. The precipitated solid was filtered, washed with water and dried to give **8** (125 mg, 58%) as a pale yellow powder. An analytical sample was prepared by recrystallization from water, mp 233° dec; nmr (deuterium oxide-sodium deuterium oxide): δ 27.8, 28.5 (iso-β), 29.4 (glu-β), 35.0 (glu-γ), 42.7 (iso-γ), 53.0 (iso-α), 56.1 (glu-α), 60.8 (CH_2N), 123.0, 146.7, 151.5, 154.1, 162.9, 163.7 (heteroaromatic), 178.3, 179.7, 182.9 (CO_2 × 2, CON).

Anal. Calcd. for $C_{18}H_{24}N_8O_5 \cdot 4.5H_2O$: C, 42.10; H, 6.48; N, 21.82. Found: C, 42.21; H, 6.25; N, 21.81.

Ethyl *N*-(2-Amino-3-cyanopyrazin-5-yl)methylisonipecotinate (**9**).

To an ice-cooled solution of ethyl isonipecotinate (1.05 g, 6.6 mmoles) in chloroform (20 ml), a solution of **5** (0.71 g, 3.3 mmoles) in tetrahydrofuran (5 ml) was added dropwise with stirring. After 2 hours, the reaction mixture was filtered and the solvents were removed *in vacuo*. The residue was recrystallized from benzene and **9** was isolated as a light yellow solid, 0.86 g (90%), mp 148-149° dec; nmr (deuteriochloroform): δ 14.6 (CH_3), 28.5 (C-3), 41.2 (C-4), 53.3 (C-2), 60.7 (CH_2O), 61.5 (CH_2N), 115.7 (CN), 112.1, 144.8, 147.3, 155.8 (heteroaromatic), 175.2 (CO_2).

Anal. Calcd. for $C_{14}H_{19}N_5O_2$: C, 58.11; H, 6.62; N, 24.21. Found: C, 57.96; H, 6.81; N, 24.12.

Ethyl *N*-(2,4-Diaminopteridin-6-yl)methylisonipecotinate (**10**).

Guanidine hydrochloride (1.38 g, 0.014 mole) was added to a stirred solution of sodium ethoxide prepared from sodium metal (330 mg, 0.014 g-atom) and ethanol (50 ml). After the mixture was stirred for 1 hour the precipitated sodium chloride was removed by filtration and rinsed with ethanol (50 ml). Ester **9** (3.5 g, 0.012 mole) was added and the mixture was refluxed for 6 hours. On cooling, **10** was isolated as a yellow solid, 3.6 g (90%), mp 230-233°.

Anal. Calcd. for $C_{15}H_{21}N_7O_2$: C, 54.36; H, 6.39; N, 29.59. Found: C, 54.25; H, 6.40; N, 29.33.

N-[2-Amino-4(3*H*)-oxopteridin-6-yl]methylisonipecotinic Acid (**11**).

A slurry of **10** (3.3 g, 0.01 mole) in 5% sodium hydroxide (100 ml) was heated on the water bath for 5 hours. After cooling, the resulting yellow solution was acidified to *p*H 6 with 50% acetic acid and kept overnight in a refrigerator, **11** was isolated as a pale yellow powder, 3.2 g (95%), mp 290°; nmr (deuterium oxide-sodium deuterium oxide): δ 29.2 (C-3), 44.7 (C-4), 53.5 (C-2), 61.4 (CH_2N), 129.3, 146.5, 149.8, 156.3, 165.0 (heteroaromatic), 173.8 (CO), 185.5 (CO_2).

Anal. Calcd. for $C_{13}H_{16}N_6O_3 \cdot 2H_2O$: C, 45.87; H, 5.92; N, 24.70. Found: C, 45.52; H, 5.92; N, 24.65.

Diethyl *N*-[(*N*-[2-Amino-4(3*H*)-oxopteridin-6-yl]methyl)isonipecotinoyl]-L-glutamate (**12**).

Compound **11** (608 mg, 2 mmoles) was dried over phosphorus pentoxide *in vacuo* at 60° for 1 hour and then suspended in dry 1-methyl-2-pyrrolidinone (25 ml). Triethylamine (202 mg, 2 mmoles) was added to the mixture. After 1 hour of stirring at room temperature, ethyl chloroformate (217 mg, 2 mmoles) was added and stirred for 1 hour. After this period a solution of diethyl L-glutamate (406 mg, 2 mmoles) in dry dimethyl formamide (2 ml) was added. Stirring was continued at room temperature for 1 hour and the reaction mixture was filtered. The filtrate was concentrated *in vacuo* and the residue was dissolved in chloroform and the solution was poured into a column packed with silica gel (60-230 mesh) in chloroform. Then the column was washed with chloroform until no compound was present in the washings. Elution with 10% methanol in chloroform gave pure **12**, 370 mg (38%), mp 261° dec.

Anal. Calcd. for $C_{22}H_{31}N_7O_6 \cdot 0.5H_2O$: C, 53.00; H, 6.47; N, 19.67. Found: C, 52.93; H, 6.62; N, 19.77.

N-[(*N*-[2-Amino-4(3*H*)-oxopteridin-6-yl]methyl)isonipecotinoyl]-L-glutamic Acid (**13**).

The hydrolysis of **12** (245 mg, 0.5 mmole) to give **13** (136 mg, 63%) was carried out as described above for **8** except that the time of reaction was 24 hours, mp 220° dec; nmr (deuterium oxide-sodium deuterium oxide): δ 28.1, 28.8 (iso-β × 2), 29.3 (glu-β), 25.0 (glu-γ), 42.9 (iso-γ), 53.0 (iso-α), 61.3 (CH_2N), 129.4, 146.4, 150.0, 156.4, 165.0 (heteroaromatic), 173.9 (aromatic CO), 178.5, 179.8, 183.0 (CO_2 × 2, CON).

Anal. Calcd. for $C_{18}H_{23}N_7O_6 \cdot 3.5H_2O$: C, 43.54; H, 6.09; N, 19.75. Found: C, 43.35; H, 5.73; N, 19.67.

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