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Synthesis, antimycobacterial evaluation and pharmacophore modeling of analogues of the natural product formononetin

Peggoty Mutai[a], Elumalai Pavadai[a], Ian Wiid[b], Andile Ngwane[b], Bienyameen Baker[b], Kelly Chibale[a,c,d*]

[a]*Department of Chemistry, University of Cape Town, Rondebosch 7701, South Africa*
[b]*DST-NRF Centre of Excellence for Biomedical Tuberculosis Research, SAMRC Centre for Tuberculosis Research, Division of Molecular Biology and Human Genetics, Faculty of Medicine and Health Sciences, Stellenbosch University, PO Box 19063, Tygerberg 7505, South Africa*
[c]*South African Medical Research Council Drug Discovery and Development Research Unit, University of Cape Town, Rondebosch 7701, South Africa*
[d]*Institute of Infectious Disease and Molecular Medicine, University of Cape Town, Rondebosch 7701, South Africa*

*Corresponding author

Email address: Kelly.Chibale@uct.ac.za (K. Chibale)

Abstract

The synthesis and antimycobacterial activity of formononetin analogues is hereby reported. Formononetin and its analogue **11E** showed 88% and 95% growth inhibition, respectively, against the H37Rv strain of *Mycobacterium tuberculosis.* Pharmacophore modeling studies indicated that the presence of a hydroxyl group in formononetin and its analogues, is crucial for maintaining activity.

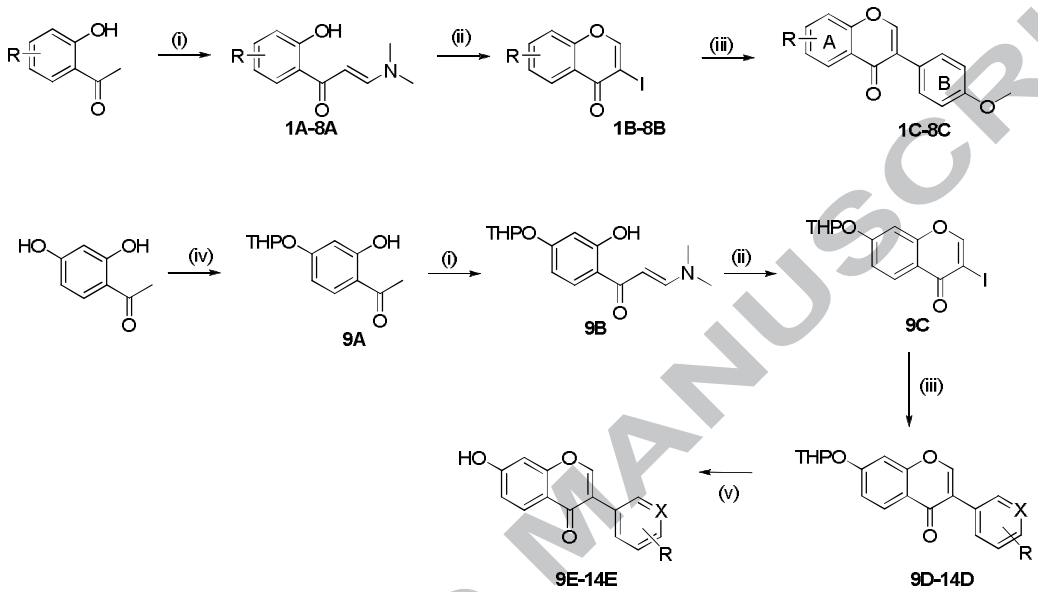
Keywords Antimycobacterial, Formononetin, Pharmacophore, Structure, Activity

Tuberculosis (TB) is a leading cause of mortality and morbidity, being estimated to infect about one third of the world's population [1]. The Global tuberculosis report by WHO in 2013 reported that there were an estimated 8.6 million incidents of TB in 2012 and 1.3 million TB related deaths [2].The HIV scourge has made the TB situation worse, especially in sub-Saharan Africa, with the co-epidemic being particularly concentrated in the Southern African countries where HIV prevalence is high [3]. Increasing rates of drug-resistant tuberculosis are a significant concern and pose serious implications for current and future treatment of the disease[4]. Resistance has been reported for new drugs such as bedaquiline[5], indicating that there is an urgent need for more new drug candidates to raise the probability of developing a novel, short-course and safe 'universal' regimen applicable to drug-susceptible and all forms of drug-resistant TB[6].

Natural products have played a prominent role in the management of tuberculosis. A number of phytochemicals have been found to possess antimycobacterial activity while many of the currently used drugs in the treatment of TB such as streptomycin and rifampicin are derived from microbial natural sources [7,8]. Formononetin is an isoflavone that is widespread in the Leguminoseae family. It has previously been reported to possess various pharmacological activities such as the inhibition of proliferation of prostate, breast and colon cancer cells, wound and bone fracture healing, antihypertensive activity as well as anti-infective activities especially against giardiasis where it is reported to be more potent than metronidazole, the drug of choice in the treatment of giardiasis[9–12]. In addition, formononetin has been reported to have strong multi-drug resistance reversal effects by inhibiting the P-gp efflux pump that is responsible for drug resistance [13]. This is of particular interest in tuberculosis as drug efflux has been highlighted as an important mechanism of drug resistance by *Mycobacterium tuberculosis*[14].

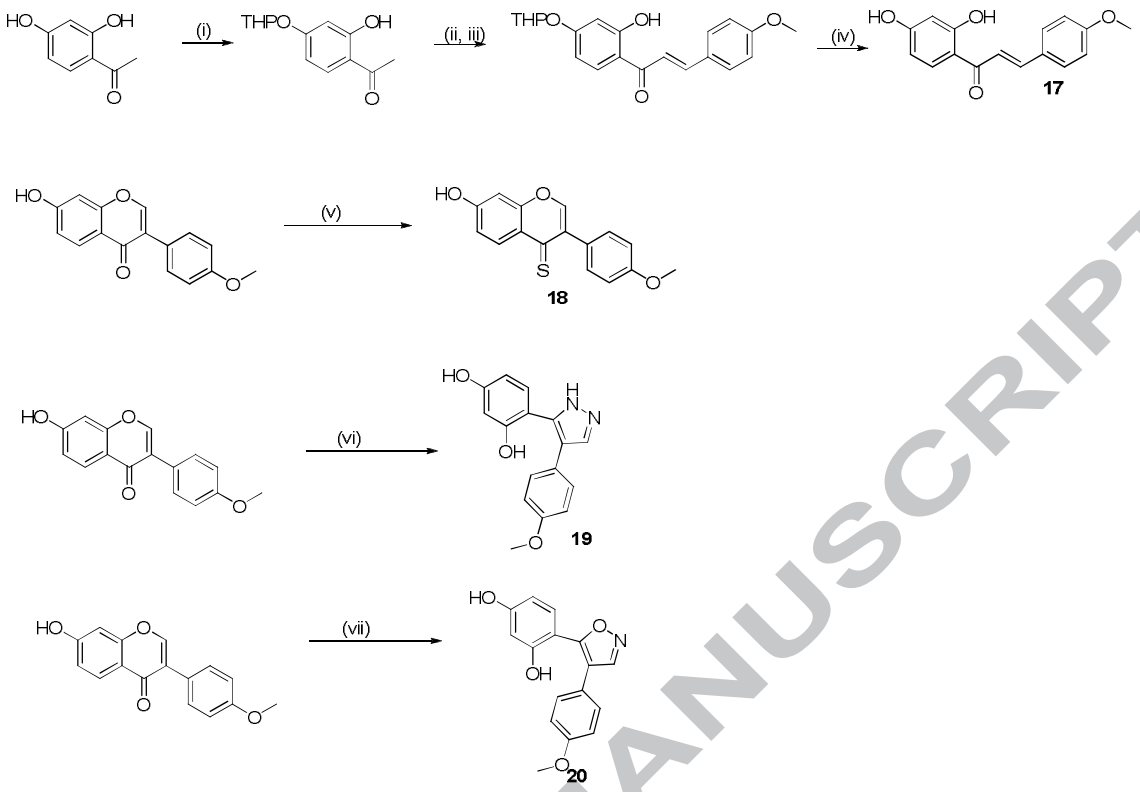
This paper reports the antimycobacterial activity of formononetin and derives a tentative structure activity relationship of its analogues based on the percent inhibition of the H37Rv strain of *Mycobacterium tuberculosis.* Further, pharmacophore modeling studies on the formononetin analogues were performed to elucidate the structural determinant responsible for the biological activity.

Synthesis of formononetin analogues was carried out by modification of rings A and B as well as the benzopyranone linker. Modifications on ring A and ring B were achieved through similar synthetic schemes. For ring A modifications, the starting materials had varied substituents at either position 5 or 6 (compounds **1**-**8**) while for ring B modifications, the scheme started with protection of the para-hydroxyl group on ring A (compounds **9**-**14**) (Scheme 1). Compounds **15** and **16** were synthesized using scheme 1 but varying both R_1 and R_2.



Scheme 1: *Reagents and conditions*: (i) DMF-DMA, 95°C, 3h (ii) I_2, pyridine, $CHCl_3$, rt, 12h, (iii) $ArB(OH)_2$, Pd/C, Na_2CO_3, DME/H_2O, 45°C, 4h (iv) DHP, PPTS, DCM, rt, 4h (v) p-TsOH, MeOH, THF, 60°C, 2h

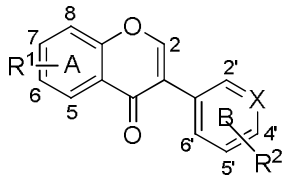
Modifications on the benzopyrone linker were achieved through various synthetic schemes. (Scheme 2)



Scheme 2: *Reagents and conditions*: (i)DHP, PPTS, DCM, rt, 4h; (ii) EtOH, aq. K_2CO_3, rt, 30 min; (iii) p-methoxybenzaldehyde, 80°C reflux, 8h (iv) p-TsOH, MeOH, THF, 60°C, 2h (v) Lawesson's reagent, Toluene, reflux, 110°C, 1h; DMF, 10h (vi) $N_2H_4.H_2O$; EtOH, reflux, 60-80°C, 2h (vii) Hydroxylamine hydrochloride, N-methylmorpholine, EtOH, Reflux, 80°C, 8h

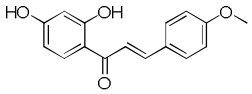
Twenty analogues of formononetin were synthesized and tested for antimycobacterial activity against *Mycobacterium tuberculosis* H37Rv (ATCC 27294) using the Microtiter Alamar Blue assay (MABA) as previously described [15] (Table 1& 2).

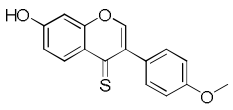
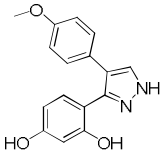
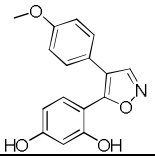
Table 1: Antimycobacterial activity of formononetin analogues with modifications on rings A and B



Compound	R^1	R^2	X	% inhibition at 10µM
Formononetin	7-OH	4'-OCH_3	C	88
1C	7-H	4'-OCH_3	C	0
2C	6-F	4'-OCH_3	C	0
3C	7-F	4'-OCH_3	C	0
4C	6-Cl	4'-OCH_3	C	0
5C	6-OCH_3	4'-OCH_3	C	0
6C	6-Br	4'-OCH_3	C	5
7C	6-CH_3	4'-OCH_3	C	7
8C	6-OH	4'-OCH_3	C	28
9E	7-OH	4-CN	C	16
10E	7-OH	4-Cl	C	54
11E	7-OH	4-$C(CH_3)_3$	C	95
12E	7-OH	4'-OCH_3	N	0
13E	7-OH	3'-F, 4'-OCH_3	C	13
14E	7-OH	3'-OCH_3, 4'-OCH_3	C	14
15	7-H	4'-H	C	0
16	7-F	4'-OCH_3	N	2

Table 2: Antimycobacterial activity of formononetin analogues:

Compound	Structure	% inhibition at 10µM
17		58

18		4
19		40
20		15

When all compounds were tested at a concentration of 10µM, the parent compound, formononetin, exhibited 88% inhibition of the H37Rv strain of *Mycobacterium tuberculosis*. All modifications on ring A led to a decrease or loss of activity. When the hydroxyl group was moved from position 7 to position 6 (**8C**), there was a marked drop in activity from 88% to 28% inhibition. When the OH was replaced by other groups such as F, Cl, Br, CH_3, OCH_3, activity was lost. Replacing the OCH_3 group in ring B of formononetin with a *tert*-butyl group (**11E**) improved activity from 88% to 95% inhibition. When the hydrophobic electron withdrawing chloro substituent was introduced (**10E**) activity dropped to 54% inhibition. A change to hydrophilic groups such as CN (**9E**) drastically reduced activity to 16%. The unsubstituted isoflavone skeleton (**15**) was devoid of antimycobacterial activity at 10µM. Compound **16**, which is a hybrid of compound **3C** and **12E**, did not display any antimycobacterial activity, just like **3C** and **12E**. When the benzopyrone linker in formononetin was replaced by an open chain linker, activity dropped from 88% inhibition to 58% inhibition. When the benzopyrone was replaced by an unfused heterocyclic ring, there was a further drop in activity as is noted with the pyrazole linked compound (**19**) and the isoxazole linked compound (**20**), which displayed 40% and 15% inhibition, respectively.

In order to elucidate the structural determinant(s) responsible for the biological activity of formononetin analogues, pharmacophore modeling studies were performed on the 21 formononetin analogues using Catalyst/HipHop of Discovery Studio 4.0. (see supplementary information). The HipHop run resulted in 10 ranked five-feature models, which are presented in Supplementary Table S1. It was observed that all models have the same pharmacophore features. However, the spatial locations and statistical parameters are different. A top-ranked model selected as the best pharmacophore model on the basis of fit values of compounds,

which contains five features; two hydrogen-bond acceptors, a hydrogen-bond-donor, an aromatic ring and a hydrophobic feature, as shown in **Fig 1 (A)**. The model fits well on the most active compound **11E** and its fit value of 13.95 as expected (Table 3). The mapping on **11E** shows that the two hydrogen-bond acceptors located on the oxygen atom and keto group of the furan ring on the chromone moiety, one hydrogen bond donor located on the hydroxyl group of chromone moiety, an aromatic feature located on the benzyl group as well as a hydrophobic feature corresponding to the tertiary butyl substituent, as shown in **Fig 1 (B).** In addition, the mapping of the model was performed on inactive compounds, which suggested that the hydrogen-bond donor feature is missing in most of the inactive compounds, indicating that the hydrogen-bond donor feature is important for biological activity. Additionally, the pharmacophore fit values of inactive compounds are significantly reduced in the absence of the hydrogen-bond donor feature (see Table 3). The mapping of one of the inactive compounds **3C**, with a fit value of 3.91, is shown in **Fig 1 (C)**. The fit values for the 21 compounds obtained by the pharmacophore model are presented in Table 3. The pharmacophore model distinguishes all active compounds from inactive ones with high fit values. The model successfully predicted all 4 active compounds (100 % success, above the fit value cut off of 13 for above the antibacterial activity of 50 %) whereas 13 out of the 17 inactive compounds are predicted by values below this threshold (76% success, below the fit value cut off of 13 for below the antibacterial activity of 50 %).

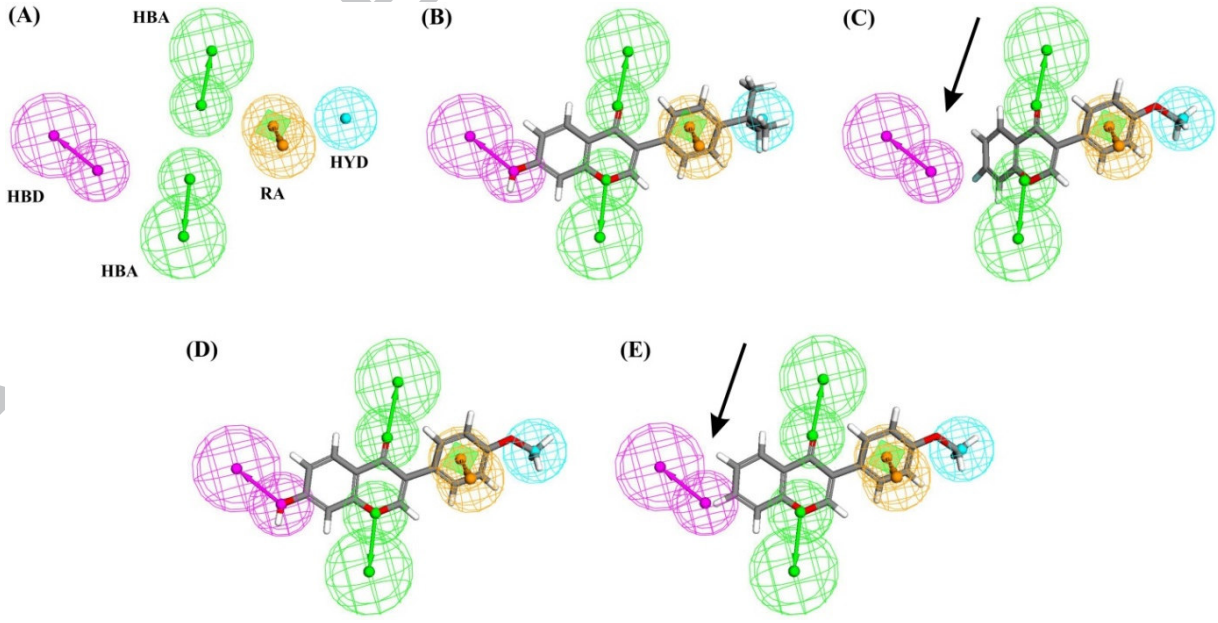


Fig 1. Pharmacophore model and its mapping with active and inactive compounds. **(A)** Common feature pharmacophore model generated by HipHop algorithm. Pharmacophore features are color-coded with green

spheres for hydrogen-bond acceptor (HBA), purple sphere for hydrogen-bond donor (HBD), orange sphere for aromatic ring (RA) and cyan sphere for hydrophobic (HYD). **(B)** The model mapping with the most active compound **11E** and its pharmacophore fit value of 13.95. **(C)** Mapping of the model with one of the inactive compounds 3C and its pharmacophore fit value of 3.91. (**D**) Mapping of the model with the parent compound formononetin and its fit value of 14. (**E**) Mapping of the model with 1C and its fit value of 3.97. The missed pharmacophore HBD feature is indicated by an arrow in (C) and (E).

Importantly, the pharmacophore model is capable of explaining the activity difference between formononetin (active) and its most inactive analogues, for example **1C**. These two compounds differ by a single functional group, hydroxyl (-OH), in formononetin that maps into the hydrogen-bond donor pharmacophore feature [**Fig 1 (D**)]. This feature is missing in **1C** owing to absence of the hydroxyl group [**Fig 1 (E)**]. As a result, there is a big difference in these fit values of the two compounds and further signifies that the hydrogen-bond donor feature is responsible for activity differences between formononetin and **1C**.

Table 3. Qualitative activity data (active/inactive) and fit values based on the best pharmacophore model for all 21 formononetin analogues.

Compound Name	Experimental Activity (%)	Experiment Scale	Estimated Scale	Pharmacophore Fit value
11E	95	+	+	13.95
Formononetin	88	+	+	14.00
17	58	+	+	13.35
10E	54	+	+	13.62
19	40	-	-	10.69
8C	28	-	-	12.79
9E	16	-	-	12.96
20	15	-	-	10.74
14E	14	-	+	13.87
13E	13	-	+	13.75
7C	7	-	-	3.97
6C	4	-	+	13.14
16	5	-	-	3.97
18	2	-	-	3.99
12E	0	-	+	13.89
5C	0	-	-	4.00

1C	0	-	-	3.97
4C	0	-	-	3.97
2C	0	-	-	3.97
3C	0	-	-	3.91
15	0	-	-	1.05

[a]Experimental activity scale: +, % of activity > 50 (active); - , % of activity < 50 (inactive).

[b]Estimated scale based on pharmacophore fit value: +, fit value > 13 (active); -, fit value < 13 (inactive).

[c]Fit value indicates how well the features in the pharmacophore overlap the chemical features in the molecule and was calculated by the equation (1).

In conclusion, formononetin analogues were synthesized and evaluated for their antimycobacterial activity against *M. tuberculosis*. This is the first report of the antimycobacterial activity of formononetin. All modifications on formononetin led to decreased activity, apart from the ring B modification with a *tert*-butyl substituent, which improved activity from 88% to 95% inhibition. Pharmacophore modeling analysis on formononetin analogues showed that the presence of a hydroxyl group in formononetin is essential for high potency. The model will be used as a guide to direct future synthetic efforts aimed at developing more potent formononetin-based antimycobacterial compounds.

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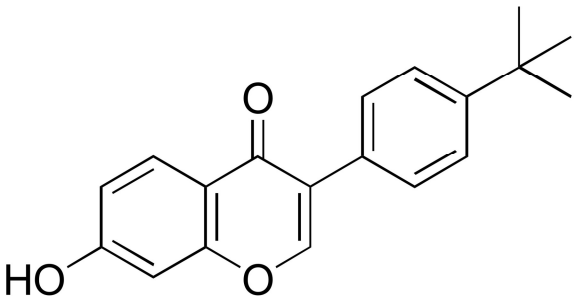

11E