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Synthesis of various 3-nitropropionamides as *Mycobacterium tuberculosis* isocitrate lyase inhibitor

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ABSTRACT

Various 3-nitropropionamides were synthesized and evaluated for in vitro activities against log and starved phase culture of two mycobacterial species and *Mycobacterium tuberculosis* (MTB) isocitrate lyase (ICL) enzyme inhibition studies. Among 22 compounds, 1-cyclopropyl-7-(3,5-dimethyl-4-(3-nitropropanoyl)piperazin-1-yl)-6-fluoro-8-methoxy-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (**22**) was found to be the most active compound in vitro with MICs of 0.16 and 0.04 μM against log- and starved-phase culture of MTB. Compound **22** also showed good enzyme inhibition of MTB ICL with IC_{50} of 0.10 ± 0.01 μM. The docking studies also confirmed the binding potential of the compounds at the ICL active site.

Mycobacterium tuberculosis (MTB) claims more human lives each year than any other bacterial pathogen. With approximately 3 million annual deaths in the 1990s, tuberculosis (TB) remains a leading cause of mortality worldwide into the 21st century.[1] It is estimated that one-third of the world population harbor a latent infection by the causative pathogen, MTB. One of the hallmarks of TB is the persistent phase of infection. During this phase the bacteria are thought to be in a slow growing or non-growing state and are recalcitrant to treatment by conventional anti-TB drugs.[2,3] One of the drawbacks of existing TB drugs is that they target actively growing bacteria in cell processes such as cell wall biogenesis protein synthesis and chromosome replication. Patients who carry a latent infection are at risk of reactivation of the disease and this is one of the factors which cause a major obstacle to the global control of TB. Most of the available TB drugs were discovered by the in vitro screening compounds against actively growing MTB under rich nutrient and oxygen conditions. In an infection, however, MTB is often in an environment where nutrients and oxygen are limiting.[4] Under these conditions, MTB is in a reduced state of growth and can persist for long periods of time, avoiding being killed by most TB drugs. This phenomenon of persistence is believed to be the main reason why TB requires a lengthy therapy. A new agent that targets the persistent state of growth would have a significant impact on the treatment of TB by shortening the duration of therapy.

To establish or maintain a persistent infection, MTB appears to require the glyoxylate pathway to bypass the energy-generating tricarboxylic acid cycle (TCA). The glyoxylate pathway uses isocitrate lyase (ICL) and malate synthase to incorporate carbon during growth of microorganisms on acetate or fatty acids as the primary carbon source.[5] A recent study found that a double deletion of both *icl1* and *icl2* resulted in complete impairment of mycobacterial intracellular replication and rapid elimination of the bacteria from the lungs, validating ICL and the glyoxylate pathway as a target for compounds that would eliminate persistent bacteria.[6] The requirement of ICL to a persistent infection makes it an attractive target for drug discovery.[5] 3-Nitropropionate and bromopyruvate were previously shown to inhibit mycobacterial ICL.[7] In this work we have reported synthesis of various 3-nitropropionamides and its in vitro activities against log and starved phase culture of two mycobacterial species and MTB ICL enzyme inhibition studies.

The synthetic protocol of 3-nitropropionamides are depicted in Scheme 1 and briefly, equimolar ratio of 3-nitropropionic acid, dicyclohexylcarbodiimide, and corresponding amines were dissolved in dichloromethane (DCM), dimethyl formamide or DCM–methanol mixture depending upon solubility of amines. The reaction mixture exposed to microwave irradiation of 420 W for 2–3 min. The reaction is monitored by TLC and after completion of the reaction precipitated dicyclohexyl urea was removed by filtration and filtrate is evaporated to gives target compounds **1–22** in 46–86% yield. The purity of compounds was checked by TLC and elemental analyses. Both analytical and spectral data (^{1}H NMR, ^{13}C NMR, and mass spectra) of all the synthesized compounds were in full agreement with the proposed structures.[8]

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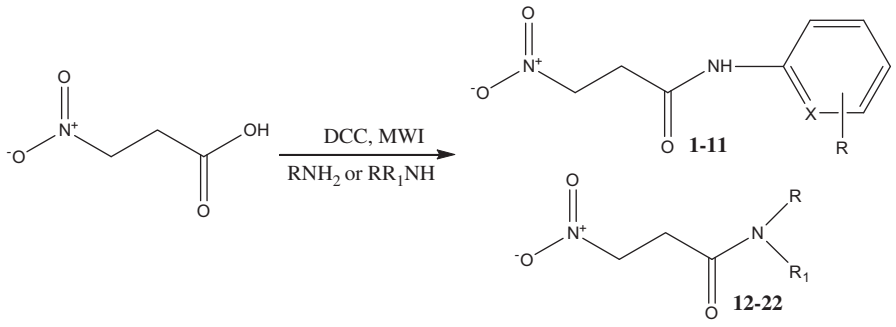
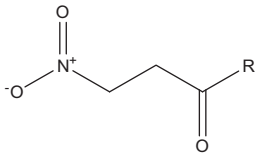


Scheme 1. Synthetic protocol for 3-nitropropinamides.

Lipophilicity of the synthesized derivatives and that of the parent compound 3-nitropropionic acid, are expressed in terms of their log *P* values (Table 1). These values were computed with a routine method called calculated log *P* (*C* log *P*) using ChemBioDraw Ultra 11.0 software.

The compounds were screened for their in vitro antimycobacterial activity initially against log-phase cultures of *M. tuberculosis* H37Rv (OD 600), and *Mycobacterium smegmatis* ATCC 14468 (MS) by agar dilution method similar to that recommended by the National Committee for Clinical Laboratory Standards for the determination of MIC in duplicate.[9] The minimum inhibitory concentration (MIC) is defined as the minimum concentration of compound required to give complete inhibition of bacterial growth. MICs of the synthesized compounds along with the standard drugs for comparison are reported (Table 1). In the initial screening against log-phase MTB, 3-nitropropionamides showed good activity with MICs ranging from 0.16 to 287 μM. Four compounds (**19–22**) showed excellent activity with MIC of <5 μM. When compared to isoniazid (INH) (MIC: 0.72 μM) two compounds (**20** and **22**) were found to be more active and all the compounds were more active than parent 3-nitropropionic acid (MIC: >419.92 μM). Among 22 compounds screened, 1-cyclopropyl-7-(3,5-dimethyl-4-(3-nitropropanoyl)piperazin-1-yl)-6-fluoro-8-methoxy-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (**22**) was found to be the most active compound in vitro with MICs of 0.16 μM against log-phase culture of MTB and was 4.5 times more potent than standard first line anti-TB drug INH. With respect to structure-MTB activity, for the phenyl substituted compounds (**1–9**) compound **1** without any substituent in phenyl ring displayed an MIC of 257.48 μM serves as the standard for potency comparison. The activity slightly improved by the introduction of electron donating groups (**2 and 3**) like methyl and methoxyl group. Whereas introduction of electron withdrawing groups (**3–6**) such as chloro, bromo, and fluoro enhances activity approximately 2 times. Surprisingly introduction di-substitution with both halogen and methyl groups (**8 and 9**) enhances activity to five folds with MIC ranging from 43.51–51.51 μM. Similar kind of SAR found in pyridyl substituted amides (**10 and 11**) also. Compounds with open chain secondary amides showed very poor activity (**12** and **13**) with MIC of >250 μM. Compounds (**19–22**) bearing fluoroquinolone moiety showed excellent activity with MIC of <4 μM. All the compounds were also screened for another mycobacterial species MS, the synthesized compounds inhibited MS with MICs ranging from 0.08 to

Table 1
Physical constants and antimyobacterial activities of 3-nitropropionamides



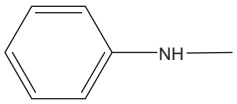
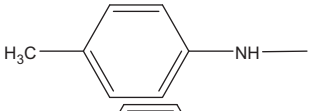
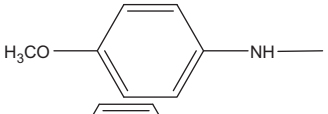
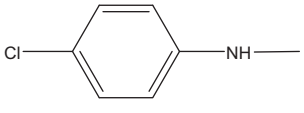
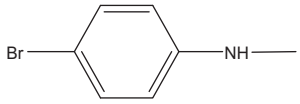
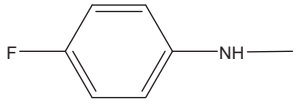
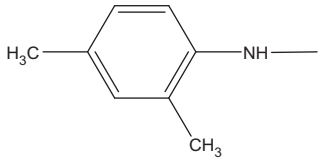
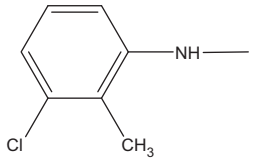
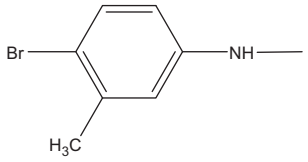
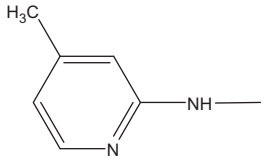
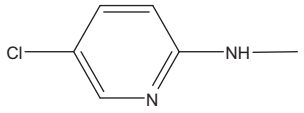
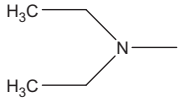
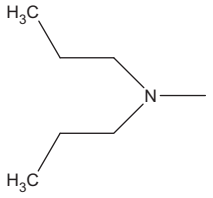
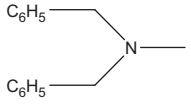
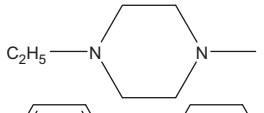
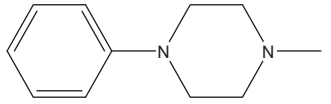
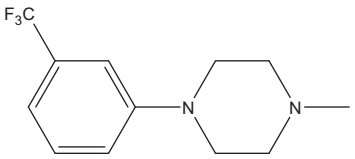
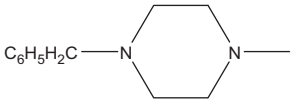
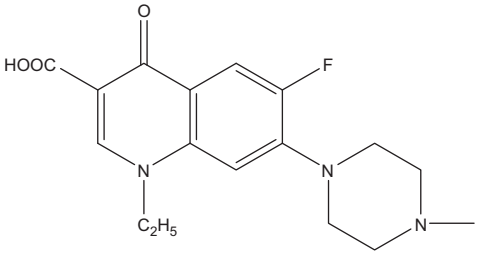
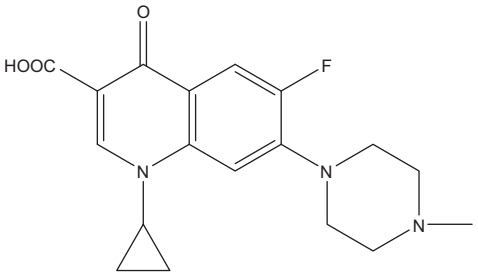
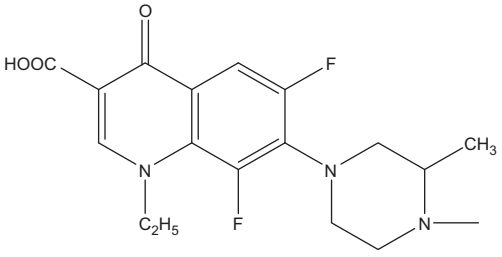
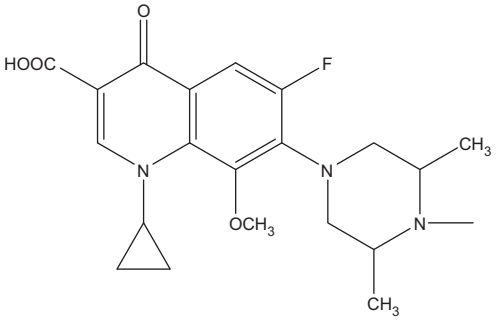
No.	R	Yield (%)	Mp (°C)	*c* log *P*	MIC in μM			
					Active phase		Starved phase	
					MS[a]	MTB[b]	MS	MTB
1		84	197–199	0.81	128.73	257.48	>257.48	64.36
2		67	188–190	1.37	120.07	240.14	>240.14	60.03
3		65	154–155	0.95	111.50	223.00	>223.00	55.75
4		73	174–175	0.99	54.67	109.34	13.69	27.33

Table 1 (*continued*)

No.	R	Yield (%)	Mp (°C)	*c* log *P*	MIC in μM			
					Active phase		Starved phase	
					MS[a]	MTB[b]	MS	MTB
5		82	138–140	1.11	91.54	183.09	91.54	45.77
6		78	204–205	0.67	117.82	117.82	235.64	58.91
7		86	161–162	1.17	224.98	224.98	>224.98	74.99
8		55	133–135	1.49	206.04	51.51	206.04	51.51
9		83	118–120	1.84	87.07	43.53	43.53	43.53
10		59	161–162	0.70	>239.00	239.00	239.00	59.75
11		52	148–149	1.03	217.75	108.87	108.87	54.43
12		83	238–240	0.40	287.02	>287.02	>287.02	>287.02
13		52	220–221	1.46	>247.21	>247.21	>247.21	>247.21
14		51	90–92	2.70	167.59	>167.59	>167.59	>167.59
15		50	120–122	0.53	58.07	58.07	116.14	29.03
16		78	113–115	1.43	189.90	189.90	>189.90	47.47

(*continued on next page*)

Table 1 (*continued*)

No.	R	Yield (%)	Mp (°C)	*c* log *P*	MIC in μM			
					Active phase		Starved phase	
					MS[a]	MTB[b]	MS	MTB
17		56	230–232	2.62	75.46	150.92	75.46	37.73
18		46	164–165	1.72	>180.29	>180.29	>180.29	90.14
19		70	222–223	1.34	7.44	1.85	0.95	0.47
20		85	238–239	1.39	0.09	1.80	0.09	0.09
21		74	224–226	2.01	6.91	3.44	1.72	1.72
22		62	231–232	3.37	0.08	0.16	0.08	0.04
3-Nitropropionic acid				−0.59	>419.92	>419.92	>419.92	>419.92
Isoniazid					45.57	0.72	>364.67	>364.67
Ciprofloxacin					4.71	NT	2.35	NT

[a] *Mycobacterium smegmatis.*
[b] *Mycobacterium tuberculosis*, NT indicates not tested.

287.02 μM and four compounds were more potent than INH (MIC: 45.57 μM). Compound **22** was found to be more potent with MIC of 0.08 μM and was 569 times more potent than INH against log-phase culture of MS.

All the compounds were further screened against six-week nutrient starved cells of MTB according to the literature procedure[10] in duplicate and MICs were reported in Table 1. Against dormant MTB synthesized novel 3-nitropropionic acid derivatives

Table 2
ICL inhibitory studies and cytotoxicity of 3-nitropropionamides

Compounds	IC_{50} (μM)	CC_{50} (μM)
4	21.32 ± 0.80	NT
5	32.46 ± 0.42	NT
9	36.80 ± 0.31	NT
15	12.60 ± 0.21	NT
16	41.21 ± 1.2	NT
17	30.18 ± 0.6	NT
19	0.20 ± 0.04	>148.74
20	0.12 ± 0.01	>144.53
21	1.46 ± 0.04	>138.14
22	0.10 ± 0.01	>127.42
3-NP	116.0 ± 1.1	<524.90

NT indicates not tested.

Table 3
Computational data for the compounds

Molecule	Affinity	Moldock score	Hydrogen bonding energy	Interacting amino acid residues
11	−32.26	−107.37	−19.29	Ser191, Gly192, His193, Tyr89, Ser91, Gly92, Trp93
21	−31.89	−83.07	−15.57	Tyr89, Ser91, Gly92, Trp93, His180, Ser191, Gly192, Arg228, Asn313, Ser317
22	−30.9	−107.71	−18.86	Tyr89, Ser91, Trp93, His180, Ser191, Gly192, His193, Asn313, Ser315, Ser317
3-NP	−30.43	−89.75	−14.21	Ser315, Ser317, Ser191, His193, Asn313, Leu194
4	−29.98	−103.65	−15.16	Ser315, Ser191, Gly192, His193, Asn313, Leu194

showed good activity with MICs ranging from 0.04 to >287 μM. Four compounds (**19–22**) showed excellent activity with MIC of <2 μM. When compared to standard MTB ICL inhibitor 3-nitropropionic acid (MIC: >419.92 μM) 19 compounds were found to be more active. When compared to INH (MIC: >364.67) which is not active against dormant MTB, most of the synthesized compounds showed good activity. Among the compounds screened, **22** was found to be the most active compound in vitro with MICs of 0.04 μM against starved-phase culture of MTB and was 4 times more potent towards starved-phase MTB than log-phase MTB. The presence of persistent and dormant MTB is thought to be the cause for the lengthy TB chemotherapy, since the current TB drugs are not effective in eliminating persistent or dormant bacilli. Therefore, these drugs active against slowly growing or non-growing persistent bacilli are thought to be important to achieve a shortened therapy. In starved MS culture the tested compounds inhibited growth with MIC ranging from 0.04 to >287 μM and most of the compounds were more potent than standard 3-nitropropionic acid and INH.

Several mechanisms may be involved in the killing of non-growing cells. The lack of efficacy of INH (which targets cell wall mycolic acid biosynthesis) in starved cells suggests that cell wall synthesis is not important during starvation, a phenomenon that has been commonly observed in other bacteria.[11] Drugs could target processes that are critical for survival even when bacteria are not replicating, such as transcription, allowing drugs like rifampin to retain some activity. Non-growing cells might have alterations in their cell walls that result in changed permeability to antibiotics. Finally, a drug might associate with the bacteria during incubation and not be removed by washing, thus manifesting its antibacterial effects during outgrowth. Any or all of these mechanisms might be functional in vivo, where a persistent antibacterial effect might lead to more-rapid clearance of infection during treatment. The strategy for survival of TB during chronic stages of infection is thought to involve a metabolic shift in the bacteria's carbon source to C_2 substrates generated by the β-oxidation of fatty acids.[12] Under these conditions, glycolysis is decreased and the glyoxylate shunt is significantly up-regulated allowing anaplerotic maintenance of the TCA cycle.[13] The glyoxylate shunt converts isocitrate to succinate and glyoxylate, catalyzed by the enzyme ICL, followed by the addition of acetyl-CoA to glyoxylate to form malate by malate synthase. It has been shown that expression of ICL is up-regulated under certain growth conditions[14] and during infection of macrophages by *Mycobacterium* spp.[15] Furthermore, ICL is required for the survival of bacteria in activated macrophages but not in

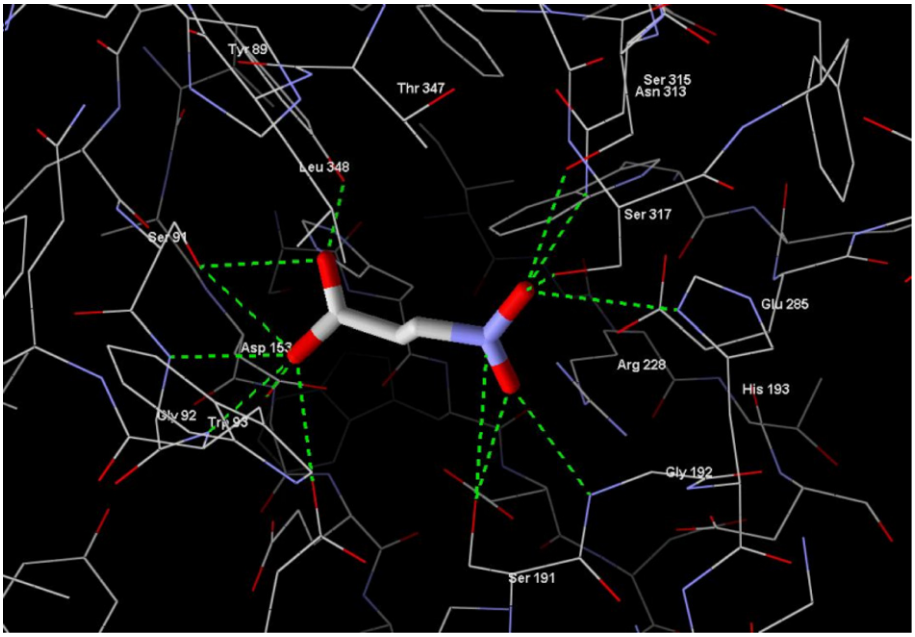


Figure 1. Figure depicting binding site of 3-nitropropionate in ICL.

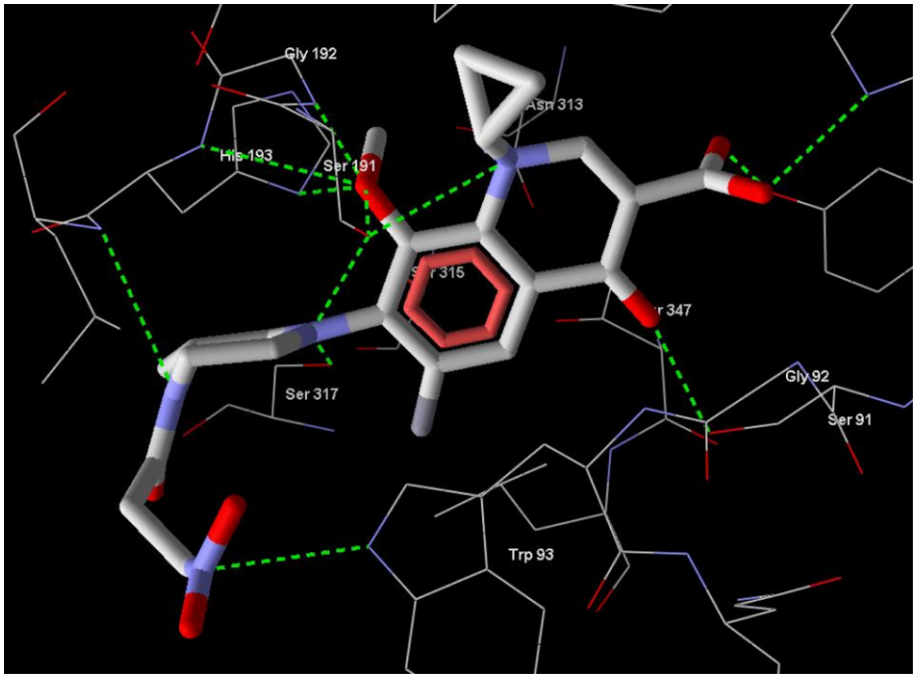


Figure 2. Figure depicting binding of **22** to ICL at the active site of 3-nitropropionate.

resting macrophages.[6] It has also been demonstrated that ICL is important for survival of MTB in the lungs of mice during the persistent phase of infection (2–16 weeks), but is not essential during the acute phase (0–2 weeks) of infection.[6]

As these synthesized compounds showed activity against dormant mycobacterium, we decided to explore the possible mechanism by screening some compounds against MTB ICL enzyme (Table 2).[16] Among 10 compounds screened, three compounds (**19, 20**, and **22**) inhibited ICL with IC_{50} of less than 1 μM. All the 10 screened compounds showed better activity (IC_{50} 0.1–36.80 μM) than standard 3-nitropropionic acid (IC_{50} 116 μM).

Four compounds were further examined for cytotoxicity (CC_{50}) in a mammalian Vero cell line at single concentration of 62.5 μg/mL. After 72 h of exposure, viability was assessed on the basis of cellular conversion of MTT into a formazan product using the Promega Cell Titer 96 non-radioactive cell proliferation assay. Most of the compounds were not cytotoxic to Vero cells (Table 2). Compound **22** was not toxic up to 127.42 μM with selectivity index of 3185 for starved MTB. CC_{50} of standard anti-TB compound INH is >455.73 μM.

In this study we have designed and developed various small molecule inhibitors of MTB ICL based on 3-nitripropionate. A series of derivatives of 3-nitropropionate were synthesized and docked into catalytic core of ICL and coordinates were taken from PDB ID: 1F8I. Docking studies using Molegro Virtual Docker 2.2.5 program,[17] showed that **4**, **11**, **21**, **22** has given better affinity, Moldock score, hydrogen bonding energy compared to 3-nitropropionate (Table 3). Moreover the interacting amino acid residues were also almost same (Figs. 1 and 2).

Acknowledgments

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8. Spectroscopic data for representative compound **22** is given below. ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm): 0.28–0.46 (m, 4H, cyclopropyl), 1.32 (m, 1H, cyclopropyl), 1.15 (d, 6H, $-CH_3$ J = 9.8 Hz), 2.86 (t, 2H, $-CH_2$ J = 7.1 Hz), 3.44–3.51 (m, 4H, $-CH_2$), 3.53 (s, 3H, $-OCH_3$), 4.02 (m, 2H, –CH), 4.42 (t, 2H, $-CH_2$ J = 7.4 Hz), 6.71 (s, 1H, C5-H), 7.95 (s, 1H, C2-H), 12.00 (s, 1H, COOH); ^{13}C NMR (DMSO-d_6) δ (ppm): 176.4 (1C═O of 4-quinolone), 171.1 (1C═O of amide),, 166.2 (1C═O of 3-COOH), 158.6 (1CH of 6-benzene), 146.9 (1C of 2-quinolone), 146.3 (1CH of 8-benzene), 132.5 (1CH of 7-benzene), 125.9 (1C of 8a-quinolone), 123.8 (1C of 4a-quinolone), 109.3 (1C of 3-quinolone), 104.5 (1CH of 4-benzene), 72.0 (3rd CH_2 of nitropropanoyl), 55.8 (1C of methoxy), 54.4 (2×C of piperazine), 53.1(2×C of piperazine), 36.1(1×C of cyclopropane), 28.6 (2nd CH_2 of nitropropanoyl), 18.7 (2×C of methyl), 7.7 (2×C of cyclopropane). Anal ($C_{23}H_{27}FN_4O_7$) C, H, N.
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