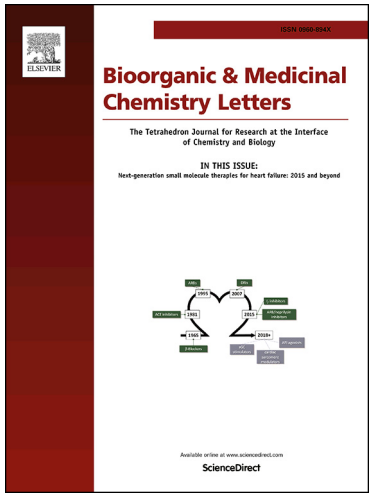
Accepted Manuscript



Design, synthesis, and bioevaluation of a novel class of *(E)*-4-oxo-crotonamide derivatives as potent antituberculosis agents

Jinfeng Ren, Jian Xu, Guoning Zhang, Changliang Xu, LiLi Zhao, XueFu You, Yucheng Wang, Yu Lu, Liyan Yu, Juxian Wang

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Design, synthesis, and bioevaluation of a novel class of *(E)*-4-oxo-crotonamide derivatives as potent antituberculosis agents

Jinfeng Ren[a,#], Jian Xu[a,b,#], Guoning Zhang[a], Changliang Xu[a,c], LiLi Zhao[a], XueFu You[a], Yucheng Wang[a], Yu Lu[b], Liyan Yu[a,*] and Juxian Wang[a,*]

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c Jiangsu Protein Drug Engineering Laboratory, Food & Drug Analysis and Testing Center, Jiangsu Food & Pharmaceutical Science College, Huai'an 223003, Jiangsu, China.

Abstract: A series of novel *(E)*-4-oxo-2-crotonamide derivatives were designed and synthesized to find potent antituberculosis agents. All the target compounds were evaluated for their *in vitro* activity against *Mycobacterium tuberculosis* H_{37}Rv(MTB). Results reveal that 4-phenyl moiety at part A and short methyl group at part C were found to be favorable. Most of the derivatives displayed promising activity against MTB with MIC ranging from 0.125 - 4 μg/mL. Especially, compound **IIIa16** was found to have the best activity with MIC of 0.125 μg/mL against MTB and with MIC in the range of 0.05 - 0.48 μg/mL against drug-resistant clinical MTB isolates.

Keywords: *Mycobacterium tuberculosis*; antituberculosis agents; *(E)*-4-oxo-2-crotonic acid derivatives; drug-resistant-TB.

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#These authors contributed equally to this work.

Tuberculosis (TB) is an airborne, highly contagious, infectious disease caused by the *Mycobacterium tuberculosis* (MTB), which kills about 1.3 million people

worldwide in 2016.[1] The recent upsurge in the incidence of multidrug-resistant TB (MDR-TB) and extensively drug-resistant TB (XDR-TB) has reaffirmed TB as a serious public health problem worldwide.[2-4] Besides, MTB-HIV coinfection further emphasizes the desperate need for new anti-TB agents.[5-7] However, the fact is that few new TB drugs have been introduced to the repertoire of anti-TB therapies over the past 50 years.[8-10] Although the discovery of bedaquiline and delamanid has renewed hope for the treatment of TB and especially MDR-TB, it is strictly recommended for the treatment of MDR-TB owing to side effects such as QT prolongation and liver function abnormalities.[11-15] Therefore, it is urgently and highly valuable to discover and develop antimycobacterial molecules with novel scaffolds as effective anti-TB drug candidates.

In our previous research, leading compound IMB-YH-8 (**3a**, Fig. 1), was identified as a protein kinase B (PknB) inhibitor from a high throughput screen (HTS), which is one of the most important serine/threonine protein kinases (STPKs) for MTB and plays a critical role in the growth of mycobacteria.[16-20] It exhibited good anti-tuberculosis activity (MIC: $H_{37}Rv$ 0.25 μg/mL, MDR-TB 0.25-1 μg/mL) and low toxicity (LD_{50} 568 mg/kg, BALB/c mice, p.o.), which encouraged us to explore its structure-activity relationship (SAR) against MTB as a new scaffold.[21-22] In the preceding work,[23] we reported the biological activity and SAR of series (*E*)-4-oxo-crotonester derivatives of IMB-YH-8 as potential PknB inhibitors. The results indicated that α,β-unsaturated ketone scaffold and "trans-" configuration are essential for the activity against PknB (Fig. 1).

Based on the above research results, considering the amide analogs had stable metabolism, it was decided to replace the ester group with the amide group at part C, and meanwhile introduce various substituents to the benzene ring at part A in this study (Fig. 1)**.** Thus, a series of novel (*E*)-4-oxo-crotonamide derivatives were designed, synthesized, the targeted compounds and the esters were evaluated *in vitro* anti-tuberculosis activities. Our primary objective was to optimize the potency of

these compounds against MTB including MDR-TB. A preliminary SAR study was also explored to facilitate the further development of the *(E)*-4-oxo-2-crotonyl scaffold.

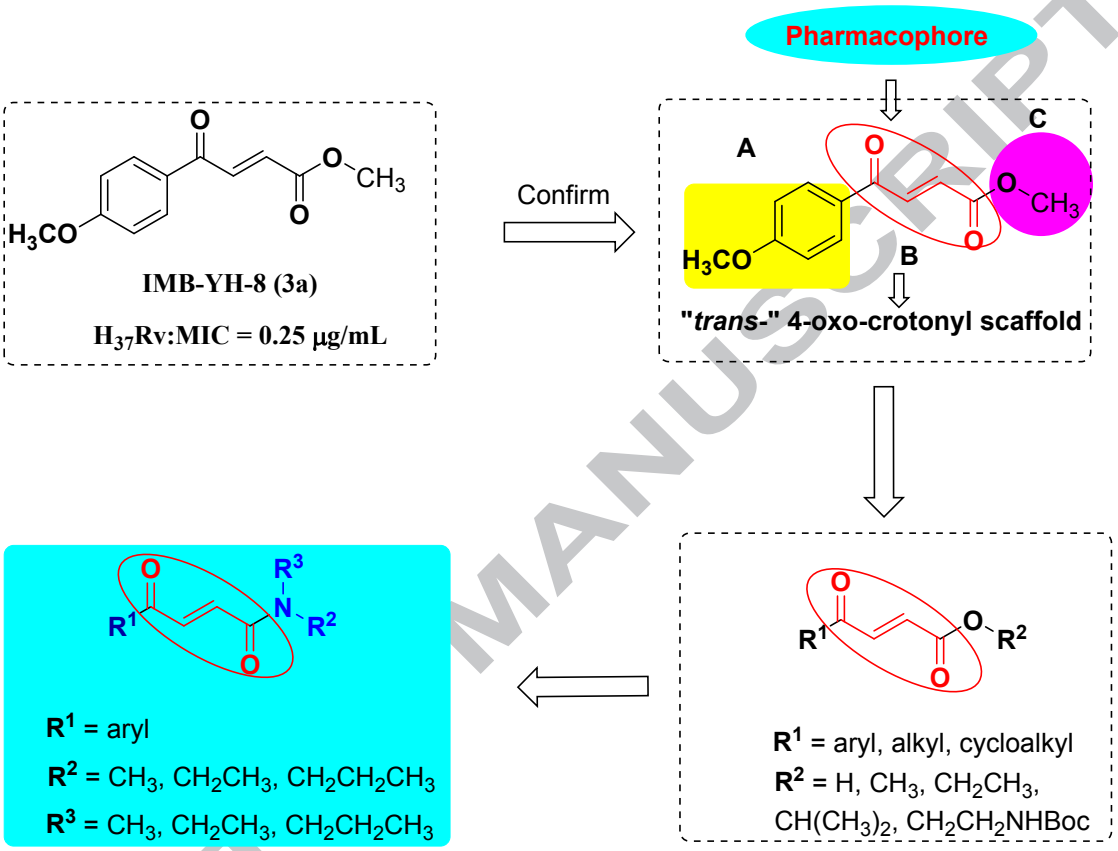
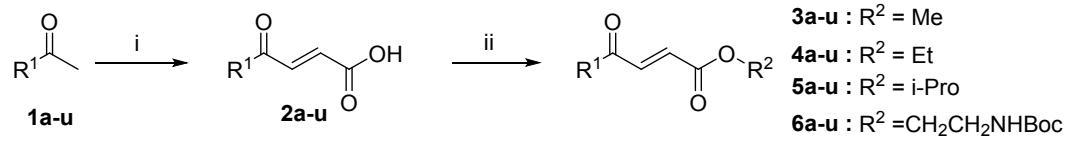


Fig. 1 Illustration of Compounds Design Strategy

The ester analogs were easily prepared based on the *(E)*-4-oxo-2-crotonyl scaffold of IMB-YH-8 for SAR investigations in the preceding work[23] (Scheme 1).

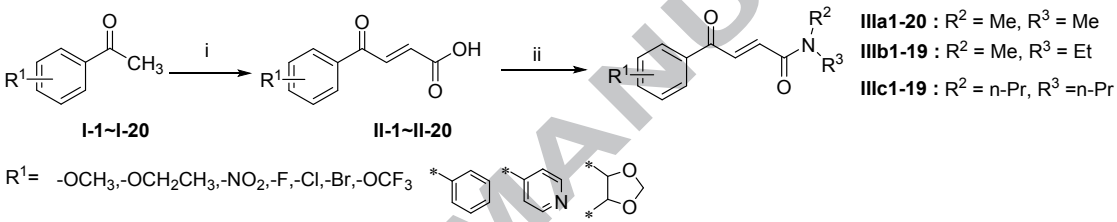


R1 = aryl, alkyl, cycloalkyl.

Reagents and conditions: (i) glyoxylic acid, sulfuric acid (Cat.), AcOH, reflux, 8h, for **2a-q** and **2s-u**; glyoxylic acid, morpholine hydrochloride (Cat.), reflux, overnight, for **2r**; (ii) ROH, sulfuric acid (Cat.), reflux, overnight, for **3a-u**, **4a-u**, **5a-u**; IBCF, TEA, anh. DCM, -15 °C, then N-Boc-ethanolamine, rt, 6 h, for **6a-u**.

Scheme 1. Synthesis of (*E*)-4-oxo-crotonester derivatives

Synthetic pathways to novel (*E*)-4-oxo-crotonamide derivatives **IIIa1-20**, **IIIb1-19** and **IIIc1-19** (58 compounds in total) are depicted in Scheme 2. A series of substituted 4-phenyl-4-oxo-crotonic acids (**II-1~20)** were directly prepared from corresponding substituted acetophenone **I-1~I-20** and glyoxylic acid in 85%-93% yields. Amidation of acids **II-1~20** with different amines through an EDCI/HOBT/DIEA mediated coupling afforded the amides **IIIa1-20**, **IIIb1-19** and **IIIc1-19** in around 50% yield.



Reagents and conditions: (i) glyoxylic acid,conc. H_2SO_4 (Cat.),AcOH, reflux, 6 h; (ii) R_2R_3NH, EDCI,HOBt, DBU, dry DMF, r.t., 24 h.

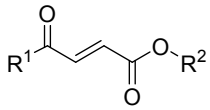
Scheme 2. Synthesis of (*E*)-4-oxo-crotonamide derivatives

All target compounds were preliminarily screened for *in vitro* activity against MTB $H_{37}Rv$ ATCC27294 strain using the Microplate Alamar Blue Assay (MABA)[24-25], including the esters synthesized in preceding work.[23] The minimum inhibitory concentration (MIC) is defined as the lowest concentration effecting a reduction in fluorescence of >90% relative to the mean of replicate bacterium-only controls. The MIC values of them along with the lead compound IMB-YH-8 (**3a**), and isoniazid (INH) for comparison were presented in μg/mL in Table 1and Table 2.

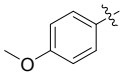
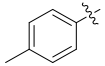
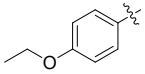
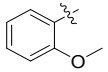
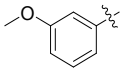
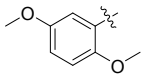
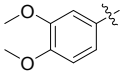
The data for esters in Table 1 reveal that the carboxylic acids and N-Boc ethyl esters are inactive against this strain (MIC: >4 μg/mL), while most of the esters show only moderate activity (MIC: <1μg/mL), weaker than IMB-YH-8 (MIC: 0.25 μg/mL). Only compounds **3k**, **4k** and **5k** with 4-phenyl have a little better activities (MIC: 0.2-0.24 μg/mL), suggesting the presence of the 4-phenyl moiety at part A is found to be favorable.

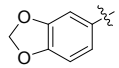
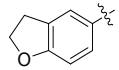
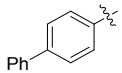
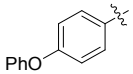
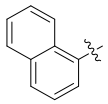
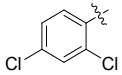
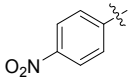
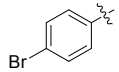
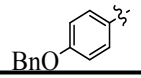
For the esters groups at part C, methyl (**3**) makes more contribution to anti-tuberculosis activity than ethyl (**4**), while isopropyl (**5**) is the lowest with few exceptions and N-Boc ethyl (**6**) eliminates the activity in vitro. Mostly, compounds containing an aryl group at part A show higher potency than that substituded with alkyl analogs (**3r-t**, **4r-t** and **5r-t**). Compounds bearing a substituent on the *para*-position of the phenyl (R^1) generally show higher activities than the corresponding *meta*- and *ortho*-substituent analogs (**3a**,**4a** vs **3e-3f**, **4e-4f**), whereas benzodiazepyl moiety possesses antitubercular activity among the double substituted derivatives (**3g-3j**, **4g-4j** and **5g-5j**). There are small differences in activity between the electron-donating groups and the electron-withdrawing groups. The contribution of the electron-donating groups is generally as follows: phenyl > methoxyl > methyl > ethyoxyl > naphthyl > H > benzyloxyl > phenoxyl . On the other hand, the activity of the electron-withdrawing groups is in the order: Br > NO_2 > 2,4-dichloro. Overall, the potency of the derivatives is related to the substituents on benzene ring at part A and the size of alkyl groups at part C.

Table 1 Structures of ester analogs and their a*in vitro* activity against MTB $H_{37}Rv$



2a-u: R^2 = H
3a-u: R^2 = Me
4a-u: R^2 = Et
5a-u: R^2 = i-Pr
6a-u: R^2 = $BocNH(CH_2)_2$

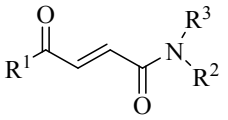
R^1	Compd.	MIC(μg/mL)	Compd.	MIC(μg/mL)	Compd.	MIC(μg/mL)	Compd.	MIC(μg/mL)	Compd.	MIC(μg/mL)
	**2a**	>4	**3a**	0.25	**4a**	0.46	**5a**	1.64	**6a**	>4
	**2b**	>4	**3b**	0.86	**4b**	0.82	**5b**	0.78	**6b**	>4
	**2c**	>4	**3c**	0.37	**4c**	>4	**5c**	0.77	**6c**	>4
	**2d**	>4	**3d**	0.41	**4d**	0.54	**5d**	0.47	**6d**	1.45
	**2e**	>4	**3e**	0.99	**4e**	1.82	**5e**	1.91	**6e**	>4
	**2f**	>4	**3f**	0.39	**4f**	0.82	**5f**	0.92	**6f**	>4
	**2g**	>4	**3g**	0.88	**4g**	1.05	**5g**	1.48	**6g**	>4
	**2h**	>4	**3h**	0.44	**4h**	0.82	**5h**	1.94	**6h**	>4

	**2i**	>4	**3i**	0.38	**4i**	0.49	**5i**	0.87	**6i**	>4
	**2j**	>4	**3j**	0.84	**4j**	0.94	**5j**	1.02	**6j**	>4
	**2k**	>4	**3k**	0.20	**4k**	0.23	**5k**	0.24	**6k**	1.23
	**2l**	>4	**3l**	0.87	**4l**	1.52	**5l**	1.69	**6l**	>4
	**2m**	>4	**3m**	0.45	**4m**	0.61	**5m**	0.69	**6m**	>4
	**2n**	>4	**3n**	1.85	**4n**	1.09	**5n**	1.01	**6n**	>4
	**2o**	>4	**3o**	0.54	**4o**	0.48	**5o**	0.50	**6o**	>4
	**2p**	>4	**3p**	0.49	**4p**	0.52	**5p**	0.41	**6p**	>4
	**2q**	>4	**3q**	0.52	**4q**	1.09	**5q**	0.65	**6q**	>4
Me	**2r**	>4	**3r**	0.86	**4r**	3.51	**5r**	2.50	**6r**	>4
	**2s**	>4	**3s**	1.20	**4s**	>4	**5s**	>4	**6s**	>4
	**2t**	>4	**3t**	>4	**4t**	>4	**5t**	0.68	**6t**	1.45
	**2u**	>4	**3u**	0.67	**4u**	0.96	**5u**	1.14	**6u**	1.91

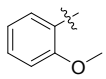
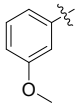
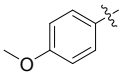
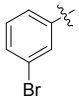
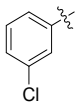
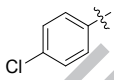
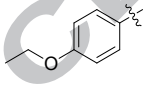
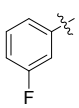
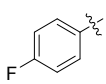
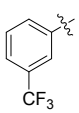
Positive control: Isoniazid: MIC=0.025 μg/ml; IMB-YH-8(3a): MIC=0.25 μg/ml.

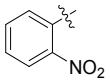
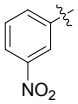
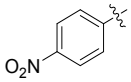
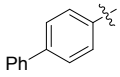
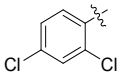
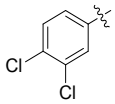
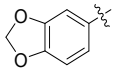
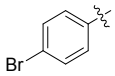
A further strategy to increase activity and improve pharmacokinetic properties was to replace ester group by N-substituted amide groups at part C containing an aryl group at part A. Thus, we synthesized a series of N-substituted amide analogs and evaluated their anti-tuberculosis activities against MTB $H_{37}Rv$ (Table 2). Though most of the analogs had considerable activity against this strain, they all were much less than IMB-YH-8 except **IIIa16** (MIC: 0.125 μg/mL). Only considering the para-substitued groups, compounds **IIIa1-20** with simple and short methyl group on the N atom exhibits higher activity than the amides **IIIb1-19** and **IIIc1-19** with long alkyl substituents (ethyl and propyl), except for fluoro substitution compounds. Therefore, it indicates that introduction of a longer alkyl substituents would be detrimental to the activity. In addition introduction of the substituents at the meta-position of the benzene ring also leads to decreased activity (e.g. **IIIa1-IIIa3**, **IIIa5-IIIa7**). In the N,N-diethyl series of compounds (**IIIb1-19**), quite surprisingly, compounds with electron-withdrawing groups (F, Cl, NO_2) show good activity (MIC: 0.24 μg/mL), which is more active than that with electron-donating groups (methoxyl, ethyoxyl, phenyl, benzodiazepyl), and comparable to IMB-YH-8 (**3a**). However, it is difficult to distinguish whether an electron-donating group contributes more to the activity or an electron-withdrawing group in the N,N- dimethyl series of compounds (**IIIa1-20**), and the activity of the para-substitued groups is in the order: phenyl > bromo > methoxyl ≈ cloro > fluoro ≈ ethyoxyl > nitro.

Table 2 Structures of N-substituted amides and their *in vitro* activity against MTB $H_{37}Rv$



IIIa1-20: $R^2 = R^3 = Me$
IIIb1-19: $R^2 = Me$, $R^3 = Et$
IIIc1-19: $R^2 = R^3 = n\text{-}Pr$

R^1	Compd.	MIC (μg/mL)	Compd.	MIC (μg/mL)	Compd.	MIC (μg/mL)
	**IIIa1**	0.92	**IIIb1**	0.95	**IIIc1**	>2
	**IIIa2**	1.62	**IIIb2**	1.88	**IIIc2**	>2
	**IIIa3**	0.38	**IIIb3**	0.93	**IIIc3**	1.89
	**IIIa4**	0.96	**IIIb4**	1.52	**IIIc4**	>2
	**IIIa5**	0.84	**IIIb5**	0.65	**IIIc5**	>2
	**IIIa6**	0.89	**IIIb6**	0.97	**IIIc6**	1.91
	**IIIa7**	0.38	**IIIb7**	0.24	**IIIc7**	1.67
	**IIIa8**	0.48	**IIIb8**	0.48	**IIIc8**	1.91
	**IIIa9**	0.95	**IIIb9**	0.24	**IIIc9**	0.94
	**IIIa10**	1.84	**IIIb10**	0.48	**IIIc10**	0.96
	**IIIa11**	0.47	**IIIb11**	0.25	**IIIc11**	0.95
	**IIIa12**	1.95	**IIIb12**	>2	**IIIc12**	>2

	**IIIa13**	1.51	**IIIb13**	0.24	**IIIc13**	1.24
	**IIIa14**	>2	**IIIb14**	>2	**IIIc14**	>2
	**IIIa15**	0.93	**IIIb15**	1.70	**IIIc15**	1.89
	**IIIa16**	0.125	**IIIb16**	0.49	**IIIc16**	1.4
	**IIIa17**	0.41	**IIIb17**	>2	**IIIc17**	>2
	**IIIa18**	0.84	**IIIb18**	0.24	**IIIc18**	0.88
	**IIIa19**	0.79	**IIIb19**	>2	**IIIc19**	>2
	**IIIa20**	0.25	-	-	-	-

- Compound was not synthesized.
- Positive control: Isoniazid: MIC=0.025 μg/ml; IMB-YH-8(3a): MIC=0.25 μg/ml.

As shown in Table 1 and 2, converting the acids into esters and N-substituded carboxamides enhanced the anti-tuberculosis activity. Generally, the activity of the derivatives against Mycobacterium tuberculosis as follows: methyl ester > ethyl ester > isopropyl ester > amide > Boc aminoethyl ester. The date reveal that 4-phenyl moiety at part A and short methyl group at part C are found to be favorable and introduction of the substituents at the *para*-position of the benzene ring at part A is acceptable.

While most of esters and N-substituded amides retained activity against $H_{37}Rv$ (MIC: 0.125 - 4 μg/mL), the most active compounds **IIIa16** (MIC: 0.125 μg/mL) were found more potent than that of IMB-YH-8 (MIC: 0.25 μg/mL). Then we selected compounds **IIIa16** for further *in vitro* anti-tuberculosis activity against some drug-resistant TB strains. MIC values indicated that **IIIa16** also showed good activity

(MIC: 0.05 - 0.48 μg/mL) against drug-resistant clinical isolates (Table 3).

Table 3 *In vitro* activity of IIIa16 against drug-resistant clinical isolates of *M. tuberculosis*

Clinical isolates	IIIa16 (MIC: μg/mL)	INH	RFP	SM	EMB	MXF	Amoxicillin/ Clavulanic acid
19809	0.48	S	S	S	S	R	R
19852	0.19	S	S	S	S	S	R
19874	0.23	R	R	S	S	R	R
19769	0.43	R	R	S	S	R	R
19675	0.45	S	S	S	S	S	R
19863	0.23	S	S	S	S	S	R
19805	0.25	S	S	S	S	R	R
19806	0.23	S	S	S	S	R	R
19810	0.12	R	R	R	R	R	R
11168	0.05	R	R	S	R	S	S

S: Susceptible; R: Resistant; INH: Isoniazid; RFP: Rifampicin; SM: Sterptomycin; EMB: Ethambutol; MXF: Moxifloxacin.

In summary, a series of novel *(E)*-4-oxo-crotonamide derivatives were designed and synthesized as new anti-TB agents, including esters synthesized in preceding work. In part A, the aryl group had been replaced with an alkyl group, reducing their antituberculosis activities, and the diphenyl group may be a preferred structure. In part C, the ester and N-substituted amide analogs had generally considerable activity against MTB $H_{37}Rv$ (MIC: 0.125 - 4 μg/mL). Among all tested compounds, compound **IIIa16** displayed the best activity of MIC 0.125 μg/mL against MTB $H_{37}Rv$ and also exhibited potent anti-tuberculosis activity against drug-resistant clinical MTB isolates (MIC: 0.05-0.48 μg/mL) and it may serve as a new and promising lead compound for further antitubercular drug discovery.

Acknowledgments

This work was supported by the major New Drug Innovation (2014ZX09201001-011, 2015ZX09102007-016-001), the National Natural Science Foundation of China (Nos. 81473099, 81373452, 81473098 and 81621064), Microbial Resources (No.NIMR-2018-3), and CAMS Innovation Fund for Medical Sciences (2016-I2M-2-002, 2016-I2M-3-014).

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Design, synthesis, and bioevaluation of a novel class of *(E)*-4-oxo-crotonamide derivatives as potent antituberculosis agents

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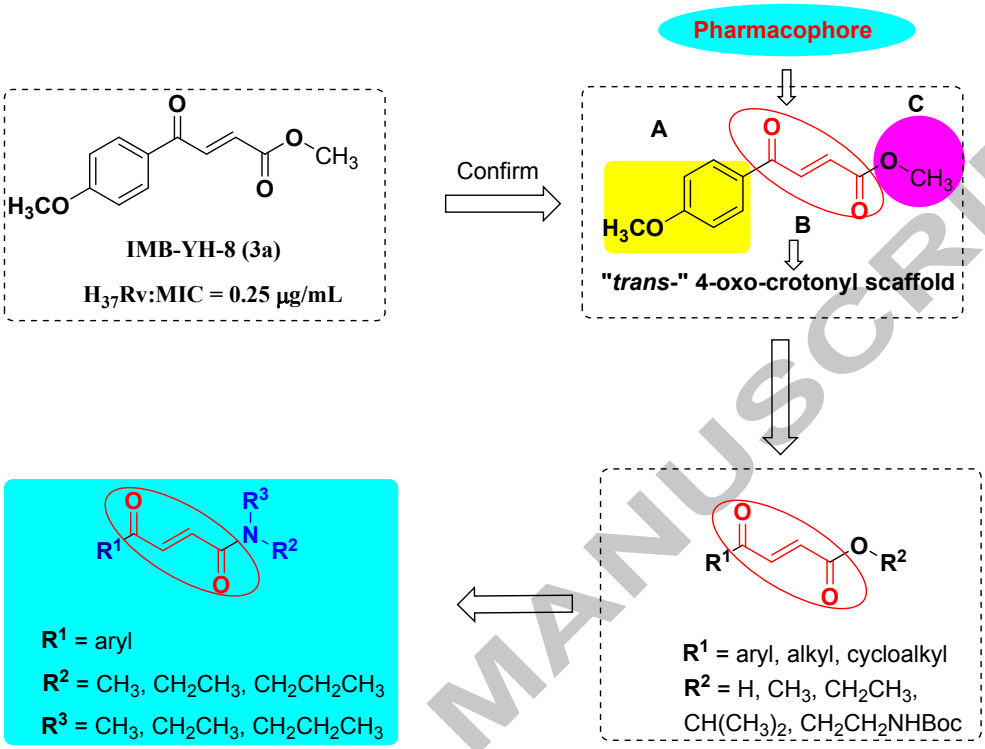
A series of novel *(E)*-4-oxo-2-crotonamide derivatives were designed and synthesized to find potent antituberculosis agents and evaluated for their biological activity. Our results reveal that compound **IIIa16** was found to have the best activity with MIC of 0.125 μg/mL against MTB and with MIC in the range of 0.05 - 0.48 μg/mL against

drug-resistant clinical MTB isolates. Results reveal that 4-phenyl moiety at part A and short methyl group at part C were found to be favorable.



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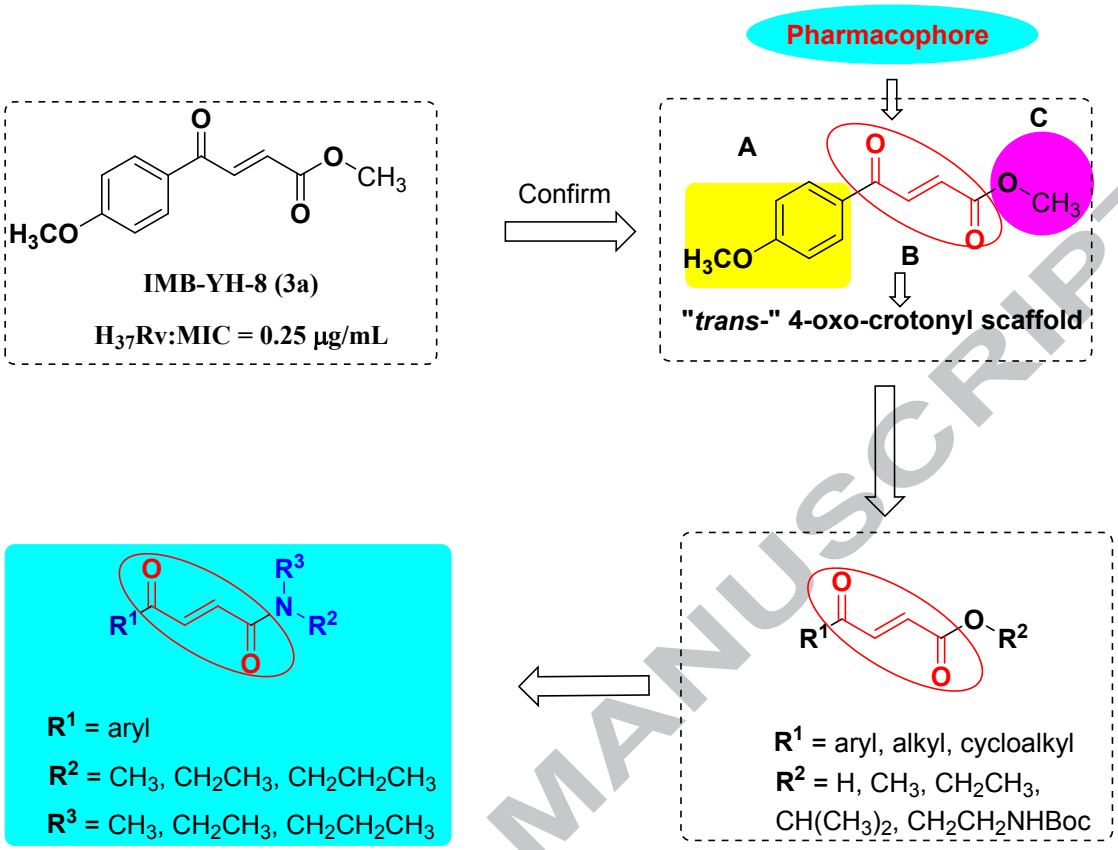
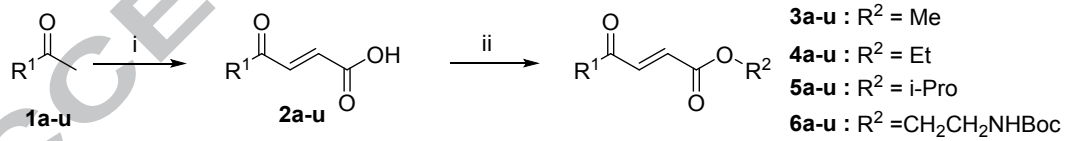


Figure.1 Illustration of Compounds Design Strategy

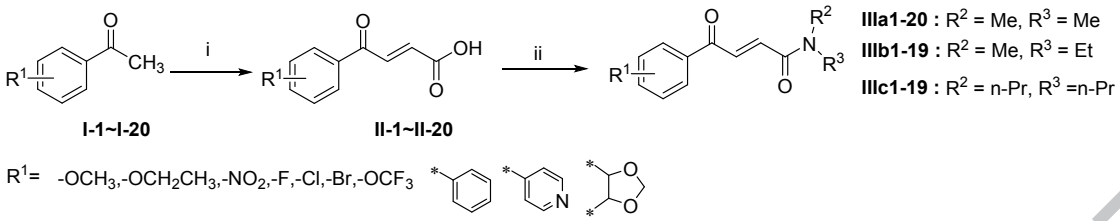
27.



R^1 = aryl, alkyl, cycloalkyl.

Reagents and conditions: (i) glyoxylic acid, sulfuric acid (Cat.), AcOH, reflux, 8h, for **2a-q** and **2s-u**; glyoxylic acid, morpholine hydrochloride (Cat.), reflux, overnight, for **2r**; (ii) ROH, sulfuric acid (Cat.), reflux, overnight, for **3a-u**, **4a-u**, **5a-u**; IBCF, TEA, anh. DCM, -15 °C, then N-Boc-ethanolamine, rt, 6 h, for **6a-u**.

Scheme 1. Synthesis of (*E*)-4-oxo-crotonester derivatives



Reagents and conditions: (i) glyoxylic acid,conc. H_2SO_4 (Cat.),AcOH, reflux, 6 h; (ii) R_2R_3NH, EDCI,HOBt, DBU, dry DMF, r.t., 24 h.

Scheme 2. Synthesis of (*E*)-4-oxo-crotonamide derivatives

28.

1. Novel *(E)*-4-oxo-crotonamide derivatives containing different substituents were designed and synthesized.
2. The SAR were summarized for further antitubercular drug discovery.
3. Compound **IIIa16** exhibit potent MIC values against MTB $H_{37}Rv$ and some MDR-MTB strains.

29.