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Original article

Synthesis and biological evaluation of 4-nitro-substituted 1,3-diaryltriazenes as a novel class of potent antitumor agents

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ABSTRACT

We describe the synthesis and biological activity of a new class of 1,3-diaryltriazenes, namely 4-nitro-substituted 1,3-diaryltriazenes. Structure–activity relationship analysis reveals that 1,3-diaryltriazenes can be modified from inactive to highly cytotoxic compounds by the introduction of two nitro groups at the *para* positions of benzene rings and two additional electron-withdrawing groups (bromo, chloro, trifluoromethyl or fluoro substituents) at their *ortho* position. In order to increase the solubility of the modified compounds, we introduced various acyl groups to their triazene nitrogen. The results of LC-MS/MS analysis showed that *N*-acyltriazenes can be considered as prodrugs of non-acylated triazenes. Selected 3-acetyl-1,3-*bis*(2-chloro-4-nitrophenyl)-1-triazene (**8b**) is highly cytotoxic against different tumor cell lines, including cisplatin-resistant laryngeal carcinoma cells. Notably, its antiproliferative activity is significantly higher against tumor cells than against normal cells. DNA binding analysis suggests that neither **8b** nor its non-acylated derivative **8a** bind into the minor groove of DNA. Instead, **8b** induces reactive oxygen species that could provoke endoplasmic reticulum (ER^d) stress finally leading to apoptosis. Our data suggest that 4-nitro-substituted 1,3-diaryltriazenes are a new class of anticancer molecules which preferentially target malignant cells and may serve as potential antitumor agents.

1. Introduction

Various triazenes have an important application in the development of anticancer molecules [1]. Triazene-derived compounds of high clinical interest are the alkylating agents dacarbazine (5-[3,3-dimethyl-1-triazeno] imidazole-4-carboxamide) and temozolomide (8-carbamoyl-methylimidazo[5,1-*d*]-1,2,3,5-tetrazin-4(3*H*)-one), which have similar chemical, physical, antitumor and mutagenic properties. Their therapeutic potential and their specific mode of action have already been extensively studied [2–4]. They undergo proteolytic decomposition under physiological conditions, leading to highly reactive methyldiazonium ion which is capable of alkylating DNA. In quantitative terms, the most frequent site of DNA alkylation for both drugs is the N^7 position of guanine. Nevertheless, DNA O^6-methylguanine adducts are the main lesions responsible for the cytotoxic and the mutagenic effect because they can generate incorrect base pairing. Eventually, these adducts trigger cell death, or, if cell survives, provoke somatic point mutations [5]. One of the first alkyltriazenes used for anticancer therapy is dacarbazine [6,7]. For many years, dacarbazine has been one of the most widely used drugs to treat malignant melanoma [8,9]. The other clinically very important triazene is temozolomide. Due to its lipophylic character, it is capable to cross the blood–brain barrier. For this reason it is the first line therapeutic option in the treatment of primary and metastatic brain tumors [10–12]. In addition, temozolomide has been found suitable for the treatment of metastatic melanoma as well [11,13].

The other well-known representative of triazenes is a diaryltriazene derivative diminazene aceturate (Berenil®), the salt of

Abbreviations: AO, acridine orange; ct-DNA, calf thymus DNA; DAPI, 4′,6′ diamino-2-phenylindole dihydrochloride; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; ER, endoplasmic reticulum; EtBr, ethidium bromide; FACS, flow cytometry; FBS, fetal bovine serum; LC-MS/MS, liquid chromatography -mass spectrometry/mass spectrometry; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ROS, reactive oxygen species; SAR, structure–activity relationship; T.I., therapeutic index.

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1,3-*bis*(4-amidinophenyl) triazene (Fig. 1). Its capacity to bind to DNA has been recognized very early [14]. The binding to DNA occurs via complexation into the minor groove of AT-rich domains of DNA double helices. In addition, diminazene aceturate can also bind to RNA and to DNA duplexes, exhibiting characteristic properties of both intercalation as well as minor groove binding [15]. Diminazene aceturate can act as an antiviral compound [16], but has been mainly used as an anti-trypanosomal drug [17–19]. In addition, diminazene aceturate exhibits a relatively weak cytotoxic activity against L1210 leukemia cells (the concentration of diminazene aceturate that reduced the cell growth to 50% is 32 μM) [20].

In order to improve the antitumor efficacy of diaryltriazenes, we synthesized and examined cytotoxic activity of 1,3-diaryltriazenes bearing typical electron acceptors on both benzene rings (Scheme 1). Namely, diminazene aceturate has two amidinium moieties that act as strong electron-withdrawing functionalities. We found that halo- substituted 1,3-diaryltriazenes themselves are inactive but can be modified to highly cytotoxic compounds by the introduction of two nitro groups at the *para* positions of benzene rings if two electron-withdrawing groups are at their *ortho* position (Fig. 1; Y: Br, Cl, CF_3 or F). This allows us to conclude that 4-nitro-substituted 1,3-diaryltriazenes represent a new class of potential antitumor agents.

2. Chemistry

To keep the electron-withdrawing character of the substituents in the molecule, as in the case of diminazene aceturate, we decided to study several 1,3-diaryltriazenes bearing electron acceptors on both benzene rings. Our first choice was a set of fluoro-, trifluoromethyl- and chloro- substituted diaryltriazenes **1a**–**1h** (Fig. 2). They were prepared from the corresponding haloanilines in reaction with either nitrous acid or isoamyl nitrite. Some of them were then acylated to give the corresponding *N*-acyl-1,3-diaryltriazenes. Acylated triazenes were chosen because they are known to act as compounds that are able to selectively transfer an acyl group to various amino functionalities even in aqueous or alcohol solutions [21]. Thus, an acetylation of the appropriate triazenes with acetyl chloride was performed leading to the products **2a** and **2b**. Furthermore, an introduction of the substituted cinnamoyl group on the triazene nitrogen to obtain compounds **3**–**5** was inspired by the effect described for the acyl group, originated from ferulic acid (4-hydroxy-3-methoxycinnamic acid). This group is an essential part of a simple amide that selectively induces apoptosis in leukemia and lymphoma cells but not in normal white blood cells [22].

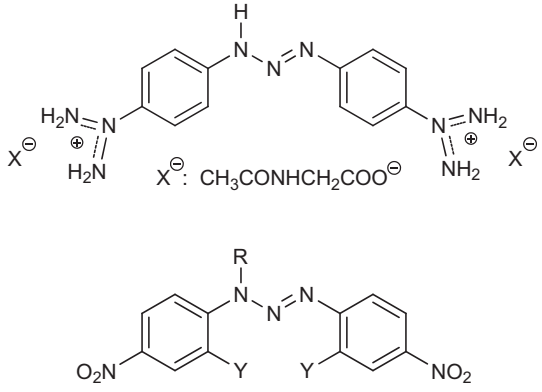
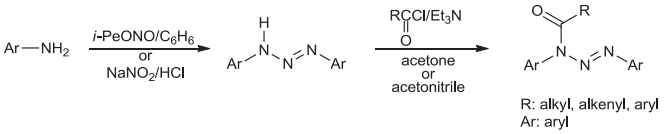


Fig. 1. Structures of diminazene aceturate (above) and 1,3-*bis*(4-nitrophenyl)triazenes (below).



Scheme 1. Synthesis of 1,3-diaryltriazenes and their *N*-acyl derivatives.

Next we focused on *bis*(4-nitrophenyl)triazenes **6** (Fig. 3), since the nitro groups were expected to cause a similar effect as the amidinium moieties in diminazene aceturate. Namely, amidinium moiety is positively charged while nitro group is neutral and of very polar functionality. In spite of this difference both are strong electron-withdrawing groups. The introduction of electron-withdrawing substituents such as F, Cl, Br, and CF_3 on two aryl rings did not lead to the active 1,3-diaryltriazenes. For this reason, the more electron-withdrawing nitro group was attached to benzene rings at their *para* position as in the case of diminazene aceturate. It means that two amidinium groups or two nitro substituents were expected only to exhibit a similar electronic effect to the central triazene moiety. However, this fact does not allow us to suggest similar binding of diminazene aceturate and 4-nitrophenyl-substituted 1,3-diaryltriazenes to DNA or other targets.

To make the triazene hydrogen more acidic, which would be beneficial for the acylation of the triazene nitrogen, the additional electron-withdrawing groups were introduced at *ortho* positions of benzene rings. So, triazenes **7a**, **8a**, **9a**, and **10a**, possessing bromo, chloro, trifluoromethyl or fluoro substituents were prepared and then used for the synthesis of their *N*-acetyl derivatives **7b**, **8b**, **9b**, and **10b**. In addition, as non-acylated triazenes show low solubility in water and partially precipitate in the cell culture growing medium, while N-acetylated triazenes demonstrate substantially improved solubility, we introduced various acyl groups to the triazene nitrogen in order to increase the solubility of the modified compounds. The structures of *N*-acyl-1,3-*bis*(4-nitroaryl)triazenes are presented in Fig. 4.

3. Results and discussion

3.1. Antiproliferative activity and structure–activity relationship (SAR)

All compounds were evaluated for their cytotoxic activity *in vitro* against human cervical carcinoma HeLa cells. We screened first the antiproliferative effect of either diaryltriazenes **1** or their acylated derivatives **2**–**5**, but the results we obtained were quite disappointing as they showed very low or no cytotoxic activity (Table 1).

Screening of 1,3-*bis*(4-nitrophenyl)triazenes and their *N*-acetyl derivatives showed that *bis*(4-nitrophenyl)triazene **6a** was not cytotoxic against HeLa cells, but the introduction of an acetyl group, leading to its analog **6b**, induced some cytotoxicity. However, either Cl, Br, or CF_3 moieties were beneficial when added to the *ortho* position of both triazene benzene rings, as their cytotoxic activity against HeLa cells significantly increased when compared to those of **6a** and **6b** (Table 1). In addition, introduction of the acetyl group increased the cytotoxicity of **7b**, **8b** and **9b** compounds further.

We learned from our initial studies shown in Table 1 that the introduction of the trifluoromethyl group to the benzene rings was beneficial for enhancing cytotoxicity of the triazene. Namely, the only active compounds (although slightly active), presented in Fig. 1, were **1e**, **2b**, and **4b**, all of them possessing trifluoromethyl groups. Furthermore, as shown in Table 1, 1,3-diaryltriazenes can be modified from inactive to highly cytotoxic compounds by an introduction

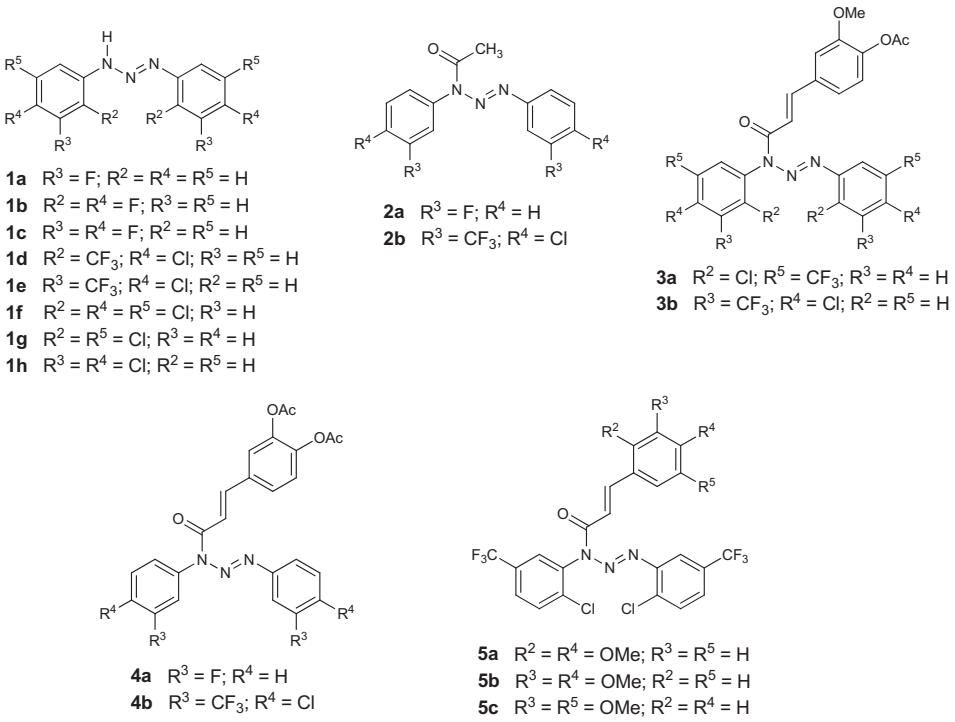


Fig. 2. Structures of halo- substituted 1,3-diaryltriazenes and their *N*-acyl derivatives.

of two nitro groups at the *para* positions of benzene rings and two other electron-withdrawing groups (bromo, chloro, trifluoromethyl or fluoro substituents) at their *ortho* position. Generally, the trifluoromethyl substituent has the strongest influence on antiproliferative activity, while fluoro substituted triazenes were the least active. Acetylation of the *ortho* substituted *bis*(4-nitrophenyl) triazenes increased the cytotoxicity of these compounds with the exception of the fluoro derivative **10b** if compared with **10a**.

Screening of *ortho* substituted *N*-acyl-1,3-*bis*(4-nitroaryl)triazenes revealed them all as highly cytotoxic against HeLa cells (Table 2). A comparison of their activity has shown that in most of the cases the trifluoromethyl group exhibited somewhat higher activity when compared to chloro or bromo substituents. For *N*-benzoyl derivatives, the antiproliferative activity of chloro and trifluoromethyl analogs was almost the same. It also turned out that either *ortho* chloro or *ortho* bromo substituted triazenes were more effective than their trifluoromethyl counterparts only in two cases. In addition, a type of an acyl group ($R^1C{=}O$), attached to the triazene nitrogen, can influence the effectiveness of the compound. For example, it was observed that cytotoxicity was lower for the compounds having 2-trifluoromethylbenzoyl functionality placed at N-3 of the triazene, comparing to the other *N*-acyl derivatives, irrespective of the substituents at the *ortho* position of the benzene rings attached on both ends of the triazene chain.

Some of *N*-acyltriazenes were found to donate their acyl groups to various amino compounds, giving the corresponding non-acylated triazenes [21]. Since the growth medium used for cell maintenance in culture (and in the cytotoxicity assay, accordingly) is abundant in amino-containing compounds reflecting the conditions found *in vivo*, we checked the possibility that N-acyltriazenes are deacylated into a parent triazene before entering cells. For that reason, non-acylated triazenes **8a** and **9a** as well as their *N*-acetyl or *N*-benzoyl analogs **8b**, **8c**, **9b**, and **9c** were dissolved in DMEM growing medium, supplemented with 10% fetal bovine serum (FBS) and kept at room temperature. The concentration of each triazene was monitored by LC-MS/MS technique within 26.25 h as follows: immediately after the preparation of the solution, after 3.75 h, 7.5 h, 11.25 h, 18.75 h, 22.5 and 26.25 h, respectively. The experiments

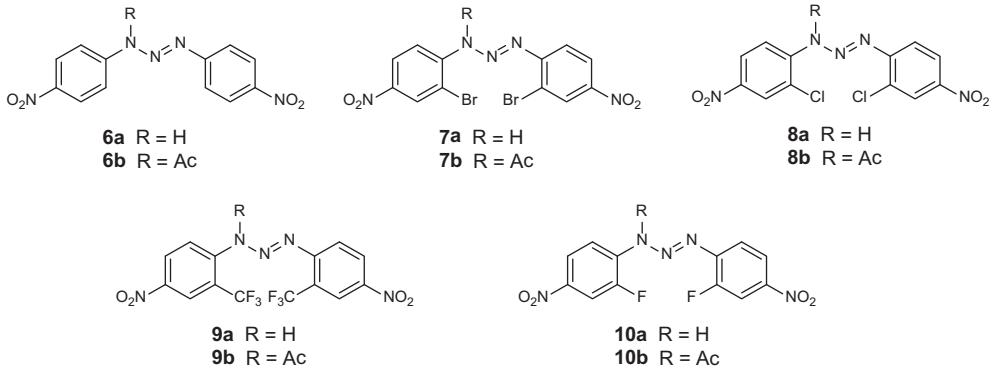
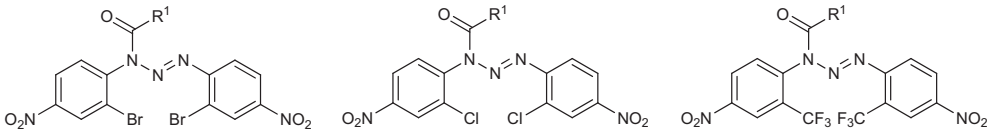


Fig. 3. Structures of 1,3-*bis*(4-nitrophenyl)triazenes and their *N*-acetyl derivatives.



7c R^1 = Ph
7d R^1 = 2-F-C_6H_4
7e R^1 = 2-CF_3-C_6H_4
7f R^1 = 3-CF_3-C_6H_4
7g R^1 = 4-F-C_6H_4
7h R^1 = $CH_3CO_2CH_2$

8c R^1 = Ph
8d R^1 = 2-F-C_6H_4
8e R^1 = 2-CF_3-C_6H_4
8f R^1 = 3-CF_3-C_6H_4
8g R^1 = 4-CF_3-C_6H_4
8h R^1 = 3-F-C_6H_4
8i R^1 = $ClCH_2$
8j R^1 = $PhCH_2$
8k R^1 = $CH_3CO_2CH_2$
8l R^1 = MeO

9c R^1 = Ph
9d R^1 = 2-F-C_6H_4
9e R^1 = 2-CF_3-C_6H_4
9f R^1 = 3-CF_3-C_6H_4
9g R^1 = 4-CF_3-C_6H_4
9h R^1 = 3-F-C_6H_4
9i R^1 = 4-F-C_6H_4
9j R^1 = $ClCH_2$
9k R^1 = $EtOCOCH_2$

Fig. 4. Structures of other *N*-acyl-1,3-*bis*(4-nitrophenyl)triazenes applied in our study.

showed that the concentration of either **8a** or **9a** remained unchanged. The solutions of *N*-acyltriazenes behaved differently. Namely, concentrations of *N*-acyltriazenes decreased within 26.25 h to reach 57% of the initial value in the case of **8b**, 9% for **8c**, 51% for **9b**, and 63% for **9c**. These processes were always accompanied by the formation of the corresponding non-acylated triazene (**8a** or **9a**) whose concentration permanently increased. Therefore, the above mentioned *N*-acyltriazenes can be considered as prodrugs of non-acylated triazenes.

To examine the cytotoxicity of 4-nitro-substituted 1,3-diaryltriazenes on different tumor cell lines in more detail and to get some insight into the molecular mechanisms involved in their cytotoxicity, we chose one of the derivatives (**8b**) as a representative compound. Namely, during the course of the cytotoxicity assay, we always carefully observed the morphology of the treated cells and found that all tested compounds induced the same morphological alterations with the same time course of their appearance, the only difference being the concentration of the compound that has been applied. In addition, the fact that *N*-acyltriazenes can be considered as prodrugs of non-acylated triazenes in the cell growth medium, partly explains their similar biological activity. The results presented in Table 3 clearly show that **8b** strongly inhibited the growth of all examined tumor cell lines, as well as one normal cell line (human fibroblasts). However, it was significantly more cytotoxic (up to >10-fold) to tumor than to normal cells. Therapeutic index, calculated from the ratio of cytotoxicity (IC_{50}) on tumor cells and cytotoxicity (IC_{50}) on normal fibroblasts was similarly low for carcinoma cell lines from different origin (higher only for urinary bladder carcinoma RT-112 cells). It is important to point out that the triazene **8b** was similarly cytotoxic to parental laryngeal carcinoma HEp-2 cells as to their cisplatin-resistant $CA3_{ST}$ subline.

Table 1
Antiproliferative activity of 1,3-diaryltriazenes and their acylated derivatives against HeLa cells.

Compound	IC_{50} (μM)	Compound	IC_{50} (μM)
1a	NA (214.4)	**5a**	NA (84.4)
1b	NA (185.7)	**5b**	NA (28.1)
1c	NA (185.7)	**5c**	NA (24.1)
1d	NA (62.2)	**6a**	NA (43.5)
1e	58.38 ± 2.96	**6b**	62.99 ± 2.18
1f	NA (41.3)	**7a**	0.94 ± 0.46
1g	NA (37.3)	**7b**	0.55 ± 0.02
1h	NA (74.6)	**8a**	3.01 ± 1.86
2a	NA (181.6)	**8b**	0.63 ± 0.05
2b	50.57 ± 0.58	**9a**	1.13 ± 0.26
3a	NA (40.3)	**10a**	3.30 ± 0.09
3b	NA (16.8)	**10b**	4.76 ± 0.26
4a	NA (52.1)	Diminazene aceturate	183.25 ± 12.78
4b	43.03 ± 2.99		

IC_{50} is the concentration of the test compound inducing 50% cell growth inhibition after 72 h incubation; NA = not active. The highest concentrations (in μM) that can be evaluated (due to the solubility of the compounds in DMSO and their further dilution in the growth medium which is limited by the influence of DMSO itself on cell growth), are indicated in the brackets.

3.2. DNA binding activity

Since compound **8b** preferentially targets malignant cells, we investigated the molecular mechanisms underlying its biological effects further. It was assumed that **8a** and **8b** could cause similar biological effects as diminazene aceturate due to similar characteristics of their electron-withdrawing substituents. Some diminazene aceturate analogs as well as some other heterocyclic bis-amidine derivatives can intercalate into DNA (e.g. 4′,6′ diamino-2-phenylindole dihydrochloride (DAPI) intercalates into G-C sequences) [23]. For instance, Wilson et al. showed for several un-fused heterocyclic bis-amidine derivatives that the ability of intercalation into DNA is controlled by the angle between aromatic moieties, whereby only closely planar-like structures were able to intercalate [24]. We have previously found that the triethylammonium salt of **8a** in the solid state is not highly planar but nearly planar [25]. However, the ^1H and ^{13}C NMR spectra of its solution in DMSO-d_6 indicate that the aryl groups are equivalent, meaning that its structure in the solution is indeed planar. Furthermore, the structure of **8a** can also be compared with **6a**: the latter is almost planar in the solid

Table 2
Antiproliferative activity of *N*-acyl-1,3-*bis*(4-nitroaryl)triazenes against HeLa cells.

Compound	IC_{50} (μM)	Compound	IC_{50} (μM)
7c	1.53 ± 1.01	**8j**	0.53 ± 0.08
7d	1.2 ± 0.52	**8k**	0.35 ± 0.01
7e	13.34 ± 6.51	**8l**	0.98 ± 0.11
7f	1.12 ± 0.62	**9c**	0.60 ± 0.35
7g	0.58 ± 0.14	**9d**	0.80 ± 0.32
7h	0.49 ± 0.22	**9e**	5.35 ± 0.49
8c	0.59 ± 0.06	**9f**	0.75 ± 0.21
8d	2.35 ± 1.58	**9g**	0.69 ± 0.27
8e	10.59 ± 4.77	**9h**	0.88 ± 0.34
8f	1.18 ± 0.25	**9i**	1.0 ± 0.51
8g	1.06 ± 0.16	**9j**	0.80 ± 0.28
8h	2.50 ± 0.46	**9k**	0.30 ± 0.07
8i	0.55 ± 0.08		

IC_{50} is the concentration of the test compound inducing 50% cell growth inhibition after 72 h incubation.

Table 3
Antiproliferative activity of **8b** on a variety of cancer and normal cell lines (IC_{50}, μM ± SD) and therapeutic index (T.I.)

Cell line	HeLa	HEp-2	$CA3_{ST}$	SW480	SW620	MIA PaCa2	RT-112	Fibro
IC_{50}/μM	0.63 ± 0.05	0.62 ± 0.04	0.63 ± 0.08	0.53 ± 0.05	0.45 ± 0.20	0.52 ± 0.01	7.2 ± 0.2	14.0 ± 1.8
T.I.	0.045	0.044	0.045	0.038	0.032	0.037	0.514	/

T.I. = therapeutic index: T.I. was calculated from the ratio of cytotoxicity (IC_{50}) on tumor cells and cytotoxicity (IC_{50}) on normal fibroblasts.
HeLa = cervical carcinoma cells, HEp-2 = laryngeal carcinoma cells, $CA3_{ST}$ = cisplatin-resistant HEp-2 subline, SW480 = primary colorectal carcinoma cells, SW620 = metastatic colorectal carcinoma cells, MIA PaCa-2 = pancreatic carcinoma cells, RT-112 = urinary bladder carcinoma cells, Fibro = human fibroblasts.

state [26], while its ^1H and ^{13}C NMR spectra in DMSO-d_6 solution show only one set of aromatic protons thus indicating a planar structure.

Therefore, we analyzed **8a** (non-acylated triazene) and **8b** (acylated triazene) for DNA binding activity. We examined their potential to bind double stranded (ds) DNA by checking their influence on the thermal stability of *calf thymus* DNA (ct-DNA). It is well known that upon heating at well-defined temperature (Tm value) ds- helices of polynucleotides dissociate into two single stranded polynucleotides. Biologically relevant non-covalent binding of small molecules to ds-polynucleotides usually has a certain effect on the thermal stability of helices thus giving different Tm values. The difference between the Tm value of free polynucleotide and polynucleotide complexed with small molecule (ΔTm value) is an important factor in characterization of small molecule and ds−polynucleotide interactions, whereby the absence of any effect strongly suggests negligible interaction of studied compound with DNA under biologically relevant conditions [27–29].

In thermal denaturation experiment **8a** and **8b** were mixed with ct-DNA in a ratio $r_{[compound]/[ct\text{-}DNA]} = 0.3$ at which both, intercalation and minor groove binding should give a measurable change in DNA melting point transition. However, both, **8a** and **8b** did not influence the thermal stability of ct-DNA within the error of the method (Fig. 5), indicating that at biologically relevant conditions (pH 7, c(compound) ≤ 10 μM) they do not form stable non-covalent complex with double stranded DNA.

3.3. Effect on cell cycle progression

To shed more light on the mechanisms responsible for the cytotoxic effect of **8b**, we examined its influence on cell cycle progression. As shown in Table 4, compound **8b** arrested the cells in the G0/G1 phase of the cell cycle, which was accompanied by a dose-dependent increase of the cells present in the subG1 fraction, which represents the apoptotic cells.

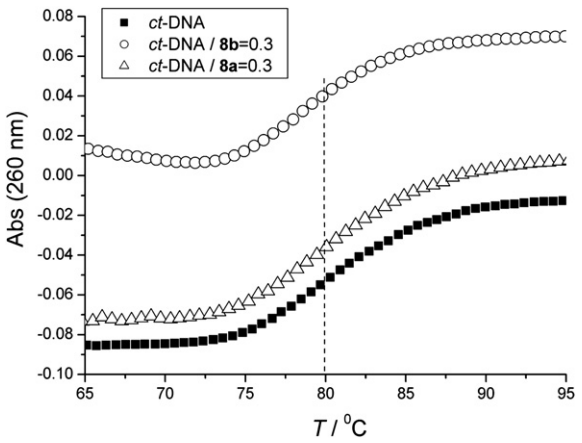


Fig. 5. Thermal denaturation curves of ct-DNA (c(ct-DNA) = 2 × 10^{-5} mol dm^{-3}, $r_{[compound]/[ct\text{-}DNA]} = 0.3$) at pH 7.0 (sodium cacodylate buffer, $I = 0.05$ M) upon addition of **8a** (Δ) and **8b** (○). Error in Δ*Tm* values: ± 0.5 °C.

3.4. Induction of reactive oxygen species

As we have shown that **8a** and **8b** do not bind DNA, alternative mechanisms of action were examined. Literature data indicate that diverse compounds can induce the formation of reactive oxygen species (ROS) [30]. Compound **8b** induced ROS formation (Fig. 6A) in a dose-dependent manner. Elevated level of ROS was detectable already 6 h after onset of treatment (Fig. 6B).

3.5. Stress of endoplasmic reticulum

ROS can either cause ER stress or can be its consequence [31,32]. To determine whether **8b** provokes the induction of ER stress we examined the expression of the prototypical ER stress markers CHOP and Grp78. Both markers were expressed 48 h after treatment with **8b** (Fig. 7A). Their expression was not detectable until 8 h after the treatment (data not shown), but increased at longer periods of time (Fig. 7B). Induction of ER stress was confirmed by using tunicamycin, which blocks protein glycosylation, therefore being a prototypical ER stress inducer (data not shown). Thus, we assume that treatment with **8b** results in ROS formation subsequently leading to ER stress.

3.6. Induction of cell death

Since endoplasmic reticulum stress can induce cell death [33,34], we examined whether the treatment of HeLa cells with **8b** will have the same consequence. The experiments showed that 48 h after treatment with **8b**, classical morphological characteristics of apoptosis were observed in HeLa cells (Fig. 8A), accompanied with the cleavage of apoptotic proteins PARP and procaspase-9, -8 and -3 (Fig. 8B). These data, alongside the observed increase in subG1 fraction (Table 4), show that **8b** efficiently triggers apoptotic death.

4. Conclusions

We have shown that 4-nitro- substituted 1,3-diaryltriazenes with two additional electron-withdrawing groups at their *ortho* position (bromo, chloro, trifluoromethyl or fluoro substituents) are highly cytotoxic new compounds. In order to increase the solubility of the modified compounds, we introduced various acyl groups to their triazene nitrogen. The results of LC-MS/MS analysis showed that *N*-acyltriazenes can be considered as prodrugs of non-acylated triazenes. The antiproliferative activity of new compounds depends on the type of the substituent introduced at the *ortho* position of the benzene rings (trifluoromethyl has the strongest influence). The selected compound **8b** is a promising representative of the 4-nitro-substituted 1,3-diaryltriazenes because of its high cytotoxicity against different tumor cell lines, including drug-resistant carcinoma cell line and its higher cytotoxicity against tumor cells as

Table 4
Effects of **8b** on the cell cycle of HeLa cells.

Conc/μM	24 h				48 h				72 h			
	G0/G1	S	G2/M	subG1	G0/G1	S	G2/M	subG1	G0/G1	S	G2/M	subG1
0	60.6	27.7	11.7	9.4	63.8	26.3	9.9	8.5	66.9	23.5	9.7	7.2
0.78	74.4	15.1	10.5	8.7	74.5	18.5	7.0	15.8	59.1	32.5	8.4	17.6
7	69.3	20.8	10.0	13.2	76.2	14.2	9.6	23.8	77.1	15.2	7.6	33.0
21	69.3	20.2	10.5	16.4	73.0	15.2	11.9	46.5	79.0	12.6	8.4	80.2

HeLa cells were treated for the indicated time period with **8b**, stained with propidium iodide and analyzed by flow cytometry. Cell cycle distribution was assessed as described in Experimental Section.

compared to normal cells. This compound arrests tumor cells in the G0/G1 phase of the cell cycle. Furthermore, DNA binding analysis suggests that neither **8b** nor its non-acylated derivative **8a** bind into the minor groove of DNA. Instead, **8b** induces reactive oxidative species that could trigger ER stress finally leading to caspase-driven apoptosis. Our data suggest that 4-nitro- substituted 1,3-diaryltriazenes are a new class of anticancer molecules which preferentially target malignant cells. Therefore we propose these novel derivatives as potential antitumor agents.

5. Experimental Section

5.1. Chemistry

Starting materials for the synthesis of examined compounds and diminazene aceturate (Berenil®) were used as obtained from the commercial source (Aldrich). Melting points were determined on a Kofler micro hot stage and are uncorrected. ^{1}H NMR spectra were recorded with a Bruker Avance DPX 300 spectrometer at 29 °C (unless otherwise stated) and 300 MHz, using TMS as an internal standard. ^{13}C NMR spectra were recorded on the same instrument at 75.5 MHz and are referenced against the central line of the solvent signal (DMSO-d_6 septet at $\delta = 39.5$ ppm). IR spectra were obtained with Bio-Rad FTS 3000MX (KBr pellets for all compounds). MS spectra were recorded with a VG-Analytical AutoSpec Q instrument. For LC-MS/MS experiments, liquid chromatograph Perkin Elmer Series 200 from Perkin Elmer (Shelton, CT, USA) with UV detector and 3200 QTRAP LC/MS/MS System equipped with ESI ion source from Applied Biosystems/MDS Sciex (Foster City, CA, USA) were used. UV detection was performed at 254 nm, whereas mass spectrometer was operating in full scan mode in negative ionization mode. Elemental analyses (C, H, N) were performed with Perkin Elmer 2400 Series II CHNS/O Analyzer. TLC was carried out on Fluka silica-gel TLC-cards.

Some triazenes were prepared as described in the literature: **1a** [35], **1e** [36], **1f** [37], **1h** [36], **2a** [38], **6a** [39], **6b** [39], **7b** [25], **8a** [40], **8b** [21], and **8j** [21]. All tested compounds possessed a purity of ≥95% as verified by ^{1}H NMR and ^{13}C NMR spectroscopy as well as with elemental microanalysis. The figures, obtained for C, H, N analysis, were always within ±0.35% of the calculated values.

5.1.1. Typical procedures for the synthesis of 1,3-diaryltriazenes (modification of known procedures [41])

5.1.1.1. Method A. A solution of isoamyl nitrite (1.755 g, 15 mmol) in benzene (30 mL) was added drop wise to 4-chloro-2-trifluoromethylaniline (1.956 g, 10 mmol) at room temperature. The reaction mixture was stirred for 5 min and evaporated to dryness under reduced pressure. The residue was treated with petroleum ether (5 mL) and the solid material was filtered off to give **1d** in 75% yield.

5.1.1.2. Method B. A solution of sodium nitrite (35 mg, 0.5 mmol) in water (5 mL), was added drop wise to the suspension of 2,5-dichloroaniline (162 mg, 1 mmol) in 5% water solution of HCl (2.1 mL) at 0 °C. The reaction mixture was stirred at room temperature for 3 h. Then, the solid material was filtered off and washed with water leading to **1g** in 95% yield.

5.1.2. Typical procedures for the synthesis of 3-acyl-1,3-diaryltriazenes (modification of known procedures [41])

5.1.2.1. Method C. A solution of 1,3-bis[(2-chloro-5-trifluoromethyl) phenyl]triazene (402 mg, 1 mmol) and triethylamine (0.28 mL, 2 mmol) in acetone (5 mL) was treated at room temperature with

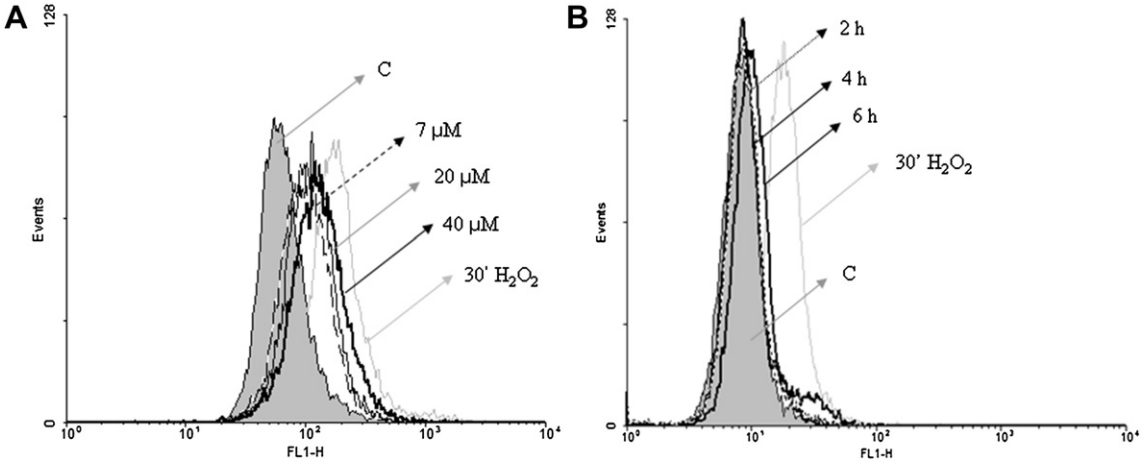


Fig. 6. Generation of ROS. A: Logarithmically growing HeLa cells were stained for 1 h with 10 μM CM-H_2DCFDA and A): treated with 7, 20 and 40 μM of **8b** for 8 h and B) treated with 7 μM of **8b** for 2, 4 and 6 h. Afterward ROS formation was determined by FACS as described in Experimental section. As positive control, HeLa cells were treated with hydrogen peroxide (H_2O_2) (0.01%) for 30 min.

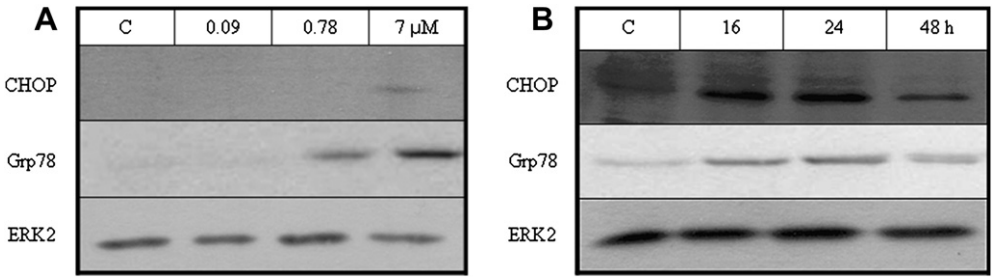


Fig. 7. Induction of endoplasmic reticulum stress. To examine the induction of ER stress, the expressions of the ER stress markers CHOP and Grp78 were analyzed by Western blot analysis. HeLa cells were treated with different concentrations of **8b** for 48 h (A), or with 7 μM of **8b** for different periods of time (B). Expression of ERK2 was determined as internal loading control.

(2E)-3-(4-acetoxy-3-methoxyphenyl)prop-2-enoyl chloride (280 mg, 1.1 mmol). The reaction mixture was stirred for 5 min and the solid (triethylammonium chloride) was filtered off. The solution was evaporated to dryness, the residue was suspended in methanol (1 mL) and the crude product was separated by filtration (3a, 73% yield).

5.1.2.2. Method D. Triethylamine (111 mg; 1.1 mmol) was added to a stirred suspension of 1,3-bis(2-bromo-4-nitrophenyl)triazene (222 mg, 0.50 mmol) in acetonitrile (5 mL) at room temperature, followed by the addition of benzoyl chloride (155 mg, 1.1 mmol). After additional stirring for 1 h, the reaction mixture was evaporated to dryness, treated with water (4 mL) and extracted with CH_2Cl_2 (3 × 5 mL). The methylene chloride solution was dried over anhydrous sodium sulfate and evaporated to dryness. The residue was dissolved in hot ethyl acetate (1 mL) and after that a hot petroleum ether was slowly added (5 mL). This solution was kept overnight at −20 °C and the crude product was filtered off (7c, 85% yield).

5.1.3. Characteristics of the synthesized compounds

*5.1.3.1. 1,3-Bis(2,4-difluorophenyl)triazene (**1b**).* Method A, reaction time: 4 h; yield 94%; mp 124–127 °C (EtOH/H_2O); IR (KBr): 3179, 1624, 1599, 1531, 1493, 1411, 1253, 1232, 1198, 1142, 969, 859, 847, 727 cm^{-1}; ^1H NMR (DMSO-d_6): 7.12 (2H, m), 7.36 (2H, m), 7.67 (2H, m), one proton not observed − exchanged; MS (EI) *m/z* 269 (M^+, 5.5), 141 (100), 113 (91), 63 (37). Anal. for $C_{12}H_7F_4N_3$: Calcd C, 53.54; H, 2.62; N, 15.61. Found C, 53.53; H, 2.40; N, 15.47.

*5.1.3.2. 1,3-Bis(3,4-difluorophenyl)triazene (**1c**).* Method A, reaction time: 4 h; yield 73%; mp 132–134 °C (EtOH/H_2O); IR (KBr): 3190, 1619, 1509, 1459, 1415, 1283, 1250, 1207, 1104, 775, cm^{-1}; ^1H NMR (DMSO-d_6) : 7.25 (2H, m), 7.45 (2H, m), 7.58 (2H, m), one proton not observed − exchanged; MS (EI) *m/z* 269 (M^+, 11), 141 (75), 113 (100), 63 (55). Anal. for $C_{12}H_7F_4N_3$: Calcd C, 53.54; H, 2.62; N, 15.61; Found C, 53.80; H, 2.61; N, 15.46.

*5.1.3.3. 1,3-Bis[4-chloro-2-(trifluoromethyl)phenyl]triazene (**1d**).* Method A, reaction time: 5 min; yield 75%; mp 146–147 °C (MeOH); IR (KBr): 3355, 1588, 1515, 1475, 1436, 1412, 1323, 1262, 1184, 1157 1101, 1049, 890, 823, 721 cm^{-1}; ^1H NMR (DMSO-d_6): δ 7.70 (2H, d, J = 8.8), 7.79 (2H, dd, J_1 = 2.0 Hz, J_2 = 8.8 Hz), 7.84 (2H, d, J = 2.0 Hz), 12.64 (1H, s); MS (EI) *m/z* 401 (M^+, 0.3), 207 (49), 179 (100), 144 (22). Anal. for $C_{14}H_7Cl_2F_6N_3$: Calcd C, 41.82; H, 1.75; N, 10.45. Found C, 41.75; H, 1.68; N, 10.33.

*5.1.3.4. 1,3-Bis(2,5-dichlorophenyl)triazene (**1g**).* Method B, reaction time: 3 h; yield 95%; mp 164–165 °C (Et_2O/toluene); IR (KBr): 3179, 3090, 1591, 1522, 1474, 1410, 1386, 1253, 1186, 1091, 1058, 980, 864, 795, 584 cm^{-1}; ^1H NMR (DMSO-d_6): δ 7.31 (2H, m), 7.57 (2H, d, J = 8.7 Hz), 7.80 (2H, d, J = 2.1 Hz), 12.60 (1H, s); MS (EI) *m/z* 333 (M^+, 0.5), 305 (3), 235 (5), 1783 (60), 145 (100), 109 (34), 75 (15). Anal. for $C_{12}H_7Cl_4N_3$: Calcd C, 43.02; H, 2.11; N, 12.54. Found C, 42.79; H, 1.95; N, 12.59.

*5.1.3.5. 3-Acetyl-1,3-bis[(4-chloro-3-trifluoromethyl)phenyl]triazene (**2b**).* Method C, reaction time: 5 min; yield 79%; mp 117.5–118.5 °C (petroleum ether); IR (KBr): 1716, 1474, 1318, 1214, 1128, 1038, 960, 661 cm^{-1}; ^1H NMR (DMSO-d_6): δ 2.66 (3H, s); 7.63 (1H, dd, J_1 = 8.5 Hz, J_2 = 2.3 Hz); 7.86 (4H, m); 7.92 (1H, d, J = 8.5 Hz); ^{13}C NMR (DMSO-d_6): δ 22.6, 121.9 (q, J = 5.4 Hz), 122.26 (q, J = 274 Hz), 122.33 (q, J = 273 Hz), 125.7, 128.5 (q, J = 5.4 Hz), 129.5 (q, J = 32 Hz), 129.7 (q, J = 32 Hz), 132.4, 132.5, 133.3 (q, J = 1.7 Hz), 133.4, 133.5, 133.7, 146.4, 172.5; MS (FAB) *m/z* 444 (MH^+, 0.7); 207

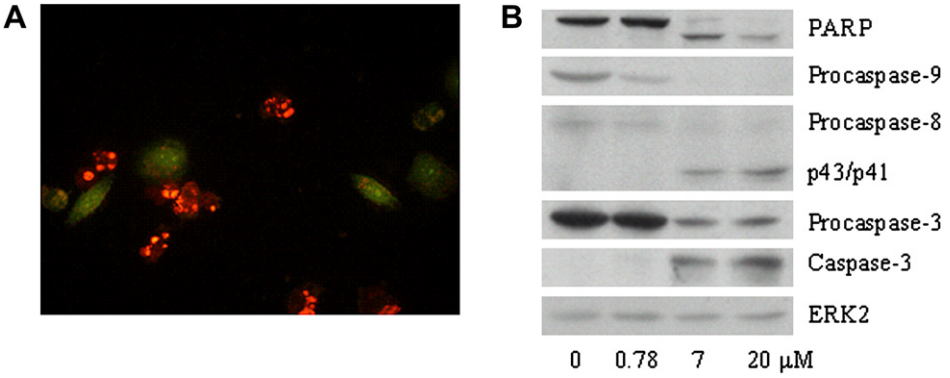


Fig. 8. HeLa cells were treated with **8b** for 48 h. The induction of apoptosis was determined by (A) staining cells treated with 7 μM **8b** with acridine orange and ethidium bromide, and viewing the characteristic morphological features under the epifluorescence microscope; and (B) by determination of the cleavage of characteristic apoptotic proteins (PARP and procaspases) by Western blot analysis (as described in Experimental section). ERK2 was used as loading control.

(100). Anal. for $C_{16}H_9Cl_2F_6N_3O$: Calcd C, 43.27; H, 2.04; N, 9.46. Found C, 43.37; H, 1.98; N, 9.39.

*5.1.3.6. 3-[(2E)-3-(4-acetoxy-3-methoxyphenyl)prop-2-enoyl]-1,3-bis [2-chloro-5-trifluoromethyl phenyl]triazene (**3a**).* Method C, reaction time: 5 min; yield 73%; mp 156–158 °C (EtOH); IR (KBr): 1763, 1695, 1624, 1508, 1420, 1360, 1329, 1217, 1203, 1177, 1156, 1131, 1082 cm^{-1}; ^1H NMR (DMSO-*d*6): δ 2.29 (3H, s), 3.88 (3H, s), 7.21 (1H, d, $J = 7.9$ Hz), 7.42 (1H, dd, *J1* = 1.8 Hz, *J2* = 8.2 Hz), 7.71 (1H, d, $J = 1.9$ Hz), 7.82 (2H, m), 7.88–8.03 (4H, m), 8.06 (1H, m), 8.23 (1H, s); ^{13}C NMR (CDCl3): δ 20.6, 55.7, 111.1, 115.6, 116.5 (q, $J = 3.8$ Hz), 122.1, 123.2 (q, $J = 272$ Hz), 123.4 (q, $J = 272$ Hz), 123.5, 126.4 (q, $J = 3.6$ Hz), 127.7 (q, $J = 3.7$ Hz), 128.1 (q, $J = 3.8$ Hz), 129.9 (q, $J = 33$ Hz), 130.4 (q, $J = 34$ Hz), 130.8, 131.4, 133.3, 134.5, 135.6 (q, $J = 1.3$ Hz), 137.0 (q, $J = 1.3$ Hz), 142.1, 145.3, 146.3, 151.6, 166.3, 168.6; MS (FAB) *m/z* 619 (M^+, 0.09), 219 (32), 177 (100). Anal. for C26H17Cl2F6N3O4: Calcd C, 50.34; H, 2.76; N, 6.77. Found C, 50.70; H, 2.84; N, 6.75.

*5.1.3.7. 3-[(2E)-3-(4-acetoxy-3-methoxyphenyl)prop-2-enoyl]-1,3-bis[(4-chloro-3trifluoromethyl) phenyl]triazene (**3b**).* Method C, reaction time: 5 min; yield 65%; mp 164.5–166.5 °C (EtOH); IR (KBr): 1767, 1692, 1622, 1509, 1480, 1419, 1360, 1312, 1258, 1206, 1176, 1145, 1122 cm^{-1}; ^1H NMR (DMSO-d_6): δ 2.29 (3H, s), 3.87 (3H, s), 7.21 (1H, d, $J = 8.1$ Hz), 7.46 (1H, dd, $J_1 = 1.9$ Hz, $J_2 = 8.1$ Hz), 7.62 (1H, d, $J = 1.9$ Hz), 7.73 (1H, dd, $J_1 = 2.4$ Hz, $J_2 = 8.8$ Hz), 7.83–7.91 (3H, m), 7.93–8.02 (4H, m); ^{13}C NMR ($CDCl_3$): δ 20.6, 55.8, 111.7, 115.9, 121.0 (q, $J = 5.5$ Hz), 121.5, 122.3 (q, $J = 274$ Hz), 122.4 (q, $J = 274$ Hz), 123.5, 126.6, 128.5 (q, $J = 5.3$ Hz), 129.4 (q, $J = 32$ Hz), 129.7 (q, $J = 32$ Hz), 132.51, 132.53, 133.2 (q, $J = 1.8$ Hz), 133.4, 133.45 (q, $J = 1.8$ Hz), 133.52 (q, $J = 0.9$ Hz), 133.9, 142.0, 146.0, 146.5, 151.6, 166.9, 168.7; MS (EI) *m/z* 619 (M^+, 1.6), 219 (46), 177 (100). Anal. for $C_{26}H_{17}Cl_2F_6N_3O_4$: Calcd C, 50.34; H, 2.76; N, 6.77. Found C, 50.50; H, 2.75; N, 6.59.

*5.1.3.8. 3-[(2E)-3-(3,4-diacetoxyphenyl)prop-2-enoyl]-1,3-bis(3-fluorophenyl)triazene (**4a**).* Method C, reaction time: 5 min; yield 61%; mp 130–131 °C (MeOH/EtOAc); IR (KBr): 3080, 1775, 1683, 1619, 1488, 1368, 1213, 1104, 1010, 680 cm^{-1}; ^1H NMR (DMSO-d_6): δ 2.31 (3H, s); 2.32 (3H, s); 7.15 (1H, d, $J = 7.7$ Hz), 7.29 (2H, m); 7.39 (3H, m); 7.56 (3H, m); 7.86 (4H, m); ^{13}C NMR (DMSO-d_6): δ 20.29, 20.33, 108.3 (d, $J = 23$ Hz), 116.1 (d, $J = 21$ Hz), 116.3 (d, $J = 22$ Hz), 116.6 (d, $J = 23$ Hz), 118.3, 119.4 (d, $J = 3$ Hz) 123.6, 124.2, 125.5 (d, $J = 3$ Hz), 127.0, 130.7 (d, $J = 9$ Hz), 131.1 (d, $J = 9$ Hz), 133.2, 137.3 (d, $J = 10$ Hz), 142.4, 143.0, 143.6, 149.6 (d, $J = 8$ Hz), 162.2 (d, $J = 245$ Hz), 162.5 (d, $J = 245$ Hz), 166.6, 168.0, 168.1; MS (FAB) *m/z* 480 (MH^+, 10); 163 (31); 123 (100); 95 (36). Anal. for $C_{25}H_{19}F_2N_3O_5$: Calcd C, 62.63; H, 3.99; N, 8.76. Found C, 62.70; H, 4.06; N, 8.60.

*5.1.3.9. 3-[(2E)-3-(3,4-diacetoxyphenyl)prop-2-enoyl]-1,3-bis[4-chloro-3(trifluoromethyl) phenyl]triazene (**4b**).* Method C, reaction time: 5 min; yield 87%; mp 136–138 °C (MeOH/EtOAc); IR (KBr): 1771, 1696, 1314, 1211, 1180, 1140, 1111, 1007 cm^{-1}; ^1H NMR (DMSO-d_6): δ 2.31 (3H, s); 2.32 (3H, s); 7.41 (1H, d, $J = 8.9$ Hz); 7.73 (1H, m); 7.78–7.90 (5H, m); 7.91–8.04 (4H, m); ^{13}C NMR (DMSO-d_6): δ 20.8, 20.9, 118.6, 122.8 (q, $J = 5.4$ Hz), 123.9, 124.72 (q, $J = 273$ Hz), 124.76 (q, $J = 273$ Hz), 124.8, 127.2, 127.7, 128.1 (q, $J = 32$ Hz), 128.2 (q, $J = 31$), 129.4 (q, $J = 5.1$ Hz), 131.8 (q, $J = 1.8$ Hz), 132.1 (q, $J = 1.9$ Hz), 133.2, 133.4, 133.7, 135.4, 135.7, 143.0, 143.7, 144.3, 147.3, 167.3, 168.6, 168.7; MS (EI) *m/z* 647 (M+, 0.08); 205 (71); 163 (100). Anal. for $C_{27}H_{17}Cl_2F_6N_3O_5$: Calcd C, 50.02; H, 2.64; N, 6.48. Found C, 50.36; H, 2.70; N, 6.46.

*5.1.3.10. 3-[(2E)-3-(2,4-Dimethoxyphenyl)prop-2-enoyl]-1,3-bis[(2-chloro-5-trifluoromethyl) phenyl]triazene (**5a**).* Method C, reaction time: 5 min; yield 77%; mp 133–135 °C (EtOH); IR (KBr): 1684, 1606, 1564, 1358, 1324, 1211, 1163, 1129, 1081, 1007 cm^{-1}; ^1H NMR ($CDCl_3$): δ 3.87 (3H, s), 3.93 (3H, s), 6.51 (1H, d, $J = 2.4$ Hz), 6.56 (1H, dd, $J_1 = 2.4$ Hz, $J_2 = 8.5$ Hz), 7.51 (1H, d, $J = 8.5$ Hz), 7.52–7.58 (3H, m), 7.68 (2H, m), 7.84 (1H, d, $J = 15.7$ Hz), 7.85 (1H, m), 8.14 (1H, d, $J = 15.7$ Hz); ^{13}C NMR ($CDCl_3$): δ 55.3, 55.5, 98.6, 105.4, 113.7, 116.3 (q, $J = 3.8$ Hz), 116.8, 125.1 (q, $J = 273$ Hz), 125.3 (q, $J = 273$ Hz), 126.1 (q, $J = 3.6$ Hz), 127.4 (q, $J = 3.6$ Hz), 128.1 (q, $J = 3.8$ Hz), 129.8 (q, $J = 33$ Hz), 130.2 (q, $J = 34$ Hz), 130.7, 131.3, 133.2, 135.1, 135.8 (q, $J = 1.3$ Hz), 137.1 (q, $J = 1.3$ Hz), 143.5, 145.5, 160.9, 163.3, 167.5; MS (EI) *m/z* 591 (M^+, 1.1), 191 (100). Anal. for $C_{25}H_{17}Cl_2F_6N_3O_3$: Calcd C, 50.69; H, 2.89; N, 7.09. Found C, 50.91; H, 2.90; N, 6.90.

*5.1.3.11. 3-[(2E)-3-(3,4-Dimethoxyphenyl)prop-2-enoyl]-1,3-bis[(2-chloro-5-trifluoromethyl) phenyl]triazene (**5b**).* Method C, reaction time: 5 min; yield 58%; mp 173–175 °C (EtOH); IR (KBr): 1689, 1619, 1596, 1510, 1365, 1328, 1264, 1175, 1159, 1080, 1009 cm^{-1}; ^1H NMR ($CDCl_3$): δ 3.94 (3H, s), 3.93 (3H, s), 6.92 (1H, d, $J = 7.9$ Hz), 7.23 (2H, m) 7.56 (3H, m), 7.62 (1H, d, $J = 15.6$ Hz), 7.70 (2H, s), 7.85 (1H, s), 8.00 (1H, d, $J = 15.6$ Hz); ^{13}C NMR ($CDCl_3$): δ 55.6, 56.0, 109.3, 111.1, 112.9, 116.4 (q, $J = 3.9$ Hz), 123.2 (q, $J = 273$ Hz), 123.4 (q, $J = 273$ Hz), 123.9, 126.3 (q, $J = 3.6$ Hz), 127.4, 127.6 (q, $J = 3.6$ Hz), 128.1 (q, $J = 3.7$ Hz), 129.9 (q, $J = 33$ Hz), 130.3 (q, $J = 34$ Hz), 130.7, 131.4, 134.6, 135.6 (q, $J = 1.2$ Hz), 137.0 (q, $J = 1.3$ Hz), 145.4, 147.0, 149.4, 151.8, 166.6; MS (EI) *m/z* 591(M^+, 2.4), 191 (100), 179 (46). Anal. for $C_{25}H_{17}Cl_2F_6N_3O_3$: Calcd C, 50.69; H, 2.89; N, 7.09. Found C, 50.92; H, 2.91; N, 7.01.

*5.1.3.12. 3-[(2E)-3-(3,5-Dimethoxyphenyl)prop-2-enoyl]-1,3-bis[(2-chloro-5-trifluoro methyl) phenyl]triazene (**5c**).* Method C, reaction time: 5 min; yield 86%; mp 156–159 °C (EtOH); IR (KBr): 1692, 1625, 1592, 1346, 1327, 1268, 1206, 1162, 1127, 1080, 1012 cm^{-1}; ^1H NMR ($CDCl_3$): δ 3.84 (6H, s), 6.56 (1H, dd, $J_1 = J_2 = 2.3$ Hz), 6.80 (2H, d, $J = 2.3$ Hz), 7.70 (1H, d, $J = 15.7$ Hz), 7.71 (2H, d, $J = 1.3$ Hz), 7.53–7.62 (3H, m), 7.81 (1H, m), 7.96 (1H, d, $J = 15.7$ Hz); ^{13}C NMR ($CDCl_3$): δ 55.3, 103.6, 106.2, 115.9, 116.5 (q, $J = 3.9$ Hz), 123.2 (q, $J = 273$ Hz), 123.4 (q,$J = 273$ Hz), 126.4 (q, $J = 3.6$ Hz), 127.7 (q, $J = 3.6$ Hz),128.1 (q, $J = 3.8$ Hz), 129.9 (q, $J = 34$ Hz), 130.3 (q, $J = 34$ Hz), 130.8, 131.4, 134.5, 135.6 (q, $J = 1.3$ Hz), 136.2, 137.0 (q, $J = 1.3$ Hz), 145.3, 147.0, 161.1, 166.3; MS (EI) *m/z* 591 (M^+, 0.8), 191 (100), 179 (28). Anal. for $C_{25}H_{17}Cl_2F_6N_3O_3$: Calcd C, 50.69; H, 2.89; N, 7.09. Found C, 50.92; H, 3.12; N, 6.47.

*5.1.3.13. 1,3-Bis(2-bromo-4-nitrophenyl)triazene (**7a**).* Method B, reaction time: 18 h; yield 98%; mp 209–210 °C (acetone); IR (KBr): 3094, 1703, 1583, 1523, 1509, 1462, 1336, 1245, 1167, 1115, 882, 744 cm^{-1}; ^1H NMR (DMSO-d_6): δ 7.92 (2H, d, $J = 9.0$ Hz), 8.28 (2H, dd, $J_1 = 2.5$ Hz, $J_2 = 9.0$ Hz), 8.54 (2H, d, $J = 2.5$ Hz), 13.08 (1H, broad s); MS (EI) *m/z* 443 (M^+, 0.2), 230 (91), 200 (100), 75 (76); HRMS for $C_{12}H_7Br_2N_5O_4$: Calcd 442.8865; Found 442.8865. Anal. for $C_{12}H_7Br_2N_5O_4 \times 0.5C_3H_6O$: Calcd C, 34.20; H, 2.13; N, 14.77. Found C, 34.36; H, 2.23; N, 14.52.

*5.1.3.14. 3-Benzoyl-1,3-bis(2-bromo-4-nitrophenyl)triazene (**7c**).* Method D, reaction time: 1 h; yield 85%; mp 147–149 °C (EtOAc/petroleum ether); IR (KBr): 3102, 1697, 1528, 1466, 1347, 1220, 1142, 1121, 1039, 934, 895, 797, 740, 704 cm^{-1}; ^1H NMR ($CDCl_3$): δ 7.35 (d, 1H, $J = 8.9$ Hz), 7.55 (m, 3H), 7.65 (m, 1H), 7.90 (m, 2H), 8.13 (dd, 1H, $J_1 = 2.4$ Hz, $J_2 = 8.9$ Hz), 8.38 (dd, 1H, $J_1 = 2.4$ Hz, $J_2 = 8.6$ Hz), 8.47 (d, 1H, $J = 2.4$ Hz), 8.65 (d, 1H, $J = 2.4$ Hz); ^{13}C NMR (DMSO-d_6): δ 120.0, 120.7, 123.3, 123.8, 124.1, 127.7, 128.2, 128.4, 129.8, 132.3, 132.6, 132.7, 141.6, 147.5, 148.48, 149.7, 169.6; MS (ESI) *m/z* 547 (51), 444 (100), 442 ([M − 105]$^-$, 53). Anal. $C_{19}H_{11}Br_2N_5O_5$: Calcd C: 41.56; H: 2.02; N: 12.75. Found C: 41.30; H: 2.26; N: 12.54.

*5.1.3.15. 3-(2-Fluorobenzoyl)-1,3-bis(2-bromo-4-nitrophenyl)triazene (**7d**).* Method D, reaction time: 1.5 h; yield 82%; mp 131–134 °C (EtOAc/petroleum ether); IR (KBr): 3098, 1704, 1612,

1526, 1479, 1455, 1349, 1210, 1140, 1126, 1038, 934, 895, 845, 787, 752 cm^{-1}; ^1H NMR ($CDCl_3$): δ 7.25 (m, 2H), 7.35 (dt, 1H, $J_1 = 1.0$ Hz, $J_2 = 7.6$ Hz), 7.58 (d, 1H, $J = 8.6$ Hz), 7.62 (m, 1H), 7.74 (m, 1H), 8.13 (dd, 1H, $J_1 = 2.4$ Hz, $J_2 = 8.8$ Hz), 8.38 (dd, 1H, $J_1 = 2.4$ Hz, $J_2 = 8.6$ Hz), 8.45 (d, 1H, $J = 2.4$ Hz), 8.66 (d, 1H, $J = 2.4$ Hz); MS (ESI) *m/z* 446 (52), 444 (100), 442 ([M−123]$^-$, 54). Anal. for $C_{19}H_{10}Br_2FN_5O_5$: Calcd C, 40.24; H, 1.78; N, 12.35; ugotovljena: C, 40.11; H, 1.96; N, 12.23.

*5.1.3.16. 3-[2-(Trifluoromethyl)benzoyl]-1,3-bis(2-bromo-4-nitrophenyl)triazene (**7e**).* Method D, reaction time: 5 h; yield 74%; mp 163–166 °C (EtOAc/petroleum ether); IR (KBr): 3103, 1717, 1528, 1483, 1469, 1347, 1317, 1218, 1169, 1128, 1036, 934, 889, 825, 792, 770 cm^{-1}; ^1H NMR ($CDCl_3$): δ 7.00 (d, 1H, $J = 8.9$ Hz), 7.54 (d, 1H, $J = 8.6$ Hz), 7.72 (m, 3H), 7.84 (m, 1H), 8.04 (dd, 1H, $J_1 = 2.4$ Hz, $J_2 = 8.9$ Hz), 8.39 (dd, 1H, $J_1 = 2.4$ Hz, $J_2 = 8.6$ Hz), 8.43 (d, 1H, $J = 2.4$ Hz), 8.67 (d, 1H, $J = 2.4$ Hz); ^{13}C NMR ($CDCl_3$): δ 119.3, 122.6, 123.4, 123.74 (q, $J = 272.0$ Hz), 123.74, 124.3, 126.7 (q, $J = 4.3$ Hz), 127.4 (q, $J = 32.3$ Hz), 128.1, 128.6, 129.1, 130.7, 131.6, 132.1, 132.9 (q, $J = 2.3$ Hz), 140.7, 148.1, 149.0, 149.7, 169.2; MS (ESI) *m/z* 650 ([MCl]$^-$, 2), 446 (52), 444 (100), 442 (53). Anal. for $C_{20}H_{10}Br_2F_3N_5O_5$: Calcd C, 38.92; H, 1.63; N, 11.35. Found C, 38.82; H, 1.66; N, 11.25.

*5.1.3.17. 3-[3-(Trifluoromethyl)benzoyl]-1,3-bis(2-bromo-4-nitrophenyl)triazene (**7f**).* Method D, reaction time: 30 min; yield 67%; mp 132–134 °C (EtOAc/petroleum ether); IR (KBr): 3101, 1710, 1528, 1481, 1347, 1323, 1215, 1123, 1074, 1038, 947, 913, 782, 743, 706 cm^{-1}; ^1H NMR ($CDCl_3$): δ 7.32 (d, 1H, $J = 8.9$ Hz), 7.59 (d, 1H, $J = 8.6$ Hz), 7.71 (t, 1H, $J = 7.8$ Hz), 7.91 (d, 1H, $J = 7.8$ Hz), 8.13 (m, 3H), 8.40 (dd, 1H, $J_1 = 2.4$ Hz, $J_2 = 8.6$ Hz), 8.48 (d, 1H, $J = 2.4$ Hz), 8.67 (d, 1H, $J = 2.4$ Hz); MS (ESI) *m/z* 446 (35), 444 (70), 442 ([M−173]$^-$, 36), 389 (100). Anal. for $C_{20}H_{10}Br_2F_3N_5O_5$: Calcd C, 38.92; H, 1.63; N, 11.35. Found C, 39.13; H, 1.85; N, 11.11.

*5.1.3.18. 3-(4-Fluorobenzoyl)-1,3-bis(2-bromo-4-nitrophenyl)triazene (**7g**).* Method D, reaction time: 40 min; yield 84%; mp 145–148 °C (EtOAc/petroleum ether); IR (KBr): 3105, 3079, 1701, 1602, 1528, 1464, 1347, 1233, 1218, 1142, 1123, 1038, 933, 895, 858, 785, 604 cm^{-1}; ^1H NMR ($CDCl_3$): δ 7.24 (m, 2H), 7.35 (d, 1H, $J = 8.8$ Hz), 7.56 (d, 1H, $J = 8.6$ Hz), 7.95 (m, 2H), 8.16 (dd, 1H, $J_1 = 2.4$ Hz, $J_2 = 8.8$ Hz), 8.38 (dd, 1H, $J_1 = 2.4$ Hz, $J_2 = 8.6$ Hz), 8.48 (d, 1H, $J = 2.4$ Hz), 8.65 (d, 1H, $J = 2.4$ Hz); MS (ESI) *m/z* 600 ([MCl]$^-$, 3), 446 (54), 444 (100), 442 (54). Anal. for $C_{19}H_{10}Br_2FN_5O_5$: Calcd C, 40.24; H, 1.78; N, 12.35. Found C, 40.19; H, 1.83; N, 12.10.

*5.1.3.19. 3-(Acetoxyacetyl)-1,3-bis(2-bromo-4-nitrophenyl)triazene (**7h**).* Method D, reaction time: 25 min; yield 74%; mp 142–145 °C (EtOAc/petroleum ether); IR (KBr): 3100, 1749, 1737, 1528, 1487, 1414, 1389, 1350, 1231, 1170, 1127, 1039, 972 cm^{-1}; ^1H NMR ($CDCl_3$): δ 2.25 (s, 3H), 5.31–5.54 (m, 2H), 7.47 (d, 1H, $J = 8.6$ Hz), 7.56 (d, 1H, $J = 8.8$ Hz), 8.25 (dd, 1H, $J_1 = 2.4$ Hz, $J_2 = 8.8$ Hz), 8.33 (dd, 1H, $J_1 = 2.4$ Hz, $J_2 = 8.6$ Hz), 8.52 (d, 1H, $J = 2.4$ Hz), 8.61 (d, 1H, $J = 2.4$ Hz); ^{13}C NMR (DMSO-d_6): δ 20.2, 61.9, 120.6, 120.9, 123.3, 123.9, 124.0, 127.7, 128.4, 132.3, 140.3, 147.8, 148.6, 149.5, 168.3, 169.8; MS (ESI) *m/z* 578 ([MCl]$^-$, 3), 446 (53), 444 (100), 442 (54). Anal. for $C_{16}H_{11}Br_2N_5O_7$: Calcd C, 35.25; H, 2.03; N, 12.85. Found C, 35.41; H, 2.22; N, 12.58.

*5.1.3.20. 3-Benzoyl-1,3-bis(2-chloro-4-nitrophenyl)triazene (**8c**).* Method C, reaction time: 30 min; yield 91%; mp 131–134 °C (Et_2O/acetone); IR (KBr): 3105, 1699, 1528, 1468, 1348, 1221, 1135, 1118, 1047, 937, 928, 797, 709 cm^{-1}; ^1H NMR (DMSO-d_6): δ 7.46 (1H, d, $J = 8.9$ Hz), 7.58–7.72 (3H, m), 7.92–7.95 (2H, m), 8.06 (1H, dd, $J_1 = 8.9$ Hz, $J_2 = 2.5$ Hz), 8.25 (1H, dd, $J_1 = 8.9$, $J_2 = 2.5$ Hz), 8.38 (1H, d, $J = 2.3$ Hz), 8.45 (1H, dd, $J_1 = 8.7$ Hz, $J_2 = 2.3$ Hz), 8.61 (1H, d, $J = 2.3$ Hz); ^{13}C NMR (DMSO-d_6): δ 120.4, 123.4, 123.7, 124.9, 125.7, 128.3, 129.9, 130.8, 132.4, 132.6, 132.7, 133.1, 139.6, 147.6, 148.6, 148.8, 169.8; MS (FAB) *m/z* 460 (MH^+, 3), 307 (29), 184 (25), 154 (100), 137 (68), 105 (29), 77 (21). Anal. for $C_{19}H_{11}Cl_2N_5O_5$: Calcd C, 49.59; H, 2.41; N, 15.22. Found C, 49.84; H, 2.56; N, 14.98.

*5.1.3.21. 3-(2-Fluorobenzoyl)-1,3-bis(2-chloro-4-nitrophenyl)triazene (**8d**).* Method D, reaction time: 1.5 h; yield 68%; mp 151–154 °C (EtOAc/petroleum ether); IR (KBr): 3100, 1707, 1613, 1525, 1491, 1455, 1349, 1212, 1133, 1048, 937, 837, 790, 746 cm^{-1}; ^1H NMR ($CDCl_3$): δ 7.21–7.30 (m, 2H), 7.35 (dt, 1H, $J_1 = 1.0$ Hz, $J_2 = 7.6$ Hz), 7.62 (m, 2H), 7.73 (m, 1H), 8.08 (dd, 1H, $J_1 = 2.4$ Hz, $J_2 = 8.9$ Hz), 8.28 (d, 1H, $J = 2.4$ Hz), 8.33 (dd, 1H, $J_1 = 2.4$ Hz, $J_2 = 8.6$ Hz), 8.49 (d, 1H, $J = 2.4$ Hz); MS (ESI) *m/z* 512 ([MCl]$^-$, 8), 358 (15), 356 (71), 354 (100). Anal. for $C_{19}H_{10}Cl_2FN_5O_5$: Calcd C, 47.72; H, 2.11; N, 14.64. Found C, 47.53; H, 2.27; N, 14.44.

*5.1.3.22. 3-[2-(Trifluoromethyl)benzoyl]-1,3-bis(2-chloro-4-nitrophenyl)triazene (**8e**).* Method D, reaction time: 4.5 h; yield 76%; mp 166–167 °C (EtOAc/petroleum ether); IR (KBr): 3104, 1715, 1528, 1483, 1350, 1317, 1220, 1136, 1048, 935, 795, 770 cm^{-1}; ^1H NMR ($CDCl_3$): δ 7.03 (d, 1H, $J = 8.9$ Hz), 7.57 (d, 1H, $J = 8.6$ Hz), 7.65–7.77 (m, 3H), 7.84 (m, 1H), 8.00 (dd, 1H, $J_1 = 2.4$ Hz, $J_2 = 8.9$ Hz), 8.25 (d, 1H, $J = 2.4$ Hz), 8.35 (dd, 1H, $J_1 = 2.4$ Hz, $J_2 = 8.6$ Hz), 8.51 (d, 1H, $J = 2.4$ Hz); MS (ESI) *m/z* 562 ([MCl]$^-$, 26), 358 (12), 356 (65), 354 (100). Anal. for $C_{20}H_{10}Cl_2F_3N_5O_5$: Calcd C, 45.48; H, 1.91; N, 13.26. Found C, 45.28; H, 2.07; N, 13.20.

*5.1.3.23. 3-[3-(Trifluoromethyl)benzoyl]-1,3-bis(2-chloro-4-nitrophenyl)triazene (**8f**).* Method D, reaction time: 30 min; yield 69%; mp 139–141 °C (EtOAc/petroleum ether); IR (KBr): 3105, 1708, 1528, 1481, 1350, 1325, 1217, 1129, 1047, 948, 745, 710 cm^{-1}; ^1H NMR ($CDCl_3$): δ 7.34 (d, 1H, $J = 8.9$ Hz), 7.60 (d, 1H, $J = 8.7$ Hz), 7.70 (t, 1H, $J = 7.8$ Hz), 7.91 (d, 1H, $J = 7.8$ Hz), 8.09 (m, 2H), 8.17 (s, 1H), 8.31 (d, 1H, $J = 2.4$ Hz), 8.35 (dd, 1H, $J_1 = 2.4$ Hz, $J_2 = 8.7$ Hz), 8.51 (d, 1H, $J = 2.4$ Hz); ^{13}C NMR ($CDCl_3$): δ 119.6, 122.8, 123.0, 123.6 (q, $J = 271.0$ Hz), 125.7, 126.3, 127.3 (q, $J = 3.9$ Hz), 129.12 (q, $J = 3.6$ Hz), 129.12, 131.0 (q, $J = 32.9$ Hz), 131.8, 133.3, 133.4 (q, $J = 1.3$ Hz), 133.5, 134.5, 139.3, 148.1, 148.6, 149.1, 168.7; MS (ESI) *m/z* 562 ([MCl]$^-$, 6), 444 (100), 358 (9), 356 (46), 354 (63). Anal. for $C_{20}H_{10}Cl_2F_3N_5O_5$: Calcd C, 45.48; H, 1.91; N, 13.26. Found C, 45.61; H, 2.09; N, 13.07.

*5.1.3.24. 3-[4-(Trifluoromethyl)benzoyl]-1,3-bis(2-chloro-4-nitrophenyl)triazene (**8g**).* Method D, reaction time: 30 min; yield 69%; mp 120–123 °C (EtOAc/petroleum ether); IR (KBr): 3105, 1703, 1528, 1483, 1410, 1347, 1323, 1220, 1173, 1131, 1115, 1066, 1047, 935, 856, 789 cm^{-1}; ^1H NMR ($CDCl_3$): δ 7.32 (d, 1H, $J = 8.9$ Hz), 7.60 (d, 1H, $J = 8.7$ Hz), 7.81 (d, 2H, $J = 8.1$ Hz), 8.00 (d, 2H, $J = 8.1$ Hz), 8.11 (dd, 1H, $J_1 = 2.4$ Hz, $J_2 = 8.9$ Hz), 8.31 (d, 1H, $J = 2.4$ Hz), 8.35 (dd, 1H, $J_1 = 2.4$ Hz, $J_2 = 8.7$ Hz), 8.50 (d, 1H, $J = 2.4$ Hz); MS (ESI) *m/z* 562 ([MCl]$^-$, 15), 358 (12), 356 (66), 354 (100). Anal. for $C_{20}H_{10}Cl_2F_3N_5O_5$: Calcd C, 45.48; H, 1.91; N, 13.26. Found C, 45.64; H, 2.18; N, 13.06.

*5.1.3.25. 3-(3-Fluorobenzoyl)-1,3-bis(2-chloro-4-nitrophenyl)triazene (**8h**).* Method D, reaction time: 20 min; yield 76%; mp 133–135 °C (EtOAc/petroleum ether); IR (KBr): 3102, 1707, 1589, 1526, 1467, 1351, 1256, 1184, 1133, 1115, 1047, 961, 901, 888, 789, 743 cm^{-1}; ^1H NMR ($CDCl_3$): δ 7.34 (m, 2H), 7.49–7.61 (m, 3H), 7.67 (m, 1H), 8.11 (dd, 1H, $J_1 = 2.4$ Hz, $J_2 = 8.9$ Hz), 8.31 (d, 1H, $J = 2.4$ Hz), 8.34 (dd, 1H, $J_1 = 2.4$ Hz, $J_2 = 8.7$ Hz), 8.50 (d, 1H, $J = 2.4$ Hz); MS (ESI) *m/z* 358 (8), 356 (40), 354 ([M−123]$^-$, 59), 293 (100). Anal. for $C_{19}H_{10}Cl_2FN_5O_5$: Calcd C, 47.72; H, 2.11; N, 14.64. Found C, 47.83; H, 2.19; N, 14.41.

*5.1.3.26. 3-Chloroacetyl-1,3-bis(2-chloro-4-nitrophenyl)triazene (**8i**).* Method D, reaction time: 10 min; yield 83%; mp 129–132 °C (MeOH/acetone); IR (KBr): 3103, 2959, 1755, 1527, 1472, 1348, 1135,

1049, 978, 783, 740 cm^{-1}; ^1H NMR (DMSO-d_6): δ 5.22 (1H, d, $J = 14.9$ Hz), 5.44 (1H, d, $J = 14.9$ Hz), 7.85 (1H, d, $J = 8.7$ Hz), 7.99 (1H, d, $J = 8.9$ Hz), 8.31 (1H, dd, $J_1 = 8.9$ Hz, $J_2 = 2.5$ Hz) 8.40–8.44 (2H, m), 8.58 (1H, d, $J = 2.5$ Hz); ^{13}C NMR (DMSO-d_6): δ 43.5, 120.9, 123.42, 123.43, 124.8, 125.6, 131.3, 132.4, 133.3, 138.5, 147.8, 148.4, 148.8, 167.9; MS (FAB) *m/z* 432 (MH^+, 1), 184 (32), 107 (24). Anal. for $C_{14}H_8Cl_3N_5O_5$: Calcd C, 38.87; H, 1.86; N, 16.19. Found C, 38.49; H, 1.86; N, 16.43.

*5.1.3.27. 3-(Acetoxyacetyl)-1,3-bis(2-chloro-4-nitrophenyl)triazene (**8k**).* Method C, reaction time: 10 min; yield 77%; mp 139–142 °C (EtOH); IR (KBr): 1738, 1527, 1485, 1391, 1354, 1230, 1173, 1137, 1048, 975 cm^{-1}; ^1H NMR ($CDCl_3$): δ 2.25 (3H, s), 5.37 (1H, s), 5.48 (1H, s), 7.48 (1H, d, $J = 8.7$ Hz), 7.59 (1H, d, $J = 8.7$ Hz), 8.20 (1H, dd, $J_1 = 2.5$ Hz, $J_2 = 8.7$ Hz), 8.28 (1H, dd, $J_1 = 2.5$ Hz, $J_2 = 8.7$ Hz), 8.34 (1H, d, $J = 2.5$ Hz), 8.44 (1H, d, $J = 2.5$ Hz); ^{13}C NMR ($CDCl_3$): δ 20.4, 61.9, 120.0, 122.76, 122.80, 125.5, 126.3, 131.7, 132.8, 134.5, 138.2, 148.2, 148.7, 149.1, 168.0, 170.5; MS (FAB) *m/z* 456 (MH^+, 0.8), 154 (100), 137 (77), 71 (56), 55 (56). Anal. for $C_{16}H_{11}Cl_2N_5O_7$: Calcd C, 42.13; H, 2.43; N, 15.35. Found C, 42.30; H, 2.48; N, 15.06.

*5.1.3.28. 3-Methoxycarbonyl-1,3-bis(2-chloro-4-nitrophenyl)triazene (**8l**).* Method C, reaction time: 1.5 h; yield 86%; mp 139–140 °C (MeOH); IR (KBr): 3103, 1764, 1528, 1476, 1441, 1351, 1327, 1267, 1233, 1133, 1093, 956, 907, 779, 760 cm^{-1}; ^1H NMR (DMSO-d_6): δ 3.96 (3H, s), 7.72 (1H, d, $J = 8.8$ Hz), 7.95 (1H, d, $J = 8.8$ Hz), 8.31 (1H, dd, $J_1 = 8.9$ Hz, $J_2 = 2.5$ Hz), 8.37–8.41 (2H, m), 8.56 (1H d, $J = 2.5$ Hz); ^{13}C NMR (DMSO-d_6): δ 55.0, 120.6, 123.4, 123.7, 124.8, 125.6, 130.6, 132.5, 133.2, 139.5, 147.6, 148.69, 148.73, 151.8; MS (EI) *m/z* 385 ($M^+ - N_2$, 1.5), 350 (3), 230 (30), 195 (75), 184 (100), 156 (97). Anal. for $C_{14}H_9Cl_2N_5O_6$: Calcd C, 40.60; H, 2.19; N, 16.91. Found C, 40.90; H, 2.17; N, 16.85.

*5.1.3.29. 1,3-Bis[4-nitro-2-(trifluoromethyl)phenyl]triazene (**9a**).* Method B, reaction time: 2 days; yield 68%; mp 171–172 °C (acetone); IR (KBr): 3332, 3303, 1622, 1595, 1545, 1513, 1336, 1308, 1287, 1262, 1154, 1119, 1050, 897 cm^{-1}; ^1H NMR (DMSO-d_6): δ 7.96 (2H, d, $J = 8.9$ Hz), 8.53 (2H, d, $J = 2.6$ Hz), 8.57 (2H, dd, $J_1 = 2.6$ Hz, $J_2 = 8.9$ Hz), 13.61 (1H, broad); MS (FAB) *m/z* 424 (MH^+, 1.9), 154 (100), 136 (75), 71 (52). Anal. for $C_{14}H_7F_6N_5O_4$: Calcd C, 39.73; H, 1.67; N, 16.55. Found C, 39.71; H, 1.72; N, 16.62.

*5.1.3.30. 3-Acetyl-1,3-bis[4-nitro-2-(trifluoromethyl)phenyl]triazene (**9b**).* Method D, reaction time: 45 min; yield 79%; mp 171.8–174 °C (EtOAc/petroleum ether); IR (KBr): 3115, 3097, 1737, 1619, 1595, 1536, 1478, 1426, 1355, 1287, 1192, 1145, 1048, 965, 917, 799, 741, 613 cm^{-1}; ^1H NMR ($CDCl_3$): δ 2.78 (s, 3H), 7.42 (d, 1H, $J = 8.6$ Hz), 7.78 (d, 1H, $J = 8.8$ Hz), 8.49 (dd, 1H, $J_1 = 2.4$ Hz, $J_2 = 8.8$ Hz), 8.55–8.59 (m, 2H), 8.69 (d, 1H, $J = 2.5$ Hz); ^{13}C NMR ($CDCl_3$): δ 22.4, 119.3, 121.5 (q, $J = 272.6$ Hz), 121.9 (q, $J = 272.8$ Hz), 122.9 (q, $J = 5.5$ Hz), 123.96 (q, $J = 5.1$ Hz), 127.8 (q, $J = 0.9$ Hz), 127.9 (q, $J = 32.9$ Hz), 128.0 (q, $J = 0.75$ Hz), 130.6 (q, $J =$ Hz), 133.10, 139.7 (q, $J = 1.9$ Hz), 147.5, 148.5, 149.2 (q, $J = 0.9$ Hz), 171.9; MS (ESI) *m/z* 500 ($[MCl]^-$, 11), 422 (100). Anal. for $C_{16}H_9F_6N_5O_5$: Calcd C: 41.30; H: 1.95; N: 15.05. Found C: 41.51; H: 2.08; N: 14.95.

*5.1.3.31. 3-Benzoyl-1,3-bis[4-nitro-2-(trifluoromethyl)phenyl]triazene (**9c**).* Method D, reaction time: 1.5 h; yield 80%; mp 142–144 °C (EtOAc/petroleum ether); IR (KBr): 3095, 1693, 1622, 1598, 1545, 1535, 1481, 1426, 1354, 1290, 1216, 1144, 1118, 1048, 934 cm^{-1}; ^1H NMR ($CDCl_3$): δ 7.51 (d, 1H, $J = 8.9$ Hz), 7.57 (m, 3H) 7.68 (m, 1H), 7.87 (m, 2H), 8.38 (dd, 1H, $J_1 = 2.4$ Hz, $J_2 = 8.9$ Hz), 8.53 (d, 1H, $J = 2.4$ Hz), 8.62 (dd, 1H, $J_1 = 2.4$ Hz, $J_2 = 8.6$ Hz), 8.75 (d, 1H, $J = 2.4$ Hz); ^{13}C NMR ($CDCl_3$): δ 119.2, 121.8 (q, $J = 272.5$ Hz), 121.9 (q, $J = 272.9$ Hz), 122.8 (q, $J = 5.5$ Hz), 123.0 (q, $J = 5.0$ Hz), 127.8 (q, $J = 0.7$ Hz), 128.0 (q, $J = 0.9$ Hz), 128.2 (q, $J = 32.9$ Hz), 128.5, 130.2, 130.5 (q, $J = 33.0$ Hz), 132.3, 133.0, 133.5, 140.1 (q, $J = 1.8$ Hz), 147.4, 148.5, 148. 9 (q, $J = 0.9$ Hz), 170.5; MS (ESI) *m/z* 562 ($[MCl]^-$, 15), 422 (100). Anal. for $C_{21}H_{11}F_6N_5O_5$: Calcd C: 47.83; H: 2.10; N: 13.28. Found C: 47.88; H: 2.15; N: 13.02.

*5.1.3.32. 3-(2-Fluorobenzoyl)-1,3-bis[4-nitro-2-(trifluoromethyl) phenyl]triazene (**9d**).* Method D, reaction time: 3.5 h; yield 90%; mp 153–155 °C (EtOAc/petroleum ether); IR (KBr): 3095, 1710, 1616, 1539, 1487, 1457, 1426, 1358, 1292, 1211, 1179, 1146, 1119, 1050, 936, 855, 801, 738 cm^{-1}; ^1H NMR ($CDCl_3$): δ 7.26 (m, 1H), 7.37 (dt, 1H, $J_1 = 1.0$ Hz, $J_2 = 7.6$ Hz), 7.43 (d, 1H, $J = 8.9$ Hz), 7.58 (d, 1H, $J = 8.6$ Hz), 7.61–7.75 (m, 2H,), 8.37 (dd, 1H, $J_1 = 2.4$ Hz, $J_2 = 8.9$ Hz), 8.52 (d, 1H, $J = 2.4$ Hz), 8.62 (dd, 1H, $J_1 = 2.5$ Hz, $J_2 = 8.6$ Hz), 8.75 (d, 1H, $J = 2.5$ Hz); MS (ESI) *m/z* 580 ([MCl], 26), 422 (100). Anal. for $C_{21}H_{10}F_7N_5O_5$: Calcd C, 46.25; H, 1.85; N, 12.84. Found C, 46.22; H, 1.81; N, 12.75.

*5.1.3.33. 3-[2-(Trifluoromethyl)benzoyl]-1,3-bis[4-nitro-2-(trifluoromethyl)phenyl]triazene (**9e**).* Method D, reaction time: 5 h; yield 92%; mp 176–177 °C (EtOAc/petroleum ether); IR (KBr): 3102, 1726, 1622, 1539, 1486, 1425, 1357, 1321, 1291, 1217, 1150, 1116, 1047, 934, 829, 800, 772, 740 cm^{-1}; ^1H NMR ($CDCl_3$): δ 7.15 (d, 1H, $J = 8.9$ Hz), 7.55 (d, 1H, $J = 8.6$ Hz), 7.64 (m, 1H), 7.71–7.86 (m, 3H), 8.29 (dd, 1H, $J_1 = 2.4$ Hz, $J_2 = 8.9$ Hz), 8.50 (d, 1H, $J = 2.4$ Hz), 8.64 (dd, 1H, $J_1 = 2.4$ Hz, $J_2 = 8.6$ Hz), 8.76 (d, 1H, $J = 2.4$ Hz); ^{13}C NMR ($CDCl_3$): δ 118.9, 121.2 (q, $J = 272.0$ Hz), 121.7 (q, $J = 272.0$ Hz), 122.8 (q, $J = 5.8$ Hz), 123.1 (q, $J = 5.0$ Hz), 123.7 (q, $J = 271.9$ Hz), 126.7 (q, $J = 4.3$ Hz), 127.5 (q, $J = 32.1$ Hz), 127.86, 127.90 (q, $J = 0.8$ Hz), 128.18 (q, $J = 0.9$ Hz), 128.22 (q, $J = 33.0$ Hz), 130.86, 130.93 (q, $J = 33.2$ Hz), 132.3 (q, $J = 1.1$ Hz), 132.5 (q, $J = 2.3$ Hz), 132.9, 138.8 (q, $J = 1.8$ Hz), 147.6, 148.5 (q, $J = 0.9$ Hz), 148.8, 169.6; MS (ESI) *m/z* 630 ($[MCl]^-$, 11), 422 (100). Anal. for $C_{22}H_{10}F_9N_5O_5$: Calcd C, 44.38; H, 1.69; N, 11.76. Found C, 44.08; H, 1.72; N, 11.62.

*5.1.3.34. 3-[3-(Trifluoromethyl)benzoyl]-1,3-bis[4-nitro-2-(trifluoromethyl)phenyl]triazene (**9f**).* Method D, reaction time: 30 min; yield 82%; mp 118–121 °C (EtOAc/petroleum ether); IR (KBr): 3104, 1715, 1619, 1538, 1484, 1426, 1356, 1328, 1290, 1207, 1150, 1118, 1048, 945, 914, 800, 740 cm^{-1}; ^1H NMR ($CDCl_3$): δ 7.48 (d, 1H, $J = 8.9$ Hz), 7.59 (d, 1H, $J = 8.6$ Hz), 7.73 (t, 1H, $J = 7.8$ Hz), 7.94 (d, 1H, $J = 7.8$ Hz), 8.07 (d, 1H, $J = 7.8$ Hz), 8.14 (s, 1H), 8.39 (dd, 1H, $J_1 = 2.4$ Hz, $J_2 = 8.9$ Hz), 8.55 (d, 1H, $J = 2.4$ Hz), 8.64 (dd, 1H, $J_1 = 2.4$ Hz, $J_2 = 8.6$ Hz), 8.76 (d, 1H, $J = 2.4$ Hz); ^{13}C NMR ($CDCl_3$): δ 119.0, 121.77 (q, $J = 272.5$ Hz), 121.8 (q, $J = 272.9$ Hz), 122.9 (q, $J = 5.5$ Hz), 123.2 (q, $J = 5.1$ Hz), 123.5 (q, $J = 271.0$ Hz), 127.3 (q, $J = 4.0$ Hz), 127.9 (q, $J = 0.8$ Hz), 128.0 (q, $J = 0.8$ Hz), 128.4 (q, $J = 32.3$ Hz), 129.3, 129.4 (q, $J = 3.8$ Hz), 130.6 (q, $J = 33.0$ Hz), 131.2 (q, $J = 33.0$ Hz), 133.1, 133.36 (q, $J = 1.2$ Hz), 133.43, 139.4 (q, $J = 1.8$ Hz), 147.7, 148.6 (q, $J = 0.9$ Hz), 148.7, 169.1; MS (ESI) *m/z* 630 ($[MCl]^-$, 3), 422 (100). Anal. for $C_{22}H_{10}F_9N_5O_5 \times 0.25$ EtOAc: Calcd C, 44.74; H, 1.96; N, 11.34. Found C, 44.77; H, 1.94; N, 11.28.

*5.1.3.35. 3-[4-(Trifluoromethyl)benzoyl]-1,3-bis[4-nitro-2-(trifluoromethyl)phenyl]triazene (**9g**).* Method D, reaction time: 1 h; yield 86%; mp 145–147 °C (EtOAc/petroleum ether); IR (KBr): 3117, 3099, 1717, 1622, 1597, 1538, 1482, 1426, 1354, 1325, 1290, 1217, 1149, 1123, 1067, 1048, 934, 850, 739 cm^{-1}; ^1H NMR ($CDCl_3$): δ 7.46 (d, 1H, $J = 8.9$ Hz), 7.59 (d, 1H, $J = 8.6$ Hz), 7.84 in 7.98 (AA'XX', 4H, $J = 8.2$ Hz), 8.41 (dd, 1H, $J_1 = 2.4$ Hz, $J_2 = 8.9$ Hz), 8.55 (d, 1H, $J = 2.4$ Hz), 8.64 (dd, 1H, $J_1 = 2.4$ Hz, $J_2 = 8.6$ Hz), 8.76 (d, 1H, $J = 2.4$ Hz); MS (ESI) *m/z* 630 ($[MCl]^-$, 12), 422 (100). Anal. for $C_{22}H_{10}F_9N_5O_5$: Calcd C, 44.38; H, 1.69; N, 11.76. Found C, 44.50; H, 1.72; N, 11.56 3-(3-Fluorobenzoyl)-1,3-*bis*[4-nitro-2-(trifluoromethyl)phenyl] triazene (9h). Method D, reaction time: 1.5 h; yield 74%; mp 146–148 °C (EtOAc/petroleum ether); IR (KBr): 3095, 1710, 1591, 1539, 1481, 1441, 1424, 1356, 1292, 1176, 1148, 1118, 1048, 962, 904, 799, 739 cm^{-1}; ^1H NMR ($CDCl_3$):

δ 7,38 (ddt, 1H, J_1 = 1.1 Hz, J_2 = 2.5 Hz, J_3 = 8.3 Hz), 7.50 (d, 1H, J = 8.9 Hz), 7.57 (m, 3H), 7.66 (m, 1H), 8.41 (dd, 1H, J_1 = 2.4 Hz, J_2 = 8.9 Hz), 8.55 (d, 1H, J = 2.4 Hz), 8.63 (dd, 1H, J_1 = 2.4 Hz, J_2 = 8.7 Hz), 8.75 (d, 1H, J = 2.4 Hz); MS (ESI) *m/z* 580 ([MCl]$^-$, 24), 422 (54). Anal. for $C_{21}H_{10}F_7N_5O_5$: Calcd C, 46.25; H, 1.85; N, 12.84. Found C, 46.26; H, 1.86; N, 12.76.

*5.1.3.36. 3-(3-Fluorobenzoyl)-1,3-bis[4-nitro-2-(trifluoromethyl) phenyl]triazene (**9h**).* Method D, reaction time: 1.5 h; yield 74%; mp 146–148 °C (EtOAc/petroleum ether); IR (KBr): 3095, 1710, 1591, 1539, 1481, 1441, 1424, 1356, 1292, 1176, 1148, 1118, 1048, 962, 904, 799, 739 cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 7.38 (ddt, 1H, J_1 = 1.1 Hz, J_2 = 2.5 Hz, J_3 = 8.3 Hz), 7.50 (d, 1H, J = 8.9 Hz), 7.57 (m, 3H), 7.66 (m, 1H), 8.41 (dd, 1H, J_1 = 2.4 Hz, J_2 = 8.9 Hz), 8.55 (d, 1H, J = 2.4 Hz), 8.63 (dd, 1H, J_1 = 2.4 Hz, J_2 = 8.7 Hz), 8.75 (d, 1H, J = 2.4 Hz); MS (ESI) *m/z* 580 ([MCl]$^-$, 24), 422 (54). Anal. for $C_{21}H_{10}F_7N_5O_5$: Calcd C, 46.25; H, 1.85; N, 12.84. Found C, 46.26; H, 1.86; N, 12.76.

*5.1.3.37. 3-(4-Fluorobenzoyl)-1,3-bis[4-nitro-2-(trifluoromethyl) phenyl]triazene (**9i**).* Method D, reaction time: 8 h; yield 93%; mp 142–144 °C (EtOAc/petroleum ether); IR (KBr): 3109, 3056, 1689, 1601, 1537, 1481, 1353, 1290, 1214, 1147, 1118, 1048, 936, 849, 799, 728 cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 7.26 (m, 2H), 7.52 (d, 1H, J = 8.9 Hz), 7.56 (d, 1H, J = 8.6 Hz), 7.92 (m, 2H), 8.41 (dd, 1H, J_1 = 2.4 Hz, J_2 = 8.9 Hz), 8.55 (d, 1H, J = 2.4 Hz), 8.62 (dd, 1H, J_1 = 2.4 Hz, J_2 = 8.6 Hz), 8.75 (d, 1H, J = 2.4 Hz); MS (ESI) *m/z* 580 ([MCl]$^-$, 25), 422 (100). Anal. for $C_{21}H_{10}F_7N_5O_5$: Calcd C, 46.25; H, 1.85; N, 12.84. Found C, 46.19; H, 1.82; N, 12.68.

*5.1.3.38. 3-Chloroacetyl-1,3-bis[4-nitro-2-(trifluoromethyl)phenyl] triazene (**9j**).* Method D, reaction time: 1 h; yield 86%; mp 117–119 °C (EtOAc/petroleum ether); IR (KBr): 3117, 3100, 1759, 1733, 1621, 1535, 1481, 1427, 1354, 1309, 1287, 1176, 1143, 1116, 1048, 984, 917, 808, 739 cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 4.79 (s, 1H), 4.81 (s, 1H), 7.47 (d, 1H, J = 8.6 Hz), 7.80 (d, 1H, J = 8.8 Hz), 8.51 (dd, 1H, J_1 = 2.4 Hz, J_2 = 8.8 Hz), 8.60 (m, 2H), 8.71 (d, 1H, J = 2.4 Hz); ^{13}C NMR ($CDCl_3$): δ 41.6, 119.5, 121.5 (q, J = 272.6 Hz), 121.8 (q, J = 272.9 Hz), 123.0 (q, J = 5.5 Hz), 123.2 (q, J = 5.0 Hz), 128.0 (q, J = 0.8 Hz), 128.1 (q, J = 0.8 Hz), 128.2 (q, J = 33.1 Hz), 130.9 (q, J = 33.4 Hz), 133.0, 138.4 (q, J = 1.8 Hz), 147.8, 148.7 (q, J = 0.9 Hz), 148.8, 167.7; MS (ESI) *m/z* 534 ([MCl]$^-$, 8), 422 (100). Anal. for $C_{16}H_8ClF_6N_5O_5$: Calcd C, 38.46; H, 1.61; N, 14.01. Found C, 38.76; H, 1.81; N, 13.77.

*5.1.3.39. 3-(Ethoxycarbonylacetyl)-1,3-bis[4-nitro-2-(trifluoromethyl) phenyl]triazene (**9k**).* Method D, reaction time: 45 min; yield 71%; mp 105–107 °C (EtOAc/petroleum ether); IR (KBr): 3100, 2998, 1754, 1725, 1626, 1534, 1479, 1357, 1319, 1288, 1188, 1171, 1154, 1123, 1052, 1005, 905, 860, 799, 739 cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 1.29 (t, 3H, J = 7.1 Hz), 3.99–4.31 (m, 4H), 7.47 (d, 1H, J = 8.6 Hz), 7.74 (d, 1H, J = 8.8 Hz), 8.48 (dd, 1H, J_1 = 2.3 Hz, J_2 = 8.8 Hz), 8.58 (m, 2H), 8.70 (d, 1H, J = 2.3 Hz); ^{13}C NMR ($CDCl_3$): δ 14.1, 42.8, 62.3, 119.3, 121.2 (q, J = 272.9 Hz), 121.5 (q, J = 272.6 Hz), 22.9 (q, J = 5.4 Hz), 123.0 (q, J = 5.1 Hz), 128.0 (q, J = 0.9 Hz), 128.1 (q, J = 0.8 Hz), 128.2 (q, J = 32.8 Hz), 131.0 (q, J = 33.3 Hz), 133.0, 139.0 (q, J = 1.8 Hz), 147.7, 148.8, 148.8 (q, J = 0.8 Hz), 166.2, 167.6; MS (ESI) *m/z* 572 ([MCl]$^-$, 7), 422 (100). Anal. for $C_{19}H_{13}F_6N_5O_7$: Calcd C, 42.47; H, 2.44; N, 13.03. Found C, 42.31; H, 2.64; N, 12.87.

*5.1.3.40. 1,3-Bis(2-fluoro-4-nitrophenyl)triazene (**10a**).* Method A, reaction time: 6 h; yield 73%; mp 200–201 °C (acetone); IR (KBr): 3274, 1612, 1534, 1506, 1476, 1416, 1340, 1296, 1258, 1202, 1171, 1124, 1073, 938, 808 cm^{-1}; ^{1}H NMR (DMSO-d_6): δ 7.92–8.03 (2H, m), 8.11–8.20 (2H, m), 8.23–8.32 (2H, m), 13.87 (1H, broad s); MS (EI) *m/z* 323 (M$^+$, 1), 168 (100), 140 (75), 94 (73). Anal. for $C_{12}H_7F_2N_5O_4$: Calcd C, 44.59; H, 2.18; N, 21.67. Found C, 44.61; H, 2.21; N, 21.68.

*5.1.3.41. 3-Acetyl-1,3-bis(2-fluoro-4-nitrophenyl)triazene (**10b**).* Method C, reaction time: 1.5 h; yield 57%; mp 159–161 °C (MeOH/acetone); IR (KBr): 1724, 1535, 1489, 1352, 1247, 1181, 1143, 1116, 961, 812, 739 cm^{-1}; ^{1}H NMR (DMSO-d_6): δ 2.74 (3H, s), 7.80 (1H, dd J_1 = 7.0 Hz, J_2 = 8.6 Hz), 7.95 (1H, m), 8.19 (1H, dd, J_1 = 1.3 Hz, J_2 = 8.9 Hz), 8.24, 8.32 (2H, m), 8.39 (1H, dd, J_1 = 2.4 Hz, J_2 = 9.5 Hz); ^{13}C NMR (DMSO-d_6): δ 22.3, 112.2 (d, J = 25 Hz), 113.2 (d, J = 25 Hz), 120.3 (d, J = 4 Hz), 120.6 (d, J = 4 Hz), 121.4, 128.5 (d, J = 15 Hz), 132.3 (d, J = 1 Hz), 140.4 (d, J = 8 Hz), 148.0 (d, J = 8 Hz), 149.0 (d, J = 9 Hz), 154.5 (d, J = 61 Hz), 157.9 (d, J = 58 Hz), 172.1; MS (CI) *m/z* 366 (MH$^+$, 0.3), 323 (2), 168 (100), 140 (53), 94 (38). Anal. for $C_{14}H_9F_2N_5O_5$: Calcd C, 46.04; H, 2.48; N, 19.17. Found C, 46.22; H, 2.51; N, 18.96.

5.2. Biology

5.2.1. Cell culture

Human cervical carcinoma HeLa and laryngeal carcinoma HEp-2 cells were obtained from cell culture bank (GIBCO BRL, Invitrogen, Grand Island, USA). The development of cisplatin-resistant $CA3_{ST}$ subline from HEp-2 cells has been published previously [42,43]. Pancreatic carcinoma MIA PaCa-2, colorectal carcinoma primary SW480 and metastatic SW620 cells were obtained from American Type Culture Collection (ATCC; Manassas, VA), whereas urinary bladder carcinoma RT-112 cell line was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ; Braunschweig, Germany). Cell lines were cultured as a monolayer culture according to manufacturer's instructions at 37 °C in a humidified atmosphere containing 5% CO_2. Normal human skin fibroblasts were isolated from the upper arm of a 7-year-old female donor at the Neurochemical Laboratory, Department of Chemistry and Biochemistry, School of Medicine, University of Zagreb. Cells were grown in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO), supplemented with 10% fetal bovine serum (FBS) and sodium carbonate at 3.7 mg/ml in 5% CO_2, and used for the cytotoxicity assay at 32 and 36 population doublings.

5.2.2. Cytotoxicity assay

Cytotoxic effect of diaryltriazenes was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [44], modified as described. Cells were seeded into 96-well tissue culture plates (3000 cells/0.18 mL medium/well). Different concentrations of new compounds were added (0.02 mL) to each well on the following day. Each concentration was tested in quadruplicate. Following 72 h incubation at 37 °C, the medium was aspirated, and 20 μg of MTT dye/0.04 mL medium/well was added. Four hours later, formazan crystals were dissolved in dimethyl sulfoxide (0.17 mL/well), the plates were mechanically agitated for 5 min and the optical density at 545 nm was determined on a microtiter plate reader (Awareness Technology Inc, Palm City, FL). Each experiment was repeated three times

5.2.3. Determination of DNA binding

The *calf thymus* (ct)-DNA was purchased from Aldrich, dissolved in Na-cacodylate buffer, I = 0.05 mol dm^{-3}, pH = 7, additionally sonicated and the obtained solution was filtered through a 0.45 mm filter. Polynucleotide concentration was determined spectroscopically as the concentration of phosphates by $\varepsilon_{260\ nm}$ = 6600 dm^3 mol^{-1} cm^{-1}. Thermal denaturation curves for ct-DNA and its complexes with studied compounds were determined in Na-cacodylate buffer, I = 0.05 mol dm^{-3}, pH = 7 by following the absorption change at 260 nm as a function of temperature, as previously described [45]. Absorbance of compounds was subtracted from every curve and the absorbance scale was normalized. Measured T_m values are the midpoints of the transition curves determined from the maximum of the first derivative and were checked graphically by the tangent

method. The ΔT_m values were calculated by subtracting T_m of the free nucleic acid from T_m of the complex. Every *ΔTm* value reported here was an average of at least two measurements. The error in ΔT_m is ±0.5 °C.

5.2.4. Cell cycle analysis

HeLa cells were seeded into 6-well tissue culture plates (100 000 cells/2 ml medium/well) and treated with different concentrations of **8b** for indicated time periods on the following day. Thereafter, both adherent and floating cells were collected, washed with PBS and fixed overnight in 70% ethanol at −20 °C. Fixed cells were treated with RNase A (30 μg/ml, Sigma) for 1 h at room temperature and afterward stained with propidium iodide (16.7 μg/ml, Sigma) for 30 min in the dark. DNA content was analyzed by flow cytometry (FACSCalibur, Becton Dickinson, Mountain View, CA). Data were analyzed with ModFitLTTM program (Verity Software House Inc., Topsham, Maine).

5.2.5. Determination of ROS generation

Generation of reactive oxygen species (ROS) was determined by addition of 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA) (Invitrogen). Briefly, logarithmically growing HeLa cells were incubated with 10 μM CM-H_2DCFDA for 1 h according to manufacturer's instructions. Afterward, cells were incubated with or without different concentrations of **8b** during indicated time periods. After trypsinization and centrifugation, the cells were fixed in cold 80% methanol. Shortly before measurement, they were centrifuged and resuspended in PBS. The fluorescence of the product, developed by removal of the acetate groups from CM-H_2DCFDA by intracellular esterases and oxidation, was measured by flow cytometry (FACS). Incubation with 0.01% H_2O_2 for 30 min was used as the positive control.

5.2.6. Induction of endoplasmic reticulum (ER) stress

Expression of CHOP and Grp78, the markers for ER stress, was analyzed by Western blot analysis. Briefly, logarithmically growing HeLa cells were treated with different concentrations of **8b** for 48 h, or with 7 μM of **8b** for different periods of time. Cell extracts were prepared as described previously [46]. Proteins were separated by SDS gel electrophoresis (10% gels) and transferred onto a nitrocellulose membrane. After blocking the membrane (with 5% dry milk in TBS/0.2% Tween, 2h at RT) expression of proteins was analyzed using the corresponding CHOP (Santa Cruz Biotechnology, USA; 1:1000, in 5% BSA in TBS/0.1% Tween, overnight incubation at 4 °C) or Grp78 antibodies (Santa Cruz Biotechnology; 1:1000, in 5% BSA in TBS/0.1% Tween, overnight incubation at 4 °C). After washing (TBS/0.2% Tween 20), blots were incubated with secondary horseradish peroxidase coupled antibody (Amersham Biosciences, Piscataway, USA), (1:5000), for 2 h at room temperature (RT). Proteins were visualized by chemiluminescence using ECL™ detection reagent (AmershamBiosciences (GE Healthcare), USA). As an internal protein loading control, ERK2 protein expression was determined by re-probing the membranes with ERK2 specific antibody (Santa Cruz Biotechnology).

5.2.7. Determination of apoptosis

Apoptosis was determined by staining cells with DNA-intercalators acridine orange (AO) and ethidium bromide (EtBr), which give green fluorescence of the nuclei in live cells and red fluorescence of the nuclei in dead cells, respectively. Apoptotic cells could be discriminated from necrotic cells by typically condensed chromatin and the appearance of apoptotic bodies. In brief, adherent and floating cells were collected by centrifugation and resuspended in a small volume of culture medium, after which 2 μL of AO (15 μg/mL, Serva, Germany) and 2 μL of EtBr (50 μg/mL, Serva) were added to 10 μL of the cell suspension. Stained cells were viewed under the epifluorescence microscope (Axiovert 35, Opton, Germany). In addition, cleavage of apoptotic proteins was determined by Western blot, as described above, using monoclonal antibodies against caspase-8 (Cell Signaling Technology Inc., Danvers, MA; 1:1000), caspase-3 (Santa Cruz Biotechnology; 1:1000) and PARP (Pharmingen, San Diego, CA; 1:4000) or rabbit polyclonal antibodies against caspase-9 (Cell Signaling Technology, 1:1000) and caspase-3 (Cell Signaling Technology, 1:1000).

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