Synthesis of potential inhibitors of InhA with pyrrolidine-2,5-dione core fragment and evaluation of their biological activity

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We report here the discovery, synthesis and screening results of the series of 3-bulky substituted pyrrolidine-2,5-dione derivatives as a novel class of potential inhibitors on InhA, a key enzyme involved in the fatty acid biosynthesis pathway (type II) of *M. tuberculosis* as well as inhibitors of *Mycobacterium tuberculosis* H37Rv.

Introduction

Tuberculosis (TB) is the leading cause with malaria and AIDS of worldwide mortality [1]. The effectiveness of current anti-tuberculosis drugs to combat this infection is severely compromised by the emergence of multi- and extensively drug-resistant tuberculosis (MDR-TB [2,3] and XDR-TB [4]). Therefore, more new drugs are required to raise the probability to shortly stop all forms of drug-resistant TB. It is in this context that many studies are based on targeting the cell wall of mycobacteria and more particularly of the components essential to their survival. The fatty acid synthase system of *M. tuberculosis* contains unique signature fatty acid, the mycolic acid, which is central constituent of the mycobacterial cell wall. Mycolic acid biosynthesis is carried out by several successive enzymatic cycles corresponding to two related but distinct Fatty Acid Synthase (FAS) systems, FAS I and II [5]. The InhA protein (ENR, EC number: 1.3.1.9) is a part of FAS II and shows a NADH-dependent enoyl-ACP reductase activity. InhA is a good target as the FAS II system is present in bacteria but is absent in humans. It is already the target of the first line drug isoniazide.

In the course of our study of Michael reaction with maleimides as dienophiles [6-8], we were interested in involving succinimide fragment as promising core of new potential inhibitors of InhA protein. Michael conjugate addition is well

known as effective method in the synthesis of pharmaceutical intermediates, peptide analogues, antibiotics and other drugs [9,10]. In addition, molecules containing succinimide fragment are frequently employed in drug design. For example, 2,5-pyrrolidinedione are core structural units found in natural products and also in some approved drugs and clinical drug candidates [9,11,12]. Compounds such Moiramide B and Andrimide (**Fig. 1**) have been described as new highly specific antibiotics [13] exhibiting potent antibacterial activity against methicillin-resistant *Staphylococcus aureus* and a range of other antibiotic-resistant human pathogens. It was defined that Moiramide B and Andrimide target FAS system that is also the primary target for antitubercular drugs [14]. Moreover, Hirsutellone A (**Fig. 1**) a natural product bearing succinimide ring is reported to display significant growth inhibitory activity against *Mycobacterium tuberculosis* H37Ra strain [15].

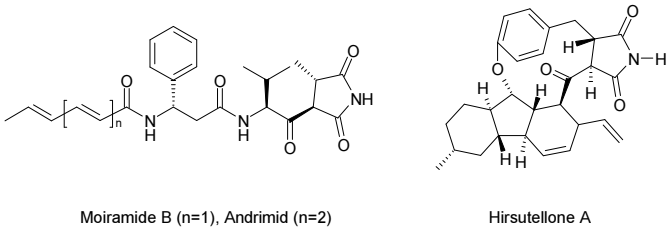


Figure 1. *Structures of natural antibiotics (Moiramide B and Andrimide) and antimycobacterial alkaloid Hirsutellone A.*

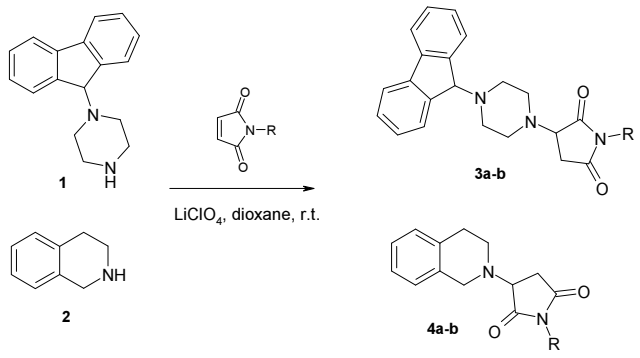
Here we report synthesis and biological evaluation of a series of bulky 3-heteryl substituted pyrrolidine-2,5-diones.

Results and discussion

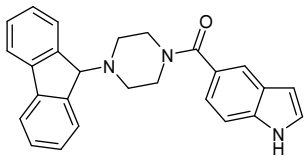
Compounds described in Table 1 were synthesized by Lewis acid catalyzed Michael reaction on the maleimide core *via* NH or C-H heterocyclic modifications. A methodological approach has already been reported by us [16, 17] then evaluated and applied in the cases presented here.

Compounds **3a-b** as well as compounds **4a-b** were obtained *via* C–N conjugate addition of 1-(9*H*-fluoren-9-yl)piperazine and 1,2,3,4-tetrahydroisoquinoline respectively to *N*-substituted maleimides (Scheme 1).

Scheme 1. *Synthesis of C–N Michael adducts.*



The best results were obtained when using a catalytic amount of lithium perchlorate ($LiClO_4$) in dry dioxane at room temperature (method A). Alternative way to the synthesis of adducts described on scheme 1 was applied. To check efficacy of our catalytic approach we provide the reaction in isopropyl alcohol with 0.5 equivalents of Hünig's base and the yields obtained in this case were lower than previously (method B). Compounds **3a** and **3b** were synthesized as analogues of known selective nanomolar inhibitor of InhA protein GEQ (Genz-10850) (**Fig 2**) [15].

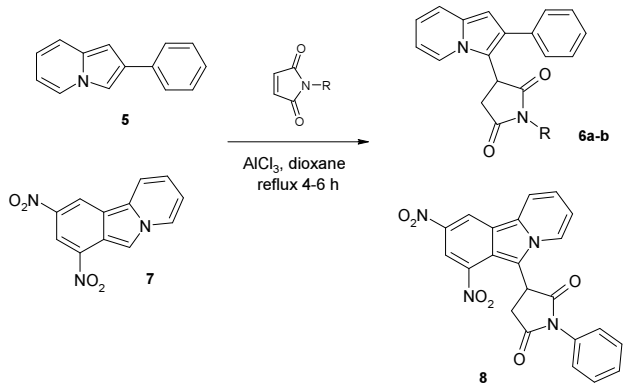


GEQ (Genz-10855)

Figure 2. *Structure of nanomolar inhibitor of InhA. IC_{50} (InhA) = 0.16 □M; MIC > 30 □M [18].*

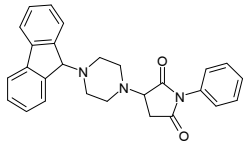
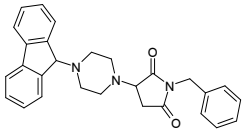
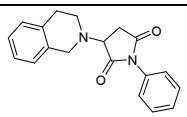
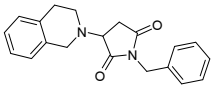
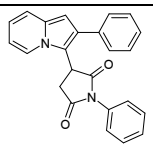
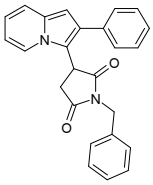
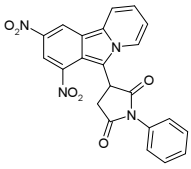
The synthesized compounds **3a** and **3b** contain a bulky and rigid fluorene along with a piperazine moiety as it is the case of GEQ. The fluorenyl ring plays as an anchor in the binding site of InhA and is responsible for extensive hydrophobic interactions [18].

Then, Michael addition was carried out between maleimide derivatives and two C-H active heterocycles i.e. 2-phenylindolizine and 7,9-dinitropyrido[2,1-a]isoindole that are shown on the scheme 2. The reaction progress was followed by TLC till consumption of the starting material. It is noteworthy that in this case when using $LiClO_4$ as catalyst, only traces of the desired products were obtained. Apparently, $LiClO_4$ is not as effective as catalyst for C–C addition. Different other Lewis acids have then been tested ($AlCl_3$, $ZnCl_2$ or $TiCl_4$). The best results were obtained when using a catalytic amount of $AlCl_3$ under reflux conditions in dry dioxane, affording compounds **6a**, **6b** and **8** in 84%, 71% and 62% yields, respectively after recrystallization (Table 1).



Scheme 2. Synthesis of C–C Michael adducts.

Table 1. *Structure of synthesized compounds and yields of the products*

Cpds	Structure	Yield (%)
3a		88 (Method A) 70 (Method B)
3b		83 (Method A) 58 (Method B)
4a		87 (Method A) 55 (Method B)
4b		79 (Method A) 60 (Method B)
6a		84
6b		71
8		62

All the synthesized compounds were evaluated by determining the minimal inhibitory

concentration (MIC) on *M. tuberculosis* H37Rv strain. They were also evaluated *in vitro* as potential InhA inhibitors at 50 µM by applying a commonly used method [18]. Recombinant *M. tuberculosis* InhA was expressed in *E. coli* and subsequently purified according to a previous reported procedure [19, 20].

The results are shown in Table 2. Triclosan and GEQ were used for comparison. Compounds **3a-b** present the best activities on InhA protein among the tested derivatives, nevertheless the values are still too modest compared to that of GEQ. Docking studies for compound **3a** were done and some possible differences in binding are shown compared to GEQ (PDB ID 1P44). From the alignment of the predictive binding modes, we could summarize that **3a** could be inserted in the active site but through a different geometry. Indeed, the fluorenyl ring could not exactly match the large hydrophobic pocket of InhA protein (**Fig 3**). It is also worth mentioned that electrostatic interactions and hydrogen bonding between **3a** and cofactor NAD^+ are missing.

Compound **8** shows an inhibitory activity equivalent to compounds **3a** and **3b**. It is noteworthy that compound **8** as compounds **3a** and **3b** bear rigid tricyclic 7,9-dinitro pyrido[2,1-a]isoindolyl and fluorene rings, while in the contrary compounds **4a**, **4b** and **6a**, **6b** have more flexible and non planar moieties.

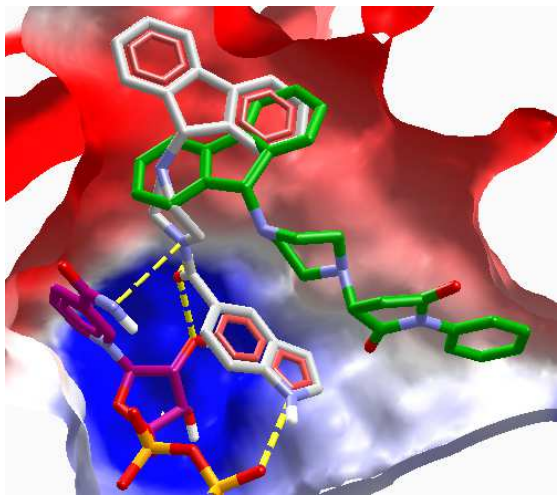


Figure 3. *Alignment of binding mode of GEQ (pdb ID 1P44, carbons in grey sticks) and predicted binding pose of **3a** (carbons in green sticks) into the binding site of InhA (surface representation colored by electrostatic interaction). Cofactor NAD^+ is in stick representation with carbons colored in violet.*

Table 2. *Enzyme inhibition values. Results are expressed as a percentage of InhA inhibition. Minimal inhibitory concentration (MIC) values on M. tuberculosis H37Rv strain.*

Compound	% Inhibition (at 50 □M)	MIC (µg/ml) / (µM)
Triclosan	>99	10 / 34.5
GEQ	>99	>60 / >150
3a	52	40 / 94.4
3b	56	40 / 91.4
4a	20	2.5 / 8.2
4b	16	10 / 31.2
6a	Nd*	40 / 109.2
6b	Nd*	20 / 52.6
8	45	>16 / >37.2

*not determined values

Finally, the MIC values of compounds **3a**, **3b**, 6**a** and **6b** are in the same range but better than that of GEQ compound. The promising results

of MIC values were obtained for compounds **4a** and **4b** that are even better than Triclosan. Thus structures of those inhibitors are point of interest for further development of effective antituberculosis agents.

Conclusion

A series of 3-bulky substituted pyrrolidine-2,5-dione derivatives have been synthesized using optimized catalytic conditions for Michael addition. Synthesized compounds were assayed for *in vitro* inhibition of InhA and *M. tuberculosis* growth.

Experimental part

Materials and Methods

Kinetic studies were perfomed on a Cary Bio 100. All chemicals were obtained from Aldrich or Acros Organics and used without further purification. Nuclear magnetic resonance spectra were recorded on a Bruker AC 300 spectrometer (^{1}H and ^{13}C NMR), solvent residue signals were used for calibration of spectral data. Mass spectrometry (MS) data were obtained on a ThermoQuest TSQ 7000 spectrometer, high-resolution mass spectra (HRMS) were recorded on a ThermoFinnigan MAT 95 XL spectrometer using electrospray ionization (ESI) methods. Melting points were measured on a Mettler Toledo MP50 melting point system and are uncorrected. 1-(9*H*-fluoren-9-yl)piperazine and GEQ compound (genz-10850) was synthesized according to the literature procedure [18].

InhA expression and purification. The production and purification were performed as described in reference [20]**.**

Inhibition Kinetics

All activity assays were performed in triplicate in accordance to the reference procedure [20].

Growth conditions

M. tuberculosis H37Rv strain was grown either

MIC determinations. The procedure was performed as described in the reference [19].

1-(9H-fluoren-9-yl)piperazine (1), 2-phenyl-indilisine (5) and 7,9-dinitropyrido[2,1-a]isoindole (7) were synthesized according to the references [18, 21, 22], respectively.

General procedure for C–N Michael addition (Method A) To a solution of *N*-substituted maleimide (0.22 mmol) in dry dioxane, $LiClO_4$ (6 mg) was added. Then a solution of 1-(9*H*-fluoren-9-yl)piperazine or 3,4-dihydroisoquinoline (0.19 mmol) in dry dioxane was added. The mixture was stirred overnight at room temperature. The reaction progress was controlled by TLC. After completion, the solvent was evaporated and the crude product was purified by flash chromatography.

Method B To a solution of amine (0.28 mmol) in *i*PrOH, *N*-phenyl maleimide (0.34 mmol) was added. After 10 min stirring, *N,N*-diisopropylethylamine (Hünig's base) was added (0.15 mmol). The reaction mixture was stirred overnight at room temperature. Then the solvent was evaporated and the corresponding crude product was purified by flash chromatography.

3-[4-(9H-Fluoren-9-yl)-1-piperazinyl]-1-phenyl-2,5-pyrrolidinedione (3a)

The crude product was purified by flash chromatography (CH_2Cl_2/EtOAc–8:2) to give lightly yellow crystals. Yield: (Method A) 88%, (Method B) 70%; mp: 191 ^{0}C. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 2.61–3.04 (m, 10H), 3.92 (dd, J = 4.8 Hz, J = 9.0 Hz, 1H), 4.85 (s, 1H), 7.24–7.49 (m, 9H), 7.63–7.10 (m, 4H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 32.1, 43.2, 48.9, 49.9, 62.6, 69.9, 119.9, 126.2, 126.6, 127.2, 128.4, 128.9, 129.3, 131.6, 141.2, 175.1, 181.8. HRMS: calculated for $C_{28}H_{29}N_3O_2$ 439.5488; found 439.5472.

1-Benzyl-3-[4-(9H-fluoren-9-yl)-1-piperazinyl]-2,5-pyrrolidinedione (3b)

Yield: (Method A) 83%, (Method B) 58%; mp: 168 ^{0}C. ^{1}H NMR (300 MHz, DMSO-d_6): δ = 2.52–2.99 (m, 10H), 3.81 (dd, J = 4.5 Hz, J = 9.0 Hz, 1H), 4.53 (s, 2H) 4.77 (br. s, 1H), 7.25–7.67 (m, 13H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 32.0, 48.9, 49.5, 50.5, 62.2, 69.8, 120.0, 126.1, 126.6, 127.1, 128.4, 128.9, 129.3, 131.5, 140.9, 174.5, 179.8.

3-(3,4-Dihydroisoquinolin-2(1H)-yl)-1-phenylpyrrolidine-2,5-dione (4a)

The product was recrystallized from isopropyl alcohol to give a white powder. Yield: 87%; mp: 149 ^{0}C. ^{1}H NMR (300 MHz, DMSO-d_6): δ = 2.79–3.10 (m, 6H), 3.72 (d, J = 14.7 Hz, 1H), 4.09 (d, J = 14.7 Hz, 1H), 4.27 (dd, J = 5.7 Hz, J = 8.7 Hz), 7.06–7.12 (m, 4H), 7.29–7.32 (m, 2H), 7.40–7.53 (m, 3H). ^{13}C NMR (75 MHz, DMSO-d_6): δ = 29.0, 31.4, 46.5, 50.9, 62.2, 125.5, 126.0, 126.4, 127.2, 128.4, 128.6, 128.9, 132.3, 133.9, 134.5, 174.6, 175.7. MS [M+H$^+$] calculated for $C_{19}H_{19}N_2O_2$ 307.37; found 307.1

1-Benzyl-3-(3,4-dihydroisoquinolin-2(1H)-yl)pyrrolidine-2,5-dione (4b)

The product was recrystallized from isopropyl alcohol to give lightly yellow crystals. Yield: 79%; mp: 106.5 ^{0}C. ^{1}H NMR (300 MHz, DMSO-d_6): δ = 2.64–2.99 (m, 6H), 3.57 (d, J = 14.7 Hz, 1H), 3.98 (d, J = 14.7 Hz, 1H), 4.18 (dd, J = 5.4Hz, J = 8.4 Hz, 1H), 4.59 (s, 2H), 6.97–7.12 (m, 4H), 7.25–7.37 (m, 5H). ^{13}C NMR (75 MHz, DMSO-d_6): δ = 29.0, 31.1, 41.2, 46.4, 50.8, 61.9, 125.5, 126.0, 127.4, 128.4, 128.5, 133.8, 134.3, 136.2, 175.3, 176.5. MS [M+H$^+$] calculated for $C_{20}H_{20}N_2O_2$ 321.39; found 321.1

General procedure for C–C Michael addition

7,9-Dinitropyrido[2,1-*a*]isoindole (100 mg, 0.39 mmol) or of 2-phenylindolizine (100 mg, 0.52 mmol) was dispersed in dry dioxane. Then *N*-substituted maleimide (0.4 or 0.53 mmol respectively to the reagents) was added. The mixture was stirred for 15 minutes. Thereafter catalytic amount of aluminium chloride was added. The reaction mixture was refluxed over 6 h with 7,9-dinitropyrido[2,1-*a*]isoindole and 4 h with 2-phenylindolizine. The reaction progress was monitored by TLC. After completion, the solvent was evaporated and the crude product was purified by crystallization.

1-Phenyl-3-(2-phenyl-3-indolizinyl)-2,5-pyrrolidinedione (6a)

The crude product was recrystallized from EtOH. Yield: 84%; mp: 210 ^{0}C. ^{1}H NMR (300 MHz, DMSO-d_6): δ = 3.06 (dd, J = 6.9 Hz, J =

17.7 Hz, 1H), 3.36 (m, 1H), 5.13 (t, J = 8.4 Hz, 1H), 6.60–7.54 (m, 14H), 7.97 (d, J = 6.6 Hz, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ = 33.6, 37.4, 99.6, 111.3, 114.5, 117.5, 119.1, 122.8, 126.9, 127.1, 128.4, 128.7, 128.9, 129.0, 129.8, 132.3, 132.5, 135.7, 174.5, 175.8.

1-Benzyl-3-(2-phenyl-3-indolizinyl)-2,5-pyrrolidinedione (6b)

The crude product was recrystallized from EtOH. Yield: 71%; mp: 192 ^{0}C. ^{1}H NMR (300 MHz, DMSO-d_6): δ = 3.14 (dd, J = 6.3 Hz, J = 16.8 Hz, 1H), 3.35 (m, 1H), 4.51 (s, 2H) 5.07 (t, J = 9.0 Hz, 1H), 6.67–7.53 (m, 14H), 7.95 (d, J = 6.9 Hz, 1H). ^{13}C NMR (75 MHz, DMSO-d_6): δ = 33.6, 37.5, 46.6, 99.8, 111.5, 114.3, 117.1, 118.9, 122.8, 124.9, 126.4, 127.1, 128.4, 128.7, 128.9, 129.0, 129.8, 132.3, 132.5, 135.7, 174.1, 175.6.

3-(7,9-Dinitropyrido[2,1-a]isoindol-6-yl)-1-phenyl-2,5-pyrrolidinedione (8)

The crude product was recrystallized from DMSO to afford a red powder. Yield: 62%; mp: 290 ^{0}C. ^{1}H NMR (300 MHz, DMSO-d_6): δ = 3.27 (dd, J = 7.8 Hz, J = 18.3 Hz, 1H), 3.79 (dd, J = 10.2 Hz, J = 18.3 Hz, 1H), 5.47 (t, J = 7.8 Hz, 1H), 7.35–7.78 (m, 7H), 8.88 (d, J = 2.1 Hz, 1H), 8.99–9.06 (m, 2H), 9.88 (d, J = 2.1 Hz, 1H). HRMS: calculated for $C_{23}H_{16}N_3O_6$ 430.1033; found 430.1036.

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