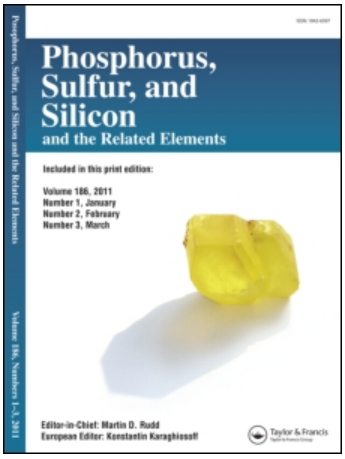

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Quinazoline Antifolate Thymidylate Synthase Inhibitors: Replacement of Glutamic Acid by Aminophosphonic Acids

Maciej Makowski[a]; Krzysztof Pawełczak[a]; Paweł Kafarski[b]; Jolanta M. Dzik[c]; Barbara Gołos[c]; Małgorzata Balinska[c]; Wojciech Rode[c]
[a] Institute of Chemistry, University of Opole, Opole, Poland [b] Technical University of Wrocław, Wrocław, Poland [c] Nencki Institute of Experimental Biology, Warszawa, Poland

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QUINAZOLINE ANTIFOLATE THYMIDYLATE SYNTHASE INHIBITORS: REPLACEMENT OF GLUTAMIC ACID BY AMINOPHOSPHONIC ACIDS

Maciej Makowski,[a] *Krzysztof Pawełczak,*[a] *Paweł Kafarski,*[b] *Jolanta M. Dzik,*[c] *Barbara Gołos,*[c] *Małgorzata Balinska,*[c] *and Wojciech Rode*[c]
Institute of Chemistry, University of Opole, Opole, Poland;[a] *Technical University of Wrocław, Wrocław, Poland;*[b] *and Nencki Institute of Experimental Biology, Warszawa, Poland*[c]

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The synthesis of six analogues of the potent thymidylate synthase (TS) inhibitor N-*[4-[*N-*[(3,4-dihydro-2-methyl-4-oxo-6-quinazolinoyl)-methyl]-*N-*prop-2-ynylamino]benzoyl]-*L*-glutamic acid* ***2*** *is described in which the glutamic acid residue has been replaced by* DL*-aminophosphonic acids. New antifolates were tested as inhibitors of TS isolated from mouse L1210 leukemic cells as well as inhibitors of growth mouse leukemic L5178Y cells. In general these modifications result in compounds that are considerably less potent than* ***2*** *as TS inhibitors with K_i's 0.17–1.10 μM. Very poor solubility in water limited their proper assay of growth cells inhibition.*

Keywords: Aminophosphonic acid analogues of antifolates; antifolates; thymidylate synthase inhibitors

Discovery, development, and clinical trial of 10-propargyl-5,8-dideazafolic acid **1**, a potent inhibitor of thymidylate synthase (TS) have stimulated and refocused antifolate cancer chemotherapy in the last two decades.[1–5] Clinically active but renal toxic drug **1** was withdrawn from the clinic and replaced with the more soluble and non toxic 2-methyl-2-desamino compound **2** with improved pharmacological properties (Figure 1).[6–10] Antifolates containing glutamyl residue (classical

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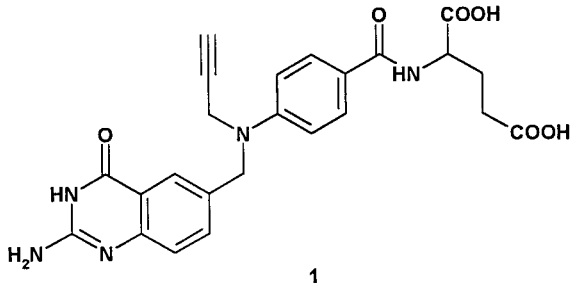
Address correspondence to Krzysztof Pawełczak, Institute of Chemistry, University of Opole, 48 Oleska Street, Opole 45052, Poland. E-mail: pawel@uni.opole.pl



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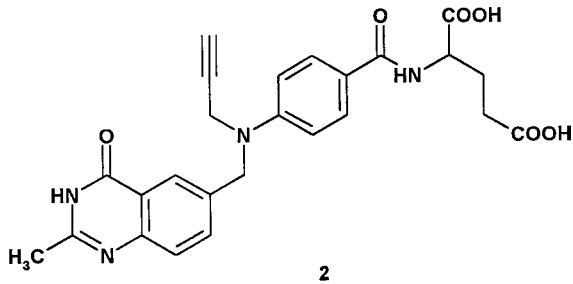

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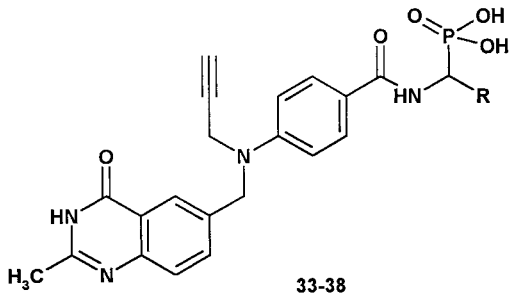


33-38

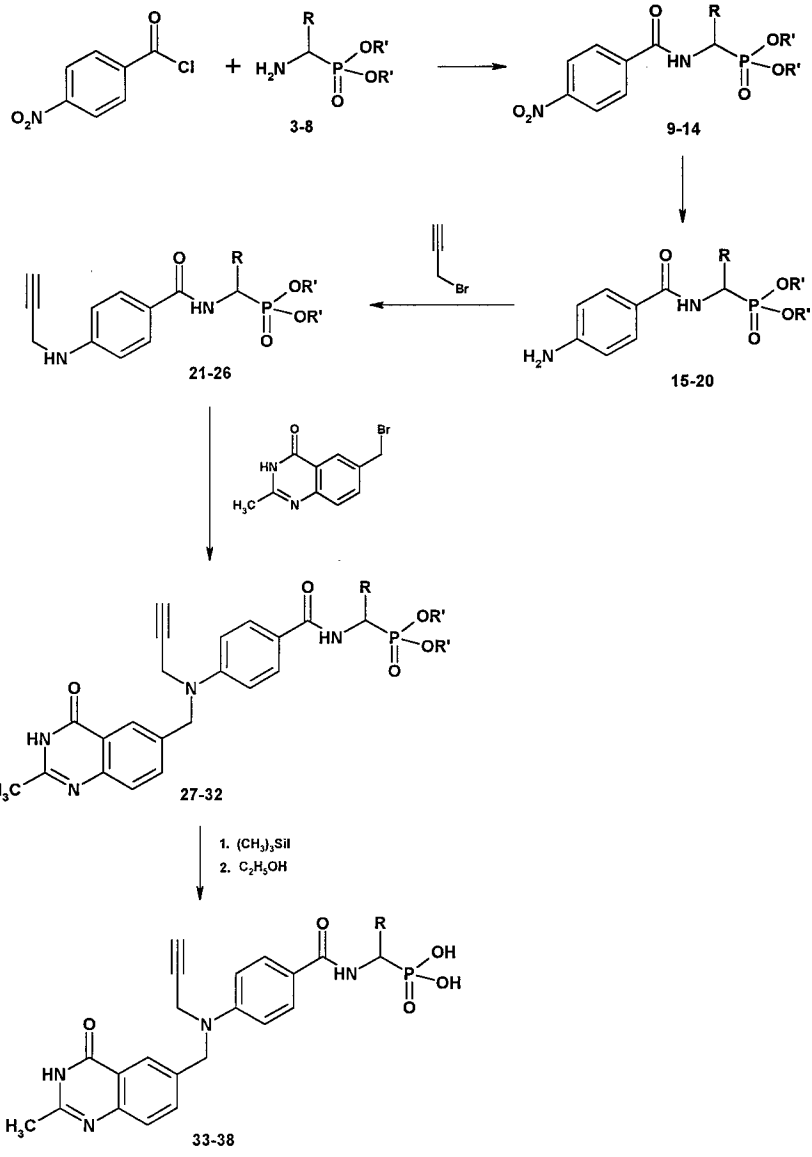
		R
33	DL-LeuP	$-CH_2CH(CH_3)_2$
34	DL-ValP	$-CH(CH_3)_2$
35	DL-NvalP	$-CH_2CH_2CH_3$
36	DL-AbuP	$-CH_2CH_3$
37	DL-Phg	$-C_6H_5$
38	DL-^{t}BugP	$-C(CH_3)_3$

FIGURE 1

TS inhibitors like **1** and **2**) require transport by the reduced folate carrier for entry into cells and are converted intracellularly into polyglutamylated species. Intracellular polyglutamylation is an important aspect of their pharmacology.[11] The fact that tumor cells can acquire resistance to classical antifolates by deletion or modification of active transport or enzymic polyglutamylation have stimulated the search for compounds that do not require these mechanisms to express antitumor activity. Compounds **1** and **2** with well defined TS inhibitory activity and cytotoxic potencies are still the starting point for molecular modifications in the search for antifolates, which do not require active transport into cells or polyglutamylation for activity. An example of such an approach is the synthesis and evaluation of biological activity of analogues of **2** in which the glutamic acid residue was replaced by other amino acids.[11] In general these modifications resulted in compounds that have equivalent TS inhibitory potency to **2**. Moreover these compounds had lower cytotoxicity if compared to **2** because of their inability to undergo intracellular conversion to polyglutamylated forms. In this article we describe the synthesis and evaluation of biological activity of six analogues (**33–38**) of TS inhibitor **2** in which the glutamic acid residue is replaced by aminophosphonic acid residues containing lipophilic α-substituents (Figure 1).

CHEMISTRY

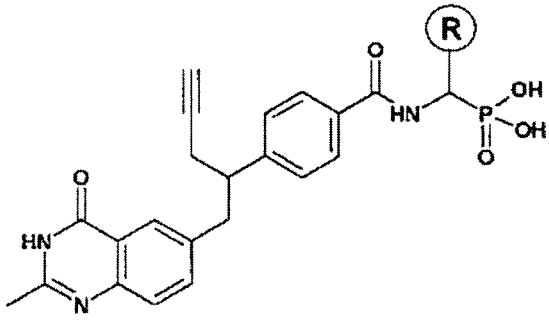
The general method for the synthesis of aminophosphonate analogues of **2** is outlined in Chart 1. 4-Nitrobenzoyl chloride was coupled with appropriate dialkyl esters of aminophosphonic acid (**3–8**) to give nitro derivatives (**9–14**) which after reduction by catalytic hydrogenation gave diesters of *N*-(4-aminobenzoyl)aminophosphonic acids (**15–20**). Alkylation with propargyl bromide yielded the appropriate secondary amines (**21–26**). Further alkylation with 2-methyl-(6-bromomethyl)-4-quinazolone gave antifolate esters. Removal of the alkyl groups from the blocked phosphonate esters was the limiting step of the syntheses and was accomplished by standard silylation procedure. This procedure caused the isolation of final product without laborious column chromatography. The purity of all compounds was established by elemental analysis; structures of phosphonate diesters (**27–32**) and final products (**33–38**) were confirmed by ^{1}H, ^{13}C, and ^{31}P NMR spectroscopy.



		R	R'						
33	DL-LeuP	$-CH_2CH(CH_3)_2$	CH_3	3	9	15	21	27	33
34	DL-ValP	$-CH(CH_3)_2$	C_2H_5	4	10	16	22	28	34
35	DL-NvalP	$-CH_2CH_2CH_3$	CH_3	5	11	17	23	29	35
36	DL-AbuP	$-CH_2CH_3$	CH_3	6	12	18	24	30	36
37	DL-Phg	$-C_6H_5$	C_2H_5	7	13	19	25	31	37
38	DL-tBugP	$-C(CH_3)_3$	CH_3	8	14	20	26	32	38

CHART 1

TABLE I Inhibition of Thymidylate Symnthase (TS) from Mouse L1210 Cells and In Vitro Cytotoxicity of Aminophosphonate Analogues



Compd.	R	TS inhibition K_i (μM) L1210	Cell growth inhibition I_{50} (mM) Mouse L5178 leukemic cells
2		0.010[a]	0.09[b]
33	—$CH_2CH(CH_3)_2$	0.83	1
34	—$CH(CH_3)_2$	0.43	1.5
35	—$CH_2CH_2CH_3$	0.71	1
36	—CH_2CH_3	0.29	3
37	—C_6H_5	0.17	5
38	—$C(CH_3)_3$	1.1	2

[a]Ref. 9.
[b]in μM units.

BIOLOGICAL EVALUATION

The antifolates **33–38** listed in Table I were tested as inhibitors of TS isolated from mouse leukemia L1210 cells. The purification and assay method used for TS from L1210 cells was previously described.[12] Mouse leukemia L5178Y cells used for I_{50} assay were plated with density 10^5 per ml, grown for 4 h exposed to different concentration of inhibitor for 48 h. Direct cell counting was conducted with inverted light microscope staining with trypan blue. Each experiment was conducted three times in triplicates.

RESULTS AND DISCUSSION

Replacement of the C-terminal carboxylic moiety of the amino acid by a phosphonic acid group is a well-established strategy for the synthesis of

new amino acids mimetics. This approach, applied in numerous cases, has shown that strong electrostatic binding of a phosphonate dianion by appropriate portions of target enzymes accounts significantly for the inhibitory action of many of the phosphonic acid analogues of amino acids and their derivatives.[13]

The synthesis of antifolates containing phosphonic acid analogues of glutamic acid has been limited so far to the compounds in which the γ-carboxylic moiety of glutamic acid was replaced by phosphonate, and thus did not give the information about the binding ability of α-carboxylic part of molecule.[14–18] In this article we synthesized analogues **33–38** of compound **2** based on the premise that the binegative phosphonate anion will be bound by the positively charged α-carboxylate binding site of the thymidylate synthase and thus act as the inhibitor of the enzyme. This appeared, however, to be not the case since data shown in Table I indicate 17–110 times weaker inhibition of enzyme caused by these compounds if compared with the parent compound **2**. The antileukemic activity of our phosphonic acid analogues of antifolates was negligible. This may result in part from very weak solubility of compounds **33–38** in water, which was a limiting factor in cell growth inhibition studies.

EXPERIMENTAL

Oxalates of dialkyl esters of aminophosphonic acids (**3–8**) were prepared according to a procedure previously reported and 6-(bromomethyl)-3,4-dihydro-2-methyl-4-quinazolone was prepared according to the known procedure.[8,12] 4-Nitrobenzoyl chloride and propargyl bromide were commercial products (Merck, 820885; Fluka, 81830). Tetrahydrofuran (THF), dioxane, diethyl ether, and triethylamine were distilled from sodium and stored over activated (250°C) 4 Å molecular sieves. Dimethylformamide was azeotropically distilled and similarly dried. The hydrogenolysis catalyst was 10% Pd/C and was used at 20% of substrate weight. Reactions were monitored and the homogeneity of products checked by TLC on silica gel 60 (Merck, 5553) with the following eluents: (A) 8% $CH_3OH/CHCl_3$; (B) 20% $CH_3OH/CHCl_3$; (C) 35% acetone/$CHCl_3$; (D) 5% acetone/$CHCl_3$; (E) acetone. Spots were visualized with chlorine-tolidine reagent. Full protected compounds **21–38** were purified by low pressure short column chromatography on silica gel 60 (Merck, 7736). Melting points were determined on a Boëtius heating block and are uncorrected. ^1H, ^{31}P, and ^{13}C NMR spectra were determined on Brucker (300 MHz) spectrometer.

The elemental analyses were performed at the Institute of Organic Chemistry, Biochemistry and Biotechnology of Technical University of Wrocław.

Preparation of Dialkyl Esters of *N*-(4-Nitrobenzoyl)aminophosphonic Acids (9–14)

To a stirred suspension of an appropriate oxalate of aminophosphonic acid alkyl ester **3–8** (1 mmol) in dioxane (4 ml), *N*-methyl-morpholine (0.33 ml, 3 mmol) was added. After 15 min, stirring at room temperature, 4-nitrobenzoyl chloride (0.186 g, 1 mmol) was added and stirring was continued for 1.5 h. Salts were filtered off and the filtrate evaporated to a dense oil that was dissolved in ethyl acetate (50 ml). The organic layer was washed with 0.2N HCl, saturated $NaHCO_3$ and brine, dried ($MgSO_4$), and concentrated to one-half volume. A white crystalline solid was obtained from AcOEt–hexane. Yields and melting points of products are given in Table II.

Preparation of Dialkyl Esters of *N*-(4-Aminobenzoyl)aminophosphonic Acids (15–20)

A solution of an appropriate dialkyl ester of *N*-(4-nitrobenzoyl)aminophosphonic acid (9–14) (1 mmol) in EtOH (10 ml) containing Pd/C in suspension was stirred under nitrogen for 30 min, whereupon TLC (system A) showed the absence of starting material. The catalyst was removed by filtration and the filtrate evaporated to dryness. The white crystalline solids after evaporation were crystallized from the solvent mixture MeOH–AcOEt–pentane. Yields and melting points of products are given in Table II.

Preparation of Dialkyl Esters of *N*-[4-(Prop-2-ynylamino)benzoyl)aminophosphonic Acids (21–26)

A mixture of appropriate dialkyl ester of *N*-(4-aminobenzoyl)aminophosphonic acid (**15–20**) (1 mmol), $CaCO_3$ (1.5 mmol), and propargyl bromide (1.5 mmol) in DMAA (0.5 ml) was stirred in the absence of light at room temperature for 24 h. The mixture was diluted with MeOH (1 ml), filtered, and the solvent removed in vacuo to give a brown oil. The oil was dissolved in 2% pyridine in $CHCl_3$ (1 ml) and purified on a column (3 cm i.d. $\times$ 7 cm L) of silica gel (22 g) using $CHCl_3$ and solutions 1–4% of pyridine in $CHCl_3$ as

TABLE II Preparation of Anifolate Syntons (**9–26**)

Compd.	Scale (mmol)	Yield (%)	m.p. (°C)	Formula	Analysis (%) Calcd (Found) C	H	N
9	10	80	160–162	$C_{14}H_{21}N_2O_6P$	48.84	6.15	8.14
					(48.52	6.23	8.31)
10	10	87	127–128	$C_{15}H_{23}N_2O_6P$	50.28	6.47	7.82
					(50.38	6.18	7.75)
11	10	76	12–122	$C_{13}H_{19}N_2O_6P$	47.28	5.80	8.48
					(47.42	5.57	8.18)
12	10	89	166–167	$C_{12}H_{17}N_2O_6P$	45.58	5.42	8.86
					(45.64	5.12	8.57)
13	10	78	144–147	$C_{18}H_{21}N_2O_6P$	55.10	5.39	7.14
					(55.37	5.34	7.18)
14	10	73	166–168	$C_{14}H_{21}N_2O_6P$	48.84	6.15	8.14
					(48.95	6.05	8.11)
15	5	93	195–198	$C_{14}H_{23}N_2O_4P$	53.50	7.38	8.91
					(53.18	7.42	8.97)
16	5	97	184–185	$C_{15}H_{25}N_2O_4P$	54.87	7.67	8.53
					(54.71	7.82	8.65
17	5	98	158–161	$C_{13}H_2N_2O_4P$	52.00	7.05	9.33
					(51.82	7.08	9.37)
18	5	98	184–187	$C_{12}H_{19}N_2O_4P$	50.35	6.69	9.79
					55.32	6.56	9.54
19	5	91	181–184	$C_{18}H_{23}N_2O_4P$	59.66	6.40	7.33
					(59.54	6.34	7.23)
20	5	94	181–184	$C_{14}H_{23}N_2O_4P$	53.50	7.38	8.91
					(53.65	7.12	8.97)
21	4	41	167–169	$C_{17}H_{25}N_2O_4P$	57.95	7.15	7.95
					(58.12	7.01	7.83)
22	5	40	119–121	$C_{18}H_{27}N_2O_4P$	59.01	7.43	7.65
					(59.34	7.12	7.82)
23	4	47	153–156	$C_{16}H_{23}N_2O_4P$	56.80	6.85	8.28
					(56.67	6.92	8.32
24	1	38	146–149	$C_{15}H_{21}N_2O_4P$	55.55	6.53	8.64
					(55.67	6.41	8.65)
25	5	53	147–148	$C_{21}H_{23}N_2O_4P$	62.99	6.29	7.00
					(63.15	6.18	7.08
26	5	37	145–147	$C_{17}H_{25}N_2O_4P$	57.95	7.15	7.95
					(58.17	7.02	7.19)

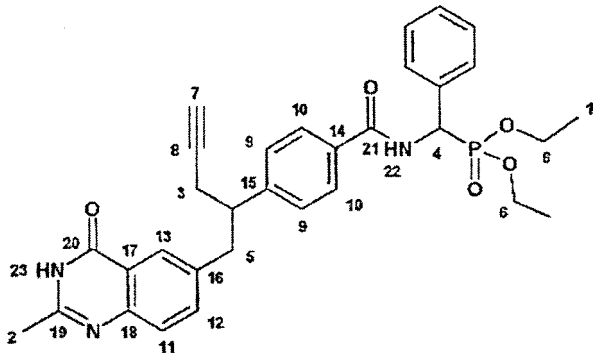
eluents. Product containing fractions were combined and evaporated in vacuo to give an oil. The oil was dissolved in MeOH (1 ml) and AcOEt (5 ml), and petroleum ether was added to turbidity. Yields, melting points, and analytical data of these products are given in Table II.

TABLE III Preparation of Antifolate Esters (**27–32**) and Antifolate Free Acids (**33–38**)

Compd.	Scale (mmol)	Yield (%)	m.p. (°C)	Formula	Analysis (%) Calcd (Found) C	H	N	^{31}P-NMR, δ
27	2	46	118–121	$C_{27}H_{33}N_4O_5P$	61.28	6.34	10.68	30.2[a]
					(61.12	6.42	10.34)	
28	4	47	112–114	$C_{28}H_{35}N_4O_5P$	62.44	6.55	10.40	28.9[a]
					(62.13	6.67	10.32)	
29	4	50	113–115	$C_{26}H_{31}N_4O_5P$	61.17	6.12	10.97	29.8[a]
					(61.25	6.18	10.87)	
30	3	36	117–120	$C_{25}H_{29}N_4O_5P$	60.48	5.89	11.28	26.6[a]
					(60.31	5.97	11.14)	
31	2	42	159–161	$C_{31}H_{33}N_4O_5P$	65.03	5.81	9.78	23.7[a]
					(64.92	5.93	9.53)	
32	1.5	53	200–203	$C_{27}H_{33}N_4O_5P$	61.82	6.34	10.68	28.5[a]
					(61.49	6.78	10.52)	
33	0.5	60	183–184	$C_{25}H_{29}N_4O_5P \cdot H_2O$	59.51	6.19	11.10	18.9[b]
					(59.54	6.23	11.08)	
34	0.5	64	168–170	$C_{24}H_{27}N_4O_5P \cdot 1.5\ H_2O$	56.56	5.93	11.00	17.5[b]
					(56.62	6.08	10.91)	22.6[c]
35	0.5	73	172–175	$C_{24}H_{27}N_4O_5P \cdot 3\ H_2O$	53.72	6.20	10.44	
					(53.61	6.12	10.32)	23.9[c]
36	0.5	78	163–167	$C_{23}H_{25}N_4O_5P \cdot H_2O$	56.79	5.59	11.52	22.8[c]
					(56.85	5.42	11.31)	
37	0.5	76	191–194	$C_{27}H_{25}N_4O_5P \cdot 1.5\ H_2O$	59.66	5.20	10.31	—
					(59.42	5.35	10.17)	
38	0.5	71	234–236	$C_{25}H_{29}N_4O_5P \cdot 2\ H_2O$	56.38	6.24	10.52	16.9[b]
					(56.24	6.32	10.63)	22.0[c]

[a] In $CDCl_3$.
[b] In D_2O + NaOD.
[c] In D_2O + D_2SO_4.

TABLE IV ^{1}H and ^{13}C NMR Data for the Main Chain of Representative Antifolate Ester

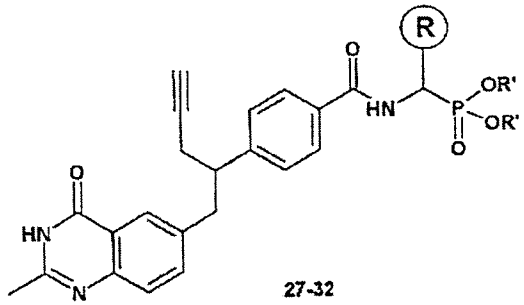


37

C or H	δ ^{1}H, ppm (m, J in Hz)[a]	^{13}C, ppm (J in Hz)[b]
1	1.152 and 1.335 (t, J = 7.1 Hz, 3H, each)	16.56 and 16.59 (5.5 Hz)
2	2.485 (s, 3H)	22.35
3	4.120 (d, J = 2.2 Hz, 2H)	40.44
4	5.91 (d-d, J_{HNH} = 9.6 Hz, J_{PH} = 21.5 Hz, 1H)	50.73 (154.6 Hz)
5	4.710 (s, 2H)	54.83
6	3.791 and 3.819 (q-q, J = J_{PH} = 8.6 Hz, 0.5H each) 3.995 and 4.005 (q-q, J = J_{PH} = 8.6 Hz, 0.5H each) 4.184 and 4.207 (q-q, J = J_{PH} = 7.5 Hz, 1H)	63.88 (7.2 Hz)
7	2.256 (t, J = 2.2 Hz, 1H)	73.30
8		79.07
9	6.863 (d, J = 8.9 Hz, 2H)	113.38
10	7.933 (d, J = 8.9 Hz, 2H)	129.01
11	7.642 (d, J = 8.4 Hz, 1H)	128.5
12	7.691 (d, J = 8.4 Hz, 0.5H each)	133.77
13	8.225 and 8.229 (s, 0.5 each)	124.85
14		123.73
15		149.36
16		121.13
17		135.70
18		151.30
19		154.10
20		163.78
21		167.11 (7.9 Hz)
22	8.146 and 8.157 (d, J_{HNH} = 9.6 Hz, 0.5H each)	
23	10.886 (s, 1H)	

[a] 300 MHz, $CDCl_3$.
[b] 75.47 MHz in $CDCl_3$.

TABLE V ^{1}H Data for the Side Chain of the Various Antifolate Esters



27-32

Compd.	Side chain R	δ ^{1}H, ppm (m, *J* in Hz)[a]
27	*i*-butyl	0.93 and 0.95 (d, J = 6.4 Hz, 3H each); 1.70 (m, 3H, $^{\gamma}$CH, $^{\beta}$CH$_2$); 4.89 (dd-t, J = 6.4 Hz, J_{NH} = 9.8, J_{PH} = 12.3 Hz, 1H, $^{\alpha}$CH$^{\alpha}$)
28	*i*-propyl	1.10 (d, J = 6.4 Hz, 3H each); 2.29 (m, J = 6.4 Hz, 1H, $^{\beta}$CH); 4.74 (d-d, J = 6.4 Hz, J_{PH} = 20.2 Hz, J_{NH} = 10.2 Hz, 1H, $^{\alpha}$CH)
29	*n*-propyl	0.92 (t, J = 7.3 Hz, 3H); 1.42 (q-q, J = 7.3 Hz, J_{PH} = 14.1 Hz, 1H each, $^{\beta}$CH$_2$); 1.75–1.9 (m, 2H, $^{\gamma}$CH$_2$); 4.84 (d-t-t, J = 5.6 Hz, J_{NH} = 9.6 Hz, J_{PH} = 19.5 Hz, 1H, $^{\alpha}$CH)
30	ethyl	1.00 (t, J = 7.3 Hz, 3H, $^{\gamma}$CH$_3$); 1.75–1.8 and 1.8–2.05 (m, 1H each); 4.71 (q-t, J = 6.1 Hz, J_{NH} = 9.8 Hz, J_{PH} = 21.9 Hz, 1H, $^{\alpha}$CH)
31	phenyl	5.91 (dd, J_{NH} = 9.6, J_{PH} = 21.5 Hz, 1H, $^{\alpha}$CH) 7.25–7.35 (m, 3H, phenyl); 7.59 (d, J = 6.3, 2H, phenyl)
32	*t*-butyl	1.11 (s, 9H, $^{\beta}$C(CH$_3$)$_3$); 4.64 (dd, J_{NH} = 10.5 Hz, J_{PH} = 18.7 Hz, 1H, $^{\alpha}$CH)

[a] 300 MHz, $CDCl_3$.

Preparation of Dialkyl Esters of *N*-[4-[*N*-[3,4-Dihydro-2-methyl-4-oxoquinazolin-6-yl)methyl]-*N*-prop-2-ynylamino]benzoyl]-aminophosphonic Acids (27–32)

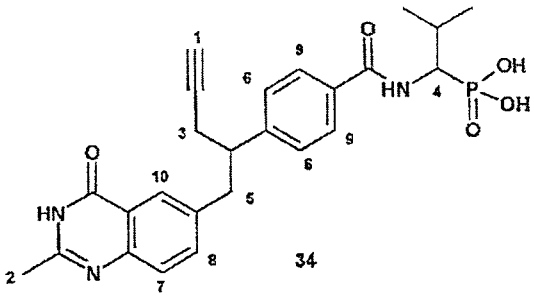
A mixture of appropriate dialkyl ester of *N*-[4-(prop-2-ynylamino)benzoyl)-aminophosphonic acid (**21–26**) (1 mmol), 6-(bromomethyl)-3,4-dihydro-2-methyl-4-oxoquinazoline (1.5 mmol), and $CaCO_3$ (2 mmol) in *N*,*N*-dimethylacetamide (DMA) (3 ml) was stirred in 50°C for 6 h in the dark under argon atmosphere. The mixture was diluted with MeOH (7 ml) and AcOEt (150 ml), and filtered. The filtrate was washed with brine (4 × 15 ml), water (4 × 15 ml), and then dried ($MgSO_4$) and evaporated in vacuo to give an oil. The oil was dissolved

in 4% MeOH in $CHCl_3$ (4 ml) and purified on a column (4 cm i.d. × 6 cm L) of silica gel (40 g) using $CHCl_3$ and solutions 0.5–3.5% of MeOH in $CHCl_3$ as the eluents. Product containing fractions were combined and evaporated in vacuo to give an oil. The products were crystallized from the mixture of AcOEt–MeOH (5:1) and petroleum ether. Yields, melting points, analytical, and ^{31}P NMR data of products **27–33** are given in Table III; ^{1}H and ^{13}C NMR data are given in Tables III and IV.

Preparation of *N*-[4-[*N*-[3,4-Dihydro-2-methyl-4-oxoquinazolin-6-yl)methyl]-*N*-prop-2-ynylamino]benzoyl]aminophosphonic Acids (33–38)

To a solution of an appropriate dialkyl ester of *N*-[4-[*N*-[3,4-dihydro-2-methyl-4-oxoquinazolin-6-yl)methyl]-*N*-prop-2-ynylamino] benzoyl]amino-phosphonic acid (**27–32**) (0.5 mmol) in $CHCl_3$ (10 ml) iodotrimethylsilane (0.27 ml, 2 mmol) was added at 0°C, and the mixture was stirred for 10 min under argon. The cooling bath was

TABLE VI ^{1}H NMR Data for the Main Chain of the Representative Antifolate Free Acid



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Atom H No.	δ ^{1}H, ppm, (m, *J* in Hz)[a]
1	2.2–2.4 (m, usually overlapped with the side chain signals)
2	2.43 (s)
3	4.02 (s)
4	4.04 (d-d, J = 6.7 Hz, J_{PH} = 17.3 Hz)
5	4.38 (s)
6	6.87 (d, J = 8.8 Hz)
7	7.25 (d, J = 8.4 Hz)
8	7.39 (d, J = 8.4 Hz)
9	7.85 (d, J = 8.8 Hz)
10	7.93 (s)

[a]300 MHz, D_2O + NaOD.

then removed and solvent was evaporated in vacuo. The residue was dissolved in EtOH (96%, 25 ml) and stirred at 40°C for 24 h in dark. The solvent was removed in vacuo to give an oil, which was treated with 1N NaOH (2 ml) and mixed for 4 h at room temperature. The mixture was acidified to pH 3.0 with 4N HCl; the precipitate was filtered, washed with water (4 × 5 ml), and dried. Yields, melting points, and analytical data of these products are given in Table III; ^{1}H and ^{31}P NMR data are given in Tables V and VI.

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