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# Diaryltriazenes as Antibacterial Agents Against Methicillin Resistant *Staphylococcus aureus* (MRSA) and *Mycobacterium smegmatis*

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*Mycobacterium smegmatis*, Antibiotic resistance, Triazenes

ABSTRACT: Antibiotic resistance poses a critical threat to global health care. Alkyl- and  
aryltriazenes compounds have found utility as DNA-modifying agents and have been previously

assessed as potential anti-cancer therapeutics. However, their potential utility as antimicrobials has only recently been appreciated. Therefore, to further investigate the efficacy of these compounds as antibiotics, we synthesized a series of forty six **diaryltriazene derivatives** and evaluated their antimicrobial properties. Initial experiments showed activity of some **diaryltriazene** compounds against both methicillin-resistant strains of *Staphylococcus aureus* (MRSA) and against *Mycobacterium smegmatis* with MICs of less than 0.02 and 1 µg/mL. Those **diaryltriazene compounds** with potent anti-staphylococcal and anti-mycobacterial activity were inactive as growth inhibitors of mammalian cell lines and yeast. Furthermore, we demonstrated that one of the most active anti-MRSA **diaryltriazene derivatives** was subject to very low frequencies of resistance at  $< 10^{-9}$ . Whole genome sequencing of resistant isolates identified mutations in the enzyme that lysylates phospholipids. This could result in the modification of phospholipid metabolism and consequently the characteristics of the staphylococcal cell membrane, ultimately modifying the sensitivity of these pathogens to triazene challenge. Our work has therefore extended the potential range of triazenes, which could yield novel antimicrobials with low levels of resistance.

## INTRODUCTION

Historically the discovery of sulphonamides and  $\beta$ -lactam antibiotics triggered the next 50 years of research, during which the majority of antimicrobial agents in use today were discovered.<sup>1</sup> However, the emergence of multidrug-resistance among bacteria including *Staphylococcus aureus* (*S. aureus*) and other members of the ESKAPE pathogens, and latterly extreme drug resistance in these and *Mycobacterium tuberculosis* (*M. tuberculosis*; XDRTB),

has challenged the effectiveness of antibiotics in the advent of modern medicine. As such, antibiotic resistance has become one of the most serious health care problems in the world.<sup>2</sup> This has been exacerbated by a collapse in antibiotic discovery by global pharma R&D, and resulted in a lack of new antibiotics coming to market.<sup>3,4</sup> Resistance arises either by alterations to the antibiotic target, by the acquisition of antibiotic modifying enzymes, or by reducing the local concentration of antibiotic *via* changes in cell wall permeability or efflux.

Considering the above, there is an urgent need to develop new effective antibacterial agents that circumvent the emergence of resistance.<sup>5</sup> Based on our recent research<sup>6</sup> we were prompted to further explore the antimicrobial activity of 1,3-diaryltriazenes. Triazenes possess a number of biological properties, including antifungal,<sup>7</sup> anorexic,<sup>8</sup> and anticancer activity.<sup>9,10</sup> The mechanism of action of alkyltriazenes is generally connected the formation of reactive diazonium species that are able to alkylate DNA.<sup>11,12</sup>

Here we describe the preparation and testing of 46 compounds from the 1,3-diaryltriene family, including 5 compounds containing an isoniazid (INH) moiety, and 31 compounds containing a fluorine atom, with the potential to be more biologically active than their non-fluorinated analogues.<sup>13</sup> As a result, several triazenes were identified as possessing potent antimicrobial activity towards methicillin resistant *S. aureus* (MRSA) and *M. Smegmatis*- a model mycobacterial representative of *M. tuberculosis*.

## RESULTS AND DISCUSSION

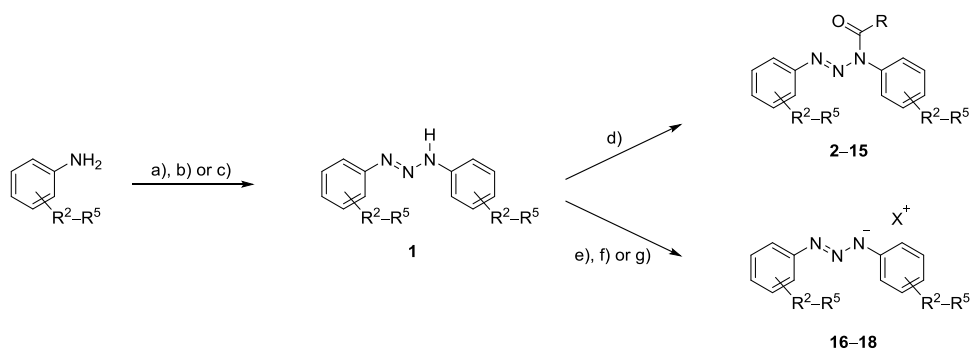
### Chemistry

A series of 46 triazene derivatives **1–19** were prepared according to Scheme 1, and their structures are depicted in Figure 1. These include 1,3-diaryltriazenes (**1**), their *N*-acyl substituted derivatives **2–15**, including the carbamate **4e**, and triazene salts **16–19**.

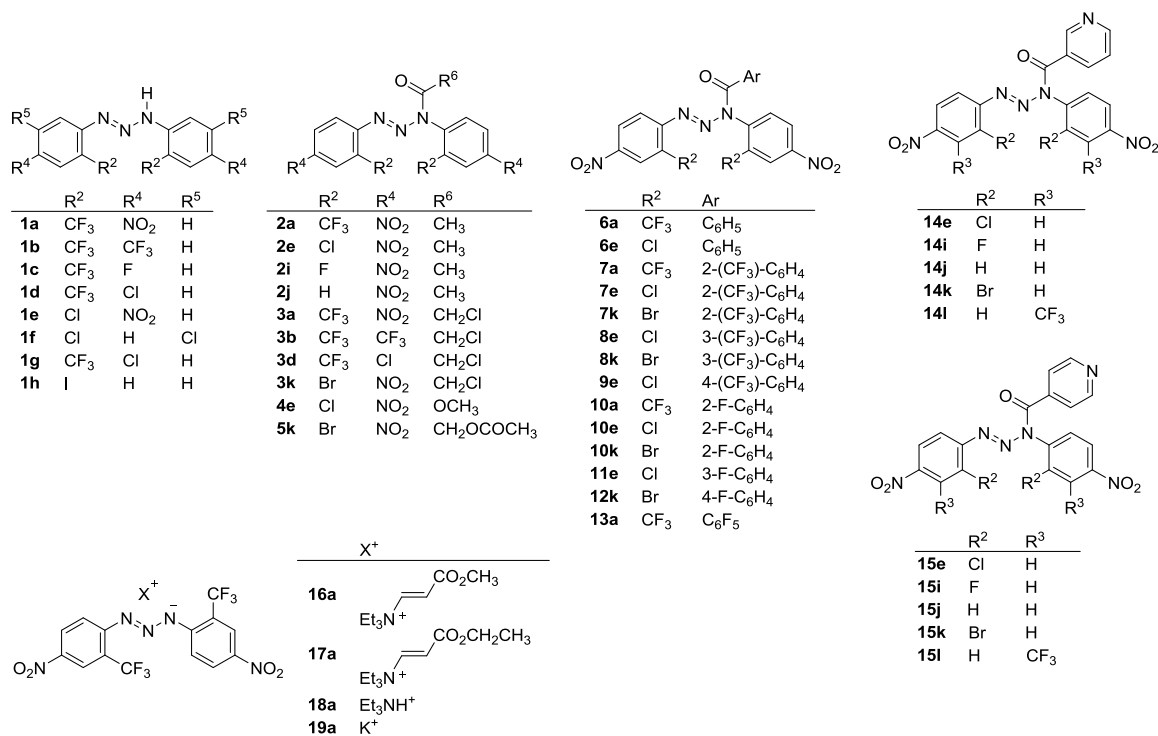
**#R4#**The selection of substituents at the triazene 1,3-diphenyl rings was mainly focused on strongly electro-withdrawing groups, in most cases possessing both –I and –R effects, whereas the electron-donating groups were not considered. This selection was stimulated by our recent observation on strong *in vitro* activity of electron-deficient triazenes against *M. tuberculosis*.<sup>6</sup> To effectively participate in modulating the electron density, *ortho*- and/or *para*- substitution pattern relative to the triazene group was selected predominantly.

**#R6#**Some 1,3-diaryltriazenes were modified by *N*-acylation. Nicotinoyl (**14**), and isonicotinoyl (**15**) derivatives were tentatively considered as potential multi-target hybrid compounds of 1,3-diaryltriazenes with isoniazid (INH) structures, potentially releasing the isonicotinoyl radical and 1,3-diaryltriazenes. INH is a highly specific first-line TB prodrug, which after activation couples isonicotinoyl radical to NADPH and binds irreversibly to enoyl-AcpM reductase, blocking cell wall synthesis.<sup>14</sup> In addition to nicotinoyl (**14**) and isonicotinoyl (**15**) derivatives, some other acyl analogues **2–13** were considered. Since alkyl triazenes are well known as DNA alkylating agents,<sup>11,12</sup> this type of derivatives was not investigated from the potential genotoxicity reasons.

**Scheme 1.** Synthesis of compounds **1–19**<sup>a</sup>



<sup>a</sup>Reagents: a) NaNO<sub>2</sub>/HCl;<sup>15</sup> b) isoamyl nitrite;<sup>15</sup> c) Na<sub>3</sub>Co(NO<sub>2</sub>)<sub>6</sub>;<sup>16</sup> d) Et<sub>3</sub>N, ClCOR;<sup>15</sup> e) Et<sub>3</sub>N, HC≡CCO<sub>2</sub>CH<sub>3</sub> or HC≡CCO<sub>2</sub>C<sub>2</sub>H<sub>5</sub>;<sup>17</sup> f) Et<sub>3</sub>N;<sup>17</sup> g) KOH.<sup>17</sup> For a key of substituents, see Figure 1.



**Figure 1.** The structures of 1,3-diaryltriazenes **1**, *N*-acylated 1,3-diaryltriazenes **2–15** and triazenide salts **16–19**.

1,3-Diaryltriazenes **1a–h** were prepared by treating the appropriately substituted anilines with either sodium nitrite in hydrochloric acid, isoamyl nitrite, or sodium hexanitrocobaltate(III). An acylation of the selected 1,3-diaryltriazenes **1** into *N*-acyl-1,3-diaryltriazenes **2–15** was achieved

by using the appropriate acyl chloride in the presence of triethylamine as a base. Acyl chlorides of nine different aliphatic, aromatic, and heteroaromatic carboxylic acids were selected, including acetyl, chloroacetyl, methoxyacetyl, acetoxyacetyl, benzoyl, fluorobenzoyl, (trifluoromethyl)benzoyl, pentafluorobenzoyl, nicotinoyl, and isonicotinoyl chlorides. In addition, four different triazenide salts were prepared from the **diaryltriazenes** **1a**. The reaction with methyl or ethyl propiolate in the presence of triethylamine afforded alkoxycarbonylvinyltriethylammonium salts **16a** and **17a**, whereas the trituration with triethylamine and potassium hydroxide gave triazenide salts **18a** and **19a** respectively (Scheme 1).

### Antimicrobial properties

Initial susceptibilities to compounds **1–19** were determined for *M. smegmatis* MC2 155, MRSA USA300 (as a representative of Gram-positive pathogen), *E. coli* DH10 $\beta$ , as a representative Gram-negative bacterium, and *Sacharomyces cerevisiae* (*S. cerevisiae*) wild strain as a representative eukaryote. The results are presented in Table 1. None of the examined compounds **1–19** however were found to be toxic towards *S. cerevisiae*.

**Table 1.** The initial MIC (minimal inhibitory concentrations) and MBC (minimal bactericidal concentration) screenings of the compounds **1–19**

Compd	<i>M. smegmatis</i> MC2 155		MRSA USA300		<i>E. coli</i> DH10 $\beta$		<i>S. cerevisiae</i> wild strain	
	MIC <sup>a</sup>	MBC <sup>a</sup>	MIC <sup>a</sup>	MBC <sup>a</sup>	MIC <sup>a</sup>	MBC <sup>a</sup>	MIC <sup>a</sup>	MBC <sup>a</sup>
<b>1a</b>	>128	/	>128	/	>128	/	>128	/
<b>1b</b>	/	/	>128	/	/	/	>128	/
<b>1c</b>	>128	/	>128	/	>128	/	>128	/

<b>1d</b>	/	/	>128	/	/	/	>128	/
<b>1e</b>	>128	/	2	4	>128	/	>128	/
<b>1f</b>	>128	/	>128	/	>128	/	>128	/
<b>1g</b>	8	128	1	1	>128	/	>128	/
<b>1h</b>	>128	/	>128	/	>128	/	>128	/
<b>2a</b>	16	128	0.25	0.25	64	>128	>128	/
<b>2e</b>	16	>128	4	4	64	>128	>128	/
<b>2i</b>	128	>128	8	32	32	128	>128	/
<b>2j</b>	>128	/	>128	/	64	128	>128	/
<b>3a</b>	32	>128	2	2	128	>128	>128	/
<b>3b</b>	/	/	>128	>128	/	/	>128	/
<b>3d</b>	/	/	>128	>128	/	/	>128	/
<b>3k</b>	>128	/	>128	/	64	128	>128	/
<b>4e</b>	128	>128	32	32	64	128	>128	/
<b>5k</b>	>128	/	8	16	64	64	>128	/
<b>6a</b>	32	>128	0.5	0.5	>128	/	>128	/
<b>6e</b>	128	>128	32	32	32	32	>128	/
<b>7a</b>	>128	/	>128	/	128	128	>128	/
<b>7e</b>	>128	/	>128	/	64	128	>128	/
<b>7k</b>	>128	/	>128	/	64	64	>128	/
<b>8e</b>	32	>128	8	8	32	64	>128	/
<b>8k</b>	128	>128	8	8	32	64	>128	/
<b>9e</b>	16	>128	8	8	64	128	>128	/
<b>10a</b>	16	128	0.25	0.5	>128	/	>128	/
<b>10e</b>	>128	/	>128	/	64	128	>128	/
<b>10k</b>	>128	/	>128	/	128	128	>128	/
<b>11e</b>	>128	/	16	16	64	64	>128	/
<b>12k</b>	>128	/	>128	/	64	64	>128	/
<b>13a</b>	32	>128	2	2	128	>128	>128	/
<b>14e</b>	64	128	32	32	32	32	>128	/



14i	>128	/	64	64	128	>128	>128	/
14j	>128	/	64	128	8	16	>128	/
14k	64	128	32	32	16	32	>128	/
14l	64	>128	4	4	32	64	>128	/
15e	128	>128	16	16	16	16	>128	/
15i	>128	/	32	32	>128	/	>128	/
15j	>128	/	128	128	16	32	>128	/
15k	128	>128	32	32	32	32	>128	/
15l	64	>128	2	2	32	32	>128	/
16a	4	16	0.0156	0.0156	>128	/	>128	/
17a	4	32	0.03125	0.03125	>128	/	>128	/
18a	0.03125	0.5	0.0156	0.03125	/	/	>128	/
19a	0.03125	0.5	0.0156	0.0156	/	/	>128	/

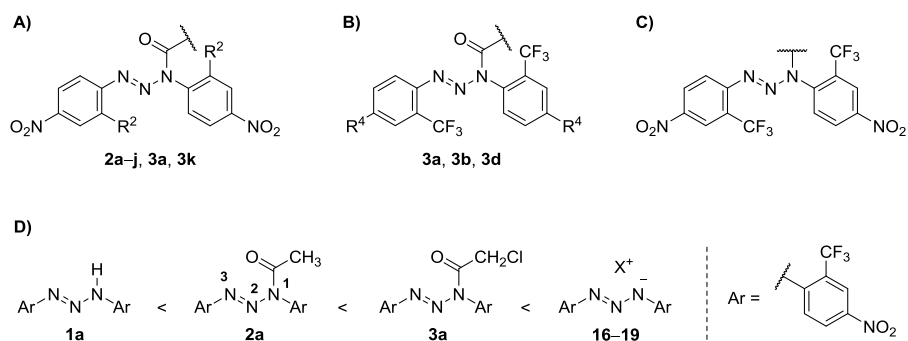
<sup>a</sup> In µg/mL.

**#R3#**The most potent of the diaryltriazene compounds against MRSA USA300 that we identified (**16a–19a** inclusive which have MIC values of <0.02 µg/mL) compare favorably with reported susceptibilities for this organism: gentamycin 0.5–8 µg/mL, kanamycin >64 µg/mL, tobramycin 0.5–2 µg/mL, rifampin <0.125–0.5 µg/mL, ciprofloxacin 0.125–>64 µg/mL, levofloxacin 0.25–32 µg/mL, gatifloxacin 0.5–4 µg/mL, vancomycin 0.25–2 µg/mL, teichoplanin <0.125–0.25 µg/mL, clindamycin <0.125–>64 µg/mL, erythromycin 8–>64 µg/mL, linezolid 2–4 µg/mL, oxacillin 16–32 µg/mL, chloroamphenicol 4–32 µg/mL, mupirocin 0.5–>64 µg/mL, fusidic acid <0.125–0.5 µg/mL, tetracycline <0.125–4 µg/mL, minocycline, and doxycycline both <0.125–0.5 µg/mL.<sup>18</sup>

Similarly, the diaryltriazenes we identified with potent antibacterial activity against *M. smegmatis* again compare favorably in potency with established antibiotics which this organism

is resistant to. For example, MIC values for  $\beta$ -lactams (amoxicillin 8–16  $\mu\text{g/mL}$ , ampicillin 128  $\mu\text{g/mL}$ , carbenicillin 512  $\mu\text{g/mL}$ , oxacillin 32  $\mu\text{g/mL}$ , cefoxitin 2–4  $\mu\text{g/mL}$  and ceftriaxone 32  $\mu\text{g/mL}$ )<sup>19</sup> and also for vancomycin (45  $\mu\text{g/mL}$ ) and D-cycloserine (105  $\mu\text{g/mL}$ )<sup>20</sup> are considerably greater than the MICs for **18a** and **19a**.

The structure-activity relationship (SAR) for compounds **1–19** was determined for MRSA USA300 strain. One can see that the MIC values, spanning from >128  $\mu\text{g/mL}$  to 0.0156  $\mu\text{g/mL}$ , are highly dependent on structure. Briefly, the comparison of antibacterial activities for compounds **2a–j**, **3a** and **3k**, having different  $R^2$  groups (H, F, Cl, Br,  $\text{CF}_3$ ) while keeping  $R^4$  ( $\text{NO}_2$ ) unchanged, reveals a decreasing trend in MIC values according to the  $R^2$  group, so that  $\text{H} < \text{Br} < \text{F} < \text{Cl} < \text{CF}_3$  (Table 1, Figure 2A). A comparison of the MIC values for the compounds **3a**, **3b**, and **3d**, having a fixed  $\text{CF}_3$  group at  $R^2$ , indicates that the  $\text{NO}_2$  group at the position 4 of the 1,3-diphenyl rings is a crucial one, and substituting this group to Cl or  $\text{CF}_3$  deactivates the compound (Figure 2B). The above results suggest that the 4-nitro-2-(trifluoromethyl)phenyl pattern may be the key structural element in identifying the “hit” diaryltriazene compound ( $R^2 = \text{CF}_3$ ,  $R^4 = \text{NO}_2$ , Figure 2C), **#R5#** at least within the derivatives under this investigation that possess electron-deficient 1,3-diaryl substituents.



**Figure 2.** A) The general structure of diaryltriazenes with a variable group R<sup>2</sup>; B) The general structure of diaryltriazenes with a variable group R<sup>4</sup>; C) The structure of the parent fragment crucial for the antibacterial activity; D) The effect of “N1 substitution pattern” on antibacterial activity of 1,3-bis(4-nitro-2-(trifluoromethyl)phenyl)triazenes.

Also evident from Table 1 is the influence of the *N*-acyl group in *N*-acyl-1,3-diaryltriazenes on MIC value. Higher activity is observed for the *N*-acyl groups that have a more electron deficient carbonyl group (for example, comparing MICs between **2a** and **3a**; Table 1, Figure 2D). The trend suggests that the liberation of a triazenide anion, presumably through the reaction of *N*-acyl-1,3-diaryltriazenes with intracellular nucleophilic reagents, may play a role in the compounds antibacterial activity. This is in line with the triazenide salts **16–19** being the most potent killers of strain MRSA USA300. The selection of the cation in the triazenide salts **16–19** appears not to be important in the activity against MRSA USA300, although this was not found for *M. smegmatis* (comparing **16a** and **17a** with **18a** and **19a**).

Interestingly, some of the examined compounds, notably **14j**, showed a significant activity against the wild type *E. coli* strain (Table 1). This observation, although not investigated further here, opens the possibility of discovering novel diaryltriazene derivatives, possessing greater potencies against Gram-negative organisms. This could be particularly important observation given the clinical concern currently associated with multi-drug resistant Gram-negative infections.

As non-specific bactericidal activity usually corresponds to the lack of prokaryotic specificity and therefore potential toxicity in mammals,<sup>21</sup> we decided to focus on identifying potentially membrane-damaging diaryltriazenes.

The BacLight™ assay is a particularly useful probe for determining the effect of compounds on bacterial membrane integrity.<sup>22</sup> Ten of the most active diaryltriazenes (**1g**, **1e**, **2a**, **3a**, **6a**, **10a**, **16a**, **17a**, **18a**, and **19a**) having MICs against MRSA USA300 of less than 2 µg/mL, were selected for testing using this assay. The results of these assays on the cell membrane of this strain are summarized in Table 2. Here it can be seen that all the tested compounds, with the exception of compound **1**, are potentially not membrane damaging.

**Table 2.** MRSA USA300 membrane integrity

Compd	MRSA USA300	
	c (µg/mL)	cells with intact membrane (%) <sup>a</sup>
<b>1e</b>	8	64.6±9.4
<b>1g</b>	2	31.2±3.2
<b>2a</b>	2	103.0±5.8
<b>3a</b>	8	100.0±2.4
<b>6a</b>	2	95.8±2.3
<b>10a</b>	2	103.8±4.7
<b>16a</b>	2	105.5±1.3
<b>17a</b>	2	107.3±3.5
<b>18a</b>	2	104.3±6.5
<b>19a</b>	2	107.6±9.6

<sup>a</sup> The compounds upon which the amount of MRSA USA300 with intact membrane was 95% and 110% are not considered to be membrane damaging. Data are shown as mean±SD obtained from at least three independent experiments.

Nine of the above compounds (**1g**, **2a**, **3a**, **6a**, **10a**, **16a**, **17a**, **18a**, and **19a**) were then selected for further evaluation of their antimicrobial activity against a variety of Gram-positive and

Gram-negative bacteria. The results are summarized in Table 3. **#R8#** All selected compounds are water soluble small molecules, stable in solid state as well as in solution of common organic solvents and water. As such, they could be relatively easily modified for further optimization.<sup>23</sup>

**Table 3.** Susceptibility (MIC, MBC) of the selected diaryltriazenes to the different Gram-positive and Gram-negative bacteria

Organism		Compd								
		1g	2a	3a	6a	10a	16a	17a	18a	19a
<b>MRSA wild strain 1653</b>	MIC <sup>a</sup>	1	0.25	0.03	0.5	0.5	0.0156	0.0156	0.0156	0.0156
	MBC <sup>a</sup>	1	0.5	0.5	1	4	0.25	0.25	0.25	0.5
<b><i>S. pneumoniae</i> R6</b>	MIC <sup>a</sup>	0.5	0.06	0.03125	0.5	0.5	0.0156	0.0156	0.0156	0.0156
	MBC <sup>a</sup>	1	0.5	0.125	1	1	0.125	0.125	0.125	0.125
<b><i>B. subtilis</i> ATCC 6051</b>	MIC <sup>a</sup>	1	0.125	0.03125	0.5	2	0.0156	0.0156	0.03125	0.03125
	MBC <sup>a</sup>	32	4	4	16	>64	2	2	2	2
<b>Vancomycin resistant <i>E. faecalis</i> 501</b>	MIC <sup>a</sup>	2	4	1	>128	>128	0.5	0.5	1	0.5
	MBC <sup>a</sup>	>128	>128	>128	/	/	>128	>128	>128	>128
<b><i>P. aeruginosa</i> PA0001</b>	MIC <sup>a</sup>	>128	>128	>128	>128	>128	>128	>128	>128	>128
	MBC <sup>a</sup>	/	/	/	/	/	/	/	/	/
<b><i>P. aeruginosa</i> ATCC 27853</b>	MIC <sup>a</sup>	/	/	>128	/	/	>128	>128	>128	>128
	MBC <sup>a</sup>	/	/	/	/	/	/	/	/	/
<b><i>K. pneumoniae</i> ATCC 13882</b>	MIC <sup>a</sup>	>128	>128	>128	>128	>128	>128	>128	>128	>128
	MBC <sup>a</sup>	/	/	/	/	/	/	/	/	/
<b><i>K. pneumoniae</i> ATCC 700603</b>	MIC <sup>a</sup>	>128	>128	>128	>128	>128	>128	>128	>128	>128
	MBC <sup>a</sup>	/	/	/	/	/	/	/	/	/
<b><i>A. baumannii</i> ATCC 19606</b>	MIC <sup>a</sup>	>128	>128	>128	>128	>128	>128	>128	>128	>128
	MBC <sup>a</sup>	/	/	/	/	/	/	/	/	/
<b><i>A. baumannii</i></b>	MIC <sup>a</sup>	>128	>128	>128	>128	>128	>128	>128	>128	>128

BAA1605      MBC<sup>a</sup>    /        /        /        /        /        /        /        /        /

<sup>a</sup> In µg/mL.

We were unable to detect activity of the selected compounds in Gram-negative strains, i.e. *E. coli* (Table 1), *P. aeruginosa*, *K. pneumonia*, *A. baumannii* (Table 3). This may have been due to a lack of penetration of the tested compounds through the outer membranes of these organisms, or possibly due to efficient efflux functioning. To decipher between these two possibilities we performed MIC evaluations of the selected compounds using an *E. coli* membrane permeable strain of D22 and an *E. coli* strain of D22 with knockout of the membrane pump N43 CGSC 5583. The results, presented in Table 4, suggest that the inactivity of the tested compounds in the case of Gram negative-bacteria, represented by the *E. coli* DH10β strain, was indeed related to low permeability of the Gram-negative outer membrane to these compounds. **#R1#** Aberrant behaviour of compounds **6a** and **10a** could arise from their structure. These compounds are unique amongst those tested from Table 3, by their possession of (substituted) *N*-benzoyl group. Although this may well be an issue that uniquely impinges upon the ability of **6a** and **10a** to penetrate cells, the explanation of the actions of **6a** and **10a** may also reside with the *E. coli* strain D22 used in these experiments. D22 contains a mutation in the LpxC gene which encodes UDP-3-*O*-((*R*)-3-hydroxymyristoyl)-*N*-acetylglucosamine deacetylase, an enzyme involved in the synthesis of lipid A.<sup>24,25</sup> Lipid A is a lipopolysaccharide appended to the outer membrane of *E. coli*. Perturbations to its synthesis caused by LpxC mutations would likely impact upon the ability of hydrophobic compounds such as **6a** and **10a** to penetrate the cell.

**Table 4.** MIC and MBC evaluation for *E. coli* membrane permeable strain D22, and an *E. coli* strain containing a knockout of the membrane pump N43 CGSC 5583

Compd	<i>E. coli</i> membrane permeable strain D22		<i>E. coli</i> membrane pump knockout N43 CGSC 5583	
	MIC <sup>a</sup>	MBC <sup>a</sup>	MIC <sup>a</sup>	MBC <sup>a</sup>
<b>1g</b>	2	>128	>128	/
<b>2a</b>	1	>128	>128	/
<b>3a</b>	0.5	16	>128	/
<b>6a</b>	>128	/	>128	/
<b>10a</b>	>128	/	>128	/
<b>16a</b>	0.25	>128	>128	/
<b>17a</b>	0.5	>128	>128	/
<b>18a</b>	0.5	>128	>128	/
<b>19a</b>	0.5	>128	>128	/

<sup>a</sup> in µg/mL.

**#R9#**Critically, resistance to methicillin and vancomycin underpins medically intractable infections caused by MRSA and vancomycin resistant enterococci (VRE). The acute diaryltriazene sensitivity of MRSA USA300, a highly resistant clinical MRSA strain, therefore prompted us to investigate the possibility that our antimicrobial diaryltriazene compounds might sensitise these pathogens to β-lactams and vancomycin, which would ultimately be of clinical interest. However, no synergism<sup>26</sup> between the compound **16a**, one of the most promising new diaryltriazene compounds, was detected when tested with methicillin against MRSA, nor with vancomycin against Vancomycin resistant *E. faecalis* (VRE). This finding led us to conclude that the mode of action of the diaryltriazene **16a** was either unrelated to the action of methicillin and vancomycin (bacterial peptidoglycan biosynthesis) or did not impact on the resistance mechanisms employed by MRSA and VRE towards β-lactams and glycopeptides.

In order to better characterise the mode of action of the compound **16a**, MRSA strain USA300 was grown continuously in the presence of this active compound, at progressively inhibitory

concentrations (2-, 4-, 6- and 8-times it's MIC value), allowing resistance to develop. Following incubation three **16a**-resistant MRSA USA300 isolates were obtained. In addition the mutation rate of the MRSA USA300 strain when exposed to the different concentrations of the compound **16a** was also determined. **#R2#**We have also obtained MICs values of all three resistant isolates (Table 5).

**Table 5.** Mutation rate assay<sup>a</sup> and resistant strains isolated

Diaryltriazene <b>16a</b>			<b>16a</b> -resistant isolate name
Concentration (µg/mL)	Mutation rate	MIC (µg/mL)	
0.03125	$3.96 \times 10^{-6}$	0.03125	<i>Isolate R1</i>
0.0625	$8 \times 10^{-9}$	0.03125	<i>Isolate R2</i>
0.09375	$8 \times 10^{-9}$	0.0625	<i>Isolate R3</i>
0.125	$< 8 \times 10^{-9}$	/	/

<sup>a</sup> Mutation rate assays were performed using MRSA USA300 strain.

MRSA USA300 isolates resistant to the compound **16a**, and sensitive parental strains were subjected to whole genome sequencing in order to identify the underlying genetic polymorphisms present in resistant isolates. Resistant isolates (identified as *Isolates R1*, *R2*, and *R3*) were compared to the parental (control) strain, allowing the genetic polymorphisms differentiating these isolates to be identified (Figure 3).

*Isolate R1* contained a single 48 base pair deletion at the position 128454 of the genome, corresponding to the position of a pseudogene in the reference, which appeared to be reactivated by this deletion. The gene was identified as Staphylococcal protein A (SpA), a known virulence protein. The deletion occurred at amino acid (AA) position 390 of the 522AA protein, causing



the protein to shift back into frame. A comparison with the NCBI blast database revealed that this was a common deletion found among *S. aureus* isolates. Cell wall associated SpA functions to bind the Fc $\gamma$  domain of human immunoglobulin.<sup>27</sup> This molecule consists of a hydrophobic C-terminal, thought to be embedded within the cell membrane, flanked at the N-terminal end by a charged tail.<sup>28,29</sup> The mechanism by which this protein reduces its sensitivity to the **diaryltriazene 16a** therefore remains unclear; its surface location could however affect the interaction or permeability of the cell to this compound.

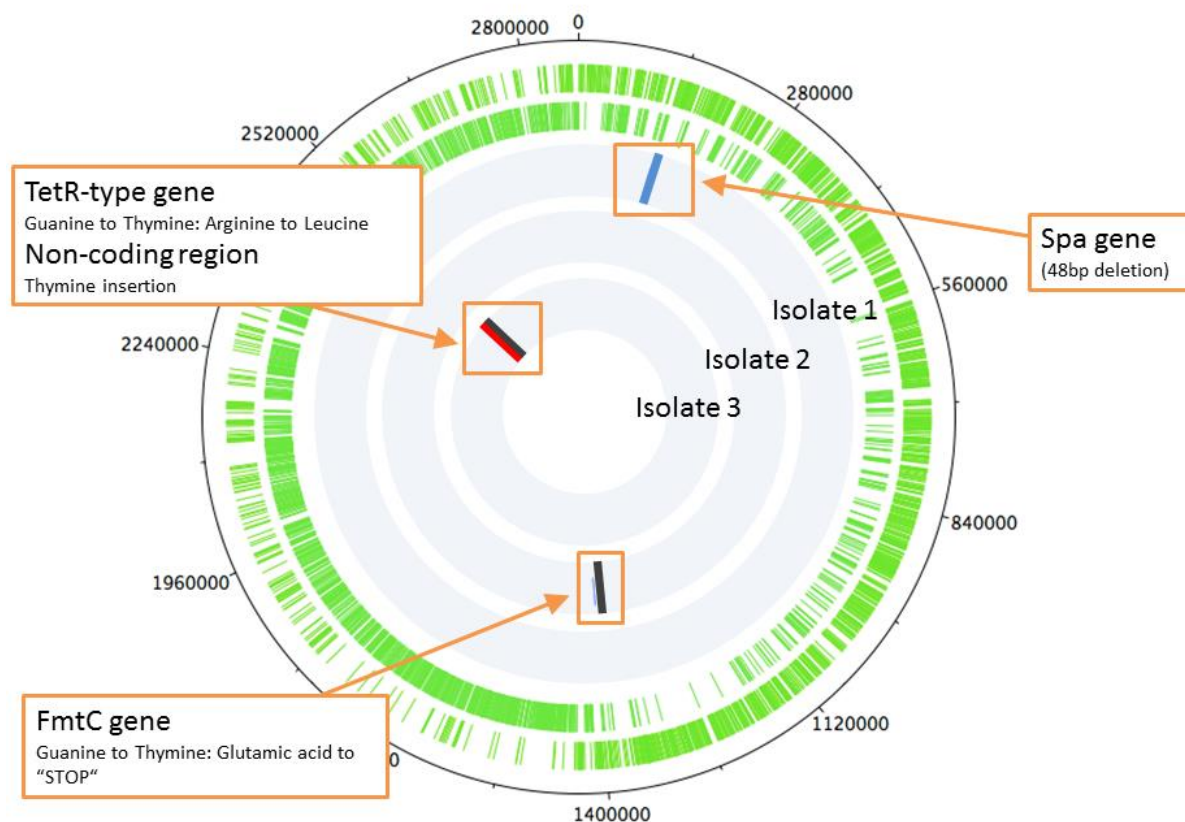
*Isolate R2* carried a single non-synonymous single nucleotide polymorphism (SNP) at position 1396319 when compared to the control strain. This guanine to thymine SNP altered the amino acid sequence of *fmtC*, a lysylphosphatidylglycerol synthetase, an 843 amino acid protein, from Glu307, to an ATG “stop” codon. This gene is not essential in *S. aureus*. The function of FmtC appears to be to impart a positive charge on the negatively charged phospholipid membrane bilayer.<sup>30</sup> This is achieved through the addition of a lysine residue, through the positive charge of its epsilon amino group. This altering of the surface charge as a route to antibiotic resistance is widely documented, particularly for the pore-forming peptides such as nisin.<sup>31,32</sup> Quenching of the membranes negative charge through the lysine addition is therefore likely to reduce a repulsion of the negatively charged **diaryltriazenes**, facilitating their penetration. A loss of FmtC function or efficacy could therefore affect the interaction between negatively charged **diaryltriazene** and the negatively charged membrane leading to resistance.

*Isolate R3* carried two mutations relative to the control, a non-synonymous SNP at position 2496871, and a single thymine insertion at position 2496195, in an intergenic region. The guanine to thymine SNP resulted in an arginine to leucine substitution at the AA-position 197 of the 210 AA DNA-binding helix-turn-helix (HTH) domain of a possible transcriptional regulator.

Using the Basic Local Alignment Search Tool (BLAST) this gene most closely resembled a Tetracycline Repressor (TetR) family transcriptional regulator- so named owing to their involvement with tetracycline resistance in *S. aureus*.<sup>33</sup>

The thymine insertion at position 2496195 occurred in an intergenic region prior to the possible transcriptional regulator described above, on the forward strand, and upstream from a hypothetical membrane protein on the reverse strand. Using the prediction software PEPPER<sup>34</sup> this region was predicted to contain a promoter sequences in the forward direction.<sup>33</sup> This suggested that expression levels for the predicted transcriptional regulator could have been affected. However, it remains unclear as to how mutations in a gene associated with tetracycline resistance impact upon **diaryltriazene** resistance.

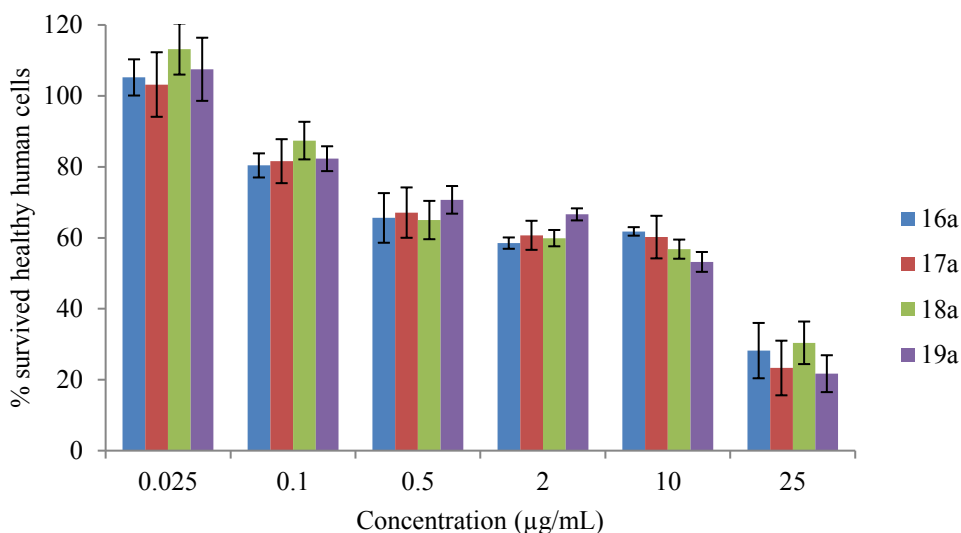
Therefore, no single target could be identified for the activity of the compound **16a**. Instead, changes in MIC appear to have resulted from independent mechanisms unique to each mutant isolate.



**Figure 3.** Mutant isolates of MRSA USA300 compared to the control. The figure illustrates the genomic locations of the different mutations relative to the control. The outer scale bar indicates the number of base pairs from the replication origin, and can be used to compare the approximate genomic locations for each of the mutations identified.

To assess the specificity and translational benefit that might arise from antimicrobials based upon the **diaryltriazene** compounds used in this research we assessed the impact of the most promising triazenide salt **16a**, as well as **17a**, **18a**, and **19a** on human keratinocytes. The results (Figure 4) show that keratinocytes survived significantly higher concentrations of triazenide salts compared to those concentrations which inhibited the growth of MRSA USA300, *M. smegmatis*

(Table 1), MRSA wild strain, *S. pneumoniae*, *Bacillus subtilis* or Vancomycin resistant *E. faecalis* (Table 3). This data clearly indicates a marked selectivity of these diaryltriazenes towards bacterial growth inhibition.



**Figure 4.** Survival of healthy human keratinocytes treated with compounds **16a**, **17a**, **18a** and **19a**.

**#R7#** The SAR conducted suggested a strong role for the nitro group on the antibacterial efficacy. Although nitroarenes are less preferred scaffolds in drug discovery due to their potential mutagenicity, certain drugs do contain this moiety.<sup>35</sup> These include chloramphenicol and metronidazole, which are on the WHO Model List of Essential Medicines.<sup>36</sup> Despite the potential threat, recent endeavor of the group of Panda has shown promise in mitigating the mutagenic nature of nitroarenes.<sup>37</sup>

It is interesting to note that the structures of 1,3-diaryl substituents at the triazenes identified in this work has a similarity with benzothiazinones (BTZ043) and related compounds.<sup>14,35,38</sup> For

antitubercular activity, it was found that these compounds need to be functionalized with nitro group as well as a *meta*- electron-withdrawing group, typically either a trifluoromethyl or another nitro. The nitroaromatic group was postulated to undergo bioreduction to a corresponding nitrosoarene which then reacts with an active site cysteine residue in the DprE1 enzyme to form a semimercaptal adduct inactivating the enzyme.<sup>14,35,38</sup> Recent chemical studies on BTZ043 confirmed the postulated reactivity, indicating that thiolates and other nucleophiles induce nonenzymatic reduction of the nitro groups to the corresponding nitroso intermediates.<sup>39</sup> We conducted NMR experiment where equimolar amounts of reduced glutathione (GSH) and triazenide salt **19a** were incubated at room temperature in a mixture of DMSO-*d*<sub>6</sub> and phosphate buffer of pH 7.4 (1:1, v/v). The reaction mixture was monitored by <sup>1</sup>H NMR spectroscopy for 24 h indicating no changes in the aromatic region of the spectra. This suggests that the molecular mechanism of action of 1,3-diarlyltriazenes under this investigation may be different to that of BTZ043.

The biological activity of alkyltriazenes has been associated to degradation products involved, forming reactive diazonium species.<sup>11,12</sup> In contrast, compounds **1a** and **19a** were found to be stable in phosphate buffer solutions at pH 7.4 with no detectable decomposition as indicated by TLC and NMR analyses. Thus, the molecular mechanism of antibacterial activity of 1,3-diarlyltriazenes is yet to be explored, and will be the subject of forthcoming investigations.

## CONCLUSIONS

Here we have described the synthesis of a library of 1,3-diarlyltriazenes, their *N*-acyl derivatives, and their salts. The structure-activity relationships (SAR) of these compounds revealed that the antimicrobial properties of these compounds depended on the type of

substituent group attached to the two constituent benzene rings. Specifically, a trifluoromethyl group at position 2, and a nitro substituent at position 4 of the triazene were found to be critical to its activity.

Regarding the biological activities of the new compounds, the most important result from our study was that the selected compounds **16a**, **17a**, **18a** and **19a** were extremely active against one of the most clinically important bacterial species, MRSA. These compounds were not found to be membrane damaging to MRSA USA300. The molecular basis of the triazene activity against *S. aureus* remained unclear, with the appearing to more than one mechanism of killing based on the observed mutations identified among resistant isolates. Where a mechanism could be characterized, it appeared that changes in the phospholipid composition of the staphylococcal cell membrane were ultimately responsible. In addition, the selected compounds were found to be very effective against other Gram-positive bacteria, such as *S. pneumoniae*, *B. subtilis*, Vancomycin resistant *E. faecalis*, and *M. smegmatis*. Promising activities of a number of the triazene family compounds towards *E. coli* were also found. The majority of the tested compounds were however inactive against Gram-negative organisms, with membrane penetration found to be limiting in *E. coli*.

These compounds were also found to be inactive against lower eukaryotes (yeast), and more importantly, exhibited only a low cytotoxicity against human keratinocytes (as compared to the cytotoxicity observed towards certain pathogenic bacteria). These characteristics are promising with respect to the potential clinical utility of these triazenes for use as novel antimicrobials. Our data suggest that 1,3-diaryltriazenes could represent a new class of antibacterial drug.

## **EXPERIMENTAL SECTION**

**Chemistry.** Starting materials and solvents for the synthesis of the examined compounds were used as obtained, and without further purification, from Aldrich, Fluka, Alfa Aesar and Maybridge Chemical Company Ltd). Melting points were determined on a Kofler micro hot stage and were uncorrected. NMR spectra were recorded with a Bruker Avance III 500 MHz instrument operating at 500 MHz ( $^1\text{H}$ ), 126 MHz ( $^{13}\text{C}$ ) at 296 K. Proton spectra were referenced to  $\text{Si}(\text{CH}_3)_4$  as the internal standard ( $\delta = 0.00$  ppm) and carbon chemical shifts were given against the central line of the solvent signal ( $\text{CDCl}_3$  at  $\delta = 77.0$  ppm). Chemical shifts were given on the  $\delta$  scale (parts per million) and coupling constants ( $J$ ) were given in Hertz. IR spectra were obtained with a Bruker ALPHA Platinum ATR spectrometer on a solid sample support (ATR). High-resolution mass spectrometry (HRMS) analysis was performed using an Agilent 6224 Accurate Mass TOF LC/MS spectrometer. Elemental analyses (C, H, N) were performed with Perkin Elmer 2400 Series II CHNS/O Analyser. The progress of all reactions was monitored on Fluka silica-gel TLC-plates (with fluorescence indicator UV254), using ethyl acetate/petroleum ether as the solvent system. A column chromatography was performed using Merck silica gel 60 (35–70  $\mu\text{m}$ ) with solvent mixtures as specified in the corresponding experiments. All the compounds tested were of  $\geq 95\%$  purity, as verified by  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR spectroscopy as well as with elemental microanalysis. The values obtained for C, H, N analyses were within  $\pm 0.40\%$  of the calculated values.

**Materials.** The compounds **1a**, **1c–1h**, **2**, **3a**, **4–13** and **18** were prepared according to known procedures.<sup>15,16,40</sup> Precursor 1,3-diaryltriazenes, i.e., 1,3-bis(2-fluoro-4-nitrophenyl)triazene, 1,3-bis(2-methyl-4-nitrophenyl)triazene, 1,3-bis(2-bromo-4-nitrophenyl)triazene, and 1,3-bis(4-nitro-3-(trifluoromethyl)phenyl)triazene, that were used for the acylation shown in Scheme 1 into **2i**, **2j**, **3k**, **5k**, **7k**, **8k**, **10k**, **12k**, **14i–l**, **15i–l**, were synthesized as described in the literature.<sup>6</sup>

**Preparation of 1,3-bis(2,4-bis(trifluoromethyl)phenyl)triazene (1b).** An aqueous solution of sodium nitrite (0.035 g, 0.5 mmol/10 mL) was added dropwise to the solution of 2,4-bis(trifluoromethyl)aniline (0.229 g, 1 mmol) in 5% aqueous HCl (2.5 mL) at 0 °C. The reaction mixture was stirred at room temperature for 20 h. Then, the solid material was filtered and washed with water.

Pale yellow solid; yield 75%, mp 91–93 °C (MeOH/H<sub>2</sub>O); IR: 3343, 3101, 1632, 1594, 1527, 1487, 1455, 1426, 1347, 1306 cm<sup>-1</sup>; <sup>1</sup>H NMR (500 MHz, DMSO-*d*<sub>6</sub>): δ (ppm) 7.89–7.95 (m, 2H), 8.08–8.18 (m, 4H), 13.25 (s, 1H); HRMS (ESI+) *m/z* for C<sub>16</sub>H<sub>8</sub>F<sub>12</sub>N<sub>3</sub><sup>+</sup> [M + H]<sup>+</sup>: calcd 470.0521, found 470.0517. Anal. calcd for C<sub>16</sub>H<sub>7</sub>F<sub>12</sub>N<sub>3</sub>: C, 40.95; H, 1.50; N, 8.96; found C, 41.02; H, 1.37; N, 9.08.

### **General procedures for the synthesis of 3-acyl-1,3-diaryltriazenes 2–15**

*Method A. (3b, 3d, 3k)* Triethylamine (0.279 mL, 2 mmol) was added to a stirred suspension of the appropriate triazene **1** (1 mmol) in acetonitrile (10 mL) at room temperature, followed by the addition of chloroacetyl chloride (0.155 mL, 2 mmol). After additional stirring for 10 min, the reaction mixture was evaporated to dryness under reduced pressure. Then, water (5 mL) was added and the water phase was washed with dichloromethane (3 × 10 mL). Combined organic phases were first washed with water (20 mL) and then dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, filtered and evaporated under reduced pressure to dryness yielding the crude products **3**. Thereafter, products were recrystallized using the appropriate solvents.

*Method B. (14, 15)* Triethylamine (0.558 mL, 4 mmol) was added to a stirred suspension of the appropriate triazene **1** (1 mmol) in dry acetonitrile (20 mL) at room temperature, followed by the



addition of nicotinoyl chloride (isonicotinoyl chloride in cases of **15**). After additional stirring for 10–20 min, the reaction mixture was evaporated to dryness under reduced pressure, treated with methanol, filtered off and washed with saturated NaHCO<sub>3</sub> solution and methanol yielding the crude products **14** and **15**. Thereafter, all products were recrystallized using the appropriate solvents.

### **General procedures for the synthesis of triazenide salts 16–19**

*Method C. (16, 17)* Triethylamine (0.558 mL, 4 mmol) was added to a stirred suspension of 1,3-bis(4-nitro-2-(trifluoromethyl)phenyl)triazene (**1a**) (0.424 g, 0.5 mmol) in acetonitrile (7 mL) at room temperature followed by the addition of methyl propiolate (ethyl propiolate in the case of **17a**). After additional stirring for 1 h, the reaction mixture was evaporated to dryness under reduced pressure, treated with diethyl ether and filtered off, yielding the crude products **16a** and **17a**. Thereafter, both products were recrystallized using the appropriate solvents.

*Method D. (19)* A solution of KOH (0.224 g, 4 mmol) in water (20 mL) was added to a stirred solution of 1,3-bis(4-nitro-2-(trifluoromethyl)phenyl)triazene in acetonitrile (10 mL) at room temperature. After additional stirring for 20 min, acetonitrile was evaporated under reduced pressure, and the precipitate was filtered off, yielding **19a**. A crude product was recrystallized from acetonitrile/water mixture.

**1-(1,3-Bis(2,4-bis(trifluoromethyl)phenyl)triaz-2-en-1-yl)-2-chloroethanone (3b).** Method A. Pale yellow solid; yield 91%; mp 85–87 °C (petroleum ether); IR: 1744, 1626, 1596, 1492, 1345, 1304, 1274, 1232, 1160, 1120 cm<sup>-1</sup>; <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>): δ (ppm) 4.73–4.90 (m, 2H), 7.35–7.42 (m, 1H), 7.71–7.77 (m, 1H), 7.88–7.98 (m, 2H), 7.98–8.03 (m, 1H), 8.07–8.12

(m, 1H);  $^{13}\text{C}$  NMR (126 MHz,  $\text{CDCl}_3$ ):  $\delta$  (ppm) 41.7, 118.7, 121.9 (q,  $J = 273$  Hz), 122.2 (q,  $J = 273$  Hz), 122.8 (q,  $J = 273$  Hz), 122.9 (q,  $J = 273$  Hz), 124.3–124.5 (m), 124.8–125.0 (m), 127.6 (q,  $J = 32$  Hz), 130.0 (q,  $J = 32$  Hz), 131.9 (q,  $J = 32$  Hz), 132.2, 133.2 (q,  $J = 32$  Hz), 136.1, 147.1, 167.8; HRMS (ESI $^-$ )  $m/z$  for  $\text{C}_{16}\text{H}_6\text{F}_{12}\text{N}_3^-$  [ $\text{M} - \text{COCH}_2\text{Cl}$ ] $^-$ : calcd 468.0376, found 468.0380. Anal. calcd for  $\text{C}_{18}\text{H}_8\text{ClF}_{12}\text{N}_3\text{O}$ : C, 39.62; H, 1.48; N, 7.70; found: C, 39.76; H, 1.43; N, 7.60.

**1-(1,3-Bis(4-chloro-2-(trifluoromethyl)phenyl)triaz-2-en-1-yl)-2-chloroethanone (3d).**

Method A. Yellow solid; yield 96%; mp 83–85 °C (diisopropyl ether); IR: 3109, 3015, 2960, 2122, 1733, 1714, 1599, 1501, 1481, 1413  $\text{cm}^{-1}$ ;  $^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$ ):  $\delta$  (ppm) 4.74 (d,  $J = 13.9$  Hz, 1H), 4.82 (d,  $J = 13.9$  Hz, 1H), 7.11–7.16 (m, 1H), 7.57–7.63 (m, 2H), 7.65–7.71 (m, 2H), 7.79–7.83 (m, 1H);  $^{13}\text{C}$  NMR (126 MHz,  $\text{CDCl}_3$ ):  $\delta$  (ppm) 41.9, 119.1, 121.8 (q,  $J = 274$  Hz), 122.2 (q,  $J = 274$  Hz), 127.2 (q,  $J = 5$  Hz), 127.9 (q,  $J = 5$  Hz), 128.6 (q,  $J = 32$  Hz), 130.4 (q,  $J = 32$  Hz), 131.2 (q,  $J = 2$  Hz), 132.6, 132.8, 133.2, 136.1, 136.8, 143.1, 167.8; HRMS (ESI $^-$ )  $m/z$  for  $\text{C}_{14}\text{H}_6\text{Cl}_2\text{F}_6\text{N}_3^-$  [ $\text{M} - \text{COCH}_2\text{Cl}$ ] $^-$ : calcd 399.9848, found 399.9852. Anal. calcd for  $\text{C}_{16}\text{H}_8\text{Cl}_3\text{F}_6\text{N}_3\text{O}$ : C, 40.15; H, 1.68; N, 8.78; found: C, 39.91; H, 1.60; N, 8.57.

**1-(1,3-Bis(2-bromo-4-nitrophenyl)triaz-2-en-1-yl)-2-chloroethanone (3k).** Method A. Yellow solid; yield 91%; mp 151–153 °C (dichloromethane/diisopropyl ether); IR: 3101, 3076, 3002, 2958, 2112, 1991, 1922, 1720, 1594, 1580  $\text{cm}^{-1}$ ;  $^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$ ):  $\delta$  (ppm) 4.76–4.90 (m, 2H), 7.44–7.49 (m, 1H), 7.59–7.65 (m, 1H), 8.23–8.29 (m, 1H), 8.33–8.38 (m, 1H), 8.50–8.54 (m, 1H), 8.60–8.65 (m, 1H);  $^{13}\text{C}$  NMR (126 MHz,  $\text{CDCl}_3$ ):  $\delta$  (ppm) 41.8, 119.8, 122.3, 123.5, 123.5, 124.1, 128.7, 129.2, 131.6, 140.4, 148.2, 148.9, 149.8, 167.2; HRMS (ESI $^-$ )  $m/z$  for  $\text{C}_{12}\text{H}_6\text{Br}_2\text{N}_5\text{O}_4^-$  [ $\text{M} - \text{COCH}_2\text{Cl}$ ] $^-$ : calcd 441.8792, found 441.8792. Anal. calcd for  $\text{C}_{14}\text{H}_8\text{Br}_2\text{ClN}_5\text{O}_5$ : C, 32.24; H, 1.55; N, 13.43; found: C, 32.31; H, 1.46; N, 13.32.

**(1,3-Bis(2-chloro-4-nitrophenyl)triaz-2-en-1-yl)(pyridin-3-yl)methanone (14e).** Method B, reaction time: 20 min. Pale yellow solid; yield 66%; mp 128–132 °C (MeOH/acetone); IR: 3098, 1703, 1584, 1528, 1474, 1351, 1226, 1144, 1134, 1120, 1047 cm<sup>-1</sup>; <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>):  $\delta$  (ppm) 7.36–7.43 (m, 1H), 7.50–7.56 (m, 1H), 7.58–7.65 (m, 1H), 8.07–8.13 (m, 1H), 8.19–8.25 (m, 1H), 8.29–8.34 (m, 1H), 8.34–8.38 (m, 1H), 8.48–8.53 (m, 1H), 8.84–8.89 (m, 1H), 9.10–9.16 (m, 1H); <sup>13</sup>C NMR (126 MHz, CDCl<sub>3</sub>):  $\delta$  (ppm) 119.7, 122.8, 122.9, 123.3, 125.6, 126.2, 128.9, 131.8, 133.2, 134.4, 137.6, 139.1, 148.1, 148.5, 149.0, 150.7, 153.0, 168.6; HRMS for C<sub>18</sub>H<sub>11</sub>Cl<sub>2</sub>N<sub>6</sub>O<sub>5</sub> [M + H]<sup>+</sup>: calcd 461.0162, found 461.0164. Anal. calcd for C<sub>18</sub>H<sub>10</sub>Cl<sub>2</sub>N<sub>6</sub>O<sub>5</sub> × 0.5 H<sub>2</sub>O: C, 46.56; H, 2.53; N, 17.61; found: C, 46.41; H, 2.70; N, 17.78.

**(1,3-Bis(2-fluoro-4-nitrophenyl)triaz-2-en-1-yl)(pyridin-3-yl)methanone (14i).** Method B, reaction time: 10 min. Brown solid; yield 76%; mp 126–128 °C (MeOH); IR: 3091, 1699, 1586, 1526, 1483, 1417, 1346, 1308, 1261, 1241, 1216 cm<sup>-1</sup>; <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>):  $\delta$  (ppm) 7.42–7.48 (m, 1H), 7.50–7.55 (m, 1H), 7.59–7.64 (m, 1H), 8.00–8.07 (m, 2H), 8.16–8.22 (m, 2H), 8.24–8.30 (m, 1H), 8.83–8.88 (m, 1H), 9.09–9.13 (m, 1H); <sup>13</sup>C NMR (126 MHz, CDCl<sub>3</sub>):  $\delta$  (ppm) 112.7 (d, *J* = 25 Hz), 113.4 (d, *J* = 25 Hz), 120.0 (d, *J* = 15 Hz), 120.0 (d, *J* = 15 Hz), 121.5, 123.3, 128.5 (d, *J* = 15 Hz), 128.8, 131.5, 137.8, 140.1 (d, *J* = 8 Hz), 148.5 (d, *J* = 8 Hz), 149.6 (d, *J* = 8 Hz), 150.8, 153.0, 156.5 (d, *J* = 260 Hz), 157.4 (d, *J* = 260 Hz), 168.5; HRMS (ESI<sup>+</sup>) *m/z* for C<sub>18</sub>H<sub>11</sub>F<sub>2</sub>N<sub>6</sub>O<sub>5</sub><sup>+</sup> [M + H]<sup>+</sup>: calcd 429.0754, found 429.0747. Anal. calcd for C<sub>18</sub>H<sub>10</sub>F<sub>2</sub>N<sub>6</sub>O<sub>5</sub>: C, 50.48; H, 2.35; N, 19.62; found: C, 50.30; H, 2.22; N, 19.42.

**(1,3-Bis(4-nitrophenyl)triaz-2-en-1-yl)(pyridin-3-yl)methanone (14j).** Method B, reaction time: 10 min. Pale violet solid; yield 58%; mp 225–227 °C (MeOH); IR: 3054, 1689, 1610, 1584, 1519, 1470, 1416, 1330, 1300, 1209, 1170, 1132 cm<sup>-1</sup>; <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>):  $\delta$  (ppm) 7.47–7.56 (m, 5H), 8.16–8.21 (m, 1H), 8.22–8.28 (m, 2H), 8.43–8.49 (m, 2H), 8.83–8.88

(m, 1H), 9.07–9.11 (m, 1H);  $^{13}\text{C}$  NMR (126 MHz,  $\text{CDCl}_3$ ):  $\delta$  (ppm) 123.2, 123.3, 124.9, 125.0, 129.5, 130.3, 137.7, 140.6, 148.2, 148.3, 150.8, 151.6, 152.8, 169.2; HRMS (ESI+)  $m/z$  for  $\text{C}_{18}\text{H}_{13}\text{N}_6\text{O}_5^+$   $[\text{M} + \text{H}]^+$ : calcd 393.0942, found 393.0939. Anal. calcd for  $\text{C}_{18}\text{H}_{12}\text{N}_6\text{O}_5$ : C, 55.11; H, 3.08; N, 21.42; found: C, 54.86; H, 2.85; N, 21.33.

**(1,3-Bis(2-bromo-4-nitrophenyl)triaz-2-en-1-yl)(pyridin-3-yl)methanone (14k).** Method B, reaction time: 20 min. Yellow-brown solid; yield 49%; mp 142–144 °C (MeOH/acetone); IR: 3098, 1702, 1583, 1531, 1519, 1485, 1469, 1418, 1345, 1289, 1250, 1223  $\text{cm}^{-1}$ ;  $^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$ ):  $\delta$  (ppm) 7.33–7.39 (m, 1H), 7.50–7.55 (m, 1H), 7.57–7.61 (m, 1H), 8.13–8.18 (m, 1H), 8.19–8.25 (m, 1H), 8.38–8.43 (m, 1H), 8.48–8.51 (m, 1H), 8.65–8.69 (m, 1H), 8.83–8.88 (m, 1H), 9.12–9.16 (m, 1H);  $^{13}\text{C}$  NMR (126 MHz,  $\text{CDCl}_3$ ):  $\delta$  (ppm) 119.5, 122.7, 123.3, 123.5, 123.5, 124.0, 128.7, 129.0, 129.2, 131.8, 137.7, 141.1, 148.1, 148.8, 149.6, 150.7, 153.0, 168.1; HRMS (ESI+)  $m/z$  for  $\text{C}_{18}\text{H}_{11}\text{Br}_2\text{N}_6\text{O}_5^+$   $[\text{M} + \text{H}]^+$ : calcd 548.9152, found 548.9147. Anal. calcd for  $\text{C}_{18}\text{H}_{10}\text{Br}_2\text{N}_6\text{O}_5$ : C, 39.30; H, 1.83; N, 15.28; found: C, 39.22; H, 1.73; N, 15.22.

**(1,3-Bis(4-nitro-3-(trifluoromethyl)phenyl)triaz-2-en-1-yl)(pyridin-3-yl)methanone (14l).** Method B, reaction time: 10 min. Pale yellow solid; yield 36%; mp 131–133 °C (MeOH); IR: 3094, 2018, 1698, 1587, 1538, 1495, 1477, 1419, 1354, 1314, 1293, 1271  $\text{cm}^{-1}$ ;  $^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$ ):  $\delta$  (ppm) 7.52–7.58 (m, 1H), 7.65–7.72 (m, 2H), 7.75–7.80 (m, 1H), 7.81–7.85 (m, 1H), 7.92–7.98 (m, 1H), 8.11–8.16 (m, 1H), 8.17–8.22 (m, 1H), 8.85–8.92 (m, 1H), 9.08–9.13 (m, 1H);  $^{13}\text{C}$  NMR (126 MHz,  $\text{CDCl}_3$ ):  $\delta$  (ppm) 121.30 (q,  $J = 274$  Hz), 121.33 (q,  $J = 274$  Hz), 123.0 (q,  $J = 5$  Hz), 123.3, 125.21 (q,  $J = 35$  Hz), 125.25 (q,  $J = 35$  Hz), 125.6, 126.6, 126.8, 128.8, 129.1 (q,  $J = 5$  Hz), 134.1, 137.7, 138.4, 147.9, 148.3, 149.6, 150.7, 153.2, 168.89; HRMS (ESI+)  $m/z$  for  $\text{C}_{20}\text{H}_{11}\text{F}_6\text{N}_6\text{O}_5^+$   $[\text{M} + \text{H}]^+$ : calcd 529.0690, found 529.0690. Anal. calcd for  $\text{C}_{20}\text{H}_{10}\text{F}_6\text{N}_6\text{O}_5$ : C, 45.47; H, 1.91; N, 15.91; found: C, 45.39; H, 1.91; N, 15.67.

**(1,3-Bis(2-chloro-4-nitrophenyl)triaz-2-en-1-yl)(pyridin-4-yl)methanone (15e).** Method B, reaction time: 20 min. Pale violet solid; yield 63%; mp 140–142 °C (MeOH/acetone); IR: 3092, 1698, 1586, 1520, 1480, 1410, 1346, 1293, 1249, 1223, 1131  $\text{cm}^{-1}$ ;  $^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$ ):  $\delta$  (ppm) 7.28–7.33 (m, 1H), 7.59–7.63 (m, 1H), 7.68–7.72 (m, 2H), 8.08–8.14 (m, 1H), 8.30–8.34 (m, 1H), 8.34–8.39 (m, 1H), 8.49–8.54 (m, 1H), 8.85–8.93 (m, 2H);  $^{13}\text{C}$  NMR (126 MHz,  $\text{CDCl}_3$ ):  $\delta$  (ppm) 119.6, 122.8, 123.0, 123.0, 125.7, 126.3, 131.7, 133.3, 134.4, 138.7, 140.4, 148.2, 148.3, 149.1, 150.3, 168.6; HRMS for  $\text{C}_{18}\text{H}_{11}\text{Cl}_2\text{N}_6\text{O}_5$   $[\text{M} + \text{H}]^+$ : calcd 461.0162, found 461.0165. Anal. calcd for  $\text{C}_{18}\text{H}_{10}\text{Cl}_2\text{N}_6\text{O}_5$ : C, 46.87; H, 2.19; N, 18.22; found: C, 46.76; H, 2.16; N, 18.01.

**(1,3-Bis(2-fluoro-4-nitrophenyl)triaz-2-en-1-yl)(pyridin-4-yl)methanone (15i).** Method B, reaction time: 10 min. Brown solid; yield 57%; mp 136–138 °C (MeOH); IR: 3087, 1720, 1704, 1592, 1531, 1482, 1405, 1349, 1310, 1262, 1243  $\text{cm}^{-1}$ ;  $^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$ ):  $\delta$  (ppm) 7.35–7.42 (m, 1H), 7.57–7.65 (m, 1H), 7.65–7.72 (m, 2H), 7.98–8.10 (m, 2H), 8.16–8.23 (m, 1H), 8.24–8.32 (m, 1H), 8.79–8.99 (m, 2H);  $^{13}\text{C}$  NMR (126 MHz,  $\text{CDCl}_3$ ):  $\delta$  (ppm) 112.7 (d,  $J = 25$  Hz), 113.4 (d,  $J = 25$  Hz), 120.0 (d,  $J = 22$  Hz), 120.0 (d,  $J = 22$  Hz), 121.4, 123.1, 128.1 (d,  $J = 15$  Hz), 131.5, 139.9 (d,  $J = 8$  Hz), 140.2, 148.6 (d,  $J = 8$  Hz), 149.7 (d,  $J = 8$  Hz), 150.2, 156.5 (d,  $J = 260$  Hz), 157.3 (d,  $J = 260$  Hz), 168.8; HRMS (ESI+)  $m/z$  for  $\text{C}_{18}\text{H}_{11}\text{F}_2\text{N}_6\text{O}_5^+$   $[\text{M} + \text{H}]^+$ : calcd 429.0754, found 429.0750. Anal. calcd for  $\text{C}_{18}\text{H}_{10}\text{F}_2\text{N}_6\text{O}_5$ : C, 50.48; H, 2.35; N, 19.62; found: C, 50.18; H, 2.22; N, 19.36.

**(1,3-Bis(4-nitrophenyl)triaz-2-en-1-yl)(pyridin-4-yl)methanone (15j).** Method B, reaction time: 10 min. Yellow solid; yield 94%; mp 156–158 °C (MeOH/acetone); IR: 3034, 1699, 1609, 1591, 1553, 1518, 1489, 1476, 1407, 1366, 1343, 1295  $\text{cm}^{-1}$ ;  $^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$ ):  $\delta$  (ppm) 7.42–7.54 (m, 4H), 7.63–7.70 (m, 2H), 8.21–8.28 (m, 2H), 8.43–8.49 (m, 2H), 8.85–8.91

(m, 2H);  $^{13}\text{C}$  NMR (126 MHz,  $\text{CDCl}_3$ ):  $\delta$  (ppm) 100.0, 123.1, 123.2, 124.9, 125.0, 130.2, 140.2, 141.0, 148.3, 150.2, 151.4; HRMS (ESI+)  $m/z$  for  $\text{C}_{18}\text{H}_{13}\text{N}_6\text{O}_5^+$   $[\text{M} + \text{H}]^+$ : calcd 393.0942, found 393.0944. Anal. calcd for  $\text{C}_{18}\text{H}_{12}\text{N}_6\text{O}_5$ : C, 55.11; H, 3.08; N, 21.42; found: C, 54.86; H, 2.88; N, 21.45.

**(1,3-Bis(2-bromo-4-nitrophenyl)triaz-2-en-1-yl)(pyridin-4-yl)methanone (15k).** Method B, reaction time: 20 min. Pale yellow solid; yield 67%; mp 162–164 °C (MeOH/acetone); IR: 3087, 2112, 1698, 1593, 1521, 1480, 1409, 1344, 1290, 1249, 1211  $\text{cm}^{-1}$ ;  $^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$ ):  $\delta$  (ppm) 7.26–7.31 (m, 1H), 7.57–7.61 (m, 1H), 7.69–7.74 (m, 2H), 8.12–8.18 (m, 1H), 8.39–8.43 (m, 1H), 8.48–8.52 (m, 1H), 8.66–8.70 (m, 1H), 8.86–8.90 (m, 2H);  $^{13}\text{C}$  NMR (126 MHz,  $\text{CDCl}_3$ ):  $\delta$  (ppm) 119.4, 122.8, 123.0, 123.5, 123.6, 124.0, 128.7, 129.2, 131.8, 140.4, 140.7, 148.2, 148.9, 149.5, 150.3, 168.4; HRMS (ESI+)  $m/z$  for  $\text{C}_{18}\text{H}_{11}\text{Br}_2\text{N}_6\text{O}_5^+$   $[\text{M} + \text{H}]^+$ : calcd 548.9152, found 548.9149. Anal. calcd for  $\text{C}_{18}\text{H}_{10}\text{Br}_2\text{N}_6\text{O}_5$ : C, 39.30; H, 1.83; N, 15.28; found: C, 39.31; H, 1.74; N, 15.18.

**(1,3-Bis(4-nitro-3-(trifluoromethyl)phenyl)triaz-2-en-1-yl)(pyridin-4-yl)methanone (15l).** Method B, reaction time: 10 min. Pale yellow solid; yield 72%; mp 128–130 °C (MeOH/acetone); IR: 3079, 1704, 1594, 1535, 1476, 1415, 1354, 1294, 1271, 1210, 1135  $\text{cm}^{-1}$ ;  $^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$ ):  $\delta$  (ppm) 7.61–7.71 (m, 4H), 7.75–7.79 (m, 1H), 7.80–7.83 (m, 1H), 7.92–7.97 (m, 1H), 8.11–8.16 (m, 1H), 8.87–8.93 (m, 2H);  $^{13}\text{C}$  NMR (126 MHz,  $\text{CDCl}_3$ ):  $\delta$  (ppm) 121.3 (q,  $J = 274$  Hz), 121.3 (q,  $J = 274$  Hz), 122.9 (q,  $J = 5$  Hz), 123.1, 125.3 (q,  $J = 35$  Hz), 125.5 (q,  $J = 35$  Hz), 125.7, 126.6, 126.8, 129.0 (q,  $J = 5$  Hz), 134.0, 138.0, 140.2, 148.1, 148.4, 149.5, 150.3, 169.2; HRMS (ESI+)  $m/z$  for  $\text{C}_{20}\text{H}_{11}\text{F}_6\text{N}_6\text{O}_5^+$   $[\text{M} + \text{H}]^+$ : calcd 529.0690, found 529.0688. Anal. calcd for  $\text{C}_{20}\text{H}_{10}\text{F}_6\text{N}_6\text{O}_5$ : C, 45.47; H, 1.91; N, 15.91. Found: C, 45.25; H, 1.76; N, 15.63.

***N,N,N*-triethyl-3-methoxy-3-oxoprop-1-en-1-aminium 1,3-bis(4-nitro-2-(trifluoromethyl)phenyl)triaz-2-en-1-ide (16a)**. Method C. Green solid; yield 94%; mp 140–142 °C (MeOH); IR: 3735, 3649, 3103, 2988, 1720, 1651, 1599, 1576, 1494, 1457 cm<sup>-1</sup>; <sup>1</sup>H NMR (500 MHz, DMSO-*d*<sub>6</sub>): δ (ppm) 1.15 (t, *J* = 7.2 Hz, 9H), 3.57 (q, *J* = 7.2 Hz, 6H), 3.78 (s, 3H), 6.65 (d, *J* = 14.4 Hz, 1H), 7.18 (d, *J* = 14.4 Hz, 1H), 7.88–7.95 (m, 2H), 8.21–8.28 (m, 2H), 8.31–8.37 (m, 2H); <sup>13</sup>C NMR (126 MHz, DMSO-*d*<sub>6</sub>): δ (ppm) 7.5, 52.5, 54.0, 116.8, 119.4 (q, *J* = 30 Hz), 122.5, 122.9 (q, *J* = 6 Hz), 123.7 (q, *J* = 273 Hz), 127.5, 139.8, 145.7, 158.3, 163.7; HRMS (ESI<sup>+</sup>) *m/z* for C<sub>10</sub>H<sub>20</sub>NO<sub>2</sub><sup>+</sup> [M]<sup>+</sup>: calcd 186.1489, found 186.1488; HRMS (ESI<sup>-</sup>) *m/z* for C<sub>14</sub>H<sub>6</sub>F<sub>6</sub>N<sub>5</sub>O<sub>4</sub><sup>-</sup> [M]<sup>-</sup>: 422.0329, found 422.0332. Anal. calcd for C<sub>24</sub>H<sub>26</sub>F<sub>6</sub>N<sub>6</sub>O<sub>6</sub>: C, 47.37; H, 4.31; N, 13.81. Found: C, 47.30; H, 4.07; N, 13.84.

**3-Ethoxy-*N,N,N*-triethyl-3-oxoprop-1-en-1-aminium 1,3-bis(4-nitro-2-(trifluoromethyl)phenyl)triaz-2-en-1-ide (17a)**. Method C. Green solid; yield 92%; mp 128–130 °C (EtOH); IR: 3085, 2948, 1715, 1649, 1596, 1574, 1494, 1455, 1436, 1396 cm<sup>-1</sup>; <sup>1</sup>H NMR (500 MHz, DMSO-*d*<sub>6</sub>): δ (ppm) 1.15 (t, *J* = 7.1 Hz, 9H), 1.27 (t, *J* = 7.1 Hz, 3H), 3.57 (q, *J* = 7.1 Hz, 6H), 4.24 (q, *J* = 7.1 Hz, 2H), 6.64 (d, *J* = 14.4 Hz, 1H), 7.16 (d, *J* = 14.4 Hz, 1H), 7.88–7.93 (m, 2H), 8.22–8.27 (m, 2H), 8.31–8.35 (m, 2H); <sup>13</sup>C NMR (126 MHz, DMSO-*d*<sub>6</sub>): δ (ppm) 7.5, 13.9, 54.0, 61.5, 116.8, 119.4 (q, *J* = 30 Hz), 122.8, 122.9 (q, *J* = 6 Hz), 123.7 (q, *J* = 273 Hz), 127.5, 139.7, 145.6, 158.6, 163.3; HRMS for C<sub>11</sub>H<sub>22</sub>NO<sub>2</sub> [M]<sup>+</sup>: calcd 200.1645, found 200.1644; HRMS (ESI<sup>-</sup>) *m/z* for C<sub>14</sub>H<sub>6</sub>F<sub>6</sub>N<sub>5</sub>O<sub>4</sub><sup>-</sup> [M]<sup>-</sup>: calcd 422.0329, found 422.0332. Anal. calcd for C<sub>25</sub>H<sub>28</sub>F<sub>6</sub>N<sub>6</sub>O<sub>6</sub>: C, 48.23; H, 4.53; N, 13.50. Found: C, 48.19; H, 4.27; N, 13.45.

**Potassium 1,3-bis(4-nitro-2-(trifluoromethyl)phenyl)triaz-2-en-1-ide (19a)**. Method D. Violet solid; yield 93%; mp 295–297 °C (MeCN/H<sub>2</sub>O); IR: 3112, 2114, 1733, 1715, 1601, 15831505, 1465, 1436, 1415 cm<sup>-1</sup>; <sup>1</sup>H NMR (500 MHz, DMSO-*d*<sub>6</sub>): δ (ppm) 7.88–7.96 (m, 2H),

8.23–8.30 (m, 2H), 8.32–8.40 (m, 2H);  $^{13}\text{C}$  NMR (126 MHz, DMSO- $d_6$ ):  $\delta$  (ppm) 117.0, 119.5 (q,  $J = 30$  Hz), 122.9 (q,  $J = 6$  Hz), 123.7 (q,  $J = 273$  Hz), 127.6, 140.0, 157.9; HRMS (ESI $^-$ )  $m/z$  for  $\text{C}_{14}\text{H}_6\text{F}_6\text{N}_5\text{O}_4^-$  [M] $^-$ : calcd 422.0329, found 422.0327. Anal. calcd for  $\text{C}_{14}\text{H}_6\text{F}_6\text{KN}_5\text{O}_4 \times 0.5 \text{H}_2\text{O}$ : calcd C, 35.75; H, 1.50; N, 14.89; found: C, 35.74; H, 1.38; N, 14.73.

**Bacterial strains and growth conditions.** *Acinetobacter baumannii* ATCC 19606, *A. baumannii* ATCC BAA-1605, *Bacillus subtilis subsp. subtilis* ATCC 6051, *Klebsiella pneumoniae* ATCC 13882, *K. pneumoniae* ATCC 700603, *Pseudomonas aeruginosa* ATCC 27853 were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Methicilin resistant *Staphylococcus aureus* (MRSA) USA300 was obtained from the culture collection of Professor C. G. Dowson (Warwick). *Escherichia coli* D22, *E. coli* N43 CGSC 5583 and *P. aeruginosa* PA0001 were generously donated from the strain collection of Biota Ltd. *Saccharomyces cerevisiae* wild strain, *Streptococcus pneumoniae* R6, Vancomycin resistant *Enterococcus faecalis* (VREF) 501, *Escherichia coli* DH10 $\beta$  (Invitrogen) pHuLUC3/DH10 $\beta$ , MRSA wild strain 1653 and *Mycobacterium smegmatis* MC2 155 were obtained from the culture collection of Professor C. G. Dowson (Warwick). The direct colony suspension method described by the Clinical and Laboratory Standards Institute (CLSI) was used for inoculum preparation.<sup>41</sup> Inoculum suspensions were prepared from isolated colonies selected from a 20 h to 24 h growth (96 h in case of *M. smegmatis* MC2 155) on tryptic soy agar (TSA) (*A. baumannii* ATCC 19606, *A. baumannii* ATCC BAA-1605, *K. pneumoniae* ATCC 13882, *K. pneumoniae* ATCC 700603, *M. smegmatis* MC2 155), Luria broth (LB) agar (*E. coli* DH10 $\beta$ , *E. coli* D22, *E. coli* N43 CGSC 5583, *P. aeruginosa* ATCC 27853, *P. aeruginosa* PA0001), yeast extract Peptone (YEP) agar (*S. cerevisiae* wild strain) and Brain-Heart Infusion (BHI) agar



supplemented with 5% (v/v) sheep blood (Thermo Scientific) (MRSA USA300, MRSA wild strain 1653, *S. pneumoniae* R6, VREF 501).

**Determination of Minimal-Inhibitory Concentrations (MICs) and Minimal-Bactericidal Concentrations (MBCs).** All MIC determinations were performed in polystyrene 96-well plates (Falcon) covered with seals (4titude, ABgene, Excel Scientific), with at least one duplicate. Growth was recorded using a plate reader (BMG Labtech Clario star, Thermo Scientific Varioskan Flash or Labsystems iEMS Reader MF). MICs were determined in accordance with the microdilution procedure recommended by EUCAST.<sup>42</sup> The MICs for *E.coli* strains, MRSA USA300 and *Bacillus subtilis* ATCC 6051 were determined using LB broth. The final bacterial inoculum was  $2 \times 10^4$  CFU/mL and the results were read after 18–20 h aerobic incubation at 37 °C. The same protocol, but with a final bacterial inoculum of  $3 \times 10^4$  CFU/mL, was used for *P. aeruginosa* strains and the MRSA wild-type strain. The MICs against *A. baumannii* and *K. pneumoniae* strains were determined using TSB broth, with a final bacterial inoculum of  $3 \times 10^4$  CFU/mL. Inoculated microtiter plates were aerobically incubated for 18–20 h at 37 °C. We also determined MICs for the tested compounds against *S. pneumoniae* R6, with a final bacterial inoculum of  $5 \times 10^4$  CFU/mL, grown in BHI broth, the results being read after 18–20 h anaerobic incubation at 37 °C. The MICs against VREF 501 were determined using BHI broth. The final bacterial inoculum was  $3 \times 10^4$  CFU/mL and the results were read after 18–20 h aerobic incubation at 37 °C. Microtiter plates inoculated with *M. smegmatis* MC2 155 in TSB broth (final inoculum  $1 \times 10^5$  CFU/mL) were incubated aerobically for 94–98 h at 37 °C prior to recording the results. YEP broth was used for MIC determination against *S. cerevisiae* wild-type strain. The final inoculum cell density was  $1 \times 10^5$  CFU/mL and the results were read after 18–20 h aerobic incubation at 30 °C. The same protocols as above were used to determine MBCs for

the tested compounds. After the appropriate incubation time, the content of the wells at concentrations corresponding to the MIC,  $2 \times \text{MIC}$ ,  $4 \times \text{MIC}$ ,  $8 \times \text{MIC}$  (or above if necessary) were mixed, and a 10  $\mu\text{L}$  portion was plated onto the corresponding (see Bacterial Strains and Growth Conditions) antibiotic-free agar plates, and incubated for 18–20 h (in case of *M. smegmatis* MC2 155 96 h) prior to visualization. When the MIC was above 128  $\mu\text{g}/\text{mL}$ , MBC was not measured.

**Membrane integrity assay.** Cell membrane damage was quantitatively determined with fluorescence measurements taken in a microplate reader (Varioskan Flash) using LIVE/DEAD<sup>®</sup> BacLight<sup>™</sup> Bacterial Viability Kit (Invitrogen), repeated at least in triplicate. Suspension of bacterial cells, previously grown on the corresponding antibiotic-free agar plate (see Bacterial Strains and Growth Conditions), in 0.85% (w/v) NaCl solution, were divided into two equal portions and centrifuged at 4000g for 20 min. One pellet, representing live cells, was suspended in 0.85% (w/v) NaCl solution to yield an absorbance ( $\text{OD}_{600}$ ) of 0.2. The same amount of isopropanol (70% v/v) was added to the pellet, composed of dead bacterial cells. Both suspensions were incubated, with gentle shaking, for 1 h at room temperature. There were then harvested by centrifugation at 4000g for 20 min. Pellets were suspended in equal amount of 0.85% (v/v) NaCl to yield a live cell suspension of  $\text{OD}_{600}$  0.2. Different proportions of live and dead cells were mixed to obtain cell suspensions containing different ratios of live and dead cells. 100  $\mu\text{L}$  of these cell mixes were distributed into separate wells of a 96-well flat bottom black microtiter plate containing 3.2  $\mu\text{L}$  DMSO in order to create a standard curve for comparison (see Supporting Information). Additionally, 100  $\mu\text{L}$  of live cells were plated into wells containing the triazene solutions in 3.2  $\mu\text{L}$  DMSO. The plate was incubated for 30 min at 37 °C, then 100  $\mu\text{L}$  dye solution (3  $\mu\text{L}$  each component in 1 mL water) was added. After 15 min

incubation in the dark at room temperature, fluorescence was measured (excitation wavelength 485 nm, emissions 530 nm and 630 nm).

**Synergistic effects of the triazenes with commercially available antibiotics.** Synergy was determined by MIC measurement as described in the “Determination of Minimal-Inhibitory Concentrations (MICs) and Minimal-Bactericidal Concentrations (MBCs)” section. Selected ranges of different antibiotic concentrations (methicillin 0.25  $\mu\text{g/mL}$  to 256  $\mu\text{g/mL}$ , and vancomycin 0.25  $\mu\text{g/mL}$  to 256  $\mu\text{g/mL}$ ) and the corresponding triazene (0.004  $\mu\text{g/mL}$  to 0.25  $\mu\text{g/mL}$ ) were tested in all possible combinations.

**Mutation frequency determination.** Assays were carried out in triplicate. MRSA USA300 inoculums were prepared in 15 mL LB broth as described above (see “Bacterial Strains and Growth Conditions” section). After a 18–20 h incubation at 37 °C, cultures were subjected to serial tenfold dilutions in sterile PBS buffer. For cell counting, 100  $\mu\text{L}$  of each dilution was plated on BHI non-selective solid media. Cell numbers were determined following a 20–24 h incubation at 37 °C, by performing colony counts on the agar plates. For mutant cell counting 100  $\mu\text{L}$  of each dilution was plated onto selective BHI solid media containing active compound **16a** (0.03125  $\mu\text{g/mL}$ , 0.0625  $\mu\text{g/mL}$ , 0.09375  $\mu\text{g/mL}$  or 0.125  $\mu\text{g/mL}$ ). Colony counts were then performed as above. The mutation rate value was calculated as the ratio between the resistant bacterial cell counts to the parental culture cell counts.

**Genomic DNA extraction.** DNA was obtained from MRSA USA300 resistant strains obtained from the mutation rate assays. First, strains were continuously grown on selective BHI solid media plates for 20–24 h at 37 °C, with the appropriate concentration of the compound **16a**, for 10 days. DNA extraction was performed using a Wizard Genomic DNA Purification Kit

(Promega) according to the manufacturer's instructions. As lytic enzymes, both lysozyme (60  $\mu$ L, 10  $\mu$ g/mL) (Sigma Aldrich) and lysostaphin (60  $\mu$ L, 1  $\mu$ g/mL) (Sigma Aldrich) were used.

**Whole genome sequencing.** 16a-Resistant MRSA USA300 strains were used. Short reads were generated using the Illumina MiSeq platform. The genome sequence of the closely related *S. aureus* USA300\_TCH1516, available from the NCBI archive,<sup>43</sup> was used as a reference. Short reads were aligned to the reference genome using SMALT.<sup>44</sup> SNP's were identified based on the criteria described by Harris and colleagues.<sup>45</sup> Briefly, only bases with a Phred quality score above 50 were assessed (corresponding to a 99.999% base calling accuracy; www.phrap.org; www.illumina.com). A SNP was called if more than 75% of four or more reads (at least two reads aligning to the forward and reverse strand) supported the SNP. The site was otherwise identified as missing data. Short sequence insertions and deletions (indels) were identified both during the SMALT alignment process, and from using the program PINDEL<sup>46</sup> to identify split reads i.e. where only part of a read mapped to the reference.

On average, over 99.09% of reads mapped to the chosen reference. Variation was then filtered so that mutant isolates could be compared directly to the control isolate (MRSA USA300) used in the laboratory assays.

***In vitro* cytotoxicity assay.** The compound cytotoxicity was determined on the culture of primary normal human keratinocytes. Cells were grown as a monolayer culture in Dulbecco's modified Eagle's medium, DMEM (GIBCO), supplemented with 10% (v/v) fetal serum (GIBCO) at 37 °C with 5% (v/v) CO<sub>2</sub>. Cellular sensitivity to the most promising compounds was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.<sup>47</sup> Cells were seeded in 96-well tissue culture plates (3000 cells/0.18 mL medium/well) and incubated for 24 h. The compounds were dissolved in DMSO and diluted with growth medium to

different concentrations, 20  $\mu$ L of each dilution being added to each well. The highest DMSO % that we applied was 0.25%, which was not toxic to cells in the MTT assay. Each concentration was tested in quadruplicate. Following a 72 h incubation at 37  $^{\circ}$ C, the medium was aspirated, and 20  $\mu$ g of MTT dye/0.04 mL medium was added to each well. Four hours later, formazan crystals were dissolved in DMSO (0.17 mL/well), and the plates were mechanically agitated for 5 min. The optical density at 545 nm was determined on a microtiter plate reader (Awareness Technology Inc.), with each experiment being repeated three times.

## ASSOCIATED CONTENT

**Supporting Information.**  $^1$ H and  $^{13}$ C NMR spectra of new compounds. **Membrane integrity assay standard curve.** This material is available free of charge via the Internet at <http://pubs.acs.org>.

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### Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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## ABBREVIATIONS USED

ATR, attenuated total reflectance; BHI, brain-heart infusion; BLAST, basic local alignment search tool; CLSI, Clinical and Laboratory Standards Institute; DMSO, dimethyl sulfoxide; DNA, DNA; ESKAPE, *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter spp.*; HRMS, high-resolution mass spectrometry, HTH, helix-turn-helix; IR, infrared; LB, luria broth; MBC, minimal-bactericidal concentration, MIC, minimal-inhibitory concentrations; MRSA, Methicillin resistant *Staphylococcus aureus*; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NMR, nuclear magnetic resonance; R&D, research and development; SAR, structure-activity relationship; SNP, single nucleotide polymorphism; TetR, tetracycline repressor; TLC, thin-layer chromatography; VREF, vancomycin resistant *Enterococcus faecalis*; XDRTB, extensively drug-resistant tuberculosis; YEP, yeast extract peptone.

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