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Evaluation of flavonoid and resveratrol chemical libraries reveals abyssinone II as a promising antibacterial lead

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Keywords

flavonoid; naringenin; resveratrol; abyssinone II; antitubercular; antibacterial mechanistic study

The growing prevalence of antibiotic-resistant pathogens continues to drive the need for antibiotics with novel modes of action. Of particular concern are infections caused by multidrug-resistant *Mycobacterium tuberculosis*, methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus* (VRE), penicillin-resistant *Streptococcus pneumoniae*, fluoroquinolone-resistant *Pseudomonas aeruginosa* (FQRP), and β -lactam-resistant Gram-negative organisms, since many of these pathogens are intransigent to multiple classes of drugs.^[1–3] Among discovery strategies, the empirical screening of chemical entities that are structurally distinct from clinically established agents represents an effective approach to developing novel antibiotics.

Historically, the majority of antimicrobial agents originate from natural products or are semi-synthetic derivatives.^[4–7] As part of our ongoing effort to discover novel antitubercular and antibacterial agents and to exploit natural product as scaffolds for chemical diversity, we have been interested in following up emerging and underexplored naturally occurring compounds showing good antimicrobial activities. These promising natural product leads may provide a valuable starting point for the discovery and development of novel antimicrobial chemotypes. In this regard, natural phytochemicals are actively being pursued for their antibacterial properties.^[8]

Flavonoids are a large family of naturally occurring polyphenolic phytochemicals, which have been reported to have various desirable pharmacological properties including anticancer^[9, 10] and cancer chemopreventive,^[11–13] antimalarial,^[14] antimicrobial,^[15, 16]

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and antioxidant^[17, 18] effects. As such, flavonoids have been the focus of research programs seeking to develop potential chemotherapeutic and chemopreventive agents, traditional Chinese medicine, and dietary supplements with favorable low toxicity profiles.^[19–23] The general chemical scaffold of flavonoids consists of a characteristic ring system A-C, chemically known as 2-phenyl-chrome-4-one or 2-phenyl-benzo- γ -pyrone, a regioisomer of coumarin (Figure 1). One such compound, (2*S*)-naringenin, was recently reported to show good antituberculosis activity with a minimum inhibitory concentration (MIC) of 2.8 $\mu\text{g}/\text{mL}$.^[24] To further explore if this novel flavonoid scaffold possesses any tractable antituberculosis and antibacterial activities based on its structure-activity relationship (SAR), we systematically evaluated a small focused compound collection consisting of commercial and in-house synthesized flavonoid analogues, as well as their structurally related resveratrol analogues. In particular, the recently described, abyssinone II, a naturally occurring prenylated flavonoid^[25] with aromatase inhibitory activity and breast cancer chemopreventive^[26] and antiproliferative^[27] properties, was included in the screen due to its close structural similarity with naringenin. Moreover, diethylstilbestrol (DES), a known pre-penicillin antibiotic was also included for comparison to further develop the SAR. Briefly, the initial screening library (Figure 2) included four compound series. The first series (compounds **1-7**) belongs to flavanone derivatives; the second compound series contains flavone (**8-19**) or isoflavone (**20**) analogues; the third compound series (**21-24**) is 3-hydroxyflavone derivatives; and the final series (compounds **25-34**) belongs to resveratrol analogues. Compounds **1-6**, **8**, **10-24**, and **26-34** were purchased from Sigma-Aldrich (purity >97%). The remaining compounds, such as 4'-bromoflavone^[11], abyssinone II and analogues^[26], and resveratrol^[28], were previously synthesized and studied in anticancer and cancer chemopreventive programs. We subsequently screened the collated flavonoid and resveratrol library against *M. tuberculosis* and a panel of Gram-positive and Gram-negative bacterial pathogens, including *Enterococcus faecalis*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Escherichia coli*, and *Pseudomonas aeruginosa*. Their anti-TB and antibacterial activities are summarized in Table 1.

Antitubercular evaluation revealed that flavanone derivatives **1**, **4**, **5**, and **7** exhibited weak to moderate anti-TB activities with MICs ranging from 50–200 $\mu\text{g}/\text{mL}$. Interestingly, abyssinone II (**7**), bearing the 3'-prenyl group, demonstrated the most potent antituberculosis activity with a MIC value of 50 $\mu\text{g}/\text{mL}$. The prototype flavanone **1** is two-fold less active (MIC = 100 $\mu\text{g}/\text{mL}$) compared with abyssinone. Introduction of a 2'-OH or a 6-OH group to the flavanone nucleus led to the complete loss of anti-TB activity by comparing compounds **1** and **2**, **3**. Further comparison of the activities of **4** and **7** indicates that the lipophilic prenyl group at the B ring is important. Contrary to the report of Chen *et al.*,^[24] racemic (\pm)-naringenin **4** only showed marginal anti-TB activity in our assay with a MIC value of 200 $\mu\text{g}/\text{mL}$. This is likely due to differences in the test methods, as the naringenin MIC was previously determined on agar with 2-weeks incubation as opposed to the recommended 3-weeks for growth, which yields results comparable to the broth MIC tests.^[29] In the flavone series, only the prototype flavone (**8**) and 2'-methoxyflavone (**10**) exhibited moderate anti-TB activity (100 and 50 $\mu\text{g}/\text{mL}$, respectively). Incorporation of a 4'-bromo group abolished the anti-TB activity by comparing **8** and **9**. All 3-hydroxyflavone compounds (**21-24**) bearing a conjugated 2,3-enol functionality with the 4-oxo group were not active against *M. tuberculosis*, which is likely due to the increased polarity from 3-OH group and poor penetration of the mycobacterial cell wall. Moreover, flavanone **1** showed about the same anti-TB activity as flavone **8** (MIC = 100 $\mu\text{g}/\text{mL}$), while 3-hydroxyflavone **21** was inactive. Notably, resveratrol, a structural relative of flavonoid, completely inhibited the mycobacteria growth at 100 $\mu\text{g}/\text{mL}$.

Further antimicrobial assessment showed that abyssinone II also exhibited relatively good activity against Gram-positive bacteria including *E. faecalis* (ATCC29212), *S. aureus* (N315), and *S. pneumoniae* (HM145), with MIC values of 25, 12.5 and 25 $\mu\text{g}/\text{mL}$, respectively. For comparison, the pre-penicillin compound DES (**34**) also demonstrated good activity against Gram-positive bacteria, with MIC values of 6.25 $\mu\text{g}/\text{mL}$ (Table 1). However, most other compounds were relatively inactive. Amid the flavonoid and resveratrol analogues evaluated, no test compounds were active against Gram-negative bacteria including *K. pneumoniae* (ATCC13883), *A. baumannii* (ATCC19606), *E. coli* (K12), and *P. aeruginosa* (PAO1). This observation is consistent with prior reports that flavonoids and DES primarily show anti-Gram-positive activities that may result from poor penetration of the Gram-negative outer-membrane by lipophilic molecules.^[30,31]

Encouraged by the promising antibacterial activity of abyssinone, a small selected number of abyssinone-based analogues **35-40**^[26] (Figure 3) were examined against the Gram-positive test panel. Their antibacterial activities are shown in Table 2. Interestingly, chalcones **35** and **36** with the opened central pyran C ring, considered as the synthetic precursors of abyssinone II, showed comparable antibacterial activities against *E. faecalis*, *S. aureus*, and *S. pneumoniae* with MIC values ranging from 25–50 $\mu\text{g}/\text{mL}$. Indeed, synthetic and naturally occurring chalcones have been reported with antiinfective and antiinflammatory properties.^[32,33] Unfortunately, removal of the 7-hydroxyl group, or substitution for 7-*O*-methylated or 7-*O*-MOM protected analogues (**37-42**), resulted in a loss of antimicrobial activity. These data suggest the free phenol hydroxyl group at the A-ring is essential for antibacterial activity, while the middle C-ring has relatively minimal impact on activity. This led us to speculate that abyssinone may act as an ionophore, resulting from sum effects of its acidic hydroxyl ion carrier and the lipophilic prenyl group that may interact with the cytoplasmic membrane.

Through membrane potential assays, with the potentiometric dye DiSC3(5), abyssinone (**7**) was found to rapidly hyperpolarize the staphylococcal membrane, in a manner similar to the proton ionophore CCCP^[34] (Figure 4). As anticipated, compound **37**, with the 7-hydroxy group replaced by a methoxyl group, failed to hyperpolarize the membrane. Changes in membrane potential status was not associated with structural damage to the membrane integrity, since hyperpolarized cells showed no uptake of propidium iodide, which only penetrates damaged membranes. Rapid hyperpolarization was also induced by the chalcone **36**, in contrast to the methoxy-analogue **35** that only had a marginal effect. Overall this suggests that the mode of action of abyssinone and related compounds might result from disruption of the membrane potential that is utilized for cellular energy production. However, this is unlikely to be the sole mechanism, since compound **36** was slightly less active than **35**. Further studies revealed that abyssinone inhibited the synthesis of key cellular macromolecules such as protein, RNA and DNA, as part of its global effects (Figure 5), which is consistent with the findings for agents targeting the membrane.^[35, 36] Indeed, the membrane-targeted drug daptomycin, which depolarizes Gram-positive bacteria, reduces the biosynthesis of several macromolecules and is one of the most potent agents in the clinic.^[35, 36]

In conclusion, the systematic screening of a focused flavonoid and resveratrol library led to the identification of abyssinone II, as an anti-Gram-positive agent that may have a multi-targeted mode of action, resulting from its ability to target the bacterial membrane. Such agents are increasingly becoming attractive therapeutic options owing to their potent actions, likely multi-target effects and limited potential for resistance development. The characterization of abyssinone II as a membrane-targeting molecule therefore presents a promising natural product lead for further medicinal chemistry optimization in an attempt to identify advanced experimental candidates with antimicrobial therapeutic potential.

Experimental Section

MIC determination

MIC values were determined against *M. tuberculosis* (H37Rv) and other bacteria using the standard microbroth dilution method exactly as previously described,^[29] which is based on the methods by the Clinical and Laboratory Standards Institute.^[37] The maximum test concentration used against *M. tuberculosis* was 200 µg/mL and 100 µg/mL against other pathogens.

Membrane potential assays

Dissipation of the transmembrane potential was measured using 3,3'-dipropylthiadicarbocyanine iodide [DiSC3(5)] from Anaspec (San Jose, CA).^[38] *S. aureus* 8325 was grown to OD₆₀₀ of 0.4 in Mueller-Hinton broth, washed twice in 50 mM potassium phosphate buffer containing 10 mM glucose. After resuspending cells in the buffer, 5 µM of DiSC3(5) was added and incubated at room temperature for 30 min, before transferring cells to a 96-well plate. When the signal for DiSC3(5) had equilibrated, cells were then exposed to compounds (12.5 and 100 µg/mL) and their fluorescence measured over 30 min in a Synergy 2 plate reader (Biotek). The uptake of propidium iodide by *S. aureus* 8325 exposed to compounds at 12.5 and 100 µg/mL was assayed as previously described.^[39]

Macromolecular synthesis

The effects of abyssinone (at 1 and 8 × MIC = 12.5 µg/mL) on DNA, RNA and protein were determined on *S. aureus* 8325 (OD₆₀₀ = 0.4), by measuring the incorporation of the radiolabeled precursors [*methy*-³H]thymidine, [5,6-³H]uridine and [4,5-³H]leucine into macromolecular fractions as previously described.^[40] The precursors (1 µCi/mL) were added to cultures 10 min before the addition of compounds. Following exposure to compounds for 30 min, cells were lysed with cold 10% TCA and precipitated macromolecules collected on GF/C filters. After washing with 95% ethanol, GF/C filters were analyzed by liquid scintillation counting. The relative % activity in cells exposed to compounds was determined. Ciprofloxacin (DNA), rifampicin (RNA) and tetracycline (protein) were used as controls.

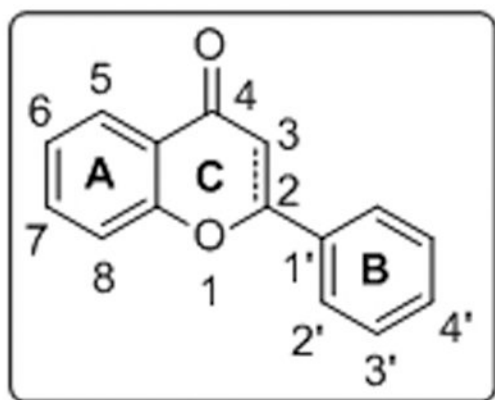
Acknowledgments

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General scaffold of flavonoid

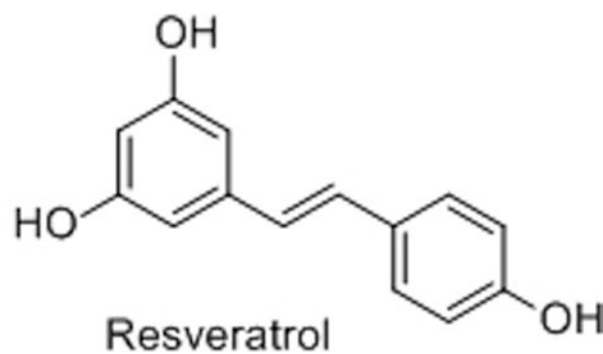
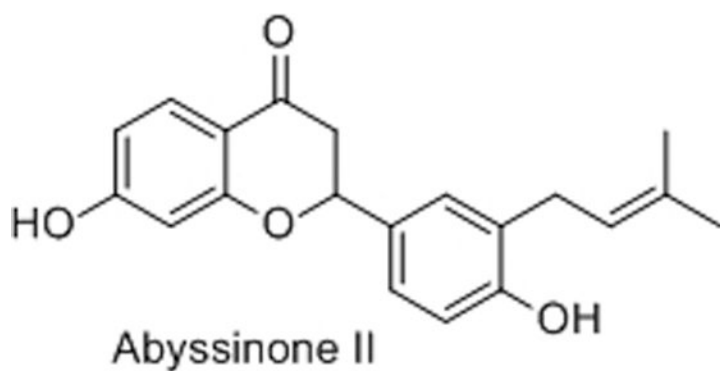
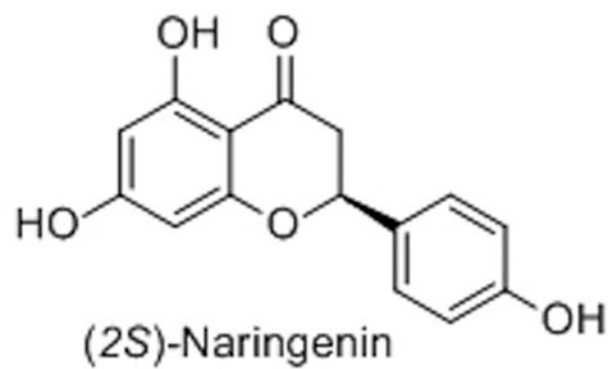


Figure 1.
General structural scaffold of flavonoid and chemical structures of naringenin, abyssinone II, and resveratrol.

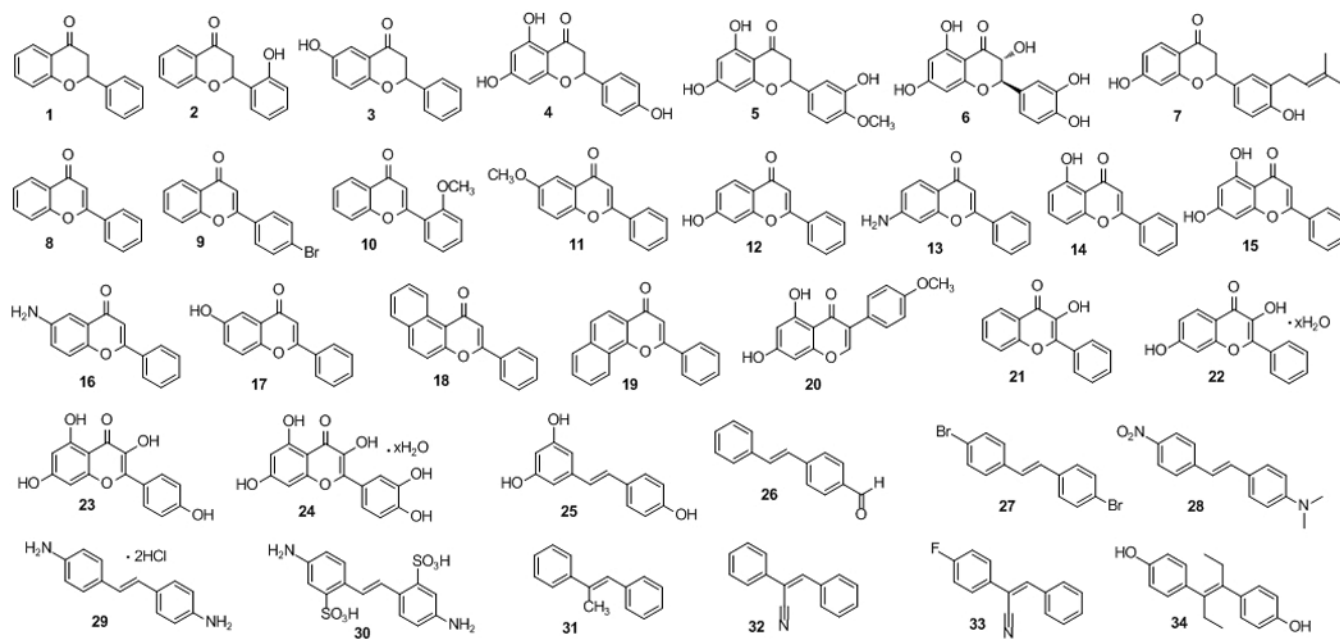


Figure 2.
The chemical structures of flavonoid and resveratrol analogues subjected to initial screening.

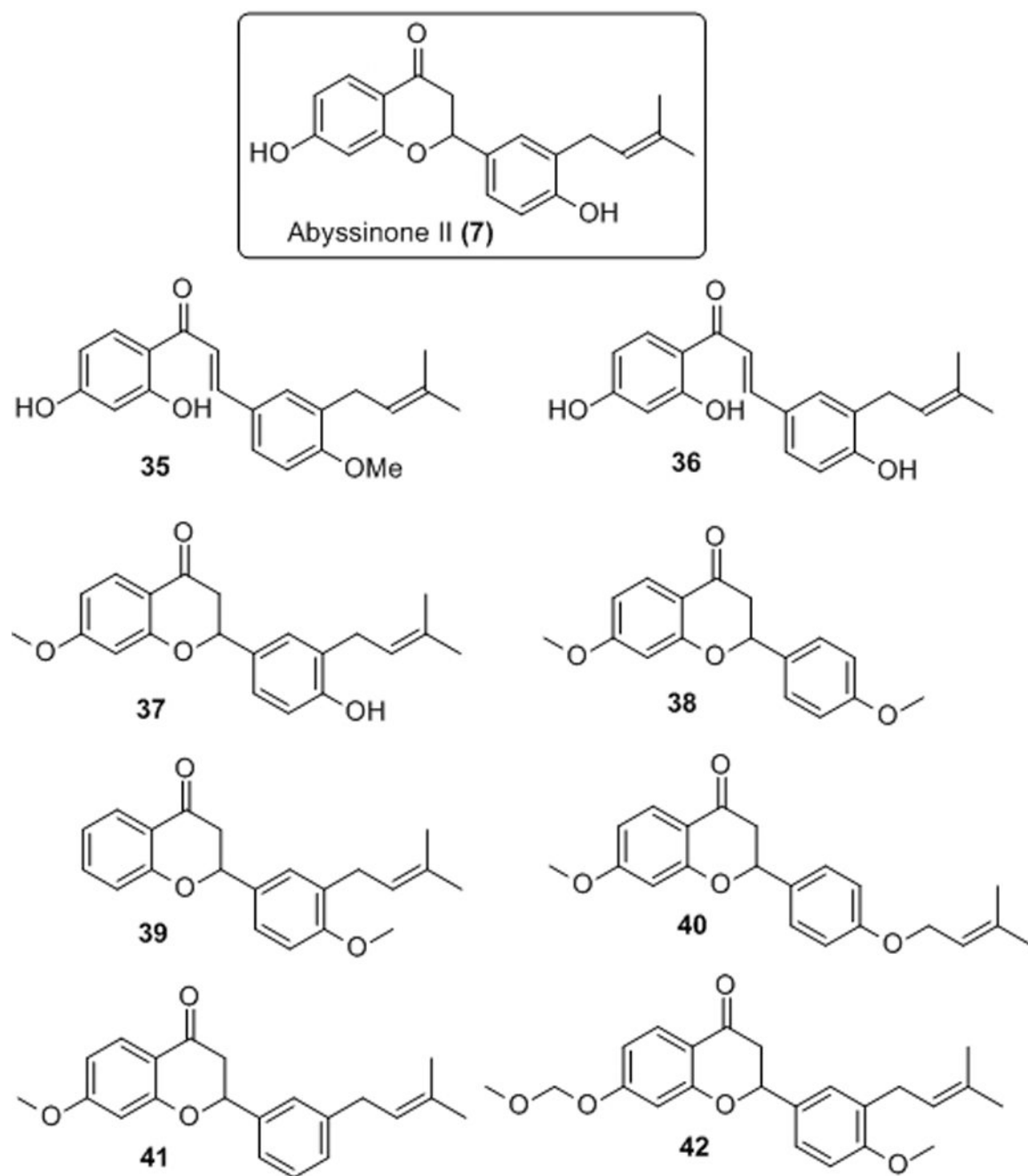


Figure 3.
Additional abyssinone II analogues evaluated.

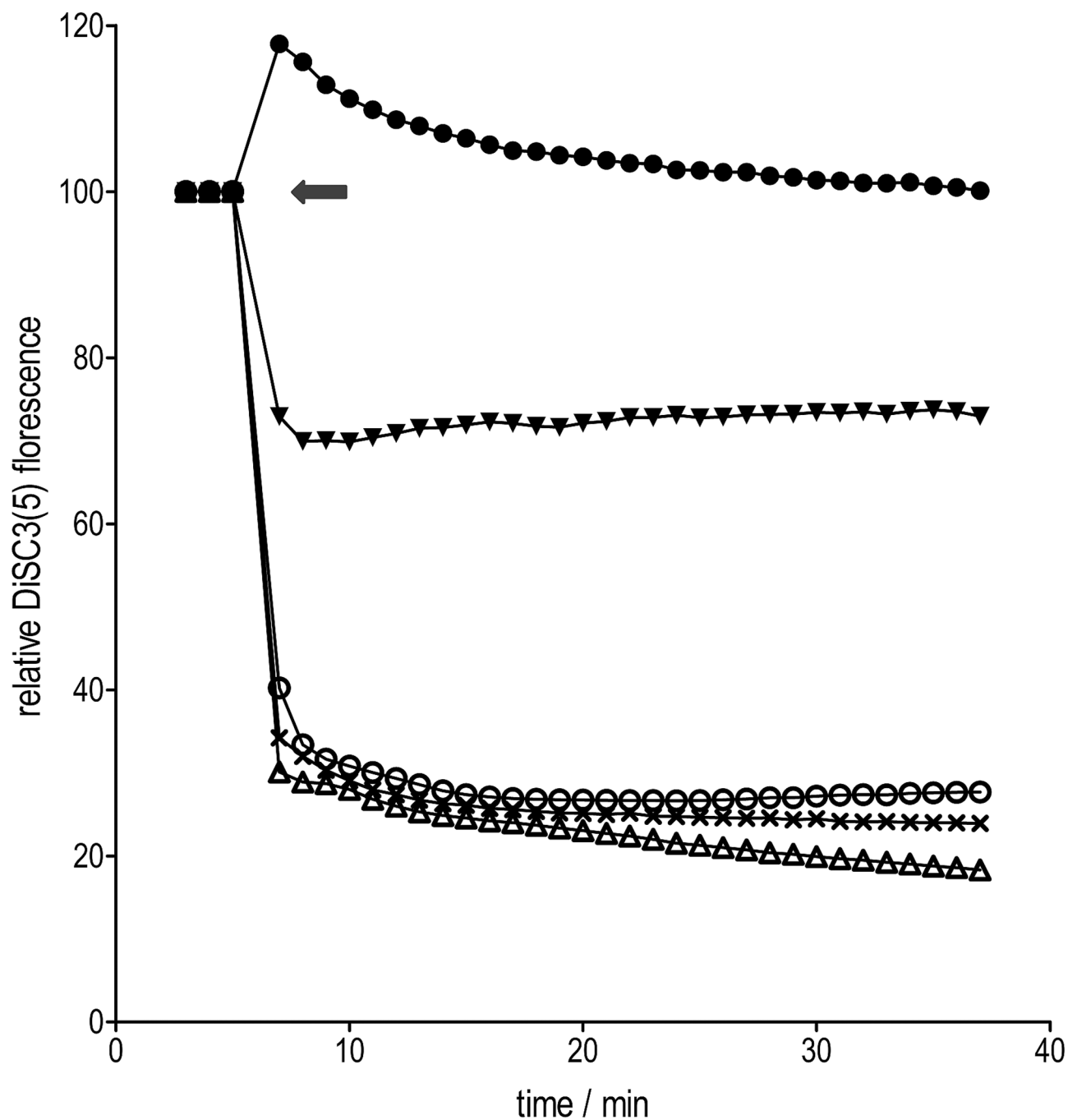


Figure 4. Effects of abyssinone and analogues on bacterial membrane potential at 100 $\mu\text{g/mL}$. Each data point represents the mean of at least three replicates. The grey arrow indicates the time of addition of compounds; compounds **37** (●), **35** (▼), **7** (○), CCCP (x), **36** (Δ). Compound **37** caused some depolarization but this effect was not sustained.

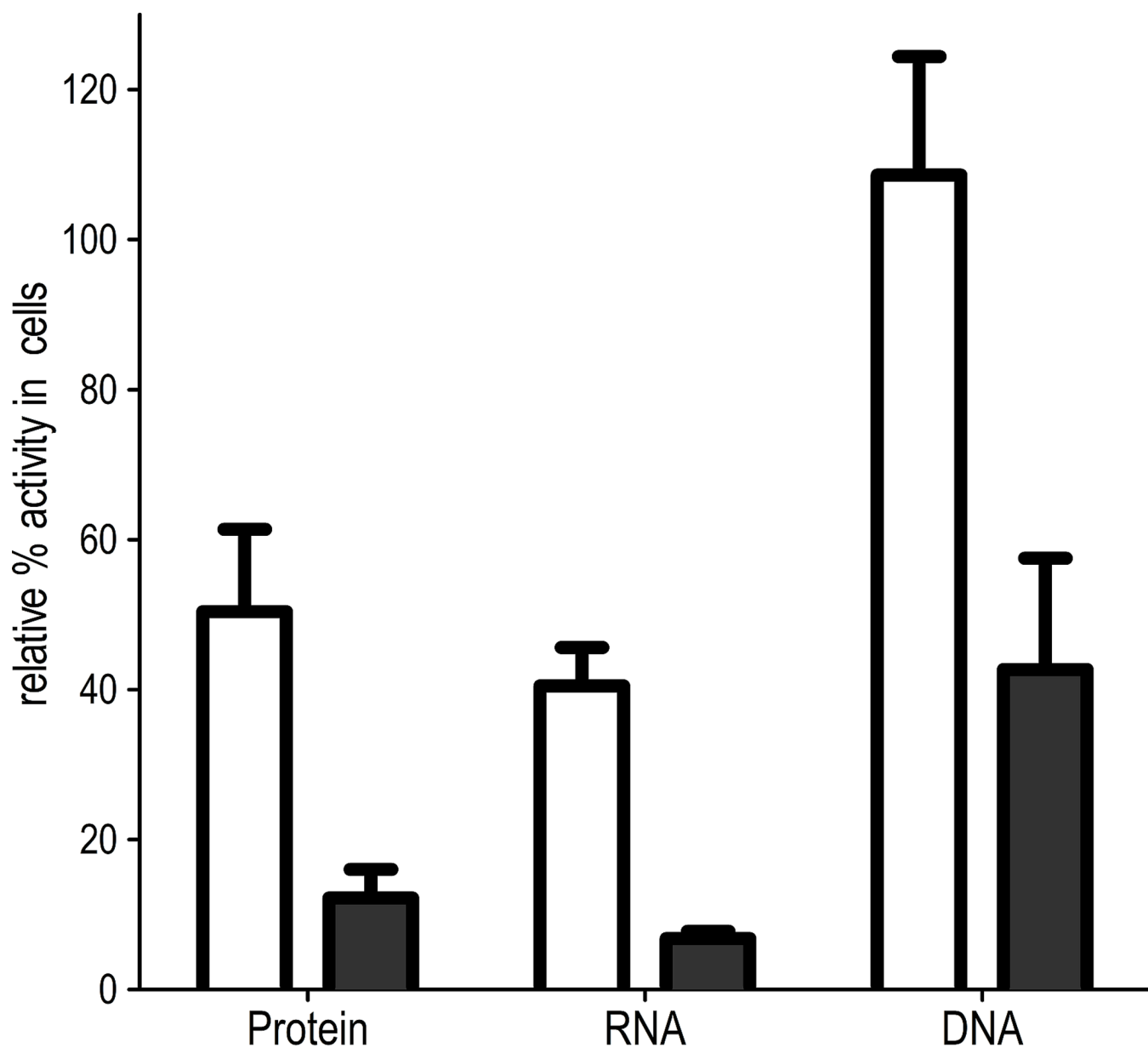


Figure 5. Abyssinone affects multiple macromolecular processes in a concentration-dependent manner, 12.5 µg/mL (□), 100 µg/mL (■). The activities of respective macromolecular processes with control antibiotics were as follows: 0.8 µg/mL tetracycline (protein, 46±1.2), 0.06 µg/mL rifampicin (RNA, 18.5±2.6), 0.8 µg/mL ciprofloxacin (DNA, 52±5.2).

Table 1

Antitubercular and antibacterial activity ($\mu\text{g/mL}$) of flavonoid and resveratrol derivatives.^{[a]/[b]}

Compound	<i>M. tuberculosis</i> (H37Rv)	<i>E. faecalis</i> (ATCC29212)	<i>S. aureus</i> (N315)	<i>S. pneumoniae</i> (HM145)	Compound	<i>M. tuberculosis</i> (H37Rv)	<i>E. faecalis</i> (ATCC29212)	<i>S. aureus</i> (N315)	<i>S. pneumoniae</i> (HM145)
1	100	>100	>100	>100	18	>200	>100	>100	>100
2	>200	>100	>100	>100	19	>200	>100	>100	>100
3	>200	>100	>100	>100	20	>200	>100	>100	>100
4	200	>100	>100	>100	21	>200	>100	>100	>100
5	100	>100	>100	>100	22	>200	>100	>100	>100
6	>200	>100	>100	>100	23	>200	>100	>100	>100
7	50	25	12.5	25	24	>200	25	>100	>100
8	100	>100	>100	>100	25	100	100	>100	100
9	>200	>100	>100	>100	26	200	>100	>100	>100
10	50	>100	>100	>100	27	>200	>100	>100	>100
11	>200	>100	>100	>100	28	>200	>100	>100	>100
12	>200	>100	>100	>100	29	100	>100	>100	>100
13	200	>100	>100	>100	30	>200	>100	>100	>100
14	>200	>100	>100	>100	31	>200	>100	>100	>100
15	>200	>100	>100	>100	32	>200	>100	>100	>100
16	>200	>100	>100	>100	33	>200	>100	>100	>100
17	>200	>100	>100	>100	34	>200	6.25	6.25	6.25

^[a]The MICs of control antibiotics were 0.032 $\mu\text{g/mL}$ (INH, *M. tuberculosis*), 0.4 $\mu\text{g/mL}$ (ciprofloxacin, *E. faecalis*), 0.4 $\mu\text{g/mL}$ (ciprofloxacin, *S. aureus*), 0.8 $\mu\text{g/mL}$ (ciprofloxacin, *S. pneumoniae*).^[b]No test compounds were active against Gram-negative bacteria including *K. pneumoniae* (ATCC13883), *A. baumannii* (ATCC19606), *E. coli* (K12), and *P. aeruginosa* (PAO1).

Table 2Antibacterial activity ($\mu\text{g/mL}$) of selected abyssinone analogues.

Compound	<i>E. faecalis</i> (ATCC29212)	<i>S. aureus</i> (N315)	<i>S. pneumoniae</i> (HM145)
7	25	12.5	25
35	50	25	25
36	50	50	>100
37	>100	>100	>100
38	>100	>100	>100
39	>100	>100	>100
40	>100	>100	>100
41	>100	>100	>100
42	>100	>100	>100