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# Identification of antibacterial and antiviral activities of novel fused 1,2,4-triazine esters

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**Abstract**—The in vitro biological activities of novel derivatives of methyl and ethyl 2-(4-oxo-8-aryl-2*H*-3,4,6,7-tetrahydroimidazo[2,1-*c*][1,2,4]triazin-3-yl)acetates (**3a, 3d–j**) have been evaluated and are reported. The final heterobicycles (**3a–j**) were obtained from monocyclic 1-aryl-2-hydrazonoimidazolidines (**2a–f**) by addition and cyclization reaction with fumaric acid esters. In particular, compounds **3d** and **3e** were found to exhibit comparable antibacterial potencies in vitro as that of ampicillin. Heterobicycles of the **3e**, **3g** and **3j** type were screened for their antiviral activities against the selected viruses' DNA (human adenovirus type 5-Ad-5) and RNA (human enterovirus-Echo-9). Simultaneously, their cytotoxicities towards HEK-293 and GMK cells were established. In particular, heterobicycle **3j**, completely non-toxic for GMK cells, was found to exhibit virucidal properties against Echo-9 virus justifying its further investigation as the potential antiviral agent.

## 1. Introduction

1,2,4-Triazines and their condensed derivatives occupy a pivotal position in modern medicinal chemistry, because of their high potential for biological activity. Thus, the 1,2,4-triazine ring is a prominent structural motif found in numerous natural and synthetic biologically active compounds. For example well-known antiviral drug—azaribine is structurally based on the 1,2,4-triazine scaffold.[1] In addition, certain azanucleosides, for example, 6-azacytosine and 6-azauracil, bearing the 1,2,4-triazine heterocycle, have displayed an impressive array of biological activities, among which antiviral,[2,3] antitumour[4,5] and antifungal[6] activities have been cited in the scientific literature. Furthermore, 6-azaisocytosine (3-amino-1,2,4-triazin-5(2*H*)-one), an isosteric isomer of 6-azacytosine and 6-azauracil, is of great biological interest due to its resistance to deaminase. Condensed 1,2,4-triazines found various applications as pharmaceuticals, herbicides, pesticides and dyes.[7–11] Pyrrolo[2,1-*f*][1,2,4] triazines (e.g., 4-aza-7,9-dideazapurines), congeners of substituted nucleic acid purines, showed an interesting broad spectrum of antiproliferative activity and a pronounced in vitro growth inhibitory activity against leukaemic cell lines (comparable to that of 9-deazaadenosine). Some derivatives of pyrrolo[2,1-*c*][1,2,4] triazine demonstrated inhibitory effects on the growth of a wide range of cancer cells generally at $10^{-5}$ M level and, in some cases, even at micromolar concentrations.[12,13] Some of pyrazolo[5,1-*c*][1,2,4]triazines have been designed and screened in vitro, revealing antitumour and antifungal activities.[14,15] It is noteworthy that many potential antiviral and anticancer drugs have been modelled on them.[16–19]

Recently, there is a widespread interest in the design and synthesis of novel imidazo[2,1-*c*][1,2,4]triazine derivatives because of their potential biological activities associated with their skeleton. For instance, certain synthetic derivatives of imidazo[2,1-*c*][1,2,4]triazin-4(1*H*)-one have been designed and obtained as novel bicyclic nucleosides related to 6-azaisocytosine.[20] In addition,

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promising fungi- and bacteriostatic activities of some previously designed derivatives with these heterobicyclic core system have been reported in the literature.[21,22]

However, our previous studies concerning the synthesis of biologically active nitrogen systems bearing the 1,2,4-triazine moiety revealed that the definite derivative of 2-[4-oxo-8-(4-chlorophenyl)-2*H*-3,4,6,7-tetrahydroimidazo [2,1-*c*][1,2,4]triazin-3-yl]acetic acid showed a significant activity against all Gram-negative bacterial strains tested.[23]

Prompted by these reports, and continuing our attempt to achieve important bioactive heterobicycles,[24–26] it seemed worthwhile to evaluate the antibacterial and antiviral properties of some novel derivatives of methyl and ethyl 2-(4-oxo-8-aryl-2*H*-3,4,6,7-tetrahydroimidazo[2,1-*c*][1,2,4]triazin-3-yl)acetates.

## 2. Chemistry

Heterobicyclic esters of the type **3a–j** have been synthesized by following our previously described methods as shown in Scheme 1.[27] Briefly, biologically active, monocyclic 1-aryl-2-methylthioimidazolines (**1a–f**) as the starting materials were prepared by patent pending according to Sztanke et al.[28] Treatment of compounds **1a–f** with hydrazine hydrate gave the corresponding 1-aryl-2-hydrazinoimidazolines of type **2a–f**. New derivatives of methyl 2-(4-oxo-8-aryl-2*H*-3,4,6,7-tetrahydroimidazo[2,1-*c*][1,2,4]triazin-3-yl)acetate (**3a–f**)[29] were afforded by the reaction of appropriate monocyclic 1-aryl-2-hydrazonoimidazolidines with dimethyl fumarate, whereas novel derivatives of ethyl 2-(4-oxo-8-aryl-2*H*-3,4,6,7-tetrahydroimidazo[2,1-*c*][1,2,4]triazin-3-yl)acetate (**3g–j**)[30] were obtained from respective 1-aryl-2-hydrazonoimidazolidines on reacting with diethyl fumarate. The reaction of 1-aryl-2-hydrazonoimidazolidine (1-aryl-2-hydrazinoimidazoline) derivatives with fumaric acid esters, as a novel synthetic route for preparing the polynitrogenated heterobicyclic imidazo[2,1-*c*][1,2,4]triazine scaffold, was patented by Sztanke.[29,30] The synthetic pathway in the synthesis of 2-(4-oxo-8-aryl-2*H*-3,4,6,7-tetrahydroimidazo[2,1-*c*][1,2,4]triazin-3-yl)acetic acid ester derivatives may be useful in view of biological interest in these classes of compounds. The above-mentioned heterobicycles (**3a–j**) can be obtained starting both from free base (method i) or hydroiodide in the triethylamine presence (method ii) with comparable yields (see Table 1). The reaction was carried out by refluxing the appropriate 1-aryl-2-hydrazinoimidazoline with fumaric acid esters in alcoholic medium for 6–7 h. Reaction conditions were established experimentally.

Forming the imidazo[2,1-*c*][1,2,4]triazine nucleus in this case has been proposed to proceed by a stepwise mechanism. At the first step of the reaction sequence, the addition of the nucleophilic reagents, such as monocyclic 1-aryl-2-hydrazonoimidazolidines of the **2a–f** type (containing the basic nitrogen atom of the hydrazono moiety (=N–$NH_2$)), to the *trans* olefin (dimethyl fumarate and diethyl fumarate, respectively) carbon–carbon double bond took place to give intermediate addition products (dimethyl-2-[2-(1-aryl-imidazolidin-2-ylidene)-hydrazinyl] succinates and diethyl-2-[2-(1-aryl-imidazolidin-2-ylidene)hydrazinyl] succinates, respectively). Because of the presence of the hydrogen atom on cyclic *N*-3 nitrogen of imidazolidine, the subsequent cyclocondensation of these intermediates was possible and finally afforded bicyclic polyazaheterocycles-methyl 2-(4-oxo-8-aryl-2*H*-3,4,6,7-tetrahydroimidazo[2,1-*c*][1,2,4]triazin-3-yl)acetic esters of the **3a–f** type and ethyl 2-(4-oxo-8-aryl-2*H*-3,4,6,7-tetrahydroimidazo[2,1-*c*][1,2,4]triazin-3-yl)acetic esters **3g–j** with concomitant loss of methanol and ethanol molecule, respectively.

Theoretically, the intermediate chain derivatives-products of the addition reaction may cyclize to the imidazo[2,1-*c*][1,2,4]triazine system of the **3a–j** type (methyl and ethyl 2-(4-oxo-8-aryl-2*H*-3,4,6,7-tetrahydroimidazo[2,1-*c*][1,2,4]triazin-3-yl)acetate derivatives) as illustrated in Scheme 1, or the concurrent imidazo-[2,1-*c*][1,2,4]triazole system (with loss of methyl or ethyl acetate).

The scrutiny of $^1H$ NMR, $^{13}C$ NMR, EI-MS and IR spectra has proved that under the reaction conditions, the formation of the bicyclic imidazo[2,1-*c*][1,2,4]triazine scaffold is accompanied with loss of methanol (**3a–f**) or ethanol (**3g–j**) molecule, respectively, and the concurrent course of the cyclization reaction was excluded.

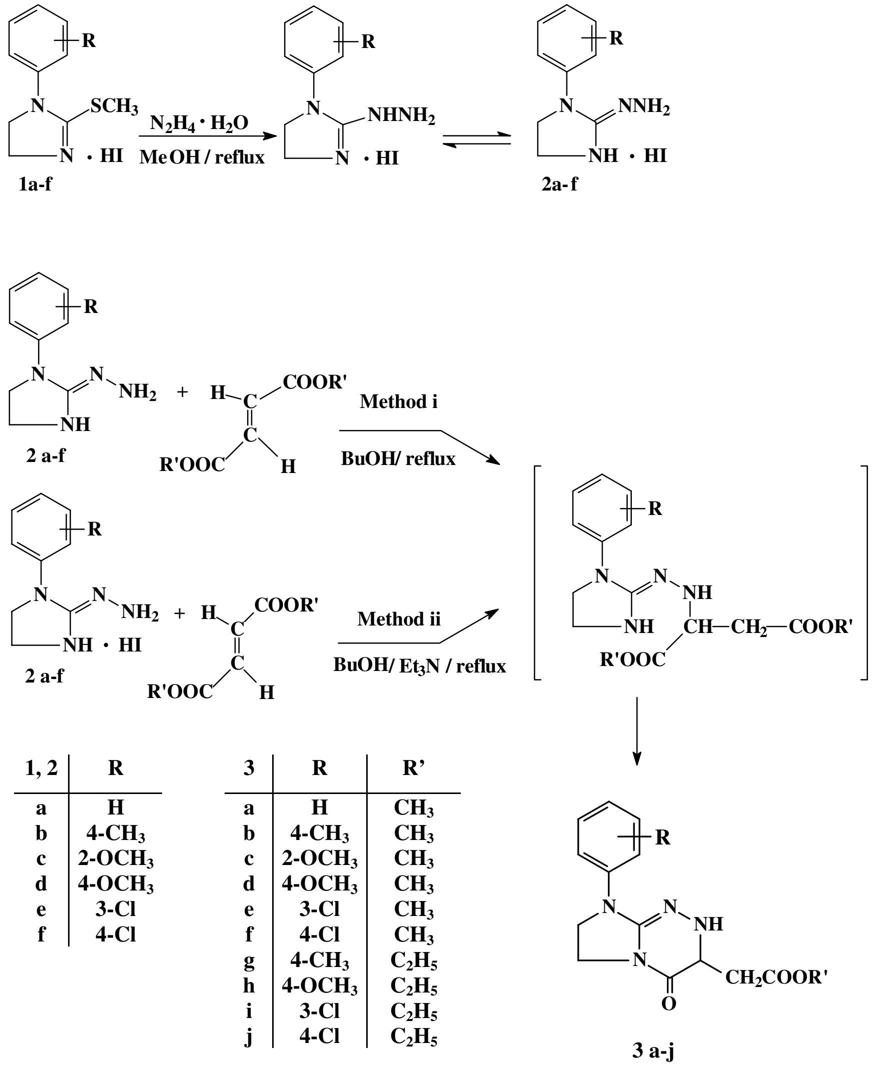
Spectral data ($^1H$ NMR, $^{13}C$ NMR, EI-MS) of all the heterobicycles synthesized, and X-ray crystallography for **3g** given in Supplementary data, were in full agreement with proposed structures (Table 1).

## 3. Results and discussion

### 3.1. Antibacterial action

Determination of the in vitro antibacterial activities of the compounds tested was performed using the disc-diffusion method by Kirby–Bauer.[31–33]

The majority of investigated heterobicycles demonstrated remarkable antibacterial activities against three (**3a**, **3f**, **3i** and **3j**) or four (**3d** and **3e**) test microorganisms (Table 2). Only compound **3h**, containing the ethoxycarbonylmethyl group on position 3 of the heterobicycle and the methoxy substituent at position 4 of the phenyl ring, revealed antibacterial effect against two of four tested strains of bacteria. It is noteworthy in this case that the replacement of the methoxycarbonylmethyl formation on position 3 of the heterobicycle in **3d** with the ethoxycarbonylmethyl moiety as in **3h** led to complete lack of activity against *Staphylococcus aureus* ATCC 25923 and *Staphylococcus epidermidis*. Simultaneously, this structural change caused slight decrease in activity against *Escherichia coli* ATCC 25922. Heterobicycles **3d** and **3e** were active against three Gram-positive (*S. aureus* ATCC 25923, *S. epidermidis*, *Streptococcus pyogenes*) and one Gram-negative (*E. coli* ATCC 25922) strains tested. Derivative **3e** was



| 1, 2 | R |
|---|---|
| a | H |
| b | 4-$CH_3$ |
| c | 2-$OCH_3$ |
| d | 4-$OCH_3$ |
| e | 3-Cl |
| f | 4-Cl |

| 3 | R | R’ |
|---|---|---|
| a | H | $CH_3$ |
| b | 4-$CH_3$ | $CH_3$ |
| c | 2-$OCH_3$ | $CH_3$ |
| d | 4-$OCH_3$ | $CH_3$ |
| e | 3-Cl | $CH_3$ |
| f | 4-Cl | $CH_3$ |
| g | 4-$CH_3$ | $C_2H_5$ |
| h | 4-$OCH_3$ | $C_2H_5$ |
| i | 3-Cl | $C_2H_5$ |
| j | 4-Cl | $C_2H_5$ |

**Scheme 1.** Synthetic pathway for the preparation of designed compounds.

**Table 1.** Physical constants of the synthesized heterobicycles

| Compound | R | R′ | M. wt. | M.F. | MP (°C) | % Yield | |
|---|---|---|---|---|---|---|---|
| | | | | | | i | ii |
| **3a** | H | 4-$CH_3$ | 288.30 | $C_{14}H_{16}N_4O_3$ | 162–164 | 63 | 57 |
| **3b** | 4-$CH_3$ | 4-$CH_3$ | 302.33 | $C_{15}H_{18}N_4O_3$ | 133–135 | 64 | 55 |
| **3c** | 2-$OCH_3$ | 4-$CH_3$ | 318.33 | $C_{15}H_{18}N_4O_4$ | 97–99 | 59 | 52 |
| **3d** | 4-$OCH_3$ | 4-$CH_3$ | 318.33 | $C_{15}H_{18}N_4O_4$ | 157–159 | 67 | 62 |
| **3e** | 3-Cl | 4-$CH_3$ | 322.75 | $C_{14}H_{15}ClN_4O_3$ | 175–176 | 61 | 56 |
| **3f** | 4-Cl | 4-$CH_3$ | 322.75 | $C_{14}H_{15}ClN_4O_3$ | 173–174 | 61 | 58 |
| **3g** | 4-$CH_3$ | 4-$C_2H_5$ | 316.35 | $C_{16}H_{20}N_4O_3$ | 142–144 | 66 | 57 |
| **3h** | 4-$OCH_3$ | 4-$C_2H_5$ | 332.35 | $C_{16}H_{20}N_4O_4$ | 133–134 | 62 | 58 |
| **3i** | 3-Cl | 4-$C_2H_5$ | 336.77 | $C_{15}H_{17}ClN_4O_3$ | 104–105 | 60 | 57 |
| **3j** | 4-Cl | 4-$C_2H_5$ | 336.77 | $C_{15}H_{17}ClN_4O_3$ | 153–154 | 64 | 57 |

found to be the most active against *S. aureus* ATCC 25923, whereas compounds **3h** and **3j** were inactive against that bacterial strain. Heterobicycles **3a**, **3d–f** and **3j** were proved to be active against *S. epidermidis*, revealing the same potencies against that bacterial strain. Simultaneously, their potencies were higher than ampicillin. However, heterobicycles **3h** and **3i** were found to be inactive against *S. epidermidis*. The majority of tested esters: **3a**, **3d** and **3h–j** were highly active against *S. pyogenes*. Simultaneously, their potencies were higher than ampicillin. Against *S. pyogenes* derivative **3f** was found to be equipotent to ampicillin, whereas **3e** showed lower activity than that of ampicillin. Heterobicycles **3d**, **3e** and **3i** demonstrated higher activities against *E. coli* ATCC 25922 than that of ampicillin. Beside this, compound **3h** was found to be equally active

**Table 2.** Antibacterial activities of evaluated compounds against the tested bacterial isolates using the disc diffusion method

| Compound | R | R′ | Microorganisms | | | |
|---|---|---|---|---|---|---|
| | | | *Staphylococcus aureus* ATCC 25923 | *Staphylococcus epidermidis* | *Streptococcus pyogenes* | *Escherichia coli* ATCC 25922 |
| **3a** | H | $CH_3$ | + | ++ | +++ | – |
| **3d** | 4-$OCH_3$ | $CH_3$ | + | ++ | +++ | +++ |
| **3e** | 3-Cl | $CH_3$ | ++ | ++ | + | +++ |
| **3f** | 4-Cl | $CH_3$ | + | ++ | ++ | – |
| **3h** | 4-$OCH_3$ | $C_2H_5$ | – | – | +++ | ++ |
| **3i** | 3-Cl | $C_2H_5$ | + | – | +++ | +++ |
| **3j** | 4-Cl | $C_2H_5$ | – | ++ | +++ | + |
| Standard | | | ++ | + | ++ | ++ |

Results were interpreted in terms of the diameter of inhibition zone: (–): <9 mm; (+): 10–15 mm; (++): 16–20 mm; (+++): >20 mm. Standard: ampicillin at concentration of 200 μg $mL^{-1}$.

as ampicillin, whereas **3j** revealed lower activity than that of ampicillin against *E. coli* ATCC 25922. However, two tested compounds: **3a** and **3f** were inactive against *E. coli* ATCC 25922.

According to the results obtained heterobicycles **3d** and **3e** were found to exhibit a comparable potency as that of ampicillin, and therefore may be considered promising for the development of novel antibacterial agents.

### 3.2. Toxicities in cell cultures and antiviral activities of the tested heterobicycles

Antiviral and cytotoxicity assays of the newly synthesized compounds **3e**, **3g** and **3j** against human adenovirus type-5 (Ad-5) in HEK-293 cell line and human enterovirus (Echo-9) in GMK cells were performed by the cytopathic effect (CPE) inhibition assay.[34] HEK-293 (Human Embryonic Kidney) cells were obtained from the American Type Culture Collection (ATCC CRL-1573). Green Monkey Kidney (GMK) cells were obtained from the European Collection of Cell Cultures (ECACC 88020401).

The toxicity of the **3e** and **3g** type heterobicycles was dependent on dose and time of incubation (Tables 3,4). Furthermore, the toxicity of all the investigated compounds depended significantly on the presence of serum. When medium supplemented with 2% foetal bovine serum (FBS) was used, the cytotoxicity of all the tested derivatives was several-fold lower than that in medium without FBS (data not shown). Compound **3j** at concentration range of 2.5–100 μg $mL^{-1}$ and 2.5–50 μg $mL^{-1}$ was totally non-toxic for GMK and HEK-293 cells, respectively, even after incubation of 24–120 h. Simultaneously, this derivative at a concentration of 200 μg $mL^{-1}$ was found to be non-toxic for GMK cells after exposure for 24–72 h. Heterobicycle of the **3e** type, in concentration range of 2.5–50 μg $mL^{-1}$, was almost non-toxic for GMK and HEK-293 cells, whereas **3g** at concentrations of 50 and 100 μg $mL^{-1}$ was partially toxic towards these normal cell lines.

The conducted experiments revealed that the examined heterobicycles of the **3g** and **3j** type possessed virucidal activity against one of both tested viruses that belong to different families: human adenovirus type-5 (Ad-5, DNA virus, ATCC CRL-1573 from the Adenoviridae family) and human enterovirus (Echo-9, RNA virus, derived from the Picornaviridae family). However, derivative **3e** revealed moderate antiviral activity against both tested viruses.

During 1 h of contact with the appropriate virus all the tested heterobicycles caused the decrease in titres of viruses by 0.27–1.17 log (Table 5). The level of inhibition of human adenovirus type 5 titre for all the solutions of compound **3g** (50, 100 and 200 μg $mL^{-1}$) was found to be about 23%, whereas for derivative **3e** it was about 8% at a concentration of 200 μg $mL^{-1}$. Derivative **3e** at a concentration of 100 μg $mL^{-1}$ evoked the decrease in the titre of Echo-9 virus by 0.33 log, which corresponds to the level of inhibition 9.9%. Moreover, this derivative caused the decrease in the titre of Ad-5 virus by 0.27 log, which corresponds to the level of inhibition 7.7%. However, heterobicycle **3j** at concentration of 50, 100 and 200 μg $mL^{-1}$ evoked the decrease in the titre of Echo-9 virus by 0.44, 1.00 and 1.17 log, respectively (which correspond to the following percentage levels of inhibition: 9.4%, 21.4% and 25.0%). Simultaneously, this compound was found to be inactive against human adenovirus type-5. However, compound **3g** was proved to be completely inactive against Echo-9 virus (Table 5).

Regarding the results obtained from both examined ethyl 2-(4-oxo-8-aryl-2*H*-3,4,6,7-tetrahydroimidazo[2,1-*c*][1,2,4]-triazin-3-yl)acetates (**3g** and **3j**), these showed that the difference of activity is due to different substituents present at the phenyl ring. Thus, the presence of lipophilic, electron-withdrawing substituent such as a chloro group at position 4 of the phenyl ring in compound **3j** seemed to be responsible for the action against Echo-9 virus and lack of activity against Ad-5 strain. However, when the position of the phenyl ring was substituted with lipophilic, weak electron-donating 4-$CH_3$-group (**3g**), good antiviral activity against adenovirus type 5 was obtained. Simultaneously, this structural change led to complete lack of activity against Echo-9 virus.

In conclusion heterobicycle **3j**, non-toxic for GMK cells, demonstrated virucidal properties against Echo-9 virus justifying its further investigation as the potential antiviral agent. Therefore, further work on this compound is in progress.

**Table 3.** Effect of tested concentrations of the examined heterobicycles (**3e**, **3g** and **3j**) on the viability of normal GMK cells

| Compound | Concentration ($\mu$g mL$^{-1}$) | Cell viability (in %) | | | | |
|---|---|---|---|---|---|---|
| | | Exposure time | | | | |
| | | 24 h | 48 h | 72 h | 96 h | 120 h |
| **3e** | 2.5 | 100 | 100 | 100 | 100 | 100 |
| | 5 | 100 | 100 | 100 | 100 | 100 |
| | 10 | 100 | 100 | 100 | 100 | 100 |
| | 25 | 100 | 100 | 95 | 95 | 95 |
| | 50 | 100 | 95 | 95 | 90 | 90 |
| | 100 | 80 | 50 | 20 | 0 | 0 |
| | 200 | 80 | 10 | 20 | 0 | 0 |
| **3g** | 2.5 | 100 | 100 | 100 | 100 | 100 |
| | 5 | 100 | 100 | 100 | 100 | 100 |
| | 10 | 100 | 100 | 100 | 95 | 90 |
| | 25 | 100 | 90 | 85 | 80 | 70 |
| | 50 | 90 | 80 | 80 | 70 | 70 |
| | 100 | 90 | 80 | 70 | 70 | 70 |
| | 200 | 0 | 0 | 0 | 0 | 0 |
| **3j** | 2.5 | 100 | 100 | 100 | 100 | 100 |
| | 5 | 100 | 100 | 100 | 100 | 100 |
| | 10 | 100 | 100 | 100 | 100 | 100 |
| | 25 | 100 | 100 | 100 | 100 | 100 |
| | 50 | 100 | 100 | 100 | 100 | 100 |
| | 100 | 100 | 100 | 100 | 100 | 100 |
| | 200 | 100 | 100 | 100 | 95 | 95 |

**Table 4.** Effect of the tested concentrations of examined heterobicycles (**3e**, **3g** and **3j**) on the viability of normal HEK-293 cells

| Compound | Concentration ($\mu$g mL$^{-1}$) | Cell viability (in %) | | | | |
|---|---|---|---|---|---|---|
| | | Exposure time | | | | |
| | | 24 h | 48 h | 72 h | 96 h | 120 h |
| **3e** | 2.5 | 100 | 100 | 100 | 100 | 100 |
| | 5 | 100 | 100 | 100 | 100 | 100 |
| | 10 | 100 | 100 | 100 | 100 | 100 |
| | 25 | 100 | 100 | 98 | 95 | 95 |
| | 50 | 95 | 95 | 95 | 90 | 90 |
| | 100 | 98 | 50 | 55 | 50 | 50 |
| | 200 | 98 | 40 | 35 | 35 | 30 |
| **3g** | 2.5 | 100 | 100 | 100 | 100 | 100 |
| | 5 | 100 | 100 | 100 | 100 | 100 |
| | 10 | 100 | 100 | 100 | 100 | 100 |
| | 25 | 100 | 100 | 100 | 95 | 95 |
| | 50 | 90 | 80 | 80 | 70 | 75 |
| | 100 | 70 | 80 | 70 | 70 | 70 |
| | 200 | 30 | 20 | 15 | 15 | 15 |
| **3j** | 2.5 | 100 | 100 | 100 | 100 | 100 |
| | 5 | 100 | 100 | 100 | 100 | 100 |
| | 10 | 100 | 100 | 100 | 100 | 100 |
| | 25 | 100 | 100 | 100 | 100 | 100 |
| | 50 | 100 | 100 | 100 | 100 | 100 |
| | 100 | 95 | 90 | 70 | 70 | 50 |
| | 200 | 50 | 30 | 25 | 25 | 20 |

## 4. Experimental protocols

### 4.1. General procedure for the preparation of methyl 2-(4-oxo-8-aryl-2*H*-3,4,6,7-tetrahydroimidazo[2,1-*c*][1,2,4]-triazin-3-yl)acetate (method i)

Free base of 1-aryl-2-hydrazonoimidazolidine (0.05 mol) was dissolved in 80 mL of *n*-butanol. Dimethyl fumarate of 0.05 mol (7.21 g) was added and the mixture was heated under reflux for 7 h. During that time precipitation of the solid started. The mixture was refrigerated overnight and the precipitation yielded was collected and purified by crystallization from DMF/methanol mixture (see Supplementary material).

**Table 5.** Virucidal activity of the tested heterobicycles (**3e**, **3g** and **3j**)

| Virus type | Compound | Concentration ($\mu$g mL$^{-1}$) | Virus titre ($TCID_{50}$ mL$^{-1}$)[a] |
|---|---|---|---|
| Ad-5 | **3e** | 0 | 3.50 ± 0.41 |
| | | 50 | 3.50 ± 0.41 |
| | | 100 | 3.50 ± 0.41 |
| | | 200 | **3.23 ± 0.49** |
| | **3g** | 0 | 3.23 ± 0.49 |
| | | 50 | **2.50 ± 0.32** |
| | | 100 | **2.50 ± 0.32** |
| | | 200 | **2.50 ± 0.32** |
| | **3j** | 0 | 3.50 ± 0.41 |
| | | 50 | 3.50 ± 0.41 |
| | | 100 | 3.50 ± 0.41 |
| | | 200 | 3.50 ± 0.41 |
| Echo-9 | **3e** | 0 | 3.33 ± 0.36 |
| | | 50 | 3.33 ± 0.36 |
| | | 100 | **3.00 ± 0.45** |
| | | 200 | 3.33 ± 0.36 |
| | **3g** | 0 | 3.67 ± 0.37 |
| | | 50 | 3.67 ± 0.37 |
| | | 100 | 3.67 ± 0.37 |
| | | 200 | 3.67 ± 0.37 |
| | **3j** | 0 | 4.67 ± 0.37 |
| | | 50 | **4.23 ± 0.49** |
| | | 100 | **3.67 ± 0.37** |
| | | 200 | **3.50 ± 0.41** |

The virus titres are shown in log ± SD.

[a] Incubation of virus with compounds—1 h at 37 °C.

### 4.2. General procedure for the synthesis of methyl 2-(4-oxo-8-aryl-2*H*-3,4,6,7-tetrahydroimidazo[2,1-*c*][1,2,4]-triazin-3-yl)acetate (method ii)

Dimethyl fumarate of 0.05 mol (7.21 g) was added to the suspension of appropriate 1-aryl-2-hydrazonoimidazolidine hydroiodide (0.05 mol) in 80 mL of *n*-butanol. The mixture was stirred and triethylamine (5 mL) was added. The reaction was carried out under reflux for 6 h. During that time precipitation of solid started. The crude product obtained after cooling was collected, washed off with cold methanol and finally purified by recrystallization from DMF/methanol mixture (see Supplementary material).

### 4.3. General procedure for preparation of ethyl 2-(4-oxo-8-aryl-2*H*-3,4,6,7-tetrahydroimidazo[2,1-*c*][1,2,4]triazin-3-yl)acetate (method i)

Free base of 1-aryl-2-hydrazonoimidazolidine (0.05 mol) was dissolved in 80 mL of *n*-butanol. Diethyl fumarate of 0.05 mol (8.61 g) was added and the mixture was heated under reflux for 7 h. During that time precipitation of the solid started. The mixture was refrigerated overnight and the precipitation yielded was collected and purified by crystallization from DMF/methanol mixture (see Supplementary material).

### 4.4. General procedure for the synthesis of ethyl 2-(4-oxo-8-aryl-2*H*-3,4,6,7-tetrahydroimidazo[2,1-*c*][1,2,4]triazin-3-yl)acetate (method ii)

Diethyl fumarate of 0.05 mol (8.61 g) was added to the suspension of appropriate 1-aryl-2-hydrazonoimidazolidine hydroiodide (0.05 mol) in 80 mL of *n*-butanol. The mixture was stirred and triethylamine (5 mL) was added. The reaction was carried out under reflux for 6 h. During that time precipitation of solid started. The crude product obtained after cooling was collected, washed off with cold methanol and finally purified by recrystallization from DMF/methanol mixture (see Supplementary material).

### 4.5. Microbiology

**4.5.1. Disc diffusion assay for in vitro testing.** Assay of antimicrobial activity in vitro. The synthesized heterobicycles of the **3a**, **3d–j** type were screened for their antibacterial activities by the disc-diffusion method according to Kirby-Bauer, using Mueller–Hinton medium for bacteria. Two reference strains of bacteria—*S. aureus* ATCC 25923 (Gram-positive bacterial strain), *E. coli* ATCC 25922 (Gram-negative bacterial strain) and two Gram-positive clinical isolates (*S. epidermidis* and *S. pyogenes*) were included in these studies. The clinical isolates were identified with conventional morphological and biochemical methods. In the disc-diffusion method, sterile paper discs ($\phi$ 5 mm) impregnated with dimethylsulfoxide (DMSO) compound at concentration of 100 $\mu g\,mL^{-1}$ were used. Discs containing DMSO were used as a control. Then, the paper discs impregnated with the solutions of the compound tested were placed on the surface of the media inoculated with the microorganism. The plates were incubated at 35 °C/24 h for the microorganism's cultures. After incubation, the growth inhibition zones around the discs were observed indicating that the examined compound inhibits the growth of microorganism.[31,32] The antibacterial activity was evaluated by measuring the zone of inhibition against the test microorganism. Ampicillin (200 $\mu g\,mL^{-1}$) was used as a standard drug. Dimethylsulfoxide (DMF) was used as a solvent control. Results were interpreted in terms of the diameter of inhibition zone: (−): <9 mm; (+): 10–15 mm; (++): 16–20 mm; (+++): >20 mm and are shown in Table 2. Each assay in this experiment was repeated three times.

The presented results were obtained from three independent measurements. The investigations were carried out in the Department of Medical Microbiology, Medical University, Lublin, Poland.

**4.5.2. Cell cultures.** GMK (Green Monkey Kidney) cells were obtained from the European Collection of Cell Cultures (ECACC 88020401). HEK-293 cells (ATCC CRL-1573) were generated by transformation of human embryonic kidney cell cultures with sheared adenovirus 5 DNA. They were grown in Eagle's Minimal Essential Medium (MEM, Sigma) supplemented with 10% foetal bovine serum (FBS, Sigma) and 100 $U\,mL^{-1}$ of penicillin (Polfa, Tarchomin) and 100 $\mu g\,mL^{-1}$ of streptomycin (Polfa, Tarchomin). The cell culture was incubated in a humidified atmosphere with 5% of $CO_2$.

**4.5.3. Toxicity in cell cultures.** All the investigated compounds were dissolved in dimethylsulfoxide (DMSO, Sigma) 10 $\mu g\,mL^{-1}$ and then diluted in cell culture media supplemented with 2% foetal bovine serum (FBS). GMK and HEK-293 cells were plated into 96-well plastic plates (Nunc, Denmark) at a cell density, $2 \times 10^4$ cells per well in Eagle's Minimal Essential Medium (MEM) supplemented with 10% foetal bovine serum (FBS, Sigma) and 100 $U\,mL^{-1}$ of penicillin (Polfa, Tarchomin) and 100 $\mu g\,mL^{-1}$ of streptomycin (Polfa, Tarchomin) to obtain the confluent growth of cells. After 24 h of incubation at 37 °C, the media from cell cultures were removed and cells were treated with derivatives, diluted in media at final concentrations of 200, 100, 50, 25, 10, 5 and 2.5 $\mu g\,mL^{-1}$. Cell cultures were incubated at 37 °C for 24–120 h. The toxicity was estimated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (e.g., MTT) that is cleaved into a coloured formazan product by metabolically active cells according to the assay previously described by Takenouchi and Munekata.[35] All experiments were done in triplicate. The investigations were carried out in the Department of Virology, Medical University, Lublin, Poland.

**4.5.4. Evaluation of virucidal activity of the tested heterobicycles.** A suspension of viruses was mixed (1:1 v/v) with derivatives, which were diluted in media without foetal bovine serum (FBS, Sigma) to final concentrations of 50, 100 and 200 $\mu g\,mL^{-1}$. Mixtures were incubated at 37 °C for 1 h and then viruses were titrated in appropriate cell cultures (Ad-5 in HEK-293 line and Echo-9 in GMK line). The viruses' suspension (Ad-5 and Echo-9) with media but without tested derivatives was a control. The cytopathic effect of virus (CPE) occurring after 24 h of incubation was determined by using the Reed–Muench method.[36,37] Median tissue culture infective doses ($TCID_{50}$) of the tested derivatives are given in Table 3. The presented results were obtained from three independent measurements. The investigations were carried out in the Department of Virology, Medical University, Lublin, Poland.

## Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2007.05.048.

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