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# Biflavonoids as Novel Antituberculosis Agents

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**Abstract**—A series of naturally occurring and synthetic biflavonoids was evaluated for inhibitory activity against *Mycobacterium tuberculosis H37Rv* (*Mtb*). Compounds **6**, **24**, and **25** demonstrated 96, 95, and 87% inhibition, respectively, at a screening concentration of 12.5 μg/mL. The type of linkage and the presence of methoxy- and nitro-substituents in biflavonoids may contribute to the observed inhibitory activity. The results of this study represent the discovery of biflavonoids as a potential new class of antituberculosis agent. 

Tuberculosis (TB), an infection of *Mycobacterium tuberculosis* (*Mtb*), is the leading cause of worldwide death among infectious diseases.[1,2] In turn, infectious disease remains the leading cause of death in the world today, greater than cardiovascular disease or cancer.[3] Currently, one-third of the world's population is infected with *Mtb*, a facultative intracellular bacillus.[4] The World Health Organization (WHO) estimates that within the next 20 years, an estimated 1 billion people will be newly infected, 200 million will become sick and 35 million will die.[5] The recent resurgence of TB is associated with the emergence of the HIV/AIDS epidemic[6] and the rapid spread of multidrug resistant TB strains. TB infection is a serious problem for hemodialysis patients,[7] and is also the leading cause of death in women of childbearing age around the world.[8]

Current treatment of TB requires a patient to take at least two antibiotics in order to decrease the risk of drug resistance. The leading combination therapy requires isoniazid and rifampicin for an extended period of time. This may be supplemented with pyrazinamide and ethambutol when isoniazid resistance is suspected. Recently, rifapentine,[9] a derivative of rifamycin, was approved by the FDA for the treatment of tuberculosis.[10] The rapid spread of TB worldwide has intensified the need for more efficient drugs to combat this disease.

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Biflavonoids are a series of naturally occurring compounds that include flavone–flavone, flavanone–flavone and flavanone–flavanone subunit linkages (Fig. 1). More than 100 biflavonoids have been identified from plants since the isolation of gingetin in 1929.[11,12] A variety of biological activities for biflavonoids have been published, including antiinflammatory, antimicrobial, antioxidant, and others.[12,13] We have recently reported the anti-HIV-1, anti-HBV, and antiviral activities of biflavonoids isolated from *Rhus succedanea* L. (Anacardiaceae) and *Garcinia multiflora* Champ. (Guttiferae).[12,14–16] In continuing our drug discovery program, we present the screening results for a series of naturally occurring and synthetic biflavonoids against *Mtb*.

Symmetric biflavones with a carbon–carbon linkage were produced by Ullmann condensation of monohalogenoflavonoids.[17,18] Unsymmetrical biflavones (**1**–**5**) were synthesized by Ullmann condensation of the corresponding iodoflavones.[19] Compound **6** (6–6″ biapigenin hexamethylether) was generated in four steps from benzyl 4-iodo-3,5-dimethoxyphenylether (**I**) (Scheme 1). 4,4′-Dibenzyloxy-2,2′,6,6′-tetramethoxybiphenyl (**II**) was prepared from the Ullmann reaction of compound **I**.[20] The Hoesch reaction was performed on compound **II** with $CH_3CN$, $ZnCl_2$ and HCl in dry $CHCl_3/Et_2O$ (1:1) to yield the key product, 4,4′-dihydroxy-3,3′-diacetyl-2,2′,6,6′-tetramethoxybihenyl (**III**). Treatment with two mols of *p*-anisaldehyde in the presence of base afforded bichalcone **IV**. Oxidative cyclization of the bichalcone with $SeO_2$ in dioxane followed by

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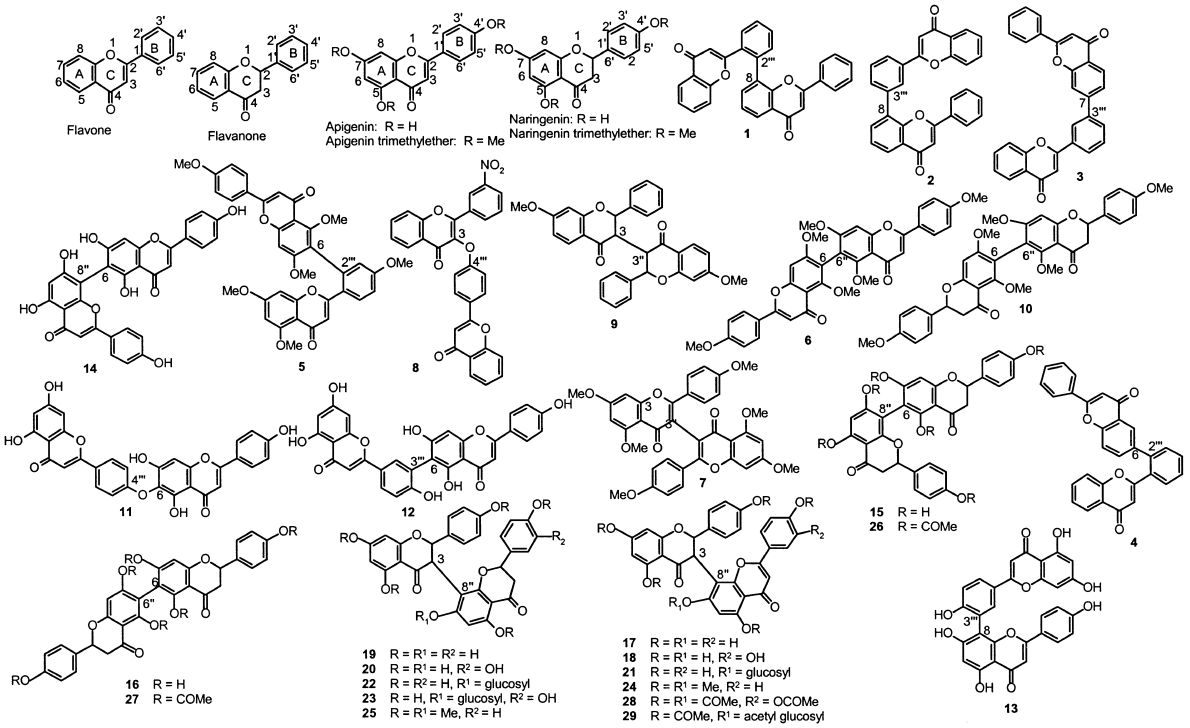
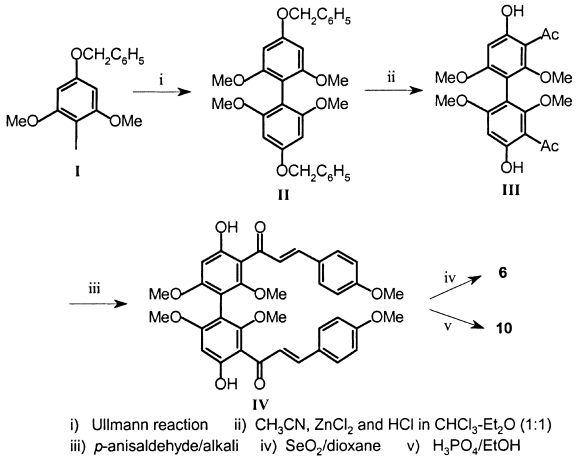



**Figure 1.** Structures of flavonoids and biflavonoids.




**Scheme 1.** (i) Ullmann reaction; (ii) $CH_3CN$, $ZnCl_2$ and HCl in $CHCl_3/Et_2O$ (1:1); (iii) *p*-anisaldehyde/alkali; (iv) $SeO_2$/dioxane; (v) $H_3PO_4$/EtOH.

purification by preparative TLC yielded 6,6″-biapigenin hexamethylether (**6**). Cyclization of bichalcone (**IV**) by refluxing with alcoholic $H_3PO_4$ under acidic conditions for 3 weeks afforded 6,6″-Binarigenin hexamethylether (**10**).

Naturally occurring biflavonoids were isolated from seed kernels of *R. succedanea* and *G. multiflora*.[21,22] Briefly, seed kernels were defatted with hexane followed by extraction with 95% ethanol and concentration of the extract solution. Hinokiflavone (**11**) and Robustaflavone (**12**) were precipitated as pigment A during the early stages of concentration due to their low solubilities. The solution was filtered to remove pigment A and the filtrate was further concentrated. Amentoflavone (pigment B, **13**), with intermediate solubility in ethanol, was precipitated and removed by filtration. The filtrate separated from pigment B was evaporated to yield pigment C, a brown solid.[21] Pigment A was purified by silica gel column chromatography with toluene/pyridine/formic acid (36:10:2) as the eluting solvent to yield hinokiflavone (**11**) and robustaflavone (**12**), successively. Alternatively, robustaflavone hexaacetate was obtained by the reaction of pigment A with acetic anhydride/pyridine and recrystallization from a mixture of methylene chloride/ethyl acetate (~9:1). Hydrolysis of this compound with 2 M NaOH produced robustaflavone (**12**). Repeated recrystallization of pigment B from MeOH afforded amentoflavone (**13**). Pigment C was purified by silica gel (toluene/ethyl acetate, 4:1–1:9) and polyamide (water/methanol 9:1–1:9) column chromatography, providing agathisflavone (**14**), rhusflavanone (**15**), and succedaneaflavanone (**16**).[21]

Biflavonoids **17**–**23** were isolated from the heartwood of *G. multiflora*.[22] Briefly, the dried heartwood shavings were extracted with hot methanol. The methanol extract was treated with toluene and ethyl acetate, successively. The ethyl acetate-soluble fraction was purified by both silica gel chromatography (toluene/ethyl acetate, 7:3–1:9) and polyamide chromatography (methanol/water, 1:9–9:1).

Volkensiflavone hexamethylether (**24**) and GB-1a hexamethylether (**25**) were prepared by methylation of volkensiflavone (**17**) and GB-1a (**19**) with dimethylsulfate in dry acetone and potassium carbonate.[22] Rhusflavanone hexaacetate (**26**), succedaneaflavanone hexaacetate (**27**), morelloflavone heptaacetate (**28**), and spicataside acetate (**29**) were prepared by acetylation of com-

pounds **15**, **16**, **18**, and **21**, with acetic anhydride and pyridine.[22,23]

The compounds were screened for TB inhibition against *Mtb H38Rv* in BACTEC 12B medium using the BACTEC 460 radiometric system.[24] Biflavones composed of two flavone units with linkages of 8–2‴ (**1**), 8–3‴ (**2** and **13**), 7–3‴ (**3**), 6–2‴ (**4** and **5**), 6–6′ (**6**), 3–3′ (**7**), 6–8′ (**14**), 6–3‴ (**12**), 3–*O*–4‴ (**8**), and 6–*O*–4‴ (**11**) (Fig. 1) were screened against *Mtb* and the results are summarized in Table 1. Among these biflavones, biflavone **6**, which is constructed with two molecules of apigenin trimethyl ether in a 6–6″ linkage, exhibited highest inhibitory activity (96%) at a concentration of 12.5 μg/mL. The MIC value was estimated to be greater than 12.5 μg/mL. It is notable that biflavones **5** and **7**, which consist of the same flavone units as **6**, but with different linkages (6–2‴ and 3–3″), were completely devoid of activity. This result suggests that the 6–6″ linkage is required for the inhibitory activity of biflavone. Biflavone **8**, which possesses a nitro-group and a linkage of 3–*O*–4‴, exhibited moderate activity with 61% inhibition against *Mtb*. In the absence of methoxy- or nitro-substituents, biflavones **1**–**4** were inactive (**1** and **3**) or weakly (**4**) to moderately active (**2**). In addition, compounds with hydroxy substituents (**11**–**14**) were completely inactive against *Mtb*. These results indicated that methoxy- and nitro-groups and a linkage of C6–C6′ or C3–*O*–C4‴ might contribute to the biflavone anti-TB activity.

The inhibitory activities of biflavonoids that are composed of flavanone–flavone subunits are listed in Table 2. Biflavonoids **17**, **18**, **21**, **28**, and **29**, with a 3–8″ linkage, were completely devoid of activity. These compounds contain polar substituents such as hydroxy- (**17** and **18**), acetoxy- (**28** and **29**) or glucosyl- (**20**). Methylation of **17** to provide hexamethylether **24** led to a significant increase in inhibitory activity of 95% against *Mtb*.

All biflavanones with a 3–8″ linkage possessing hydroxy- (**19** and **20**), acetoxy- (**26** and **27**) or glucosyl- (**22** and **23**) groups were inactive against *Mtb*. However, compound **25**, the hexamethylether of GB-1a (**19**), demonstrated an inhibitory activity of 87% against *Mtb* growth (Table 3). These results indicate that methoxy groups may play a role in the increased activity of a flavanone–flavone or a biflavanone with a 3–8″ linkage. Other biflavanones such as 6–8″ (**15**) and 6–6″ (**16**) binaringenins and their hexaacetates **26** and **27** were inactive. Compounds **9** and **10** with 3–3″ and 6–6″ linkages, respectively, were inactive, despite the presence of methoxy substituents. Both methoxy groups and a 3–8″ linkage appear to be important for the anti-TB activity of biflavanones.

It has been reported that a C-8 methoxy group increased the bacteriostatic activities of fluoroquinolones and quinolones against moderately resistant, clinical isolates of *Mtb*,[25,26] and that introducing nitro- or methoxy-substituents to 3-hydrazono-1*H*-2-indolinones increased their anti-TB activities.[27] These results coincide with our observations that methoxyl- and nitro-groups are requirements for the anti-TB activity of biflavonoids. Factors besides the lipophilic character of a compound may therefore affect inhibitory activity against *Mtb*.

The above results represent the discovery of biflavonoids as a new class of anti-TB agents. A 6–6″ linkage for biflavone hexamethylether, a 3–8″ linkage for flavanone–flavone compound and biflavanone hexamethylether, and increased lipophilic properties are required for inhibitory activity. The mechanistic and pharmacological properties and the determination of structure–activity relationships of biflavonoids are critical for the development of new anti-TB drugs.

**Table 1.** Antituberculosis activities of biflavones

| Compound number | Compound name | % Inhibition at 12.5 μg/mL |
|---|---|---|
| **1** | 8-2‴-Biflavone | 0 |
| **2** | 8-3‴-Biflavone | 49 |
| **3** | 7-3‴-Biflavone | 0 |
| **4** | 6-2‴-Biflavone | 9 |
| **5** | 6-2‴-Biapigenin hexamethylether | 1 |
| **6** | 6-6″-Biapigenin hexamethylether[a] | 96 |
| **7** | 3-3″-Biapigenin hexamethylether | 0 |
| **8** | 3′-Nitro-3-*O*-4′-biflavone | 61 |
| **11** | Hinokiflavone (6-*O*-4‴-biapigenin) | 0 |
| **12** | Robustaflavone (6-3‴-biapigenin) | 0 |
| **13** | Amentoflavone (8-3‴-biapigenin) | 0 |
| **14** | Agathisflavone (6-8″-biapigenin) | 0 |

[a]MIC > 12.5 μg/mL.

**Table 2.** Antituberculosis activities of flavanone–flavones

| Compound number | Compound name | % Inhibition at 12.5 μg/mL |
|---|---|---|
| **17** | Volkensiflavone (3-8″-naringenylapigenin) | 0 |
| **18** | Morelloflavone (3-8″-naringenylluteolin) | 0 |
| **21** | Spicataside (volkensiflavone-7-glucoside) | 0 |
| **24** | Volkensiflavone hexamethylether[a] | 95 |
| **28** | Morelloflavone heptaacetate | 0 |
| **29** | Spicataside acetate | 0 |

[a]MIC = 12.5 μg/mL.

**Table 3.** Antituberculosis activities of biflavanones

| Compound number | Compound name | % Inhibition at 12.5 μg/mL |
|---|---|---|
| **9** | 3-3″-Bi-(7-methoxyflavanone) | 0 |
| **10** | 6-6″-Binaringenin hexamethylether | 2 |
| **15** | Rhusflavanone (6-8″ binarigenin) | 0 |
| **16** | Succedaneaflavanone (6-6″-binaringenin) | 0 |
| **19** | GB-1a (3-8″ binaringenin) | ND[a] |
| **20** | GB-2a (3-8″ naringenyleriodictyol) | 0 |
| **22** | GB-1a glucoside | 0 |
| **23** | GB-2a glucoside | 0 |
| **25** | GB-1a hexamethylether[b] (3-8″-Binarigenin hexamethylether) | 87 |
| **26** | Rhusflavanone hexaacetate | 0 |
| **27** | Succedaneaflavanone hexaacetate | 0 |

[a]ND, not determined.
[b]MIC > 12.5 μg/mL.

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