Synthesis and in Vitro Antileukemic Activity of Some New 1,3-(Oxytetraethylenoxy)cyclotriphosphazene Derivatives[§]

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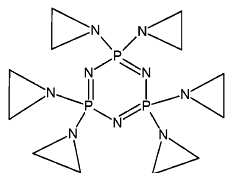
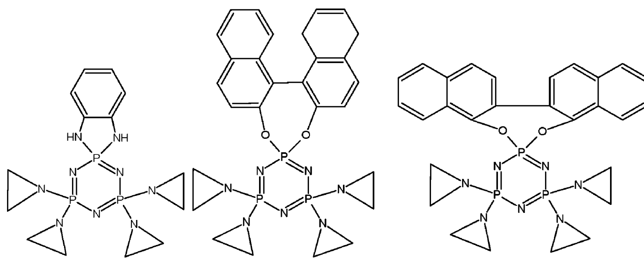
A new series of 1,3-(oxytetraethylenoxy)cyclotriphosphazene derivatives bearing 2-chloroethylamine or salicylaldehyde (2-hydroxybenzaldehyde) or its Schiff base (after condensation with 2-chloroethylamine) units and having also 2-naphthyl or anthraquinone groups as cosubstituents has been synthesized. The in vitro cytotoxic activity of these compounds against a panel of four cancer cell lines has been studied. Most of the compounds exhibited antiproliferative activity in the range of the international criterion for synthetic agents (4 μg/mL) against the MOLT4, L 1210, HL-60, and P388 cell lines chosen for testing.

Introduction

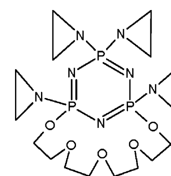
In the search for anticancer drugs many compounds with different structures have been tested. Even though some of them exhibit therapeutical properties and are widely used, the hunt for new substances especially those with improved efficiency and no side effects is still an important research goal. Among others, compounds with a cyclophosphazene core bearing different substituents seem to be good candidates for anticancer drugs. A great number of reports have described the antitumor activity of (1-aziridynyl)cyclophosphazenes,[1−9] mainly hexa-(1-aziridynyl)cyclophosphazene MYKO 63.



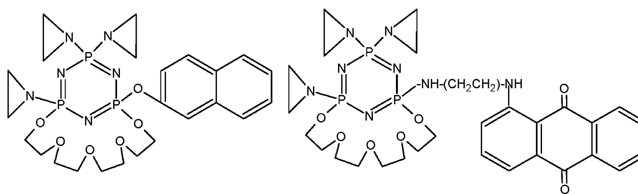
In previous works of our group a series of tetraaziridinylcyclophosphazenes bearing various spiro substituents whose structures are shown below were tested and their cytotoxic



activity against L1210 cells was confirmed.[8,9] The cytostatic activity of aziridynyl groups is believed to result from the formation of a carbocation capable of interacting with DNA via alkylation.[1,10] However, a severe disadvantage of these compounds is their high cumulative bone marrow toxicity and other undesirable side effects. Therefore, a great deal of research has been devoted to overcome these drawbacks and also to improve solubility of aziridinyl cyclophosphazene. The latter property was achieved by incorporation of a crown ether structure into the aziridinyl cyclophosphazene which caused good solubility of the compound in water (∼10% w/w) and also a possibility of metal cation complexation.



The cytostatic activity of 1,3-(oxytetraethylenoxy)-1,3,5,5-tetra(1-aziridynyl)cyclotriphosphazatriene has been tested in vitro using five AIDS-related human lymphoma cell lines and leukemia CCRF-CEM. The compound exhibited remarkable cytotoxic activity.[11] Further modification of this compound by replacement of one aziridine group with an intercalating agent, i.e., 2-naphthol or an anthraquinone derivative, has been carried out.[12]



It was found that introduction of the 2-naphthol structure decreased the in vitro antiproliferative activity of the compound against human and mouse leukemic cells (MOLT 4, P 388, L 1220, HL-60) while the anthraquinone group enhanced cytotoxic activity in comparison with the 1,3-(oxytetraethylenoxy)-1,3,5,5-tetra(1-aziridinyl)cyclotriphosphazatriene without intercalating agent. However, for the synthesis of the compounds described above, it is necessary to use aziridine which in itself is a carcinogen. Another group of compounds capable of DNA alkylation are those with a 2-chloroethylamine moiety.[10,13−15] To the authors' best knowledge there are no previous reports

[§] In memory of Krystyna Brandt.

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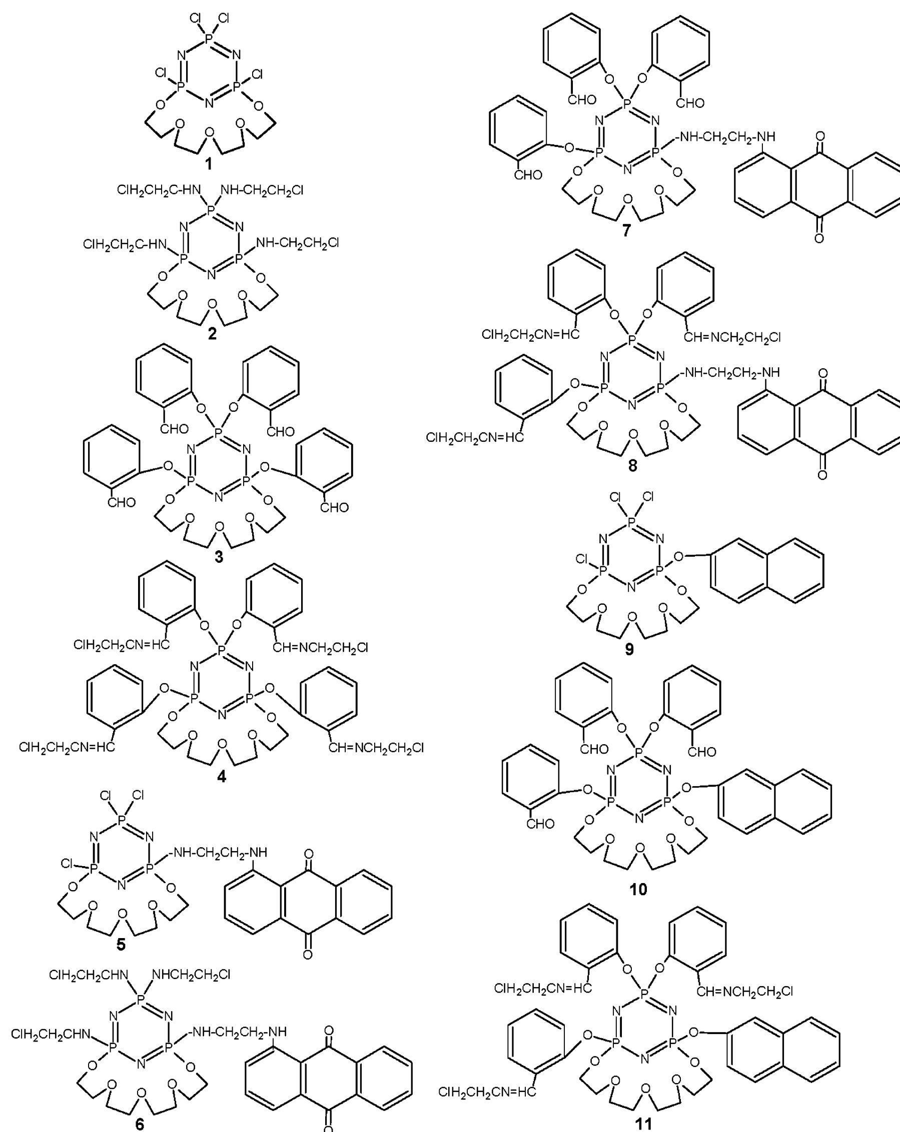

Figure 1. Structures of the compounds **1**−**11**.

concerning the synthesis and cytostatic activity of cyclophosphazatriene substituted with an oxytetraethylenoxy group (crown ether) and a 2-chloroethylamine group, as are presented in this work.

2-Chloroethylamine was bonded directly to the cyclophosphazatriene ring or was introduced in the form of a Schiff base. In the latter case 2-chloroethylamine was condensed with a previously substituted cyclophosphazene ring with 2-hydroxybenzaldehyde (salicylaldehyde). Syntheses of the compounds having additional intercalating groups (2-naphthol, anthraquinone) were also carried out.

Results and Discussion

In this work, two series of derivatives of 1,3-(oxytetraethylenoxy)-1,3,5,5-tetrachlorocyclotriphosphazatriene (**1**, precursor) have been synthesized (Figure 1) and their structures confirmed by elemental analysis, mass spectroscopy, FTIR, ^{1}H NMR, and ^{31}P NMR. In the first group, all chlorine atoms of the precursor **1** were substituted with 2-chloroethylamine being directly connected with phosphorus atoms of the cyclotriphosphazatriene ring as in **2** or 2-chloroethylamine was bonded via an oxyphenylene unit and was in the form of aldimine as in **4**. In the second group intercalating anthraquinone or 2-naphthol structures were introduced additionally (**6**, **8**). Their presence was expected to enhance cytotoxic activity of the compounds in comparison to the ones having only alkylating agent.

The compounds synthesized were tested for their antiproliferative activity in vitro against the cells of four human and mouse leukemic lines. The results are presented in Table 1. All the compounds bearing alkylating and intercalating groups, except **2**, revealed antiproliferative activity against the cells tested. However, the compounds **5** and **6** did not exhibit activity in the range of the international activity criterion for synthetic agents (4 μg/mL).[19]

In the compounds **2** and **6**, the 2-chloroethylamine moiety is directly bonded to phosphorus atoms. This might account for its low DNA alkylating activity. On the other hand, the presence of the anthraquinone structure in **6** induced antiproliferative activity similar to that of the compound **5**, having unsubstituted chlorine atoms. In contrast to **6**, a compound with aziridinyl substituents instead of 2-chloroethylamine groups exhibited antiproliferative activity against the same leukemic cells. However, the tetraaziridinyl compound (without intercalator) was also inactive similar to **2**.[12]

A sufficient cytostatic activity was exhibited by compounds **4** and **8** in which the alkylating agent is in the form of a Schiff base. The presence of an intercalating agent in **8** did not influence its activity significantly. Similarly, introduction of the

Table 1. In Vitro Antiproliferative Activity of the Compounds (Figure 1)

Comp. No as in Fig. 1.	Cell line			ID$_{50}$[μg/ml] ± SD IC$_{50}$[μmol/ml]×10^{-3} ± SD	
	MOLT4	P388	L1210	HL-60	BALB/3T3
1	negative	negative	negative	36.85±6.11 78.57±13.03	negative
2	negative	negative	negative	negative	-
3	3.2±1.2 3.94±1.5	3.2±1.0 3.94±1.2	3.2±1.3 3.94±1.6	2.7±1.3 3.33±1.6	12.12±3.35 14.94±4.13
4	2.1±1.7 1.99±1.6	3.2±.1.0 3.03±0.9	3.2±1.1 3.03±1.0	2.9±1.2 2.74±1.1	25.29±2.58 23.92±1.13
5	29.6±1.6 42.36±2.3	32.8±1.2 46.94±1.7	52.8±1.4 75.56±2.0	38.3±1.1 54.8±1.6	-
6	27.2±1.2 32.87±1.4	19.4±1.4 23.44±1.7	29.3±1.5 35.40±1.8	18.9±1.2 22.8±1.4	-
7	1.14±0.43 1.19±0.5	2.9±0.13 3.03±1.5	2.42±0.62 2.53±0.6	2.23±0.62 2.33±0.6	33.16±11.82 34.70±12.37
8	3.08±0.07 2.70±0.06	2.16±0.57 1.89±0.5	2.69±0.42 2.36±0.4	2.067±0.71 1.81±0.6	9.54±1.89 8.371.66
10	3.09±0.07 3.71±0.08	2.55±0.2 3.06±0.2	2.75±0.18 3.30±0.2	2.81±0.1 3.37±0.1	-
11	3.11±0.08 3.06±0.08	2.70±0.23 2.65±0.2	2.43±0.58 2.39±0.6	2.89±0.11 2.84±0.1	-

[a] ID$_{50}$: the dose of compound that inhibits cell proliferation by 50%.

2-naphthol structure, instead of anthraquinone, in compound **11** did not cause any change in activity. It is very interesting that compounds **3**, **7**, and **10**, having aldehyde groups in their substituents, also exhibited high antiproliferative activity that was little enhanced by the anthraquinone moiety in the compound **7**. In fact, the activity of the compounds having aldehyde and Schiff base groups differ very little.

For comparison, activity of compounds **3**, **4**, **7**, and **8** against normal cells (BALB/ 3T3) was also examined. As can be seen in Table 1, the antiproliferative activity of the compounds is much higher against the cancer cells than against the normal cells.

The following results summarize our investigations dealing with antiproliferative activity in vitro against cells of human and mouse leucemic lines (MOLT4, P388, L1210, HL-60) of the new compounds:

(a) 1,3-(Oxytetraethylenoxy)-1,3,5,5-tetrachlorocyclotriphosphazatriene **1** and its mono- aminoanthraquinone-substituted derivative **5** do not exhibit antiproliferative activity in the range of the international criterion for synthetic agents.

(b) Compound **2** in which four chlorine atoms in compound **1** are substituted with a 2-chloroethylamine moiety also is inactive in relation to the cell lines tested. Replacement of one 2-chloroethylamine residue in **2** by an aminoanthraquinone moiety enhances antiproliferative activity but it is still lower than the value of the international criterion.

(c) Substitution of four chlorine atoms in **1** with 2-oxybenzaldehyde groups (**3**) enhances the cytotoxic activity, being sufficient to meet the international criterion. Slightly better activity resulted from replacement of one 2-oxybenzaldehyde group in **3** by an intercalating agent, i.e., aminoanthraquinone (**7**) or 2-naphthol (**10**) residue.

(d) Cytotoxic activity of compound **4**, a condensation product of aldehyde groups in **3** with 2-chloroethylamine, is comparible with compound **3**. The presence of an intercalating agent does little to enhance antiproliferative activity of compounds **8** and **11** in comparison with compound **4**. These compounds also exhibit activity in the range of the international criterion.

Therefore, new compounds **3**, **4**, **7**, **8**, **10**, and **11** may be considered as the agents with highest potential antileukemic activity and appear to be good candidates for more advanced screening.

Experimental Section

Synthesis of the Investigated Compounds 1−11. Structures are presented in Figure 1.

1,3-(Oxytetraethylenoxy)-1,3,5,5-tetrachlorocyclotriphosphazatriene 1. This compound was prepared as reported previously.[16,17]

1,3-(Oxytetraethylenoxy)-1,3,5,5-tetra(2-chloroethylamine)-cyclotriphosphazatriene 2. 2-Chloroethylamine hydrochloride (2.06 g, 17.6 mmol) and triethylamine (TEA) (4.857 g, 48.0 mmol) in dry benzene (100 mL) were stirred for 1 h at room temperature under an argon atmosphere. Subsequently, **1** (0.938 g, 2.0 mmol) in dry benzene (20 mL) was added. The mixture was stirred for 29 h in boiling solvent and then cooled and filtered. The filtrate was evaporated under reduced pressure. The crude product was column-chromatographed on silica gel using hexane:THF (tetrahydrofuran) (1:1 and next 2:3 and 1:2, v/v) as eluent. The oily substance (yield 48.5%) was crystallized from a mixture of hexane:$CHCl_3$ (1:3, v/v) to provide **2**: mol mass (g/mol) found/calcd M = 642.1/641, mp 84.70 °C. Anal. ($C_{16}H_{36}Cl_4N_7O_5P_3$) C, H, N.

1,3-(Oxytetraethylenoxy)-1,3,5,5-tetra(phenyloxy-2-carboxaldehyde)cyclotriphosphazatriene 3. 1 (0.938 g, 2.0 mmol), 2-hydroxybenzaldehyde (1.495 g, 12 mmol), and sodium hydride (60%) (0.48 g, 12 mmol) in THF (80 mL) were stirred for 27 h at room temperature under an argon atmosphere. The mixture was filtered and the filtrate evaporated under reduced pressure. The crude product was column-chromatographed on silica gel using hexane: THF (3:2 next 1:1 v/v) as eluent. The oily substance (yield 49.5%) was crystallized from mixture of hexane:$CHCl_3$ (1:3, v/v) to provide **3**: mol mass (g/mol) found/calcd M = 812.6/811.48, mp 100.8 °C. Anal. ($C_{36}H_{36}N_3O_{13}P_3$) C, H, N.

1,3-(Oxytetraethylenoxy)-1,3,5,5-tetra(phenyloxy-2-{2-chloroethylimino})cyclotriphosphazatriene 4. 2-Chloroethylamine hydrochloride (0.445 g, 3.8 mmol) and triethylamine (1.154 g, 11.4 mmol) in dry benzene (20 mL) were stirred for 1 h at room temperature in an argon atmosphere. Subsequently, **3** (0.772 g, 0.95 mmol) in dry benzene (20 mL) was added. The mixture was stirred for 4 h in boiling solvent. The mixture was then filtered and benzene evaporated under reduced pressure. The yield of the oily substance **4** was 51.2%, mol mass (g/mol) found/calcd M = 1058.0/1057.48. Anal. ($C_{44}H_{52}Cl_4N_7O_9P_3$) C, H, N.

1-{2-[1,3-(Oxytetraethylenoxy)-3,5,5-tri(2-chloroethylamine)-cyclotriphosphazatrien-1-yl]aminoethylamino}anthraquinone 6. The synthesis of this compound required two steps (a, b). Step a: **1** (0.235 g, 0.5 mmol), 1-(2-aminoethylamino)anthraquinone (prepared as described in the literature[18]) (0.266 g, 1.0 mmol), and NaOH (0.2 g, 5 mmol) were dissolved in a mixture of hexane: THF (1:1, v/v; 60 mL) and heated at 40 °C for 5 h. The mixture was filtered and the filtrate evaporated under reduced pressure. The crude product was column-chromatographed on silica gel using hexane:THF (3:1, v:v) as eluent to provide 1-{2-[1,3-(oxytetraethylenoxy)-3,5,5-trichlorocyclotriphosphazatrien-1-yl]aminoethylamino}-anthraquinone **5** as a red powder. Yield 58.2%, mol mass (g/mol) found/calcd M = 699.2/698.7, mp 149.4 °C. Anal. ($C_{24}H_{29}Cl_3N_5O_7P_3$) C, H, N: calcd, 10.0; found, 9.58.

Step b: 2-Chloroethylamine hydrochloride (0.476 g, 4.07 mmol) and triethylamine (1.123 g, 11.1 mmol) in dry benzene (60 mL) were stirred for 1 h at room temperature in an argon atmosphere. Subsequently, **5** (0.432 g, 0.618 mmol) in dry benzene (60 mL) was added. The mixture was then stirred for 22 h in boiling solvent. After cooling, the mixture was filtered and the solvent evaporated under reduced pressure. The crude product was purified column-chromatographically on silica gel using hexane:THF (2:1, v/v) as eluent. Compound **6** possessed a greasy nature. Yield 18.7%, mol mass (g/mol) found/calcd M = 827.4/827.63 g/mol, mp 84.33 °C. Anal. ($C_{30}H_{44}Cl_3N_8O_7P_3$) C, H, N.

1-{2-[1,3-(Oxytetraethylenoxy)-3,5,5-tri(phenyloxy-2-carboxaldehyde)cyclotriphosphazatrien-1-yl]-aminoethylamino}-anthraquinone 7. 5 (0.699 g, 1 mmol), 2-hydroxybenzaldehyde (0.56 g, 4.5 mmol), and sodium hydride (60%) (0.18 g, 4.5 mmol) in THF (60 mL) were stirred for 29 h at room temperature in an

argon atmosphere. The mixture was then filtered and the filtrate evaporated under reduced pressure. The crude product was chromatographed on a silica gel column using hexane:THF (3:1, v:v) as eluent to provide **7** as a red oil. Yield 44.1%, mol mass (g/mol) found/calcd M = 956.0/955.52. Anal. ($C_{45}H_{44}N_5O_{13}P_3$) C, H, N.

1-{2-[1,3-(Oxytetraethylenoxy)-3,5,5-tri(phenyloxy-2-{2-chloroethylimino})cyclotriphosphazatrien-1-yl]aminoethylamino}anthraquinone 8. 2-Chloroethylamine hydrochloride (0.121 g, 1.04 mmol) and triethylamine (0.316 g, 3.1 mmol) in dry benzene (20 mL) were stirred at room temperature in an argon atmosphere. After 1 h, **7** (0.33 g, 0.345 mmol) in dry benzene (20 mL) was added and the stirring continued for an additional 4 h in boiling solvent. The mixture was filtered, and the filtrate was evaporated under reduced pressure. The yield of **8** (obtained as a red oil) was 95%, mol mass (g/mol) found/calcd M = 1140.3/1139.99.Anal. ($C_{57}H_{56}N_8O_{10}P_3$) C: calcd, 53.68; found, 53.28; H; N: calcd, 9.82; found, 9.36.

1,3-(Oxytetraethylenoxy)-1-(2-naphthyloxy)-3,5,5-trichlorocyclotriphosphazatriene 9. 1 (0.938 g, 2 mmol), 2-naphthol (0.294 g, 2 mmol), and sodium hydride (60%) (0.08 g, 2 mmol) in THF (120 mL) were stirred for 2 h at room temperature. The mixture was filtered and the filtrate evaporated under reduced pressure and finally washed with hot water. The yield of **9** was 99%, mol mass [g/mol] M = 576.5 g/mol. Anal. ($C_{18}H_{23}Cl_3N_3O_6P_3$) C, H, N.

1,3-(Oxytetraethylenoxy)-1-(2-naphthyloxy)-3,5,5-tri(phenyloxy-2-carboxaldehyde)cyclotriphosphazatriene 10. 9 (1.15 g, 1.99 mmol), 2-hydroxybenzaldehyde (1.116 g, 8.96 mmol), and sodium hydride (60%, 0,358 g, 8.96 mmol) in THF (140 mL) were stirred at room temperature for 24 h. The mixture was filtered and the filtrate evaporated under reduced pressure. The crude product was chromatographed on a silica gel column using hexane:THF (2:1, v/v) as eluent. The yield of **10** (as a red oil) was 50.8%, mol mass (g/mol) found/calcd M = 834.1/833.36. Anal. ($C_{39}H_{38}N_3O_{12}P_3$) C, H, N.

1,3-(Oxytetraethylenoxy)-1-(2-naphthyloxy)-3,5,5-tri(phenyloxy-2-{chloroethylamino})cyclotriphosphazatriene 11. 2-Chloroethylamine hydrochloride (1.184 g, 3.9 mmol) and triethylamine (1.184 g, 11.7 mmol) in dry benzene (35 mL) were stirred for 1 h at room temperature under an argon atmosphere. After 1 h, **10** (1.1 g, 1.3 mmol) in dry benzene (35 mL) was added and the mixture stirred for a further 4 h in boiling solvent. Subsequently, the solvent (benzene) was evaporated under reduced pressure. The yield of **11** (obtained as a red oil) was 95%, mol mass (g/mol) found/calcd M = 1018.4/1017.83. Anal. ($C_{51}H_{50}N_6O_9P_3$) C, H, N.

(Elemental analysis and spectroscopic date (FTIR, ^{1}H NMR, ^{31}P NMR) are reported in Supporting Information).

Measurements. ^{31}P NMR spectra were recorded with a Varian VXR 300 spectrometer operating at 121 MHz using solutions in $CDCl_3$ with 85% H_3PO_4 as an external standard and with positive shifts recorded downfield from the reference. ^{1}H NMR were recorded with a Varian VXR 300 spectrometer. Chemical shifts are expressed in ppm, relative to the internal reference TMS. Mass spectra were recorded on Finnigan LCQ ion trap mass spectrometer (Finnigan, San Jose, CA). Flash column chromatography was performed using silica gel (100−200 mesh, Merck) using different solvents as eluents. Melting points were measured using a Boethius hot stage and a DSC 2010 TA instrument. IR spectra were recorded with a BIO-RAD FTS-40A spectrometer. Elemental analyses were carried out using a Perkin Elmer 2400 series II CHNS/O analyzer.

Cell Lines. The following established in vitro human and mouse cancer cell lines were applied: MOLT4 (human acute lymphoblastic leukemia), HL-60 (acute promyelocytic leukemia), L1210 (mouse lymphocytic leukemia), P388 (mouse lymphocytic leukemia).

The MOLT-4 cell line was purchased from the American Type Culture Collection (Rockville, MD), the L1210 cell line from Flow Laboratories, and the HL-60 cell line from European Type Culture Collection by courtesy of Professor Spik and Dr. Mazurier (Laboratory of Biological Chemistry USTL, Lille, France). All the cell lines were maintained in the Institute of Immunology and Experimental Therapy, Wroclaw, Poland. The cells were cultured in RPMI medium supplemented with glutamine, antibiotics, and 10% fetal bovine serum.

Antiproliferative Assays. Cells were plated in 96-well plates (Sarstedt, Costar) at a density of 10^4 cells per well in 100 μL of culture medium. After 24 h, testing agents were added to the cells in concentrations ranging from 100 to 0.1 μg/mL. The incubation continued for an additional 72 h.

The MTT technique was applied for the cytotoxicity screening. Twenty microliters of MTT solution (MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma); stock solution: 5 mg/mL) was added to each well and incubated for 4 h. The mitochondria of viable cells convert the pale yellow MTT to a navy-blue colored formazan. After the incubation time was completed, 80 μL of the lysing mixture was added to each well (lysing mixture: 225 mL of dimethylformamide, 67.5 g of sodium dodecyl sulfate (Sigma), and 275 mL of distilled water). After 24 h, when the formazan crystals had dissolved, the optical densities of the samples were read on an Multiskan RC photometer (Labsystems) at 570 nm.

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Supporting Information Available: Spectroscopic date (FTIR, ^{1}H NMR, ^{31}P NMR) and elemental analysis of the compounds synthesized and investigated are available free of change via the Internet at http://pubs.acs.org.

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