Synthesis and Antitumor Activity of Methyltriazene Prodrugs Simultaneously Releasing DNA-Methylating Agents and the Antiresistance Drug *O*^{6}-Benzylguanine

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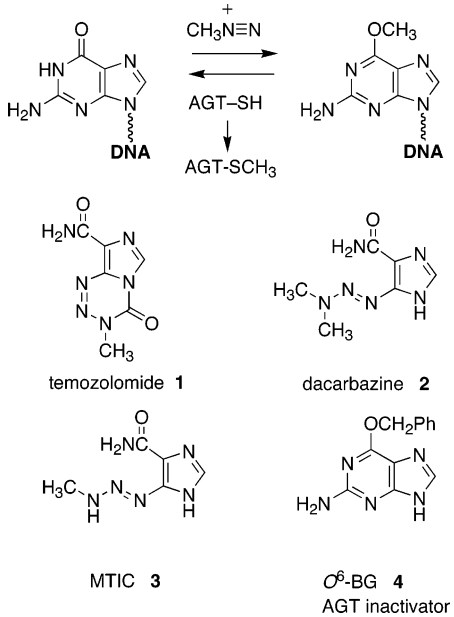
Active resistance of tumor cells against DNA alkylating agents arises by the production of high levels of the DNA repair protein *O*^{6}-alkylguanine-DNA alkyltransferase (AGT). This resistance during treatment with, for example, the anticancer agent temozolomide can be reversed by administration of *O*^{6}-benzylguanine, a purine that transfers its benzyl group to AGT and irreversibly inactivates it. Stimulated by the favorable therapeutic properties of temozolomide we designed and synthesized DNA-methylating triazenes built on the antiresistance benzylguanine ring system. The condensation reaction between 2-nitrosopurines and acylhydrazines proved to be very suitable to prepare acylated methyltriazenes. Fine-tuning of the release rate of both the methylating agent (diazomethane) and of *O*^{6}-benzylguanine was accomplished by variation of the hydrolysis-sensitive acyl substituent in **5**. Hydrolysis studies were performed with ^{1}H NMR and revealed that the *p*-nitrophenyl substituted triazene **26** showed an optimal hydrolysis rate ($t_{1/2}$ = 23 min) and almost 100% selectivity for the desired fragmentation route. In vitro antitumor studies in the 60 human tumor cell line panel of the National Cancer Institute confirmed the superior properties of *p*-nitrophenyl-protected methyl triazene **26**, showing mean IC_{50} values of 10 μM compared to 100 μM for temozolomide. In analogy with temozolomide, triazene **26** showed however low preference for each of the cancer subpanels, with IC_{50} values between 8 and 14 μM.

Introduction

Methylation of the O^6-position of guanosine residues in DNA is the major cause of cytostatic activity of antitumor agents such as the methyltriazenes temozolomide **1** and dacarbazine **2**. DNA methylation takes place preferably at purine nitrogen atoms, but the N-methylated bases are efficiently removed from the DNA by the base excision repair system (BER) and do not contribute to the antitumor activity. Only 5% of the purine methylation takes place at the guanine O^6-position which is responsible for most of the activity.[1] During DNA-replication the strand opposite the *O*^{6}-methyl guanine residue mismatches with thymidine which triggers the DNA mismatch repair system (MMR). The thymidine residue is removed; however, thymidine is reincorporated and again the MMR function is activated ultimately resulting in cell death by apoptosis[2] or by autophagy.[3,4] A third DNA repair protein, *O*^{6}-alkylguanine-DNA alkyltransferase (AGT) is more successful and repairs these lesions by simply taking over the methyl group of guanine with one of its cysteine residues (Scheme 1).[5−7] AGT is a suicide enzyme that is degraded after transferring a single methyl group, and its activity can only be recovered by its resynthesis. This demethylation of *O*^{6}-methyl guanine by AGT is responsible for the rapid increase of resistance against DNA-alkylating antitumor agents and high levels of AGT can be detected after treatment with, for example, temozolomide.[8,9] Resistance against DNA-alkylating agents based on increased AGT production is also observed during chemotherapy with the chloroethylating agent 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU). AGT-production varies not only between cells and tissue but also between individuals and depends on expression of the MGMT gene that encodes for AGT.[10]

Also the expression of MMR plays a role and in effect three genotypes can be considered in which the relative

Scheme 1. Methylation and AGT-Catalyzed Demethylation of Guanosine Residues



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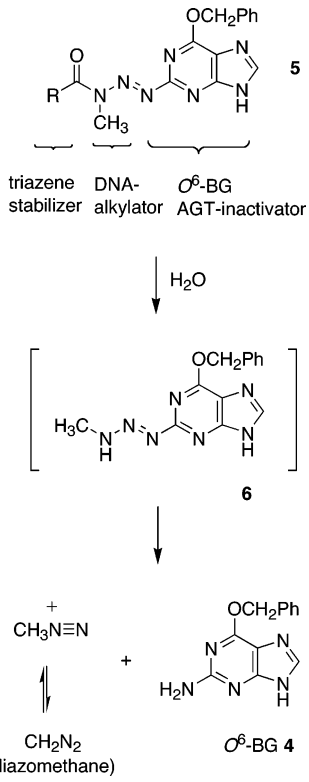
Scheme 2



expression of AGT and MMR plays a role: actively resistant (high AGT), not susceptible or passively resistant (low MMR) and susceptible (high MMR). Reversal of resistance can be accomplished by administration of O^6-benzylguanine (**4**, O^6-BG), a single base which efficiently depletes AGT levels by transferring its benzyl group to AGT.[11] In vitro studies have shown that **4** is approximately 2000 times more active then O^6-methylguanine in alkylating AGT.[7] O^6-BG enhances the action of several DNA alkylating and cross-linking agents and is currently in clinical trials in combination with temozolomide and BCNU against brain tumors and metastatic melanoma.[12]

To overcome the therapeutic disadvantages of alternating treatments with temozolomide and O^6-BG, we designed triazene prodrugs containing the DNA methylating triazenes and the antiresistance agent O^6-BG in one molecule. An important advantage of this strategy is that AGT-depletion induced by O^6-BG release and formation of the methylating species occur simultaneously and in the same environment, ensuring optimal effect from DNA methylation. There are literature examples that describe combination of DNA-targeting triazenes with epidermal growth factor receptor (EGFR) inhibitors in one molecule, that deliver interesting antiproliferate activity.[13−16] This combi-targeting principle however lacks the presence of an AGT inhibitor and is hampered by strong AGT-induced resistance against DNA methylation.[17]

Our target triazenes with general structure **5** (Scheme 2) contain an acyl substituent on the triazene to protect the purinyltriazene **6** against premature fragmentation. After hydrolysis of this R−C=O group (at physiological pH), unstable **6** is formed, which degrades further into the highly reactive methyldiazonium cation and O^6-BG **4**. The validity of our concept depends on the hydrolysis rate of acyl triazene **5** to methyltriazene **6**, preferably under physiological conditions. Fine-tuning of the hydrolysis rate of **5** can be accomplished by bringing variation in the R-group.

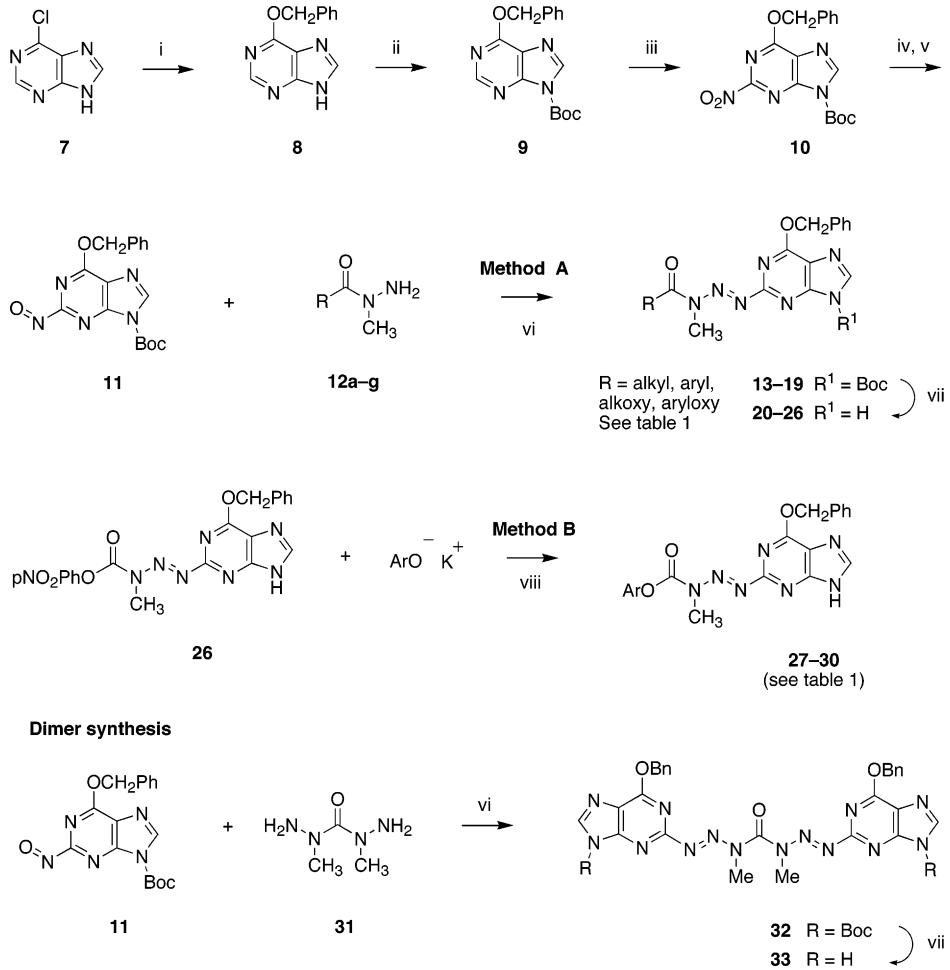
In particular the spontaneous hydrolysis of temozolomide **1** ($t_{1/2}$ = 110 min in vitro, pH 7.4) into MTIC (**3**), which further degrades into the DNA-alkylating methyldiazonium cation and AICA, stands as a model for this process.[18] Temozolomide has low to moderate toxicity and is clinically used against, for example, malignant melanoma and brain tumors.[9,19] Oral administration of temozolomide produces linear pharmacokinetics and excellent bioavailability, allowing outpatient treatment. This is in contrast to, for example, dacarbazine (DTIC, **2**), clinically in use against metastatic melanoma, which requires activation by hepatic hydrolysis, which is patient-dependent. More experimental antitumor triazenes have been developed containing acyl groups sensitive to hydrolysis, such as 1,3-dialkyl-3-acyltriazenes[20,21] and acyloxymethyl-protected arylmethyltriazenes that are sensitive toward enzymatic hydrolysis.[22−24]

Here we describe the preparation of electron-deficient, hydrolysis-sensitive triazenes **5**. The relation between hydrolytic decomposition into the DNA-methylating agent and the antiresistance drug O^6-benzylguanine and the resulting antitumor activity will be discussed.

Chemical Results and Discussion

Nitrosopurine 11. The classical pathway to prepare acylated aromatic triazenes consists of condensation reactions of amines with aromatic diazonium compounds, followed by acylation.[25] Since purinyl diazonium compounds are not stable, this procedure is not applicable. We developed a condensation reaction of heterocyclic nitroso compounds with hydrazides and used it for the synthesis of the triazene part of temozolomide.[26] This method is in particular suitable for the preparation of electron deficient triazenes.[27] Introduction of a nitroso substituent in the 2-position of purines is easily accomplished via a nitration/redox procedure, as described for the synthesis of 2-nitrosoadenosine.[28] After some experimentation with different substituents on the N-9 position, the Boc group appeared to be an excellent protecting group for the purine N−H in **8** (Scheme 3). With DMAP as catalyst, the base-labile N9-Boc purine **9** was obtained as a single regioisomer. A few Boc-protected adenine derivatives have been described in the literature; however, the utility of these compounds is restricted by the sensitivity of the 9-Boc substituent toward nucleophilic deprotection.[29] Although in general Boc-protected amines are extremely base-stable, the purine substituent in 9-Boc-purines acts as a good leaving group, and nucleophiles such as amines or even water can remove the Boc-group (vide infra). The route from **9** toward the anticipated triazenes only requires neutral or mildly acidic conditions which are well compatible with Boc protection. Nitration of **9** with tetrabutylammonium nitrate/trifluoroacetic anhydride (TBAN/TFAA)[30] gave 2-nitropurine **10** in high yield. Hydrogenation with 10% Pt/C completely stopped at the hydroxylamine stage, and after reoxidation with sodium periodate, nitrosopurine **11** was obtained as a stable and crystalline compound, representing a convenient precursor for the synthesis of all triazenes.

Scheme 3[a]



[a] Reagents and conditions: (i) (a) DABCO, DMSO, rt; (b) NaOBn, rt, 96%; (ii) Boc_2O, DMAP, DCM, rt, 82%; (iii) TBAN/TFAA, 1.3 equiv, DCM, 0 °C, 84−90%; (iv) H_2, 10% Pt/C, EtOAc, MeOH, HOAc; (v) 2 equiv of $NaIO_4$ in H_2O, EtOAc, 1 h, 84% (two steps); (vi) DCM/HOAc 3:1; rt, 18 h, 52−77%; (vii) 1 equiv of piperidine in MeCN, rt or MeCN/H_2O, reflux, 30 min, 42−83%; (viii) DMF or DMSO, rt, 53−76%.

Triazene Synthesis. Condensation reactions of aromatic nitroso compounds with nucleophiles is not always a favorable process. In particular, electron-donating nucleophiles such as phenylhydrazine give rise to redox reactions, whereby the nitroso compound is reduced to the corresponding hydroxylamine. For instance, an attempt was made to prepare the dacarbazine analogue of O^6-BG; however, reaction of **11** with 1,1-dimethylhydrazine gave only trace amounts of the desired triazene. This redox side process could be largely suppressed by condensation of **11** with electron poor, acylated hydrazines **12a**−**g**. In the literature the required hydrazides and carbonates **12a**−**g** are prepared via a variety of more or less useful procedures. In a general procedure, we acylated methylhydrazine (2 equiv) with an active carbonyl compound: (a) acetic anhydride, (b) benzoyl chloride, (c) *p*-nitrobenzoyl chloride, (d) nicotinoyl chloride, (e) ethyl chloroformate, (f) diphenyl carbonate, and (g) *p*-nitrophenyl chloroformate. In all examples, methylhydrazine was acylated on the more nucleophilic methyl-substituted nitrogen atom. Acid catalysis condensation of **12a**−**g** with **11** gave Boc-protected triazenes **13**−**19** in good to reasonable yields (Method A). Dimer **32** was obtained from bishydrazide **31**.[31] In the final step the Boc protecting group was removed under nucleophilic conditions with 1 equiv of piperidine in acetonitrile at room temperature for compounds **20**−**25** and **33**. In an effort to obtain the very sensitive *p*-nitrophenyl derivative **26**, even milder conditions were discovered for Boc-removal: 30 min reflux in aqueous acetonitrile was sufficient. Once the versatile *p*-nitrophenyl triazene **26** was obtained, several new *O*-phenyl groups could be introduced, simply by reaction of **26** with the desired phenolate anion (Method B). In this way, the para-substituted phenolic derivatives **27**−**30** were prepared in a single step.

Biological Results and Discussion

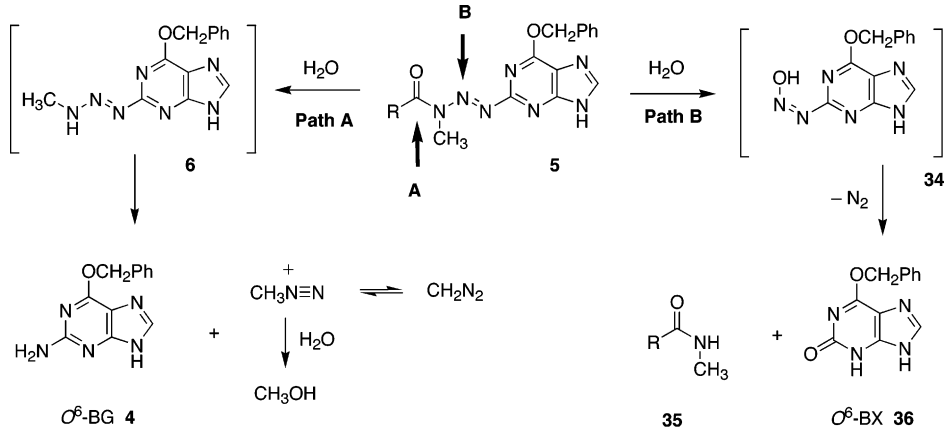
Triazene Hydrolysis. The mechanism of triazene hydrolysis was accurately followed by ^{1}H NMR spectroscopy. For many years, the chemistry and biology of antitumor triazenes have been studied, and detailed mechanistic studies of the hydrolytic decomposition of 1,3-dialkyl-3-acyltriazenes,[21] 1-aryl-3-methyl-3-acyltriazenes,[24] and temozolomide[18] in aqueous buffer solution are described in the literature. Temozolomide has, both in phosphate buffer at pH 7.4 and in plasma after intravenous or oral dosing, a half-life of 110 min, which emphasizes that the hydrolysis to MTIC is not a metabolic process. ^{1}H NMR data under physiological conditions, 37 °C, pD 7.8 in deuterated phosphate buffer, which corresponding to pH 7.4 in nondeuterated buffer,

Table 1. Triazene Hydrolysis Analyzed by ^{1}H NMR[a]

triazene	R	synthesis	hydrolysis product[b]	$t_{1/2}$ (min) pD 7.8	ratio O^6-BG/O^6-BX
			Amides		
20	methyl	A	acetate	3500	15/85
21	phenyl	A	benzoate	2300	7/93
22	*p*-nitrophenyl	A	*p*-nitrobenzoate	960	96/4
23	3-pyridyl	A	nicotinate	530	28/72
			Urethanes		
24	ethoxy	A	ethanol	3500	6/94
25	phenyloxy	A	phenol	1150	71/29
27	*p*-acetylaminoPhO	B	paracetamol	1160	66/34
29	*p*-carboxamidoPhO	B	*p*-hydroxybenzamide	385	84/16
30	*p*-sulfonamidoPhO	B	*p*-hydroxysulfonamide	169	93/7
28	*p*-methoxycarbonylPhO	B	methyl *p*-hydroxybenzoate	147	97/3
26	*p*-nitrophenyloxy	A	*p*-nitrophenol	23	99/1
33	dimer	A	CO_2	430	71/29
1	temozolomide		CO_2	25	
1	temozolomide		CO_2	110[c]	
3	MTIC			192[c]	

[a] Solvent: 2/1 mixture of DMSO-d_6 and D_2O buffer pD 7.8, 37 °C. [b] Compounds **24**−**30** also release CO_2. [c] See ref 18, D_2O buffer pD 7.8, 37 °C.

Scheme 4. Hydrolytic Pathways



produced comparable half-life values.[18] Since the water solubility of the 2-triazenyl-O^6-benzylpurines is rather low, we used mixtures of DMSO-d_6 with phosphate buffers in D_2O as solvent to analyze the decomposition products and to determine the $t_{1/2}$ values (Table 1). As a control experiment, temozolomide was also hydrolyzed under these mixed solvent conditions and showed a half-life of 25 min. From the ^{1}H NMR data, two different fragmentation processes could be identified from the end products (Scheme 4).

Route A presents the anticipated pathway and starts with hydrolytic removal of the protecting R-group from **5** to produce 2-methyltriazenylpurine **6** together with a carboxylic acid or an alcohol and CO_2. 2-Methyltriazenylpurine **6** decomposes quickly and is not observed in NMR, except during the hydrolysis of *p*-nitrophenyl derivative **26**, which hydrolyzes fast enough to allow formation of detectable concentrations of intermediate **6**. O^6-BG is formed in the next step and is easily quantified by the CH_2-signal at 5.49 ppm. The methyldiazonium/diazomethane intermediate is not detected by NMR as such, but as methanol formed by reaction with water (Scheme 4). Incorporation of deuterium in the methyl group of methanol gives a clear indication for the intermediacy of the methyldiazonium cation, which is in equilibrium with diazomethane.[18] All three deuterated forms, CH_3OD, CH_2DOD, and CHD_2OD, were observed, although the total amount of methanol was less than the theoretical amount, probably as a result of the formation of CD_3OD which is invisible in ^{1}H NMR.

Route B implies fragmentation of the triazene between two nitrogen atoms, as can be deduced from the end products O^6-benzylxanthine **36**[32] and *N*-methylcarboxamides or *N*-methylurethanes (**35**). This type of fragmentation was also observed for acylated dialkyltriazenes.[20,21] As an alternative for attack of water on nitrogen as is shown in Scheme 4, a direct N−N fragmentation mechanism can be suggested, that forms O^6-benzylxanthine **36** via the unstable 2-diazonium-purinyl cation.

Table 1 shows four amides (**20**−**23**) and seven urethanes (**24**−**30**), with a large variety in both hydrolysis half-life and preference for hydrolysis pathway A or B. Low hydrolysis rates and preference for the undesired N−N-fragmentation pathway B is observed for the amides **20**−**23**. *p*-Nitrobenzoate **22** has an acceptable O^6-BG/O^6-BX ratio, but the hydrolysis rate is unacceptably low.

A good leaving group is favored, as is present in the *p*-nitrophenylurethane **26** and methyl benzoate **28**. In particular for *p*-nitrophenyloxy derivative **26** an optimal half-life of 23 min, closely resembling that of temozolomide, is observed with 99% preference for fragmentation into O^6-benzylguanine and diazomethane. Some other derivatives with increased water solubility (**29** and

Table 2. In Vitro Antitumor Prescreen Results[a]

triazene	R	NCI-H460 (lung)[b]	MCF7 (breast)[b]	SF-268 (CNS)[b]
20	methyl	89	99	78
21	phenyl	93	107	79
24	ethoxy	90	103	91
25	phenyloxy	87	107	102
26	*p*-nitroPhO	0[c]	1[c]	4[c]
28	*p*-MeOOCPhO	53[c]	116[c]	107[c]
33	dimer	58	74	46

[a] NCI 3-cell line one dose primary anticancer assay. [b] Reduction of growth (%) compared to untreated control cells (100%) at 100 μM. [c] At 50 μM.

30) were prepared, but these compounds showed moderate hydrolysis profiles. Paracetamol is released as side product from triazene **27**, but also this compound did not produce a satisfactory hydrolysis rate or selectivity.

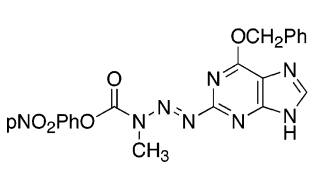
Dimer **33** was designed as a "clean" prodrug: hydrolysis of **33** produces two molecules of O^6-BG, two molecules of diazomethane, and carbon dioxide as the only waste product. Although this dimer does not have the favorable leaving group properties of the aromatic urethanes, it still displays a reasonable hydrolysis rate and O^6-BG/O^6-BX ratio.

In general there is a good correlation between the leaving group character of group R and both the hydrolysis rate and selectivity for O^6-benzylguanine and O^6-benzylxanthine formation. Hydrolysis of *p*-nitrophenyloxy derivative **26** at pH 8.2 gave side reactions and was not further investigated. Higher pH-values cause deprotonation of H-9, which complicates the reaction. At pD 7.4 the hydrolysis profile of **26** was comparable with the data obtained at pD 7.8.

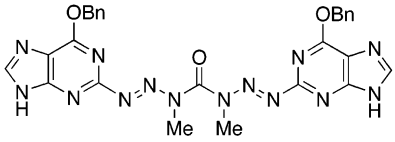
In Vitro Antitumor Tests. First a set of triazenes was prepared to examine the structural requirements for cytotoxic activity. *N*-Acyltriazenes **20** and **21**, the urethanes **24** and **25**, and dimer **33** were evaluated in the National Cancer Institute prescreen. This one dose primary anticancer assay is run on 3 cell lines, MCF7 (breast), NCI-H460 (lung) and SF-268 (CNS). From Table 2 it appears that both amides and urethanes are completely inactive while dimer **33** is slightly inhibiting the growth of all three cell lines. During the screening process we also obtained the hydrolysis data of these compounds, and a direct correlation between the biological activity and the hydrolysis profile became visible. A drastic lowering of the $t_{1/2}$ was required, and attention was directed to the synthetic part. The preparation of the highly sensitive *p*-nitrophenyl derivative **26** appeared to be an important breakthrough, giving entry to several phenolic derivatives with electron-withdrawing substituents: method B shown in Scheme 3. Other reactive acyl derivatives were not stable: compound **5** with trifluoroacetyl, formyl, and imidazolcarbonyl groups on the triazene spontaneously decomposed with N_2 evolution. A new series of triazenes was synthesized, and the hydrolysis was again examined by NMR, as is reported in Table 1. On the basis of these data, *p*-nitrophenyl triazene **26**, which displayed a superior hydrolysis profile, and benzoate ester **28** were selected for a second prescreening essay. Triazene **26** was actually the only derivative which showed good cell growth inhibition (Table 2). This implies that a minimum concentration of the active DNA-alkylating component **6** is required for activity, which can only be obtained by a fast, first-step hydrolysis. The first hydrolysis step ($t_{1/2}$ = 23 min) is followed by fragmentation of methyltriazene **6** (Scheme 4) which has an estimated $t_{1/2}$ between 20 and 40 min. The similarity between these half-life values compares well with the relation between temozolomide ($t_{1/2}$ = 25 min under our conditions) and its hydrolysis product MTIC, which has an estimated $t_{1/2}$ of ca. 40 min. Apparently a high threshold concentration of the methyldiazonium species is required for biological activity. This follows from the requirement of approximately 6000 methylated guanine residues to induce cell death. Lesions induced by chloroethylating agents such as BCNU require only 5−10 cross-links.[10]

Compounds **26** and **33** were evaluated in the NCI full, 60 human tumor cell line panel as is shown in Table 3. The antitumor activity of the triazenes is expressed by three values, the IC_{50} (the molar concentration of the compound that inhibits 50% net cell growth), TGI (the

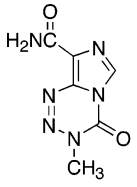
Table 3. Inhibition of in Vitro Cancer Cell Lines by *p*-Nitrophenyl Triazene **26**, Dimer **33**, and Temozolomide **1**[a]



26



33



temozolomide **1**

	IC_{50}[b] (μM)			TGI[c] (μM)			LC_{50}[d] (μM)		
cancer subpanel	**26**	**33**	**1**	**26**	**33**	**1**	**26**	**33**	**1**
leukaemia	14	55	79	50	>100	>100	50	>100	>100
non-small cell lung	11	42	100	29	>100		47		
colon	9	45	100	21	>100		43		
CNS	9	55	93	30	>100		47		
melanoma	8	47	98	23	93		40		
ovarian	12	50	91	34	>100		50		
renal	9	36	98	25	87		44		
prostate	10	40	100	23	>100		46		
breast	10	39	100	30	>100		50		

[a] Data obtained from NCI's in vitro disease-oriented tumor cells screen. [b] IC_{50} is the molar concentration causing 50% growth inhibition of tumor cells. [c] TGI is the molar concentration giving total growth inhibition. [d] LC_{50} is the molar concentration leading to 50% net cell death.

molar concentration of the compound leading to total inhibition of net cell growth), and LC_{50} (the molar concentration of the compound that induces 50% net cell death). Each cancer subpanel consists of the mean values of several individual cell-lines which are not shown here.

p-Nitrophenyl-protected triazene **26** and dimer **33** demonstrated activity on almost every cell-line tested. In agreement with both the hydrolysis studies and the prescreen results, **26** was more active than dimer **33**, probably as a result of higher levels of **6** that become available after the hydrolysis step. Also considerably higher cytotoxicity was observed for **26** when compared with temozolomide **1**, although the concentration of **1** that causes 50% inhibition of cell growth lies close to the maximal concentration tested (100 μM). This also means that the TGI and LC_{50} values of **1** are higher than the maximal dose tested and are not displayed here (data obtained from the NCI database). Since the hydrolysis rates of **26** and **1** are comparable, this effect is most likely due to the anticipated depletion of AGT-levels within the cell. A second explanation may lie in the difference in penetration properties of the hydrolyzed prodrug **6** (Scheme 2) compared to the temozolomide counterpart MTIC **3** (Scheme 1).

The selectivity of **26** for any individual cell line was remarkably low with one exception: an IC_{50} value of 1.1 μM was obtained for the melanoma LOX IMVI cell line. Also the TGI value (11 μM) and LC_{50} value (27 μM) were the lowest for this cell line.

A search in the NCI molecular database for levels of the DNA mismatch repair proteins MMR (hMSH2, hMLH1) and the alkyltransferase AGT (MGMT) in the 60 human tumor cell line panel was performed. No correlation between these levels and the in vitro screening results for **26** was found however. It should be noted that even the successful antitumor agent temozolomide shows no selectivity for any of the cell lines in the NCI panel (see Table 3).

Conclusions

^{1}H NMR hydrolysis studies on benzylguanine triazenes give an indication for in vitro biological activity. To confirm that increased antitumor activity of **26** compared to temozolomide is caused by AGT-depletion, further screening on pairs of MER± tumor cell lines expressing different AGT levels and comparison with temozolimide/O^6-BG combinations is required.

Experimental Section

Chemicals and Solvents. All reagents and solvents were used as commercially available, unless indicated otherwise.

Chromatography. Thin-layer chromatography (TLC) was carried out using silica-coated plastic sheets (Merck silica gel 60 F_{254}). Spots were visualized under UV (254 nm). Flash chromatography refers to purification using the indicated eluents and Janssen Chimica silica gel 60 (0.030−0.075 mm).

Instruments and Analysis. Elemental analysis were performed by Kolbe, Mülheim a. d. Ruhr, Germany. Infrared (IR) spectra were obtained from $CHCl_3$ solutions unless indicated otherwise, using a Bruker IFS 28 FT-spectrophotometer. Proton nuclear magnetic resonance (^{1}H NMR) spectra were determined in $CDCl_3$ at 300 K using a Bruker ARX 400 spectrometer. Mass spectra and accurate mass measurements were performed on a JEOL JMS−SX/SX 102 A tandem mass spectrometer using fast atom bombardment (FAB) or electron impact (EI). A resolving power of 10 000 (10% valley definition) for high-resolution electron impact or FAB mass spectrometry was used. Melting points were measured with a Leitz melting point microscope.

6-Benzyloxypurine 8. A mixture of 6-chloropurine **7** (6.18 g, 40.0 mmol) and DABCO (13.4 g, 120 mmol) in anhydrous DMSO (50 mL) was stirred at 20 °C for 4 h and at 35 °C for 30 min. This mixture was added to a solution of benzyl alcohol sodium salt, prepared from benzyl alcohol (16.5 mL, 160 mmol) and sodium hydride (5.6 g, 140 mmol) in DMSO (50 mL) in a 500 mL evaporating flask. After stirring this mixture at room temperature for 16 h, acetic acid (6.6 mL, 110 mmol) was added and the volatile components were removed in vacuo, starting at 30 mbar, 20 °C up to 1 mbar and 70 °C. The remaining solid was extracted with a mixture of DCM (100 mL) and methanol (8 mL). The suspension was filtered over Hyflow, and the solids were washed with the same solvent mixture (50 mL). Evaporation of the solvents and trituration of the residue (EtOAc) produced **8** (8.70 g, 38.5 mmol, 96%) which was sufficiently pure for the next step, mp 169−172 °C (lit.[33] 171−172 °C); ^{1}H NMR (DMSO-d_6) δ 13.60 (s, 1H), 8.52 (s, 1H), 8.35 (s, 1H), 7.35−7.5 (m, 5H), 5.62 (s, 2H).

6-Benzyloxy-9-Boc-purine 9. Boc_2O (12.0 g, 55.0 mmol) was added to a suspension of **8** (8.7 g, 38.5 mmol) and DMAP (0.5 g) in anhydrous DCM (150 mL). The solution which was obtained after stirring for 16 h was diluted with petroleum ether (150 mL), and silica gel (5 g) was added. The mixture was stirred for 30 min and slowly filtered over a short column of silica gel (5 g). A mixture of ethyl acetate (80 mL) and petroleum ether (80 mL) was used to wash the product from the silica, which was obtained as a crystalline solid after evaporation of the solvents and trituration with petroleum ether containing a small amount of ethyl acetate (**9**, 10.3 g, 31.6 mmol, 82%), mp 129−131 °C; IR (KBr) 3128, 1752, 1603, 1580 cm^{-1};^{1}H NMR δ 8.71 (s, 1H), 8. 35 (s, 1H), 7.55 (m, 2H), 7.35 (m, 3H), 5.68 (s, 2H), 1.70 (s, 9H).

2-Nitro-6-benzyloxy-9-Boc-purine 10. TFAA (0.915 mL, 6.5 mmol) was added over 1−2 min to a solution of **9** (1.63 g, 5.0 mmol) and tetrabutylammonium nitrate (2.13 g, 7.0 mmol) in anhydrous DCM (20 mL) at 0 °C. The amount of TFAA is important since excess of the nitrating mixture results in oxidation at the 8-position. After a reaction time of ca. 20 min, a sample was quenched and analyzed by ^{1}H NMR, and if necessary additional TFAA was added. After a total reaction time of 1 h, the reaction mixture was poured into a stirred mixture of $NaHCO_3$ (40 mL), H_2O (40 mL), and diethyl ether (25 mL). Additional diethyl ether (25 mL) was used to transfer the mixture into a separatory funnel, and the layers were separated. The aqueous layer was extracted with diethyl ether, and the combined organic layers were washed with water (2 × 10 mL). The residue obtained after drying (Na_2SO_4) and vacuum evaporation (bath temp < 30 °C) was triturated with ca. 5 mL ice-cold methanol (Attention: hot methanol removes the Boc-substituent from the purine ring). Yield: 84−90%, mp 130 °C (dec); IR (KBr) 1788, 1767, 1610, 1559 cm^{-1};^{1}H NMR δ 8.59 (s, 1H), 7.62 (m, 2H), 7.35 (m, 3H), 5.77 (s, 2H), 1.72 (s, 9H). Anal. ($C_{17}H_{17}N_5O_5$) C, H, N.

2-Nitroso-6-benzyloxy-9-Boc-purine 11. A mixture of **10** (1.86 g, 5.0 mmol), 10% Pt on carbon (0.04 g), acetic acid (0.314 mL, 5.5 mmol), ethyl acetate (75 mL), and methanol (15 mL) was vigorously stirred under 1 atm of hydrogen gas for 30 min. The precipitate that had formed was dissolved by adding DCM (ca 30 mL) and gentle heating. The catalyst was removed by filtration over Hyflow, and the filtrate was cooled in ice. An ice-cold solution of $NaIO_4$ (2.14 g, 10 mmol) in water (50 mL) was added, and the mixture was stirred vigorously at 0 °C until the hydroxylamine had disappeared (30−90 min) according to TLC analysis. The layers were separated, the organic layer was washed with water (25 mL) and dried (Na_2SO_4), and the solvents were removed in vacuo (bath temp < 30 °C). Trituration of the residue with cold methanol produced **11** (1.50 g, 4.22 mmol, 84%) after drying in vacuo, mp 200−205 °C; IR (KBr) cm^{-1}; ^{1}H NMR: at a concentration of 1 mg **11** in 0.5 mL $CDCl_3$ 18% of the nitroso compound was present as

its dimer.[28] Monomer δ 8.65 (s, 1H), 7.30−7.50 (m, 5H), 5.76 (s, 2H), 1.76 (s, 9H); dimer δ 8.35, 5.48. Anal. ($C_{17}H_{17}N_5O_4$) C, H, N.

General Procedure for the Synthesis of 1-Methylhydrazides 12a−g. Acylating agent (10 mmol) was added to an efficiently stirred mixture of methylhydrazine (1.06 mL, 20 mmol) and anhydrous DCM (20 mL) at −60 °C. The cooling bath was removed, and the suspension was stirred at room temperature for 3 h. The resulting mixture was extracted once with water and twice with dilute $NaHCO_3$. The organic layer was dried (Na_2SO_4), and the solvent was evaporated, producing almost pure hydrazide.

1-Acetyl-1-methylhydrazide 12a. Hydrazide **12a** was obtained from acetic anhydride as an oil (87%), ^{1}H NMR δ 4.73 (broad, 2H), 2.99 (s, 3H), 2.03 (s, 3H).

1-Benzoyl-1-methylhydrazine 12b. Benzhydrazide **12b** was obtained from benzoyl chloride as an oil in 90% yield, ^{1}H NMR δ 7.45−7.55 (m, 5H), 4.64 (broad, 2H), 3.20 (s, 3H).

1-*p*-Nitrobenzoyl-1-methylhydrazine 12c. Obtained from *p*-nitrobenzoyl chloride as an oil in 90% yield, ^{1}H NMR δ 7.40−7.55 (m, 4H), 4.88 (broad, 2H), 3.34 (s, 3H).

1-Nicotinyl-1-methylhydrazine 12d. Obtained from nicotinoyl chloride as an oil in 40% yield after chromatographic purification (silica, 5−10% MeOH in EtOAc),^{1}H NMR NMR (DMSO-d_6) δ 8.75 (s, 1H), 8.56 (m, 1H), 7.94 (m, 1H), 7.41 (m, 1H), 4.91 (broad, 2H), 3.21 (s, 3H).

1-Ethylcarbamoyl-1-methylhydrazine 12e. Prepared from ethyl chloroformate: oil, 82% yield, ^{1}H NMR (DMSO-d_6) δ 4.59 (broad, 2H), 4.05 (q, 2H, J = 7.2 Hz), 3.35 (s, 3H), 1.99 (t, J = 7.2 Hz, 3H).

1-Phenylcarbamoyl-1-methylhydrazine 12f. Prepared from diphenyl carbonate: 92% yield, oil which solidified in the refrigerator, ^{1}H NMR (DMSO-d_6) δ 7.10−7.40 (m, 5H), 4.89 (broad, 2H), 3.35 (s, 3H).

1-*p*-Nitrophenylcarbamoyl-1-methylhydrazine 12g.[34] Prepared from *p*-nitrophenyl chloroformate. Workup procedure: After stirring at room temperature for 3 h, diethyl ether (10 mL) was added and the precipitated salts were removed by filtration. The solvents were evaporated, and the residue was crystallized from EtOAc, 79% yield, ^{1}H NMR (DMSO-d_6) δ 8.27 (1/2 AB, 2H, J = 9.0 Hz),7.32 (1/2 AB, 2H, J = 9.0 Hz), 4.29 (broad, 2H), 3.32 (s, 3H).

General Procedure for the Synthesis of Boc-protected Triazenes 13−19 (Method A). To a solution of 2-nitrosopurine **11** (103.5 mg, 0.30 mmol) in a mixture of DCM (1.5 mL) and acetic acid (0.5 mL) was added hydrazide **12a−g** (0.36 mmol). The reaction mixture was stirred at room temperature for 18 h, diluted with diethyl ether (10 mL), and stirred with aqueous $NaHCO_3$ (5%, 20 mL) for 30 min. Extractive workup (small amounts of solid side products were removed by filtration, together with the drying agent Na_2SO_4), and after evaporation and crystallization of the residue (ethanol, 0 °C), triazenes **13−19** were obtained.

2-(3-Acetyl-3-methyltriazen-1-yl)-6-benzyloxy-9-Boc-purine 13. Prepared from hydrazide **12a**: yield 67%, mp 132−146 °C (dec); ^{1}H NMR δ 8.39 (s, 1H), 7.55 (m, 2H), 7.30−7.50 (m, 3H), 5.73 (s, 2H), 3.54 (s, 3H), 2.62 (s, 3H), 1.70 (s, 9H).

2-(3-Benzoyl-3-methyltriazen-1-yl)-6-benzyloxy-9-Boc-purine 14. Prepared from hydrazide **12b**: yield 63%, mp 145−147 °C (dec); ^{1}H NMR δ 8.35 (s, 1H), 7.92 (m, 2H), 7.30−7.50 (m, 8H), 5.55 (s, 2H), 3.76 (s, 3H), 1.67 (s, 9H).

2-(3-*p*-Nitrobenzoyl-3-methyltriazen-1-yl)-6-benzyloxy-9-Boc-purine 15. Prepared from hydrazide **12c**: yield 28%, mp 193−196 °C (dec); ^{1}H NMR δ 8.35 (s, 1H), 8.28 (1/2 AB, 2H, J = 8.8 Hz), 8.12 (1/2 AB, 2H, J = 8.8 Hz), 7.43 (m, 2H), 7.33 (m, 3H), 5.63 (s, 2H), 3.78 (s, 3H), 1.70 (s, 9H).

2-(3-Nicotinyl-3-methyltriazen-1-yl)-6-benzyloxy-9-Boc-purine 16. Prepared from hydrazide **12d**: yield 27%, mp 143−145 °C;^{1}H NMR δ 9.16 (d, 1H, J = 1.9 Hz), 8.66 (m, 1H), 8.36 (s, 1H), 8.28 (m, 1H), 7.30−7.50 (m, 6 H), 5.61 (s, 2H), 3.75 (s, 3H), 1.69 (s, 9H).

2-(3-Ethoxycarbonyl-3-methyltriazen-1-yl)-6-benzyloxy-9-Boc-purine 17. Prepared from hydrazide **12e**: yield 77%, mp130−133 °C; ^{1}H NMR δ 8.36 (s, 1H), 7.61 (m, 2H), 7.30−7.50 (m, 3H), 5.73 (s, 2H), 4.49 (q, 2H, J = 7.1 Hz), 3.61 (s, 3H), 1.70 (s, 9H), 1.44 (t, J = 7.1 Hz, 3H)

2-(3-Phenyloxycarbonyl-3-methyltriazen-1-yl)-6-benzyloxy-9-Boc-purine 18. Prepared from hydrazide **12f**: yield 74%, mp140−141 °C (dec); ^{1}H NMR δ 8.39 (s, 1H), 7.30−7.60 (m, 10H), 5.73 (s, 2H), 3.71 (s, 3H), 1.67 (s, 9H)

2-(3-*p*-Nitrophenyloxycarbonyl-3-methyltriazen-1-yl)-6-benzyloxy-9-Boc-purine 19. Prepared from hydrazide **12g**: yield 77.%, mp 193−196 °C (dec); ^{1}H NMR δ 8.39 (s, 1H), 8.33 (1/2 AB, 2H, J = 9.0 Hz), 7.59 (m, 2H), 7.47 (1/2 AB, 2H, J = 9.0 Hz), 7.30 (m, 3H), 5.73 (s, 2H), 3.71 (s, 3H), 1.68 (s, 9H).

Boc-Deprotection with Piperidine. To a solution of Boc-protected triazene (0.1 mmol) in DCM (3 mL) was added dropwise a 0.2 M solution of piperidine in DCM (0.5 mL, 0.1 mmol). After stirring for 18 h, the DCM was evaporated and the residue was triturated with EtOAc. The product was obtained by filtration and dried in vacuo.

Boc-Deprotection with Acetonitrile/Water. Water (0.5 mL) was added to a solution of Boc-protected triazene (0.1 mmol) in acetonitrile (2 mL). After 30 min reflux, crystallization was induced by the addition of 0.5 mL water and cooling in ice. The product was obtained by filtration, washed with acetonitrile/water 1/1, and dried in vacuo.

2-(3-Acetyl-3-methyltriazen-1-yl)-6-benzyloxypurine 20. From **13** by deprotection with piperidine, yield 82%, mp 200−202 °C (dec); ^{1}H NMR (DMSO-d_6) δ 13.62 (s, 1H), 8.44 (s, 1H), 7.58 (m, 2H), 7.40 (m, 3H), 5.68 (s, 2H), 3.40 (s, 3H); [found M^+ + 1, 326.1362; $C_{15}H_{16}N_7O_2$ requires, 326.1365]. Anal. ($C_{15}H_{15}N_7O_2$) C, H, N.

2-(3-Benzoyl-3-methyltriazen-1-yl)-6-benzyloxypurine 21. From **14** by deprotection with piperidine, yield 83%, mp 176−180 °C (dec); ^{1}H NMR (DMSO-d_6) δ 13.60 (broad, 1H), 8.43 (s, 1H), 7.83 (m, 2H), 7.30−7.60 (m, 8H), 5.52 (s, 2H), 3.62 (s, 3H); [found M^+ + 1, 388.1520; $C_{20}H_{18}N_7O_2$ requires, 388.1522]. Anal. ($C_{20}H_{17}N_7O_2 \cdot 2.0H_2O$) C, H, N.

2-(3-*p*-Nitrobenzoyl-3-methyltriazen-1-yl)-6-benzyloxypurine 22. From **15** by deprotection with piperidine, yield 68%, mp 212−213 °C; ^{1}H NMR (DMSO-d_6) δ 13.58 (broad, 1H), 8.43 (broad, 1H), 8.26 (1/2 AB, 2H, J = 8.8 Hz), 8.04 (1/2 AB, 2H, J = 8.8 Hz), 7.36 (s, 5H), 5.46 (s, 2H), 3.66 (s, 3H); [found M^+ + 1, 433.1375; $C_{20}H_{17}N_8O_4$ requires, 433.173]. Anal. ($C_{20}H_{16}N_8O_4$) C, H.

2-(3-Nicotinyl-3-methyltriazen-1-yl)-6-benzyloxypurine 23. From **16** by deprotection with piperidine, yield 42%, mp 148−150 °C; ^{1}H NMR (DMSO-d_6) δ 13.60 (s, 1H), 9.05 (s, 1H), 8.67 (d, 1H, J = 3.2 Hz), 8.46 (broad, 1H), 8.23 (m, 1H), 7.35−7.51 (m, 6H), 5.53 (s, 2H), 3.63 (s, 3H); [found M^+ + 1, 389.1498; $C_{19}H_{17}N_8O_2$ requires, 389.1474]. Anal. ($C_{19}H_{16}N_8O_2$) C, H, N.

2-(3-Ethoxycarbonyl-3-methyltriazen-1-yl)-6-benzyloxypurine 24. From **17** by deprotection with piperidine, yield 74%, mp 160−164 °C; ^{1}H NMR (DMSO-d_6) δ 13.58 (broad, 1H), 8.45 (s, 1H), 7.61 (m, 2H), 7.40 (m, 3H), 5.66 (s, 2H), 4.42 (q, 2H, J = 7.1 Hz), 3.48 (s, 3H), 1.33 (t, 3H, J = 7.1 Hz); [found M^+ + 1, 356.1476; $C_{16}H_{18}N_7O_4$ requires, 356.1471]. Anal. ($C_{16}H_{17}N_7O_3$) C, H, N.

2-(3-Phenyloxycarbonyl-3-methyltriazen-1-yl)-6-benzyloxypurine 25. From **18** by deprotection with piperidine, yield 83%, mp 206−207 °C (dec); ^{1}H NMR (DMSO-d_6) δ 13.13 (broad, 1H), 8.45 (s, 1H), 7.30−7.70 (m, 10H), 5.67 (s, 2H), 3.58 (s, 3H); [found M^+ + 1, 404.1461; $C_{20}H_{18}N_7O_3$ requires, 404.1471]. Anal. ($C_{20}H_{17}N_7O_3$) C, H, N.

2-(3-*p*-Nitrophenyloxycarbonyl-3-methyltriazen-1-yl)-6-benzyloxypurine 26. From **13** by deprotection with acetonitrile/water, yield 81%, mp 146−149 °C; ^{1}H NMR (DMSO-d_6) δ 13.65 (broad, 1H), 8.45 (s, 1H), 8.39 (1/2 AB, 2H, J = 9.0), 7.70 (1/2 AB, 2H, J = 9.0), 7.61 (m, 2H), 7.37 (m, 3H), 5.67 (s, 2H), 3.59 (s, 3H); [found M^+ + 1, 449.1316; $C_{20}H_{17}N_8O_5$ requires, 449.1322]. Anal. ($C_{20}H_{16}N_6O_5$) C, H, N.

General Procedure for the Synthesis of Triazenes 28−30 (Method B). Potassium phenolates were prepared by addition of potassium *tert*-butoxide (33.6 mg, 0.30 mmol) to a solution of a para-substituted phenol (0.30 mmol) in anhydrous

DMF (1 mL). After stirring at room temperature for 30 min, *p*-nitrophenyltriazene **26** (45 mg, 0.1 mmol) was added, and the yellow solution was stirred for another 10−15 min. Acetic acid (0.1 mL) was added followed by water (0.2−1 mL) to precipitate the product. The crystallization was completed in the refrigerator, and the products were obtained by filtration, washed with water, MeOH, and ether, respectively, and dried in vacuo.

2-[3-(*p*-Acetylaminophenyl)oxycarbonyl-3-methyltriazen-1-yl]-6-benzyloxypurine 27. Method B, yield 76%, mp 190−192 °C (dec) after recrystallization from DMF/water; ^{1}H NMR (DMSO-d_6) δ 13.59 (broad, 1H), 10.07 (s, 1H), 8.44 (s, 1H), 7.67 (d, 2H, J = 8.7 Hz), 7.62 (m, 2H), 7.38 (m, 3H), 7.29 (d, 2H, J = 8.7 Hz); [found $M^+ + 1$, 461.1679; $C_{22}H_{21}N_8O_4$ requires, 461.686]. Anal. ($C_{22}H_{20}N_8O_4$) C, H, N.

2-[3-(*p*-Methoxycarbonylphenyl)oxycarbonyl-3-methyltriazen-1-yl]-6-benzyloxypurine 28. Method B, yield 66.5%, mp 202−203 °C (dec); ^{1}H NMR (DMSO-d_6) δ 13.62 (broad, 1H), 8.47 (s, 1H), 8.09 (1/2 AB, 2H, J = 8.6 Hz), 7.62 (m, 2H), 7.55 (1/2 AB, 2H, J = 8.6 Hz), 7.35 (m, 3H), 5.67 (s, 2H), 3.89 (s, 3H), 3.58 (s, 3H); [found $M^+ + 1$, 462.1520; $C_{22}H_{20}N_7O_5$ requires, 462.1526]. Anal. ($C_{22}H_{19}N_7O_5$) C, H, N.

2-[3-(*p*-Carboxamidophenyl)oxycarbonyl-3-methyltriazen-1-yl]-6-benzyloxypurine 29. Method B, yield 70%, mp 199−200 °C (dec) after recrystallization from DMF/water; ^{1}H NMR (DMSO-d_6) δ 13.62 (broad, 1H), 8.46 (s, 1H), 8.05 (broad, 1H), 7.99 (1/2 AB, J = 8.6 Hz), 7.62 (m, 2H), 7.46 (1/2 AB, 2H, J = 8.6 Hz), 7.44 (broad, 1H), 7.36 (m, 3H), 5.67 (s, 2H), 3.58 (s, 3H); [found $M^+ + 1$, 447.1511; $C_{21}H_{19}N_8O_4$ requires, 447.1529]. Anal. ($C_{21}H_{18}N_8O_4$) C, H, N.

2-[3-(*p*-Sulfonamidophenyl)oxycarbonyl-3-methyltriazen-1-yl]-6-benzyloxypurine 30. Method B, but in DMSO instead of DMF, yield 53%, mp 146−149 °C; ^{1}H NMR (DMSO-d_6) δ 13.61 (broad, 1H), 8.44 (s, 1H), 7.95 (1/2 AB, 2H, J = 8.4 Hz), 7.35−7.60 (m, 9H), 5.67 (s, 2H), 3.62 (s, 3H); [found $M^+ + 1$, 483.1210; $C_{20}H_{19}N_8O_5S$ requires, 483.1199]. Anal. ($C_{20}H_{18}N_8O_5S$) C, H, N.

Carbonic Acid Bis(1-methylhydrazide) 31. Prepared as a hygroscopic solid in 78% yield from triphosgene and methylhydrazine (lit:[31] 95%), ^{1}H NMR (DMSO-d_6) δ 4.75 (broad, 4H), 3.35 (s, 6H).

3,3′-Carbonylbis[1-(6-benzyloxy-9-Boc-purin-2-yl)-3-methyltriazen-1-yl] (Boc-protected dimer 32). Prepared from 0.15 mmol **31** and 0.30 mmol **11** according to the general procedure, yield 52%, mp 154−158 °C (dec); ^{1}H NMR δ 8.23 (s, 2H), 7.20−7.40 (m, 10H), 5.32 (s, 4H), 3.74 (s, 6H), 1.66 (s, 18H)

3,3′-Carbonylbis[1-(6-benzyloxypurin-2-yl)-3-methyltriazen-1-yl] (dimer 33). Method A, yield 55%, mp 165−167 °C (dec); ^{1}H NMR (DMSO-d_6) δ 13.55 (broad, 2H), 8.37 (s, 2H), 7.25−7.35 (m, 10H), 5.34 (s, 4H), 3.62 (s, 6H); [found $M^+ + 1$, 593.2208; $C_{27}H_{25}N_{14}O_3$ requires, 593.2234]. Anal. ($C_{27}H_{24}N_{14}O_3$) C, H, N.

Hydrolysis Studies. NMR samples were prepared by dissolving 2 mg of triazene in 0.4 mL of DMSO-d_6; 0.2 mL of a 0.1 M a phosphate buffer solution of pD 7.8 was added and after mixing the NMR-tube was placed in a 400 MHz NMR spectrometer at 37 °C. Spectra were recorded at appropriate intervals, and the ratio of starting material and products was calculated from the integral of the CH_2 and CH_3 substituents. O^6-BG (**4**) and O^6-BX (**36**) were characterized by HPLC analysis and by adding pure **4** and **36** to the NMR samples of the hydrolysis mixtures.

Supporting Information Available: Elemental analysis for compounds **10** and **11** and for the target compounds **20**−**30** and **33**. Mean graphs from in vitro testing of compounds **26** and **33**. ^{1}H NMR spectra of compounds **13**−**30**, **32**, and **33**. This material is available free of charge via the Internet at http://pubs.acs.org.

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