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Short communication

Synthesis and antifolate evaluation of the aminopterin analogue with a bicyclo[2.2.2]octane ring in place of the benzene ring

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Abstract – *N*-[4-[[2,4-diamino-6-pteridinyl)methyl]amino]bicyclo[2.2.2]octane-1-carbonyl]-L-glutamic acid (**1**) was synthesized and tested for antifolate activity. *N*-(4-Aminobicyclo[2.2.2]octane-1-carbonyl-L-glutamic acid dimethyl ester (**6**), the side chain precursor to subject compound **1**, was synthesized readily via reported bicyclo[2.2.2]octane-1,4-dicarboxylic acid monoethyl ester (**2**). The side chain precursor **6** was alkylated by 6-(bromomethyl)-2,4-pteridinediamine (**7**). Subsequent ester hydrolysis then afforded **1**. Antifolate and antitumor evaluation of **1** verses L1210 dihydrofolate reductase (DHFR) and three tumor cell lines (L1210, S180, and HL60) showed it to be ineffective. Although compound **1** was very similar to aminopterin structurally, the bicyclo[2.2.2]octane ring system in place of the phenyl ring in the *p*-aminobenzoate moiety effectively negates the stoichiometric binding displayed by many classical DHFR inhibitors bearing appropriate aromatic ring systems in the side chain. © 2001 Éditions scientifiques et médicales Elsevier SAS

dihydrofolate reductase / Curtius rearrangement / *N*-(4-aminobicyclo[2.2.2]octane-1-carbonyl)-L-glutamic acid dimethyl ester / 6-bromomethyl-2,4-pteridinediamine / antifolate evaluation

1. Introduction

The potent DHFR inhibitor aminopterin (AMT) was introduced into the clinic as an antitumor agent over 50 years ago. It was, however, soon displaced by its N^{10}-methyl analogue methotrexate (MTX), which proved to be therapeutically superior. MTX also came to be used against diseases other than cancer such as psoriasis and rheumatoid arthritis [1]. During the five decades since this class of antitumor agent was introduced medicinal chemists have extensively investigated structural modifications with the aim of beneficially altering the therapeutic spectrum [1] of MTX in terms of selective uptake and activity versus tumor tissue, activity against a wider panel of tumor types, and direct clinical efficacy. In fact, numerous DHFR-inhibiting analogues are at least as potent as MTX in antitumor activity, but none has yet proven to have therapeutic advantages sufficient for it to displace or join MTX in clinical usage. Agents of such activity include AMT/MTX analogues in which the 4-aminobenzoyl group is replaced by the 4-amino-1-naphthoyl group [2]. Despite nearly 50 years of activity in the area of antifolate chemotherapy of cancer, new antitumor antifolates continue to be pursued [3–14].

Synthesis and testing of the naphthoyl analogues followed molecular modeling studies that revealed ample spatial accommodation for the naphthalene and even larger groups in models based on reported X-ray crystallographic data describing the multifacted binding of MTX to human DHFR [2]. These studies showed the naphthalene ring fitting into the hydrophobic pocket while not interfering with the other critical hydrogen bonds and electrostatic interactions associated with the extremely tight binding of the parent antifolates to DHFR. These naphthoyl analogues did in fact show high levels of both DHFR

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inhibition and antitumor activity that compared favorably with those of MTX in inhibiting tumor cell growth (L1210, HL60, S180) in culture and toward the E0771 mammary adenocarcinoma in mice [2].

While docking various antifolate synthetic candidates (such as the naphthalene analogues) into the enzyme model [2], we became interested in a structure in which the benzene ring of AMT is replaced by a cycloaliphatic group. For example, a 1,4-substituted bicyclo[2.2.2]octane system is quite similar to the phenyl ring of the PABA moiety in a classical antifolate in terms of orientation of the substituents and overall length. This ring system is more sterically demanding, but could potentially occupy the appropriate hydrophobic pocket of the enzyme even more efficiently than a phenyl substituent. Certainly, a bicyclo[2.2.2]octane system is more rigid than a simple 1,4-substituted cyclohexane which introduces conformational flexibility into the enzyme binding parameters; the cyclohexane analogue of MTX is significantly less active than MTX in terms of both enzyme inhibition and cytotoxicity [20]. Hence, we prepared the bicyclo[2.2.2]octane analog of the classical antifolate AMT in order to test whether such an analogue could bind and inhibit DHFR.

2. Chemistry

The accessibility of the 4-aminobicyclo[2.2.2]-octane-1-carboxylic acid system allowed synthesis of **1** for the testing of this notion. The key precursor, ethyl hydrogen bicyclo[2.2.2]octane-1,4-dicarboxylate (**2**), was prepared by a reported route [16]. Diphenylphosphoryl azide (DPPA) was used in two ways as first described by Shioiri et al. [17]. First, **2** was treated with DPPA to form its carbonyl azide which underwent the Curtius rearrangement in boiling toluene containing benzyl alcohol to give the benzyl carbamate **3** in a surprising overall yield (for three steps) of 96%. After mild ester hydrolysis of **3** gave carboxylic acid **4**, DPPA was used again in peptide-type coupling of **4** with dimethyl L-glutamate to give **5**. Hydrogenolysis of **5** removed the amine-protecting benzyloxycarbonyl group to give the sidechain **7**. Alkylation of **6** with 6-(bromomethyl)-2,4-pteridinediamine (**7**) [18] gave the diester **8**, which was purified by column chromatography. Mild ester hydrolysis gave the target compound **1**.

Table I. Comparison of properties of aminopterin (**1**) with bicyclo[2.2.2]octane analogue (**2**)

Compound	DHFR inhibition [a]	Cell growth inhibition IC_{50} (nM) [a]		
	K_i (pM)	S180	L1210	HL60
AMT	3.85 ± 0.3	1.01 ± 0.3	0.72 ± 0.1	1.00 ± 0.20
1	> 1000	1440 ± 220	850 ± 94	510 ± 380

[a] Methods described in [19].

3. Pharmacology

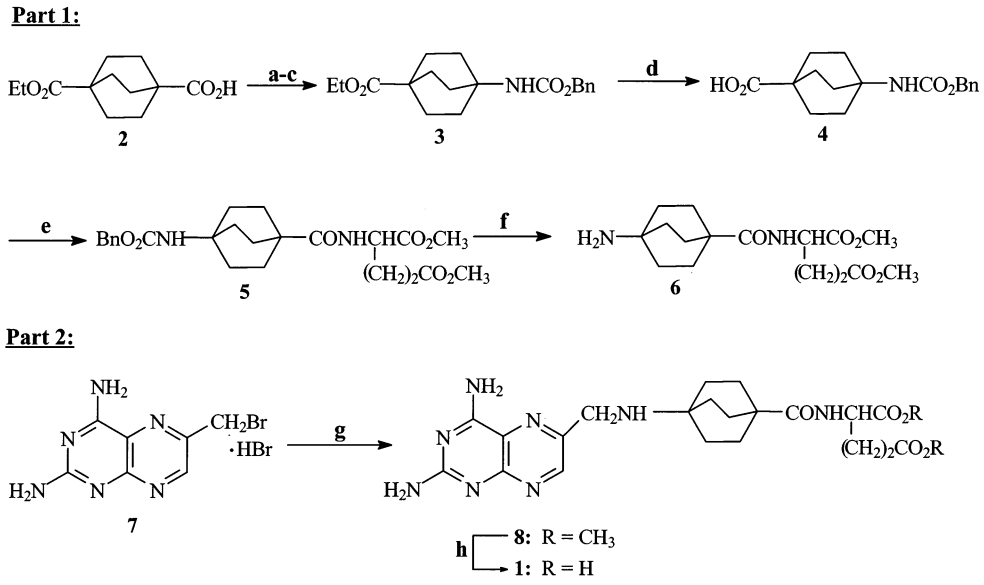
Antifolate evaluation [19] of the bicyclo[2.2.2]-octane analogue **1** revealed that it was not inhibitory toward DHFR (L1210) relative to the control drug aminopterin (AMT) nor did it appreciably inhibit cell growth in culture of three standard tumor cells lines (L1210, S180, and HL60; see *table I*).

4. Results and discussion

These results show that the rigid cycloaliphatic system, bicyclo[2.2.2]octane, cannot replace the planar aromatic ring to produce an effective DHFR inhibitor of the AMT/MTX class. Although compound **2** appeared to meet spatial requirements, the bicyclo[2.2.2]octane group was no more tolerated than the modification in the only related example that we know of, specifically the MTX analogue bearing a simple cyclohexane ring in place of the 1,4-phenylene group [20]. That modification was 740-fold less effective than MTX (IC_{50} value of 20 μM vs. 0.027 μM for MTX) in the inhibition of DHFR (pigeon liver), and it displayed no significant activity against KB cells in culture or L1210 leukemia in mice.

5. Conclusion

Substitution of the bicyclo[2.2.2]octane ring in place of the benzene ring in AMT analogue **1** renders the structure incapable of the tight binding to DHFR displayed by the parent AMT and analogues bearing



Reagents & Conditions: a) $(C_6H_5O)_2PON_3$, Et_3N, rt.; b) 1hr; Δ to 110°C, 45 min; c) BnOH, 110°C, 1 hr, 96% in three steps; d) aq EtOH, rt, 20 hr; D, 60°C, aq HCl, 85%; e) DPPA, L-Glu-diME, Et_3N, DMF, -5°C to 0°C, 2 hr then rt 18 hr, 67%; f) 10% Pd/C, H_2, MeOH, 1 atm, rt, 6 hr, 100%, g) **6**, Me_2NAc, rt, 48 hr, 41%; h) 10% aq NaOH, rt, 20 hr, aq HCl (to pH 4.4) at 0°C, 56%.

Figure 1. Synthetic route to compound **2**, the bicyclo[2.2.2]octane analogue of aminopterin.

appropriate aromatic ring systems in the sidechain [4, 5]. The electronic features of an appropriate aromatic ring may be required for a potential inhibitor of the AMT class to be fixed in position for the several other critical binding sites of the structure to form the hydrophobic, hydrogen, and salt bridge bonds that collectively produce effective inhibition of the enzyme [15].

6. Experimental

6.1. Methods

Examinations by TLC were performed on Analtech precoated (250 μm) silica gel G(F) plates. High-performance liquid chromatography (HPLC) assays were made with a Waters Associates ALC-242 liquid chromatograph equipped with an ultraviolet detector (254 nm) and a M-6000 pump using a 30×0.29 cm C_{18} μBondapak column. Purity assays were done in the reversed-phase isocratic mode with a mobile phase consisting of MeCN (10 or 15% by volume) in 0.1 M NaOAc (pH 3.6). Saponification of **9** to give **2** was monitored using a 20-min linear gradient system with the combination MeCN+0.1 M NaOAc (pH 3.6) changing from 15% MeCN to 50%. Purifications by preparative TLC were done on Analtech silica gel G(F) plates (2 mm). Column chromatographic purifications were done with silica gel (Merck, 60 A, 230–400 mesh for flash chromatography). Evaporations were performed with a rotary evaporator, higher boiling solvents (DMF, *N*,*N*-dimethylacetamide or DMAc, DMSO) were removed in vacuo (<1 mm, bath to 35°C), and more volatile solvents with a water aspirator. Products were dried in vacuo (<1 mm) at 22–25°C over P_2O_5 and NaOH pellets. The final product was dried, and then allowed to equilibrate with ambient conditions of the laboratory. Analytical results indicated by element symbols were within ±0.4% of the theoretical values. Spectral determinations and elemental analyses were performed in the Molecular Spectroscopy Section of Southern Research Institute under the direction of Dr J.M. Riordan. The ^{1}H-NMR spectra were determined in DMSO-d_6 with a Nicolet NMC 300 NB spectrometer using Me_4Si as internal reference. Chemical shifts (δ) listed for multiplets were measured from the approximate centers, and relative integrals of peak areas agreed with those expected for the assigned structures. Mass spectra were recorded on a Varian MAT 311A mass spectrometer in the fast-atom-bombardment (FAB) mode.

*6.1.1. Synthesis of sidehain precursor **6** (figure 1, part 1)*

*6.1.1.1. Ethyl hydrogen bicyclo[2.2.2]octane-1,4-dicarboxylate (**2**)*

This compound was prepared essentially as described by Roberts et al. [16]. Our sample had a m.p. 146–150°C (lit. [16] m.p. 149.0–150.5°C) and was homogeneous by TLC ($CHCl_3$–MeOH–AcOH, 95:5:0.5; detection by I_2 vapor). ^{1}H-NMR: $\delta = 1.16$ (t, 3H, CH_2CH_3), 1.70 (s, 12H, cycloaliphatic CH_2), 4.03 (q, 2H, $-CH_2CH_3$).

*6.1.1.2. Ethyl 4-[[(benzyloxy)carbonyl]amino]-bicyclo[2.2.2]octane-1-carboxylate (**3**) from **2** in steps a–c (figure 1, part 1)*

Step a: a solution of 30.0 mmol each of **2** (6.78 g), DPPA (8.28 g), and Et_3N (3.03 g) in dry toluene (120 mL) was stirred at room temperature for 1 h.

Step b: the solution of the resulting azide was then slowly heated over 45 min to boiling.

Step c: the solution, now containing the isocyanate, was cooled slightly and treated with an excess of benzyl alcohol (30 mL, 0.29 mol). The resulting solution was then refluxed 18 h, cooled, and washed successively with solutions of 5% citric acid, 5% $NaHCO_3$, and saturated NaCl. The dried (Na_2SO_4) and filtered organic phase was then evaporated in vacuo (finally at <1 mm, both at 80°C) to constant weight to leave **3** as a nearly colorless oil (9.53 g, 96% overall yield). ^{1}H-NMR: $\delta = 1.15$ (t, 3H, CH_3), 1.75 (s, 12H, CH_2 of ring), 4.00 (q, 2H, CH_2CH_3), 4.94 (s, 2H, $CH_2C_6H_5$), 7.02 (s, 1H, NH), 7.3–7.4 (m, 5H, C_6H_5).

*6.1.1.3. 4-[[(Benzyloxy)carbonyl]amino]bicyclo[2.2.2]-octane-1-carboxylic acid (**4**)*

A stirred solution of **3** (4.56 g, 13.8 mmol) in EtOH (100 mL) was treated with 1 N NaOH (70 mL). The resulting solution was kept at room temperature for 20 h before being warmed at 55–65°C (bath temp) for 1 h. EtOH was then removed by evaporation (H_2O aspirator) until the concentrate became cloudy, indicating incomplete saponification. EtOH (about 30 mL) was added to clarify, and the solution was kept at room temperature about 3 h longer. The solution was again evaporated until nearly free of EtOH; addition of H_2O (250 mL) produced only faint turbidity indicating virtually complete saponification. Extraction with Et_2O clarified the aqueous phase and evaporation (H_2O aspirator) removed residual Et_2O. The aqueous solution was acidified with stirring to pH 3.5 by dropwise addition of 12 N HCl to cause precipitation of **5** as a white solid; yield 85% (3.56 g), m.p. 189–191°C. ^{1}H-NMR: $\delta = 1.74$ (s, 12H, CH_2 of ring), 4.96 (s 2H, $CH_2C_6H_5$), 7.00 (s, 1H, NH), 7.3–7.4 (m, 5H, C_6H_5), 12.04 (s, 1H, CO_2H). Anal. ($C_{17}H_{21}NO_4$) C, H, N.

*6.1.1.4. N-[4-[[(Benzyloxy)carbonyl]amino]bicyclo[2.2.2]-octane-1-carbonyl]-L-glutamic acid dimethyl ester (**5**)*

A stirred solution of **4** (2.00 g, 6.59 mmol) and dimethyl L-glutamate·HCl (1.68 g, 7.94 mmol) in DMF (50 mL) was chilled to −5–0°C (bath temperature) and treated with a solution of DPPA (1.7 mL, 2.17 g, 7.89 mmol) in DMF (5 mL) followed by Et_3N (1.90 mL, 1.38 g, 13.7 mmol). The reaction mixture was kept at −5–0°C for 2 h before it was allowed to warm to room temperature and left for 18 h. DMF was removed in vacuo (<1 mm, bath to 30°C). The residual mixture of solid and oil was stirred with $CHCl_3$ (150 mL) and H_2O (100 mL). The H_2O layer was extracted again with $CHCl_3$ (150 mL). After the $CHCl_3$ layers were combined, the solution was washed successively with 5% $NaHCO_3$, H_2O, 0.1 N HCl, and again with H_2O before it was dried (Na_2SO_4) and evaporated to give crude **5** as a clear oil. The oil was dissolved in cyclohexane–EtOAc (1:1), and the solution was applied to a silica gel column. Elution with the same solvent afforded fractions homogeneous by TLC (R_f 0.3, detection by I_2 vapor). The combined and evaporated homogeneous fractions afforded **5** as clear oil (2.00 g, 67% yield). Mass: $m/z = 461$ (MH^+). ^{1}H-NMR: $\delta = 1.74$ (s, 12H, cycloaliphatic ring CH_2), 1.85, 1.98 (2m, 2H, $CHCH_2CH_2$, nonequivalent), 2.33 (t, 2H, $CHCH_2CH_2$), 3.58, 3.59 (2s, 6H, $-CO_2CH_3$), 4.20 (m, 1H, $NHCHCH_2$), 4.93 (s, 2H, $CH_2C_6H_5$), 6.99 (s, 1H, –OCONH), 7.34 (m, 5H, C_6H_5), 7.64 (d, 1H, $CONHCH$). In a second run, same scale and procedure as above, the product crystallized after evaporation following purification by chromatography; yield 2.18 g (72%), m.p. 95–98°C. Mass: $m/z = 461$ (MH^+). A portion of the oily product from the first run crystallized rapidly when seeded and had identical m.p. with the product from the second run.

*6.1.1.5. N-(4-Aminobicyclo[2.2.2]octane-1-carbonyl)-L-glutamic acid dimethyl ester (**6**)*

Hydrogenolysis of **5** (945 mg, 2.05 mmol) was carried out in stirred MeOH (20 mL) containing 10% Pd on C

(50 mg) at ambient conditions. Uptake of H_2 (observed by replacement with H_2O in a gas buret) had stopped after 6 h. The filtered solution was evaporated (final conditions 1 mm, bath 25°C) to give **7** as a clear oil. Further drying (1 mm over P_2O_5 at 23–25°C) brought the oil to constant weight (670 mg, 100% yield), but it proved to be hygroscopic under ambient conditions. MS: $m/z = 327$ (MH^+). ^{1}H-NMR: $\delta = 1.44$, 1.70 (2m, 6H each, CH_2 due to bicycloaliphatic ring), 1.85, 1.98 (2m, 2H, $CHCH_2CH_2$, nonequivalent), 2.34 (t, 2H, $CHCH_2CH_2$), 3.58, 3.59 (2s, 6H, $-CO_2CH_3$), 4.20 (m, 1H, $NHCHCH_2$), 7.62 (d, 1H, $CONHCH$). Anal. ($C_{16}H_{26}N_2O_5 \cdot H_2O$) C, H, N.

6.1.2. Alkylation of **6** with **7**

6.1.2.1. N-[4-[[(2,4-Diamino-6-pteridinyl)methyl]amino]-bicyclo[2.2.2]octane-1-carbonyl]-L-glutamic acid dimethyl ester (**8**)

A solution of **6** (670 mg, 2.05 mmol) and 6-(bromomethyl)-2,4-pteridinediamine hydrobromide (**7**, 251 mg of 87% purity[1], 0.650 mmol) in DMAc (10 mL) was stirred at room temperature under N_2 in a stoppered flask wrapped in Al foil. After 48 h, silica gel (1.4 g of 60–200 mesh) was added, and the mixture was then evaporated in vacuo (<1 mm, bath to 35°C) to a dry yellow dispersion. The dispersion was pulverized and dried further in vacuo (<1 mm over P_2O_5) before it was placed atop a column (200 cm of 3 cm diameter) of silica gel (230–400 mesh) that had been poured from $CHCl_3$–MeOH (9:1) containing concentrated NH_4OH (0.5% by volume). Elution by the same solvent system afforded fractions homogeneous by TLC ($R_f \sim 0.5$, 1:1 $CHCl_3$–MeOH). Pure **8** obtained as a yellow solid on evaporation of the pooled fractions weighed 100 mg (20% yield). Because the yield was less than expected, the column was extruded and divided into approximate thirds. The middle and lower thirds were then extracted by stirring with elution medium. Evaporation of the filtered extract from the middle third gave 80 mg of **8** homogeneous by TLC and the lower third gave 25 mg of essentially homogeneous **8**. The total yield was 205 mg (41%). MS: $m/z = 501$ (MH^+ for $C_{23}H_{32}N_8O_5$). ^{1}H-NMR: $\delta = 1.55$, 1.74 (2m, 6H each, cycloaliphatic CH_2), 1.85, 1.98 (2m, 2H, $CHCH_2CH_2$, nonequivalent), 2.34 (t, 2H, CH_2CH_2CO), 3.58, 3.59 (2s, 6H, both CO_2CH_3), 3.81 (s, 2H, CH_2N), 4.22 (q, 1H, $NHCHCO$), 6.52 (br s, 2H, NH_2), 7.65 (d, 1H, CONH) overlapping with 7.0 (s, 2H, NH_2), 8.71 (s, 1H C^7–H).

6.1.2.2. N-[4-[[2,4-Diamino-6-pteridinyl)methyl]amino]-bicyclo[2.2.2]octane-1-carbonyl]-L-glutamic acid (**1**)

A stirred suspension of the ester **8** (90 mg, 0.18 mmol) in H_2O (1 mL) was treated with 1*N* NaOH (0.40 mL, 2.2 molar equiv.). Solution occurred within 20 min. After 20 h at 20–23°C, HPLC analysis showed complete conversion to a single product. The solution was cooled in an ice-H_2O bath and carefully acidified to pH 4.4 using 1 N HCl (0.4 mL) to cause **1** to precipitate as a pale yellow solid. The mixture was kept in a refrigerator overnight before the solid was collected and washed sparingly with cold H_2O; yield 56 mg (56%). MS: $m/z = 473$ (MH^+). ^{1}H-NMR: $\delta = 1.6$–2.0 (overlapping m, 14H; 1.70, 1.78 cycloaliphatic CH_2 over most of m due to $CH_2CH_2CO_2H$), 2.22 (m, 2H, $CH_2CH_2CO_2H$), 3.9–4.2 (overlapping m, CH_2NH and $CHCH_2$), 6.64 (s, 2H, NH_2), 7.36 (d, 1H, CONH), 7.68, 7.80 (2 bs, 2H, NH_2), 8.73 (s, 1H, C^7-H). HPLC: single peak in gradient and isocratic mode; respective retention times 2.28 and 5.80 min. Anal ($C_{21}H_{28}N_8O_5 \cdot 4.8H_2O$) C, H, N.

[1] Compound **8**, prepared as described in [9], was estimated from its integrated ^{1}H-NMR spectrum to be of 87% purity; other matter present was the 6-methyl analogue (2%) and 2-PrOH (11%).

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