European Journal of Medicinal Chemistry 74 (2014) 664–670

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Antimycobacterial activity of nitrogen heterocycles derivatives: Bipyridine derivatives. Part III [13,14]

Ramona Danac, Ionel I. Mangalagiu*

"Al. I. Cuza" University of Iasi, Organic and Biochemistry Department, Bd. Carol 11, 700506 Iasi, Romania

ARTICLE INFO

Article history:
Received 31 May 2013
Received in revised form
2 September 2013
Accepted 4 September 2013
Available online 7 November 2013

Keywords:
Antimycobacterial
Bipyridine
Bis-fused pyridine
MIC
MBC
LORA
MTT

ABSTRACT

Three classes of fused bipyridine heterocycles were designed, synthesized and evaluated for their antimycobacterial activities. The method for preparation of fused bipyridine derivatives is straight and efficient. The primary antimycobacterial screening reveals that mono-indolizine mono-salts are displaying potency superior to the second-line antitubercular drugs Cycloserine and Pyrimethamine and, equal as the first line anti-TB Ethambutol. The data from Cycle-2 screening assay (MIC, MBC, LORA, intracellular (macrophage) drug screening, and MTT cell proliferation) confirm the promising anti-TB results from Cycle-1 for mono-indolizine mono-salts. These data indicate that mono-indolizine mono-salt **6d** is a potent compound against both replicating and non-replicating *Mycobacterium tuberculosis*, is active against both extracellular and intracellular organisms, has a bacteriostatic mechanism of action and has basically no toxicity. We see no influence concerning the anti-TB activity of the fused-pyridine substituents.

1. Introduction

Throughout history, tuberculosis (TB), a contagious disease caused by *Mycobacterium tuberculosis*, has claimed the lives of over one billion people and currently infects one third of the world's population. According to the World Health Organization (WHO), TB remains the leading infectious disease among humans, annually being registered about 9 (nine) million new cases and about 1.5 million people are dying from TB [1]. In spite of major advances that have been made in the drug discovery process over the last 40 years, no new drugs have been developed specifically against *M. tuberculosis* [2]. More than ever, there is an urgent need to develop new antitubercular drugs to combat the spread of TB, to reduce the total duration of treatment and to provide more effective treatment against multidrug resistance (MDR), extensive drug resistance (XDR) and latent tuberculosis infection [3]. In addition, tuberculosis is one of the opportunistic infections in AIDS patients, when *M. tuberculosis* enters into contact with highly immunocompromised HIV-infected populations, the mortality rate within a few weeks of infection approaches 100% [4]. There are currently two main strategies for the development of new anti-TB drugs [3,5]. One of them is based on the synthesis of analogs of existing drugs, with the aim of shortening and improving TB treatment. The other involves a search for novel structures that *M. tuberculosis* has never been challenged before. Many of these novel structures that *M. tuberculosis* has never encountered with before, are fused nitrogenv heterocycles, especially pyridine fused systems [6,7]. Particularly, the imidazo-pyridine class emerged as the most promising by having potency similar to isoniazid against replicating *M. tuberculosis* H37Rv [7].

In our previous research work we showed that some fused pyridine and diazine derivatives posses antimicrobial activity [8–12], antimycobacterial including [13,14]. In these studies, we noted that particular fused pyridine and diazine moieties display appreciable antimicrobial and anti-TB activity.

As continuation of our work in the class of fused nitrogen heterocycles and as part of our concern in the field of biologically active compounds, we report here the synthesis, structure and *in vitro* antimycobacterial activity of some fused 4,4′-bipyridine derivatives.

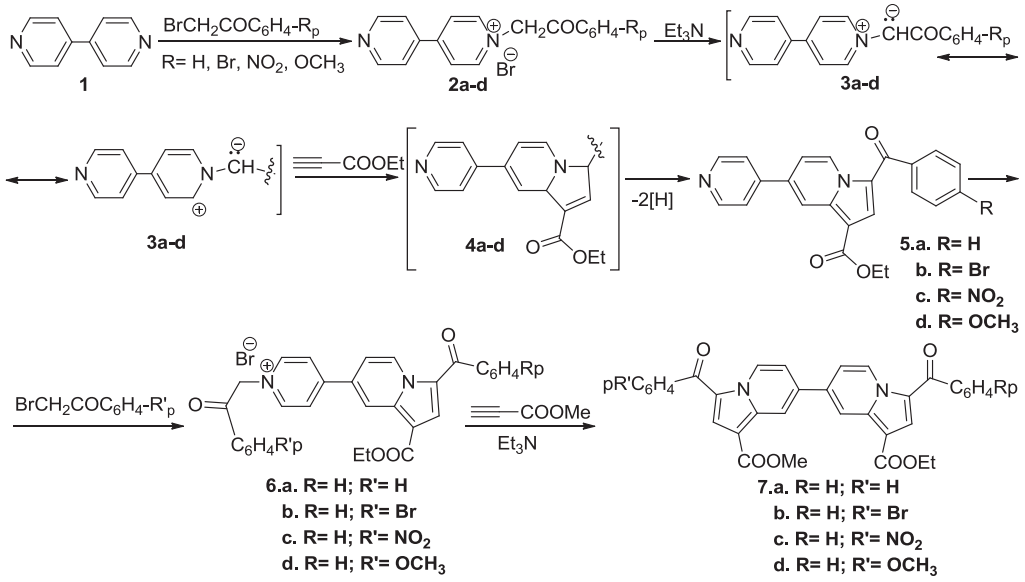
2. Results and discussion

2.1. Chemistry

The strategies adopted for synthesis of fused 4,4′-bipyridine derivatives, are depicted in Schemes 1 and 2. Synthesis of the

* Corresponding author. Tel.: +40 232 201343; fax: +40 232 201313.
E-mail address: ionelm@uaic.ro (I.I. Mangalagiu).

0223-5234/$ – see front matter © 2013 Elsevier Masson SAS. All rights reserved.
http://dx.doi.org/10.1016/j.ejmech.2013.09.061



Scheme 1. Reaction pathway to obtain fused 4,4′-bipyridine derivatives **5**–**7**.

compounds **5**–**7**, Scheme 1, has been described previously by part of our group [9,15].

In this respect, 4,4′-bipyridinium mono-ylides **3a**–**d** (generated *in situ* from the corresponding mono-salts **2a**–**d**, in alkaline medium Et_3N), react with ethyl propiolate via a Huisgen [3 + 2] dipolar cycloaddition, leading in the first step to dihydroindolizines intermediates (**4a**–**d**), and finally to the fully aromatized mono-indolizines **5a**–**d**. Following the same algorithm (obtaining of salts **6a**–**d**, then cycloaddition), bis-indolizines **7a**–**d** were obtained, Scheme 1.

Synthesis of the compounds **9a**,**b**, Scheme 2, is described herein and involves a similar strategy: obtaining the corresponding mono-salts **8a**–**d** [16,17], followed by a Huisgen [3 + 2] dipolar cycloaddition.

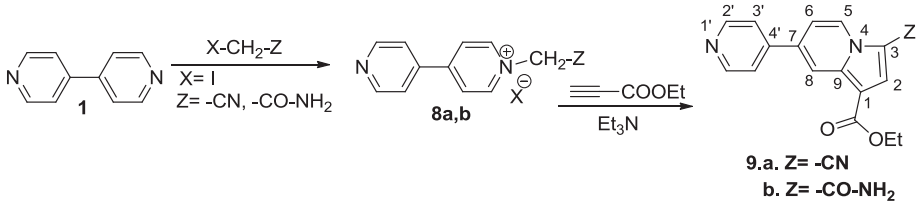
The structure of compounds was assigned by elemental and spectroscopic analysis: IR, ^{1}H NMR, ^{13}C NMR, COZY, HMQC, HMBC and X-ray diffraction for compound **9a**. The ^{1}H NMR spectra of **9a**,**b** are characterized by protons of the ester (a triplet at δ = 1.37–1.45 ppm and a quartet at δ = 4.34–4.35 ppm) and amide (7.04 and 7.35 ppm for compound **9b**) groups. The ^{1}H NMR spectrum of compound **9b** shows a low field absorption (δ = 9.83 ppm) for the proton H5 of indolizine ring. The same deshielding was as well observed in the case of other indolizines having a C=O group at position 3 [15,18], and it is caused by the proximity of nearby carbonyl group. Interestingly, the same proton H5 appears more shielded in NMR spectrum (8.42 ppm) of compound **9a** which has a linear –C≡N group as substituent at position 3. In ^{13}C NMR spectra the most important data are furnished by carbonyl ester groups, carbonyl amide group (compound **9b**) and nitrile group (compound **9a**) and those ones furnished by carbons from pyrrolo ring (C_1, C_2, C_3, C_9). Thus, the most deshielded carbons are those of carbonyl ester groups which appear around 163.1 ppm (**9a**) and 163.3 ppm (**9b**), followed by carbon of carbonyl amide group which appears around 162.5 ppm (**9b**). Carbon of nitrile group appears at 112.3 ppm (**9a**). The signals of carbon atoms (C_1, C_2, C_3, C_9) in the new pyrrolo ring appear at high chemical shifts (97–138 ppm), which is also a strong evidence for the aromatized structures. All the remaining signals from NMR spectra are in accordance with the proposed structures.

To establish unequivocally the structure of these fused pyrrolo–pyridine derivatives, the X-ray data analysis was performed in the case of compound (**9a**), Fig. 1.

The X-ray structure fully confirms the proposed structure, this molecule being a flat coplanar fused pyrrolo–pyridine system (the torsion angles related to the two fused cycle are closely to 0° or 180°) and with the ethyl group from carboxylate moiety slightly rotated out of plane [torsion angle C16–O2–C15–O1 (−3.5°), C16–O2–C15–C11 (176.5°)]. Full information concerning X-ray structure could be found in the Cambridge Crystallographic Data Centre, the CCDC 941837 structure.

2.2. Design, biological activity and mechanism of action

Taking into consideration the promising antimycobacterial activity of some fused pyridine derivatives [6,7], as well as our previous results in the field of anti-TB derivatives [13,14], we have focused on the design of novel structures that incorporate a bis-fused pyridine unit in the molecular scaffold. In this respect, we chose 4,4′-bipyridine heterocycle as main skeleton for synthesis of mono-indolizines, bis-indolizines and mono-indolizine mono-salts derivatives. In equal measure we were interested to study the



Scheme 2. Reaction pathway to obtain fused 4,4′-bipyridine derivatives **9**.

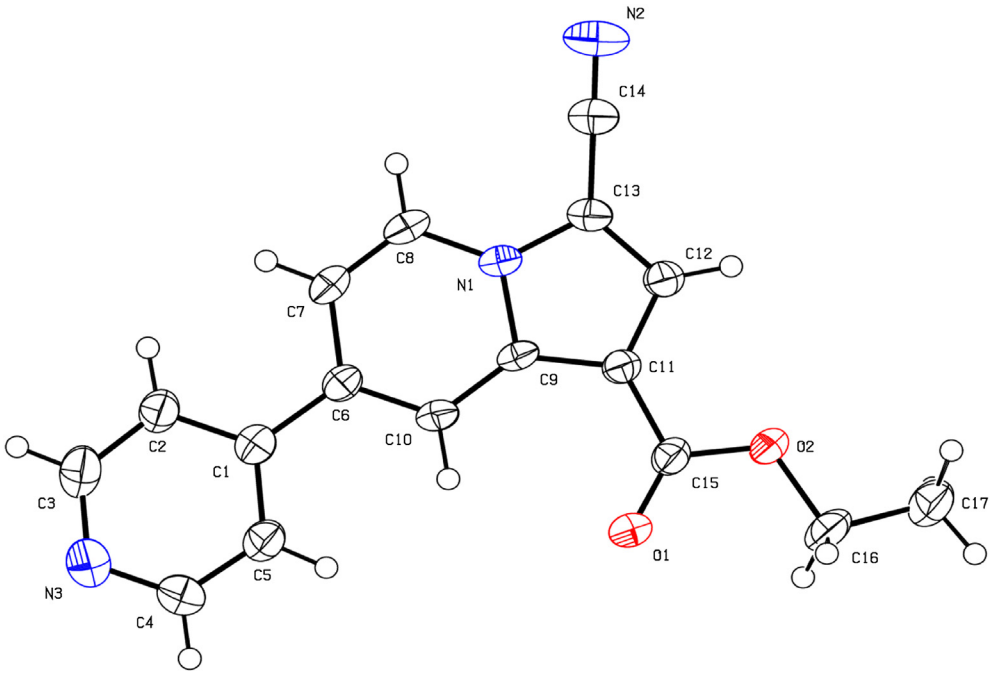
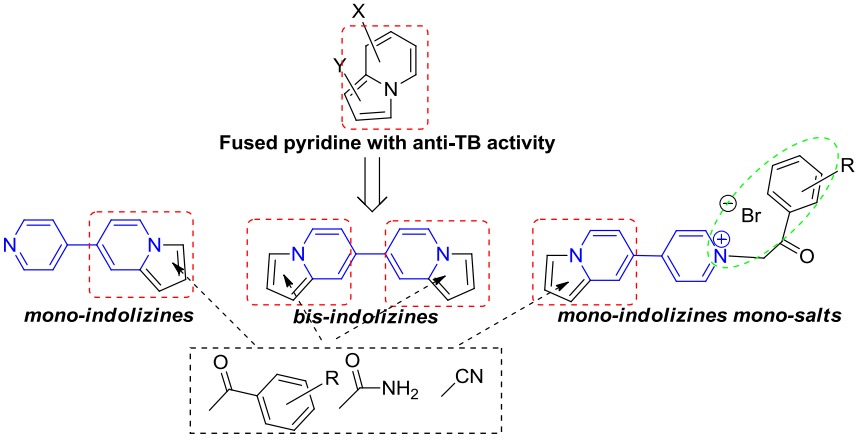


Fig. 1. The ORTEP diagram of the compound (**9a**) including atom numbering scheme. Acquisition parameters. Radiation source: fine-focus sealed tube. Graphite monochromator. Detector resolution: 16.15 pixels mm^{-1}, ω scans. Intensity data were collected using Mo-Kα radiation (λ = 0.71073 Å) with the crystal cooled in a stream of nitrogen from a Cryostream collar (200.00 K during data collection). Crystal and experimental data. Crystal system: monoclinic; space group Cc with a = 12.6169(11) Å, b = 17.3233(11) Å, c = 6.8645(6) Å, α = 90.00°, β = 109.003(9)°, γ = 90.00°, V = 1418.57(19). Goodness of fit for all reflections 1.030.

influence of the fused-pyridine substituents concerning the antimycobacterial activity, Scheme 3.

A selection of compounds, one representative per each series, was evaluated for *in vitro* antimycobacterial activity against *M. tuberculosis*, as a part of the TAACF TB screening program under direction of the US National Institute of Health, the NIAID division. A standard primary *in vitro* screen was conducted against *M. tuberculosis* H37Rv (ATCC 27294), in BACTEC 12B medium using the Microplate Alamar Blue Assay (MABA) [16,17]. Compounds that are active in this assay are reconfirmed using a BACTEC 460 Radiometric System. Compounds demonstrating a percent inhibition of bacterial growth □90% in the primary screen were tested in the next stage of the procedure, against *M. tuberculosis* H37RV to determine the *Minimum Inhibitory Concentration* (MIC) in the MABA. Compounds were tested in 10 twofold dilutions, typically from 100 μg/mL to 0.195 μg/mL. Rifampicin, Pyrimethamine, Isoniazid, Ethambutol, Cycloserine and Amikacin were used as reference compounds in these assays. MIC was defined as the lowest concentration effecting a reduction in fluorescence of 90%, relative to control. This value was determined from the dose–response curve as the IC_{90} using a curve-fitting program. IC_{90} is defined as the concentration effecting a reduction in fluorescence of 90% relative to controls. Compounds with IC_{90} values □10 μg/mL were considered active antitubercular agents. The results are summarized in Table 1.

As shown in Table 1, compounds **5a**, **7d**, and **9b** did not exhibit antimycobacterial activity (having MIC >100 μg/mL when tested in MABA), while compound **9a** showed weak inhibitory activity against *M. tuberculosis* (IC_{50} = 33.35, IC_{90} = 59.52). Notably, **6d** was identified as a potent inhibitor against *M. tuberculosis*, with a MIC value of 12.50 μg/mL in the MABA assay and its antimycobacterial activity was superior to the second-line antitubercular drugs Cycloserine (MIC = 25 μg/mL) and Pyrimethamine



Scheme 3. Design in the class of fused 4,4′-bipyridine derivatives.

Table 1
Antimycobacterial activity against *M. tuberculosis* H37Rv in the MABA assay.

Compd	Antimycobacterial activity % inhibition[a] (μg/mL)	IC_{50} (μg/mL)	IC_{90} (μg/mL)	MIC (μM)
6d	100, 50, 25, 12.5(98.50), 6.25 (65.10), 3.13 (38.60), 0.79 (9.90)	3.88	13.07	**12.50**
9a	100(97.20), 50(84.90), 25(21.50), 12.5 (15.30), 0.79 (8.60)	33.35	59.52	>100
5a	100(-8.60), 6.5 (0.20), 3.13(3.90), 1.56 (3.0)	>100	>100	>100
7d	100(-4.50), 50(5.30), 12.5(0.80), 1.56 (−2.0)	>100	>100	>100
9b	100(-17.0), 50(2.0), 12.5(4.0), 1.56 (2.80)	>100	>100	>100
Cycloserine	100(97.70), 50(96.30), 25(93.90), 12.5(51)	12.47	13.49	**25.00**
Pyrimethamine	100(95.60), 50(69.70), 25(26.70)	37.35	74.96	**100.00**
Amikacin	5, 2.5, 1.25(98), 0.63(97.30), 0.31, 0.16(96), 0.08(79.80)	0.07	0.08	0.16
Isoniazid	5, 2.5, 1.25(94), 0.63(93.90), 0.31(92.90)	0.18	0.29	0.31
Ethambutol	100(90.00), 50, 25, 12.5(89), 6.25 (86.60), 3.13 (84.30), 1.56 (69.10)	<1.56	32.79	12.50
Rifampicin	5, 2.5, 1.25, 0.63, 0.31, 0.16(99.50), 0.08(97.60), 0.04(91.90), 0.02(61.20)	0.02	0.02	0.04

[a] Antimycobacterial activity was typically determined at concentrations 100, 50, 25, 12.5, 6.25, 3.13, 1.56, 0.79, 0.4 and 0.2 μg/mL.

(MIC = 100 μg/mL), Table 1. Moreover, **6d** have a MIC value equal to the first line anti-TB Ethambutol.

The data from Table 1 illustrate that mono-indolizine mono-salt derivative **6**, displays a better antimycobacterial activity comparative with bis-indolizine **7**, suggesting a possible influence of bromine anion concerning antimycobacterial activity.

Compound **6d** found promising in primary TB assay, was then subjected to the secondary assay for evaluation of antimycobacterial activity. These assays included MIC, Minimal Bactericidal Concentration (MBC), Low-Oxygen Recovery Assay (LORA), intracellular (macrophage) drug screening, and MTT cell proliferation.

Minimum Inhibitory Concentration (MIC). The MIC screening was conducted for *M. tuberculosis* H37Rv (SRI 1345), isoniazid (INH)-resistant *M. tuberculosis* (SRI 1369), rifampin (RMP)-resistant *M. tuberculosis* (SRI 1367), and ofloxacin (OFX)-resistant *M. tuberculosis* (SRI 4000). MBC, LORA and intracellular drug screening assays were conducted using only *M. tuberculosis* H37Rv (SRI 1345). The MIC for each compound was determined by testing ten, two-fold dilutions in the following concentration ranges: 75 to 0.146 μg/mL for MIC and LORA, respectively 75, 7.5 and 0.75 μg/mL for macrophage and MTT.

MIC is reported as the lowest concentration (μg/mL) of drug that visually inhibited growth of the organism. In addition, the percentage of inhibition at MIC is provided. These data are briefly presented in Table 2.

The MIC was 2.34 μg/mL and the percentage of inhibition at MIC concentration was 51%, suggesting that compound **6d** is potent against replicating *M. tuberculosis* H37Rv (SRI 1345). The results obtained against drug resistant strains exhibited similar values of MIC.

Minimal Bactericidal Concentration (MBC). The MBC is determined subsequent to MIC testing by sub-culturing diluted aliquots from wells that fail to exhibit macroscopic growth. The MBC is defined as the lowest concentration (μg/mL) of compound exhibiting 99.9% kill over the same time period used to determine MIC (18–24 h). MBC values 16 times higher than MIC, typically indicate antimicrobial tolerance. The established rejection value of ≥40 colonies for the MBC assay was based on the calculated concentration of *M. tuberculosis* in the MIC plates. The results, reported as μg/mL concentration, are determined based on Colony Forming Units (CFUs) enumerated from agar plates. Only agar plates with countable colonies have reportable counts. If a compound lacks bactericidal activity, many times the CFUs are too numerous to count (TNTC) and are thus reported accordingly. As far for compound **6d**, the MBC value (Table 2) are above the established rejection value of ≥40 colonies and the CFUs are too numerous to count. These results suggest an antimicrobial tolerance.

Low-Oxygen Recovery Assay (LORA). Traditional screening of drugs against *M. tuberculosis* only addresses or targets the organism in an active replicating state. It is well known that *M. tuberculosis* can reside in a state of non-replicating persistence (NRP) which has not been adequately assessed in the development of new antimicrobials. The LORA assay tests drugs for activity against *M. tuberculosis* in a state of NRP. LORA test was conducted in duplicate and the following controls were included in each test plate: i) medium only (sterility control); ii) organism in medium (negative control); and iii) rifampin or isoniazid (positive control). Results for the LORA assay are reported as the lowest concentration (μg/mL) of drug that visually inhibited growth of the organism. The data are summarized in Table 3.

The LORA assay test for compound **6d** displays a value of 1.17 μg/mL, suggesting that compound **6d** was potent against non-replicating *M. tuberculosis*.

The Intracellular (macrophage) Drug Screening, and MTT cell proliferation (MTT). This assay evaluates intracellular drug effectiveness. Briefly, the purpose of this assay is to assess the inhibition of *M. tuberculosis* H37Rv phagocytised by J774.A1 macrophages when exposed to a battery of test compounds. J774.A1 is an

Table 2
MIC and MBC results.

Compd	MIC $H_{37}Rv$ (μg/mL)	Inh[a], %	MBC $H_{37}Rv$ (μg/mL)	MIC INH-R[b] (μg/mL)	Inh, %	MIC RMP-R[c] (μg/mL)	Inh, %	MIC OXF-R[d] (μg/mL)	Inh, %
6d	2.34	51	NA	4.69	60	1.17	65	1.17	61
Rifampin (pos control)	0.049	63	1.56	0.02	56	NA[e]	NA	0.05	80
Isoniazid (pos control)	NA	NA	NA	NA	NA	0.02	92	NA	NA

Inh[a] – Percent inhibition at MIC concentration.
INH-R[b] – Isoniazid resistance.
RMP-R[c] – Rifampin resistance.
OFX-R[d] – Oxofloxacin resistance.
NA[e] – Not applicable: compound not used in assay.
NA – Not applicable: colony counts above the established rejection value of ≥40.
Inh – inhibition.

Table 3
LORA, Macrophage and MTT results.

Compd	LORA (μg/mL)	Macrophage *log reduction* (low conc.)	Macrophage *log reduction* (mid conc.)	Macrophage *log reduction* (high conc.)	MTT % viability (low conc.)	MTT % viability (mid conc.)	MTT % viability (high conc.)
6d	1.17	1.19	2.65	3.11	86	71	<10
Rifampin	0.13	0.98	0.81	1.49	94	93	85

adherent macrophage cell line originating in mice routinely used for the determination of inhibition of intracellular pathogens. The macrophages are subsequently lysed and plated onto Middlebrook 7H10 agar plates for bacterial enumeration. The number of mycobacteria surviving treatment is compared to the number of mycobacteria not receiving treatment in order to determine the intracellular activity of the test compounds.

Each compound was tested in duplicate, using 3 concentrations, and rifampin was used as the positive control drug. The three concentrations chosen were based on the MIC data generated in HTS primary screen. The mid concentration bracketed the reported MIC with the lower concentration ten-fold below the mid and the higher concentration ten-fold above the mid. Intracellular drug activity is reported as *log reduction* value calculated as reduction in *M. tuberculosis* concentration from zero hour to 7 days post-infection. The data are presented in Table 3. A drug cytotoxicity control plate assay (MTT proliferation) was also conducted in parallel using uninfected macrophages to confirm that concentrations utilized for testing were not toxic to the macrophages. Drug cytotoxicity is reported as cell proliferation, or percentage of viability. These data are summarized in Table 3.

As we may notice from Table 3, the *log reduction* value for compound **6d**, is almost equal to control drug rifampin at low concentration and almost double at mid and high concentrations. These results suggest that **6d** is a potent compound against intracellular *M. tuberculosis*.

We may also notice from Table 3 that the MTT viability for compound **6d** (at low and intermediate concentrations) is close to control drug rifampin, suggesting that compound **6d** has basically no toxicity at these concentrations. However, taking into consideration that at high concentrations the MTT viability for compound **6d** is lower than 10%, we may only to presume that the activity of compound **6d** is not due to cytotoxicity of compound. Further studies remain to be done in this respect.

3. Conclusions

In conclusion, we describe herein the design, synthesis, and evaluation of antimycobacterial activities of some fused 4,4′-bipyridine heterocycles. Three classes of bipyridine heterocycles (namely, mono-indolizines, bis-indolizines and mono-indolizine mono-salts) were synthesized and their structure was assigned by elemental and spectral analysis, X-ray analysis including. The primary cycle high throughput screening reveals that mono-indolizines and bis-indolizines did not exhibit any significant anti-TB activity, while the mono-indolizine mono-salts are displaying potency superior to the second-line antitubercular drugs Cycloserine and Pyrimethamine and, equal to the first line anti-TB Ethambutol. The data from Cycle-2 screening assay (MIC, MBC, LORA, intracellular (macrophage) drug screening, and MTT cell proliferation) confirm the promising antimycobacterial results from Cycle-1 for mono-indolizine mono-salts. The MIC and LORA assay illustrate that mono-indolizine mono-salt **6d** is a potent compound against both replicating and non-replicating *M. tuberculosis* while the MBC assay indicates a bacteriostatic mechanism of action. The intracellular (macrophage) drug screening demonstrates that compound **6d** is an effective drug against intracellular *M. tuberculosis*. The MTT assay illustrates that compound **6d** has basically no toxicity at low and intermediate concentrations, suggesting that the activity is not due to cytotoxicity of compound. The mono-indolizine mono-salts derivatives have shown a better antimycobacterial activity comparative with bis-indolizines, suggesting a possible influence of bromine anion concerning antimycobacterial activity. We see no influence of the substituents on the fused-pyridine rings concerning the antimycobacterial activity.

4. Experimental protocols

4.1. Chemistry

All the reagents and solvents employed were used without further purification. Melting points were determined using an electrothermal apparatus (MELTEMP II) and were uncorrected. The NMR spectra were recorded on a Bruker Avance 400 DRX spectrometer operating at 400 MHz. The following abbreviations were used to designate chemical shift multiplicities: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet. The IR spectra were recorded on a FTIR Shimadzu Prestige 8400s spectrophotometer. The microanalyses were in satisfactory agreement with the calculated values: C, ±0.15; H, ±0.10; N, ±0.30. X-ray analysis was recorded with an Agilent Xcalibur Eos diffractometer equipped with a Mo (Kα radiation, $\lambda = 0.71073$ Å) fine-focus sealed X-ray tube and a graphite monochromator. The following crystal structure has been deposited at the Cambridge Crystallographic Data Centre and allocated the deposition number: (**9a**), CCDC 941837. Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK, (fax: +44 (0)1223 336033 or email:deposit@ccdc.cam.ac.uk).

4.1.1. General procedure for preparation of compounds (**9**)

The cycloimmonium salt **(8a**, **b)** (1 mmol, 1 equiv., 0.28 g **8a**, 0.34 g **8b**), and ethyl propiolate (1.1 mmol, 1.1 equiv., 0.11 g) were added to 10 mL of anhydrous acetone and the obtained suspension was stirred at room temperature. Then, a solution of triethylamine (TEA) (3 mmol, 3 equiv., 0.30 g) in anhydrous acetone (3 mL) was added drop-wise over 2 h (magnetic stirring) and the resulting mixture was then stirred over the night at rt. Water (10 mL) is added and after stirring for another 15 min, the solid was collected by filtration to give a powder which was washed with 10 mL methanol. The product was crystallized from ethanol–chloroform (1:1, v/v).

*4.1.1.1. Ethyl 3-cyano-7-(pyridine-4-yl)indolizine-1-carboxylate (**9a**).* Yellow crystals, mp 158–160 °C; Anal. $C_{17}H_{13}N_3O_2$: C, H, N; IR (KBr): ν/cm^{-1}: 2209 (C≡N), 1687 (C=O$_{ester}$), 1206, 1068 (C–O); ^{1}H NMR (400 MHz, $CDCl_3$): δ_{ppm}: 1.45 (t, J = 7.2 Hz, 3H, CH_3), 4.35 (q, J = 7.2 Hz, 2H, CH_2), 7.31 (dd, $J_{6,5}$ = 7.2 Hz, $J_{6,8}$ = 1.6 Hz, 1H, H_6), 7.60 (d, $J_{3',2'}$ = 5.6 Hz, 2H, $H_{3'}$, $H_{5'}$), 7.82 (s, 1H, H_2), 8.42 (d, $J_{5,6}$ = 7.2 Hz, 1H, H_5), 8.66 (s, 1H, H_8), 8.72 (d, $J_{2',3'}$ = 5.6 Hz, 2H, $H_{2'}$, $H_{6'}$). ^{13}C NMR (100 MHz, $CDCl_3$): δ_{ppm}: 14.5 (CH_3), 60.5 (CH_2), 97.0 (C_3), 107.3 (C_1), 112.3 (CN), 113.6 (C_6), 118.1 (C_8), 121.1 ($C_{3'}$,$C_{5'}$), 125.7 (C_2), 126.1 (C_5), 135.7 (C_7), 137.6 (C_9), 144.8 ($C_{4'}$), 150.7 ($C_{2'}$,$C_{6'}$), 163.1 (COO).

*4.1.1.2. Ethyl 3-carbamoyl-7-(pyridine-4-yl)indolizine-1-carboxylate (**9b**).* Yellow crystals, mp 275–277 °C; Anal. $C_{17}H_{15}N_3O_3$: C, H, N; IR

(KBr): ν/cm^{-1}: 3401 (NH_{amide}), 1686 (C=O_{ester}), 1605 (C=O_{amide}, NH)), 1396 (C–N); ^{1}H NMR (400 MHz, DMSO-d_6): δ_{ppm}: 1.37 (t, J = 7.2 Hz, 3H, CH_3), 4.34 (q, J = 7.2 Hz, 2H, CH_2), 7.04 (s, 1H, NH_b), 7.35 (s, 1H, NH_a), 7.53 (dd, $J_{6,5}$ = 7.6 Hz, $J_{6,8}$ = 1.6 Hz, 1H, H_6), 7.80 (d, $J_{3',2'}$ = 5.6 Hz, 2H, $H_{3'}$, $H_{5'}$), 8.14 (s, 1H, H_2), 8.56 (s, 1H, H_8), 8.72 (d, $J_{2',3'}$ = 5.6 Hz, 2H, $H_{2'}$, $H_{6'}$), 9.83 (d, $J_{5,6}$ = 7.6 Hz, 1H, H_5). ^{13}C NMR (100 MHz, DMSO-d_6): δ_{ppm}: 14.4 (CH_3), 59.5 (CH_2), 104.7 (C_1), 112.2 (C_6), 115.8 (C_8), 117.9 (C_3), 120.6 (C_2), 120.8 ($C_{3'}$,$C_{5'}$), 128.3 (C_5), 133.2 (C_7), 136.9 (C_9), 144.4 ($C_{4'}$), 150.5 ($C_{2'}$,$C_{6'}$), 162.5 (<u>C</u>ONH_2), 163.3 (<u>C</u>OO).

4.2. Microbiology

Compounds were evaluated for antimycobacterial activity against *M. tuberculosis*, as a part of the TAACF TB screening program under direction of the US National Institute of Health, the NIAID division. Antimycobacterial activities of the compounds were performed by Center of Tuberculosis Antimicrobial Acquisition and Coordinating Facility (TAACF) at Southern Research Institute.

4.2.1. The primary cycle high throughput screening (HTS) – Cycle 1 determination of 90% inhibitory concentration (IC_{90}), 50% inhibitory concentration (IC_{50}) and Minimum Inhibitory Concentration (MIC)

Compounds were tested for *in vitro* antitubercular activity against *M. tuberculosis* H37Rv (ATCC 27294), in BACTEC 12B medium using a broth microdilution assay, the Microplate Alamar Blue Assay (MABA) [19,20]. The *M. tuberculosis* HTS assay used black, clear-bottom, 384-well microtiter plates and 7H12 broth. Compounds stocks of 10 mM in 100% DMSO were diluted in media, adjusted with DMSO to maintain the final 1% DMSO concentration throughout, to create ten serial-dilution compound source plates at 100× the targeted test concentration, ranging from 10 mM to 0.0195 mM. The compounds were diluted in assay media to 2× final test concentration and 25 μL of these diluted compounds were transferred to 384-well plates. Amikacin was included in the positive control wells in every assay plate at two concentrations, 0.17 and 3.20 μM. The low concentration was the approximate MIC and it is an indicator of proper assay performance of each plate. The high concentration completely inhibits growth and was used in lieu of uninoculated medium (background) to calculate percent inhibition by the test compounds for each plate. Positive and negative control wells were included in each plate. Plates were placed in stacks of two and incubated for 7 days at 37 °C with approximately 95% humidity. After 7 days of incubation, autofluorescence of any test compounds was determined by pre-reading the high dose plate by a bottom read for fluorescence using a Perkin Elmer Envision plate reader at 535 nm excitation and 590 nm emission. The IC_{90} is defined as the concentration effecting a reduction in fluorescence of 90% relative to controls. Any compound with IC_{90} value of ≤10 μg/mL is considered 'active' for antitubercular activity. The Minimum Inhibitory Concentration (MIC) is defined as the lowest concentration effecting a reduction in fluorescence of 90% relative to controls.

4.2.2. In vitro assessments for antimicrobial activity-preclinical anti-tubercular testing – Cycle 2

4.2.2.1. Minimal inhibitory concentration (MIC). The broth microdilution assay format following guidelines established by the *Clinical and Laboratory Standards Institute* (CLSI) is routinely utilized for MIC testing. Briefly, testing was conducted using 96-well, U-bottom microplates with an assay volume of 0.2 mL/well. First, the test media, Middlebrook 7H9 broth supplemented with OADC Enrichment (BD BioSciences; Sparks, MD), was added (0.1 mL/well) to each well. The test compounds, solubilized in appropriate solvent and subsequently diluted in test media, were subsequently added (0.1 mL/well) to appropriate wells at twice the intended starting concentration and serially diluted two-fold across the plate. The plates were then inoculated (0.1 mL/well) with a target concentration of 1.0×10^6 CFU/mL *M. tuberculosis* and incubated at 37 °C for 7 days in approximately 90% humidity. Following incubation, the plates were read visually and individual wells scored for turbidity, partial clearing or complete clearing. Testing was conducted in duplicate and the following controls were included in each test plate: i) medium only (sterility control); ii) organism in medium (negative control); and iii) rifampin or isoniazid (positive control). The MIC is reported as the lowest concentration (μg/mL) of drug that visually inhibits growth of the organism.

4.2.2.2. Minimal Bactericidal Concentration (MBC). MBC is determined subsequent to MIC testing by sub-culturing diluted aliquots from wells that fail to exhibit macroscopic growth. The sample aliquots were inoculated onto Middlebrook 7H10 agar plates and subsequently incubated for 16–21 days at 37 °C. Once growth was readily apparent, the bacterial colonies were enumerated. MBC is defined as the lowest concentration (μg/mL) of compound exhibiting 99.9% kill over the same time period used to determine MIC (18–24 h). MBC values 16 times higher than MIC typically indicate antimicrobial tolerance.

4.2.2.3. Low-Oxygen Recovery Assay (LORA). Traditional screening of drugs against *M. tuberculosis* only addresses or targets the organism in an active replicating state. It is well known that *M. tuberculosis* can reside in a state of non-replicating persistence (NRP) which has not been adequately assessed in the development of new antimicrobials. Briefly, microplates were prepared in the same manner as MIC testing format. Instead of incubating aerobically, the plates are placed under anaerobic conditions using a MACS MIC automated jar gassing system and incubated for 7 days at 37 °C. The plates were subsequently transferred to an ambient gaseous condition (5% CO_2) for 7 days after which the plates are read visually and individual wells scored for turbidity, partial clearing or complete clearing. Testing was conducted in duplicate and the following controls were included in each test plate: i) medium only (sterility control); ii) organism in medium (negative control); and iii) rifampin or isoniazid (positive control). Results are reported as the lowest concentration (μg/mL) of drug that visually inhibits growth of the organism.

4.2.2.4. Intracellular (macrophage) drug screening, and MTT cell proliferation. Briefly, the murine J774 cell line was propagated in RPMI 1640 supplemented with L-glutamine and fetal bovine serum (FBS). Cells were maintained in tissue culture flasks at 37 °C in the presence of 5% CO_2. For infection studies, J774 cells were transferred to 12-well tissue culture chambers in 1 mL volumes at a density of 2.0×10^5 in the presence of 10% FBS. After overnight incubation, the medium was replaced with fresh medium containing 1% FBS to stop macrophage division while maintaining cell viability. Twenty-four hours later, the macrophage monolayer was enumerated with an ocular micrometer for total number of cells per well to determine the infection ratio. The medium was removed and replaced with 1 mL of fresh medium with 1% FBS containing *M. tuberculosis* at a multiplicity of infection (MOI) of 5 Mycobacteria/macrophage. The cells are infected for 4 h and then nonphagocytosed Mycobacteria were washed from the monolayers and fresh medium was added. Drugs were then added, using 3 concentrations, and infection allowed to proceed for 7 days. At 0 and 7 days, the macrophages were lysed with sodium dodecyl sulfate, treated with DNAase, diluted and plated onto 7H10 agar to determine the cell number or colony forming units (CFU). Each drug concentration was tested in duplicate and rifampin was used as the positive control drug (at 3 concentrations: 0.01, 0.1 and 1.0 μg/mL). A drug cytotoxicity control plate assay (MTT

proliferation) was also conducted in parallel using uninfected macrophages to confirm that concentrations utilized for testing were not toxic to the macrophages.

Bacteria. MIC screening was conducted for *M. tuberculosis* H37Rv (SRI 1345), isoniazid (INH)-resistant *M. tuberculosis* (SRI 1369), rifampin (RMP)-resistant *M. tuberculosis* (SRI 1367), and ofloxacin (OFX)-resistant *M. tuberculosis* (SRI 4000). MBC, LORA and intracellular drug screening assays were conducted using only *M. tuberculosis* H37Rv (SRI 1345).

Acknowledgments

Authors are also thankful to CNCSIS Bucuresti, Romania, grant PN-II-DE-PCE-2011-3-0038, no. 268/05.10.2011, for financial support. Part of this work (biological tests) was supported by National Institutes of Health and the National Institute of Allergy and Infectious Diseases, Contract No. HHSN272201100012I.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2013.09.061.

References

[1] World Health Organization Tuberculosis Programme Available from: Global tuberculosis report/WHO/HTM/TB/2012.6.
[2] L.V. Sacks, R.E. Behrman, Tuberculosis 88 (2008) 93–100.
[3] Z. Ma, C. Lienhardt, H. Mclleron, A.J. Nunn, X. Wang, Lancet 5 (2010) 1–10.
[4] W.J. Burman, B.E. Jones, Am. J. Respir. Crit. Care Med. 164 (2001) 7–12.
[5] C. Crabb, Bull. World Health Organ. 80 (2002) 518–519.
[6] B. Dulla, B. Wan, S.G. Franzblau, R. Kapavarapu, O. Reiser, J. Iqbal, M. Pal, Bioorg. Med. Chem. Lett. 22 (2012) 4629–4635.
[7] G.C. Moraski, L.D. Markley, M. Chang, S. Cho, S.G. Franzblau, C.H. Hwang, H. Boshoff, M.J. Miller, Bioorg. Med. Chem. 20 (2012) 2214–2220.
[8] R. Gradinaru, A. Luca, I. Cretescu, R. Danac, Rev. Chim. (Bucharest) 61 (2010) 903–906.
[9] A. Rotaru, R. Danac, I. Druta, G. Drochioiu, I. Cretescu, Rev. Chim. (Bucharest) 56 (2005) 179–183.
[10] A. Rotaru, M. Ungureanu, R. Danac, A. Poeata, I. Druta, Ann. Pharm. Fr. 62 (2004) 428–430.
[11] R. Butnariu, I.I. Mangalagiu, Bioorg. Med. Chem. 17 (2009) 2823–2829.
[12] M. Caprosu, R. Butnariu, I.I. Mangalagiu, Heterocycles 65 (2005) 1871–1879.
[13] D. Mantu, C. Luca, C. Moldoveanu, G. Zbancioc, I.I. Mangalagiu, Eur. J. Med. Chem. 45 (2010) 5164–5168.
[14] C. Moldoveanu, G. Mangalagiu, G. Drochioiu, M. Caprosu, M. Petrovanu, I.I. Mangalagiu, Stiint. Univ. "Al.I.Cuza" Iasi 11 (2003) 367–374 (Chem. Abstr. 142 (2004) 56249).
[15] A. Rotaru, I. Druta, E. Avram, R. Danac, Arkivoc 13 (2009) 287–299.
[16] R. Kannappan, C. Bucher, E. Saint-Aman, J.C. Moutet, A. Milet, M. Oltean, E. Metay, S. Pellet-Rostaing, M. Lemaire, C. Chaix, New J. Chem. 34 (2010) 1373–1386.
[17] D.S.N. Rao, F. Donald, Helv. Chim. Acta 81 (1998) 902–915.
[18] A. Rotaru, R. Danac, I. Druta, J. Heterocycl. Chem. 41 (2004) 893–897.
[19] L.A. Collins, S.G. Franzblau, Antimicrob. Agents Chemother. 41 (1997) 1004–1009.
[20] J. Secrist, S. Anathan, C. Kwong, J. Maddry, R. Reynolds, A. Poffenberger, M. Michael, L. Miller, J. Krahenbuhl, L. Adams, A. Biswas, S.G. Franzblau, D. Rouse, D. Winfield, J. Brooks, I. Orme, Antimicrob. Agents Chemother. 45 (2001) 1943–1946.