



Article

The Antimicrobial and Cytotoxicity Properties of New Dibrominated 1,3-Dithiolium Flavonoids

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Abstract

Background/Objectives: Antimicrobial resistance (AMR) presents a medical risk as well as a significant global socioeconomic challenge. Key contributors to AMR include the excessive use and incorrect application of antibiotics in humans and agriculture, nosocomial infections, and the absence of new classes of antibiotics. **Methods:** Novel dibrominated tricyclic flavonoids have been synthesized from the corresponding 3-dithiocarbamic flavanones and their antimicrobial and cytotoxicity properties have been investigated. **Results:** It has been found that these tricyclic flavonoids exhibit strong antimicrobial properties against clinically relevant pathogens such as *Staphylococcus aureus*, *Acinetobacter baumannii*, and *Escherichia coli* with MIC and MBC values against *S. aureus* ATCC 25923 as low as 0.12 µg/mL and 1.9 µg/mL, respectively. **Conclusions:** The synthetic tricyclic flavonoids exhibit strong antibacterial activity against selected WHO priority pathogens, including *Staphylococcus aureus* and *Acinetobacter baumannii*, surpassing the efficacy of both natural and synthetic flavonoids and several conventional antibiotics.

Keywords: benzopyran; dithiocarbamates; synthetic flavonoids; ESKAPE pathogens

1. Introduction

In 2019, approximately 4.95 million deaths were linked to antimicrobial resistance (AMR) [1], a phenomenon regarded by the World Health Organization (WHO) as one of the most significant threats to public health and development. Key contributors to AMR include the excessive use and incorrect application of antibiotics in humans and agriculture, nosocomial infections, and the absence of new antibiotic classes. Without sufficient measures, the worldwide burden of AMR will rise significantly, potentially resulting in around 10 million deaths annually by 2050 [2]. AMR presents a medical risk as well as a significant global socioeconomic challenge, resulting in an approximate loss of 3.8% of the worldwide gross domestic product anticipated by the World Bank until 2050 [3].

Microorganisms that are resistant can tolerate and even prosper despite the presence of antibiotics [4]. Consequently, antimicrobial medications grow progressively less

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effective and numerous infections become harder, if not impossible to manage, placing greater strain on healthcare systems globally [5]. Additionally, the rise and dissemination of multidrug-resistant (MDR) microbes or *superbugs* undermine the effectiveness of existing antibiotics, jeopardizing the management of infectious diseases and posing a significant challenge for contemporary medicine [6]. Pathogens such as *Staphylococcus aureus*, *Acinetobacter baumannii*, and *Escherichia coli* are developing resistance to multiple antibiotic classes, making the treatment of infections increasingly difficult and leading to higher morbidity, mortality, and healthcare costs.

This AMR crisis is further amplified by the lack of progress in new antibiotic development, largely due to economic and regulatory barriers [7]. A recent WHO report indicates that since 2017, just 13 new antibiotics have been approved, with only vaborbactam and lefamulin belonging to novel chemical classes [8]. However, the recently approved medications show no greater efficacy and do not present new mechanisms of action against current threats. This concerning trend, along with the increased incidence of superbugs, highlights the pressing necessity for creative strategies to produce new antimicrobials effective in combating infectious diseases.

In this perspective, flavonoids—an extensive category of naturally occurring heterocyclic organic substances—are likely effective candidates as antimicrobial agents. Linked to the various substitution patterns on the C6-C3-C6 framework, over 13,000 flavonoids are recognized [9]. The focus they garner is a direct result of the numerous biological actions that this group of compounds displays. Flavonoids exhibit a significant variety of chemical and biochemical traits that give them antimicrobial characteristics [10–12]. These substances are likewise recognized for their effective antioxidant properties [13–18]. Additionally, some research indicates that flavonoids may provide defense against cancer and heart diseases [19–28].

Despite their promising properties, natural flavonoids face challenges in medical applications due to limited bioavailability, poor solubility, and instability [29]. The flavonoid scaffold is highly amenable to chemical modification, allowing the design of derivatives with enhanced antimicrobial potency, selectivity, and pharmacokinetic properties. By combining natural bioactivity with rational synthetic optimization, flavonoids offer versatile solutions for developing new antibacterial agents capable of addressing the AMR crisis.

In the last twelve years, our research group has managed to design a new type of tricyclic flavonoids featuring a 1,3-dithiolium ring (Figure 1) with different substituents on the C6-C3-C6 framework. We have established that this new class of synthetic flavonoids displays significant antibacterial activity against both Gram-positive and Gram-negative strains, including ESKAPE pathogens [30], and investigated their mechanisms of action.

Based on these promising results, by changing the substituents' nature on rings **A** and **B**, we are presenting herein an expansion of our research on the development of novel tricyclic flavonoids exhibiting strong antimicrobial properties against clinically relevant pathogens such as *S. aureus*, *A. baumannii*, and *E. coli*. The overarching goal is to identify highly potent antibacterial agents capable of addressing the growing threat of antimicrobial resistance.

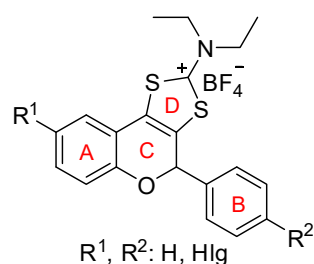


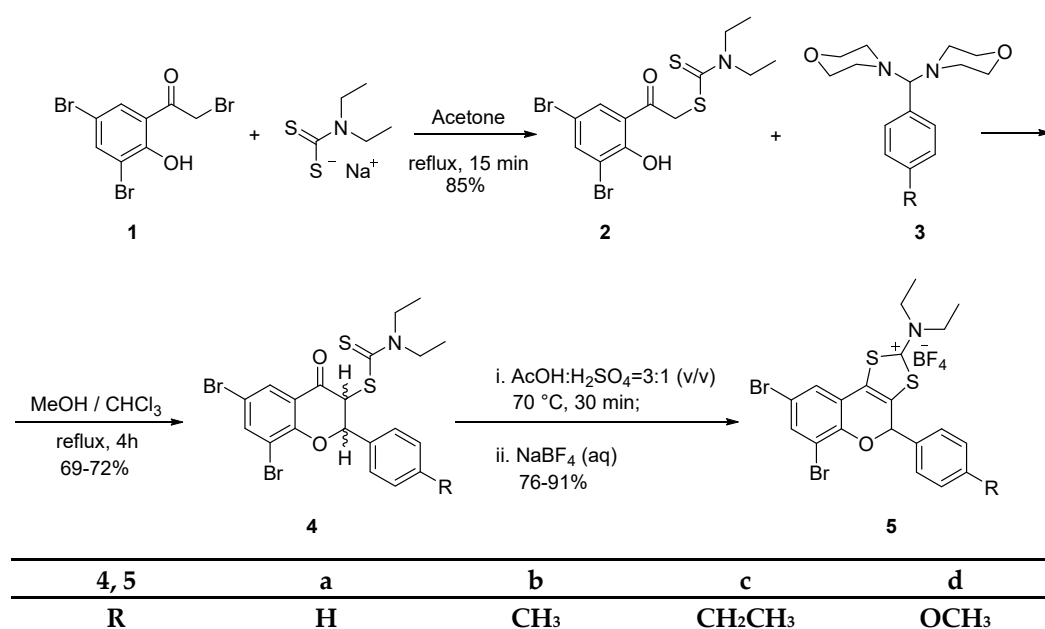
Figure 1. Molecular structure of tricyclic 1,3-dithiolium flavonoids.

2. Results and Discussion

2.1. Tricyclic Flavonoids

As previously reported, flavanone derivatives of type **4** have been synthesized by the reactions of phenacyl dithiocarbamates with amins **3** [31]. Thus, by reacting 2-bromo-1-(3,5-bromo-2-hydroxyphenyl)ethan-1-one (**1**) [32] with sodium *N,N*-diethyldithiocarbamate in acetone, 1-(3,5-dibromo-2-hydroxyphenyl)ethan-1-oxoethan-2-yl *N,N*-diethylaminocarbodithioate (**2**) has been successfully synthesized in good yield (85%) [33]. 3-Dithiocarbamic flavanones **4a–d** have been prepared through the reaction of phenacyl *N,N*-diethylaminocarbodithioate **2** with amins **3** derived from unsubstituted and methyl, ethyl, and methoxy substituted benzaldehydes (Scheme 1) [34].

NMR spectroscopy has confirmed the benzopyran ring closure. The ^1H NMR spectra indicated the disappearance of the singlet provided by the methylene group from dithiocarbamate **2** (4.84 ppm) and the appearance of the two pairs of signals between 5.8 and 6.3 ppm corresponding to the vicinal hydrogen atoms at the C-2 and C-3 positions of the benzopyran ring. Mass spectrometry also provided evidence for the ring closure to the benzopyran ring.



Scheme 1. Synthesis of 3-dithiocarbamic flavanones **4a–d** and tricyclic flavonoids **5a–d**.

Due to the relative orientation of H-2 and H-3 hydrogen atoms of the flavanones **4**, two diastereoisomers are obtained as a mixture. They originate from the position of the mentioned hydrogen atoms that can be oriented either to the same side (*syn*) or to opposite (*anti*) sides of the benzopyrane core (Figure 2). The relative positions of these two atoms are reflected in the size of the 3J coupling constants. According to the basic rules of

stereochemistry, the most stable isomer should be that with an *anti* orientation of the two hydrogen atoms **4'** as opposed to the *syn* diastereoisomer **4''**. The diastereoisomeric ratio and coupling constants of flavanones **4a–d**, presented in Table 1, support the above assumption, the *anti* diastereoisomer being the major one for all 3-dithiocarbamic flavanones.

2-*N,N*-Dialkylamino-1,3-dithiolium-2-yl cations are easily synthesized from phenacyl dithiocarbamates [35,36]. Under acidic conditions, the latter undergo cyclocondensation to the corresponding 1,3-dithiolium ring [37]. The cyclocondensation of flavanones **4** to 1,3-dithiolium flavonoids **5** has been demonstrated by analytical and spectral data.

Thus, the carbonyl absorption bands from the infrared spectra (1682–1698 cm^{−1}) disappeared, while at the same time new broad absorption bands (ca. 1010–1090 cm^{−1}) characteristic of the tetrafluoroborate anion were identified. The ¹H NMR spectra of 1,3-dithiolium flavonoids **5** indicated the absence of C-3 hydrogens from the benzopyran moiety. Simultaneously, new singlets belonging to the C-2 hydrogens appeared at approximately 6.99 ppm. The ¹³C NMR spectra indicated the disappearance of the signals for carbonyl and thiocarbonyl and the presence of a new signal (ca. 185 ppm) corresponding to the carbenium atom of the 1,3-dithiol-2-ylum ring.

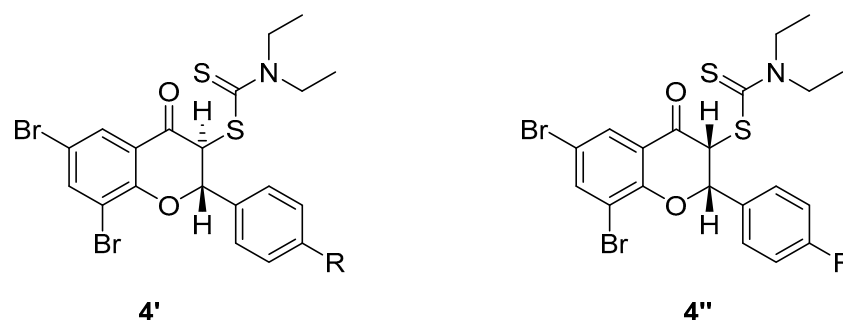


Figure 2. Diastereoisomers of flavanones **4**.

Table 1. Coupling constants and diastereoisomeric ratio of flavanones **4**.

Flavanones 4	a	b	c	d
³ J _{H2-H3} <i>anti</i> (Hz)	7.6	6.8	7.0	7.7
³ J _{H2-H3} <i>syn</i> (Hz)	4.1	4.4	4.4	4.5
<i>anti:syn</i> ratio	69:31	85:15	81:19	74:26

2.2. Antimicrobial Activity of Tricyclic Flavonoids

All novel flavonoids exhibited notable antibacterial activity against the tested strains, as summarized in Table 2. The **5b** and **5c** derivatives exhibited superior potency against the Gram-positive strain *S. aureus* ATCC 25923, reaching a minimum inhibitory concentration (MIC) as low as 0.12 µg/mL. Gram-negative bacteria were less susceptible to the tested compounds than Gram-positive strains, with MIC values ranging from 3.9 to 31.25 µg/mL. Among the tricyclic flavonoids, compounds **5b** and **5c** displayed the highest activity (3.9 µg/mL) against *E. coli* ATCC 25922 and *A. baumannii* medbio3 2013, respectively. The synthetic flavonoids also showed significant inhibitory effects against antibiotic resistant isolates. Specifically, **5d** was the most active compound against the methicillin-resistant *S. aureus* (MRSA) clinical isolate (MIC = 0.9 µg/mL), whereas **5c** exhibited the strongest activity against the *A. baumannii* strain (MIC = 3.9 µg/mL).

Table 2. In vitro antibacterial activity (MIC) of tricyclic flavonoids **5a–d** against selected bacterial strains.

Microbial Strains	MIC (µg/mL)				DMSO (%)	Control
	5a	5b	5c	5d		
<i>Staphylococcus aureus</i> ATCC 25923	0.24	0.12	0.12	0.48	24.87	1.95 ^a /7.81 ^{chl}
<i>Escherichia coli</i> ATCC 25922	7.8	3.9	15.6	7.8	12.43	62.50 ^a /7.81 ^k
<i>S. aureus</i> medbio1-2012	3.9	3.9	1.9	0.9	24.87	7.81 ^{chl}
<i>Acinetobacter baumannii</i> medbio3 2013	15.6	7.8	3.9	31.25	24.87	<0.9 ^g

^a—ampicillin; ^{chl}—chloramphenicol; ^g—gentamicin; ^k—kanamycin. The values are means for at least three replicates.

Significant bactericidal activity was observed for flavonoids **5a** and **5b** against *S. aureus* ATCC 25923, with minimum bactericidal concentration (MBC) values as low as 1.9 µg/mL (Table 3). The synthetic flavonoids exhibited higher MBCs against Gram-negative strains (7.8–62.5 µg/mL). *A. baumannii* medbio3-2013 was the least susceptible strain, showing the highest MBC value for **5d**, indicating a milder bactericidal efficacy compared to Gram-positive strains.

Table 3. Minimum bactericidal concentrations of flavonoids **5a–d** against tested bacterial strains.

Microbial Strains	MBC (µg/mL)				Control
	5a	5b	5c	5d	
<i>Staphylococcus aureus</i> ATCC 25923	1.9	1.9	7.8	3.9	7.8 ^a
<i>Escherichia coli</i> ATCC 25922	31.25	31.25	31.25	31.25	125 ^a
<i>S. aureus</i> medbio1-2012	31.25	3.9	3.9	7.8	31.25 ^{chl}
<i>Acinetobacter baumannii</i> medbio3 2013	31.25	7.8	15.6	62.5	<0.9 ^g

^a—ampicillin; ^{chl}—chloramphenicol; ^g—gentamicin. The values are means for at least three replicates.

Our results indicate that the novel flavonoids, particularly **5b** and **5c**, exhibit strong inhibitory and bactericidal effects, especially against Gram-positive bacteria. The tested Gram-negative strains were generally less susceptible to the novel flavonoids compared to Gram-positive bacteria, a finding that is consistent with known differences in bacterial cell envelope architecture. In particular, the presence of an outer membrane in the Gram-negative bacteria wall is widely recognized as an intrinsic permeability barrier that can limit the activity of many antimicrobial compounds. Nevertheless, the low MIC values recorded against *E. coli* suggest that these synthetic tricyclic flavonoids may possess enhanced membrane permeation properties, potentially contributing to their antibacterial activity. However, direct experimental evaluation of membrane permeability or integrity was beyond the scope of the present study, and future investigations will be required to validate this hypothesis.

The antibacterial potency of the synthetic tricyclic flavonoids appears higher than that reported for many natural flavonoids, including flavones, flavonols, and flavanones, which typically exhibit MIC values in the range of 250–1000 µg/mL against common bacterial strains such as *Staphylococcus aureus* and *Escherichia coli* [38]. Literature reports further indicate that natural flavonoids such as quercetin or kaempferol often display MIC values in the range of 25–125 µg/mL against *S. aureus* [39]. In comparison with highly potent natural flavonoids such as Panduratin A—previously reported as one of the most active flavonoids with MIC values of 0.5–1 µg/mL—compounds **5a–d** nonetheless exhibit notable inhibitory activity against MRSA [40]. Moreover, the tested compounds demonstrate relevant antibacterial activity relative to active natural compounds such as

isobavachalcone, particularly against *E. coli*, for which reported MIC values often exceed 256 µg/mL [41].

Compounds **5a–d** exhibit antibacterial activity comparable to, or exceeding that of previously reported halogenated tricyclic flavonoids, underscoring the effectiveness of rational scaffold modification (particularly halogenation and methoxy/alkyl substitution) in enhancing antibacterial potency relative to both natural and synthetic flavonoids. Relative to synthetic chalcones containing diphenyl ether moieties, tricyclic flavonoids **5a–d** show 2–5-fold greater activity against *E. coli* (MICs = 10–33 µg/mL) and markedly superior inhibition of *S. aureus* (MICs between 7.5 and 25 µg/mL) [42]. Moreover, our tested synthetic flavonoids outperform nitrogen-containing synthetic flavonoids, which typically display MIC values > 32 µg/mL [43]. The synthetic flavonoids exhibited remarkable activity against *A. baumannii* medbio3 2013 strain, with **5c** achieving an MIC of 3.9 µg/mL. This potency surpasses that of other synthetic scaffolds, including halogenated nitrochromenes (8–32 µg/mL) [44], and markedly exceeds the activity of natural flavonoids such as quercetin (256 µg/mL) [45] and norwogonin (128 µg/mL) [46]. While these comparisons provide a useful contextual reference, direct quantitative comparisons should be interpreted with caution, as MIC values reported across different studies may vary due to differences in experimental conditions.

For most of the tested flavonoids, the MBC/MIC ratio was ≤ 4 , indicating significant bactericidal activity. Notably, for **5b**, the MIC was equal to the MBC against antibiotic-resistant strains such as *S. aureus* MedBio1-2012 and *A. baumannii* MedBio3-2013, underscoring its exceptional antimicrobial potential. Collectively these findings position the tricyclic flavonoids **5a–d** as highly promising candidates for the treatment of infections caused by multidrug-resistant priority pathogens, including *S. aureus* and *A. baumannii*. Nonetheless, the antibacterial activity observed against resistant bacterial strains should be regarded as preliminary, as it was evaluated using only two clinical isolates. Further studies involving a larger and more diverse collection of clinical strains will be required to assess the reproducibility and spectrum of activity of these compounds against clinically important pathogens.

The pronounced inhibitory and bactericidal activities of synthetic flavonoids **5a–d** are further confirmed by comparison with the antibiotics employed as reference controls. Notably, all tested compounds exhibited considerably enhanced inhibitory activity against *S. aureus* ATCC 25923, surpassing ampicillin by up to 16-fold and chloramphenicol by up to 65-fold. Comparable trends were observed against the MRSA strain, where compounds **5c** and **5d** displayed MIC values up to ninefold lower than those of chloramphenicol. Against *E. coli*, the synthetic flavonoids demonstrated moderate yet significant activity, with compound **5b** showing up to 16-fold lower potency than ampicillin, while exhibiting activity comparable to kanamycin. In contrast, all tested compounds were less active against *A. baumannii* than gentamicin.

The strong antimicrobial activity is likely attributable to a donor–acceptor interaction between nucleophilic moieties of wall and/or membrane constituents (e.g., peptides, peptidoglycans) and the electrophilic C(2) atom of the 1,3-dithiolium ring. The lower MICs and MBCs may suggest that the inhibitory and bactericidal activity are related to the impairment of the cell membrane integrity, as our previous studies showed for similar halogenated sulfur containing tricyclic flavonoids [47]. The potential of the novel flavonoids reported here is underscored by their synthetic, non-natural nature, making them excellent candidates for antimicrobial development. Their synthetic structures allow them to circumvent existing resistance mechanisms and act via novel modes of action, enhancing their efficacy against multidrug-resistant strain. Nevertheless, further investigations are required to elucidate the precise mechanisms of action.

2.3. Cytotoxicity study

In order to evaluate the effect of tricyclic flavonoids **5a–d** on human cells, the MTS assay was used. The tested derivatives were found to have little negative effect on HDF-Ad cells at concentrations up to 7.8 µg/mL. However, when tested at a concentration of 15.6 µg/mL, cell viability was significantly reduced to approximately 60% for **5b** and **5d** and plummeted to 20% and 0% for **5a** and **5c**, respectively (Figure 3). When compared to the MIC and MBC values presented in Tables 2 and 3, it is worth noting that all four tested derivatives are active against *S. aureus* ATCC 25923 at concentrations lower than those found to induce cytotoxicity in HDF-Ad cells. For *E. coli* ATCC 25922, three of the four tested flavonoids, namely **5a**, **5b**, and **5d**, were determined to have MIC values lower than or equal to 7.8 µg/mL, at which HDF-Ad cell viability is arguably unaffected. However, all four derivatives present MBC values of 31.25 µg/mL, a 4-fold increase when compared to 7.8 µg/mL. In the case of *S. aureus* medbio1-2012, all four flavonoids presented MIC values lower than 7.8 µg/mL, but only three of them, derivatives **5b–d**, had MBC values lower than or equal to 7.8 µg/mL. Finally, for *A. baumannii* medbio3 2013, flavonoids **5b** and **5c** have MIC values of 7.8 and 3.9 µg/mL, while **5b** also has an MBC value of 7.8 µg/mL.

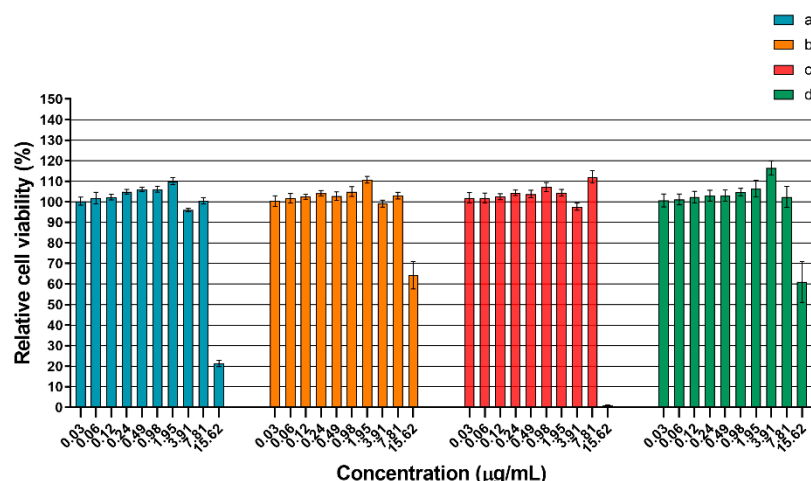


Figure 3. Graphical representation of cell viability for tricyclic flavonoids treated HDF-Ad cells, relative to untreated ones. The data are expressed as mean $\pm$ SD.

3. Materials and Methods

Melting points were obtained on a *KSPI* melting-point meter (A. KRÜSS Optronic, Hamburg, Germany) and are uncorrected. IR spectra were recorded on a Bruker Tensor 27 instrument (Bruker Optik GmbH, Ettlingen, Germany). NMR spectra were recorded on a Bruker 500 MHz spectrometer (Bruker BioSpin, Rheinstetten, Germany). Chemical shifts are reported in ppm downfield from TMS. Mass spectra were recorded on a Thermo Scientific ISQ LT instrument (Thermo Fisher Scientific Inc., Waltham, MA, USA). All reagents were commercially available and used without further purification. Elemental analyses (Table S1), ^1H NMR and ^{13}C NMR spectra are presented in the Supplementary Materials.

3.1. Chemistry

3.1.1. 6,8-Dibromo-2-phenyl-4-oxochroman-3-yl *N,N*-diethyldithiocarbamate (**4a**);

General Procedure

To a solution of 1-(2,5-dibromo-2-hydroxyphenyl)-1-oxoethan-2-yl *N,N*-diethyldithiocarbamate (**2**) (0.441 g, 1 mmol) in a mixture of $\text{CHCl}_3/\text{MeOH}$ (14 mL, 1:1 *v/v*)

aminal **3a** (0.276 g, 1 mmol) was added and the reaction mixture was heated under reflux for 4 h. After cooling, the solid material was filtered off, dried and recrystallized from ethanol to give **4a** (0.365 g, 69%) as colorless crystals. M.p. 148–149 °C. IR (ATR, cm^{-1}) 2972, 1683, 1488, 1423, 1262, 1188, 811, 714, 637, 562. ^1H NMR (CDCl_3 , selected data for the major *anti* isomer) δ 7.97 (d, J = 2.4 Hz, 1H), 7.91 (d, J = 2.4 Hz, 1H), 7.59–7.50 (m, 2H), 7.39–7.35 (m, 3H), 6.05 (d, J = 7.6 Hz, 1H), 5.80 (d, J = 7.6 Hz, 1H), 3.97 (q, J = 6.6 Hz, 2H), 3.69 (q, J = 7.1 Hz, 2H), 1.23 (m, 6H). ^{13}C NMR (CDCl_3 , selected data for the major *anti* isomer) δ 191.0, 186.2, 156.0, 141.5, 135.7, 129.3, 128.9, 128.6, 127.2, 123.2, 114.3, 113.1, 83.1, 58.2, 50.6, 47.3, 12.6, 11.4. MS (EI) m/z : 526.9 (M^+ , 12%) for $\text{C}_{20}\text{H}_{19}\text{Br}_2\text{NO}_2\text{S}_2$.

3.1.2. 6,8-Dibromo-2-(4'-methylphenyl)-4-oxochroman-3-yl *N,N*-diethyldithiocarbamate (**4b**)

Colorless crystals, m.p. 157–158 °C, 0.390 g, 72%. IR (ATR, cm^{-1}) 2978, 2728, 1690, 1494, 1416, 1273, 1200, 809, 723, 644, 551. ^1H NMR (CDCl_3 , selected data for the major *anti* isomer) δ 7.96 (d, J = 2.4 Hz, 1H), 7.90 (d, J = 2.4 Hz, 1H), 7.39 (d, J = 7.8 Hz, 2H), 7.18 (d, J = 8.0 Hz, 2H), 6.01 (d, J = 6.8 Hz, 1H), 5.78 (d, J = 6.8 Hz, 1H), 3.98 (q, J = 7.0 Hz, 2H), 3.70 (q, J = 7.7 Hz, 2H), 2.35 (s, 3H), 1.25 (t, J = 7.1 Hz, 6H). ^{13}C NMR (CDCl_3 , selected data for the major *anti* isomer) δ 191.1, 186.3, 156.0, 141.4, 138.8, 132.8, 129.3, 129.1, 127.1, 123.2, 114.2, 113.1, 83.0, 58.1, 50.5, 47.3, 21.2, 12.6, 11.4. MS (EI) m/z : 540.9 (M^+ , 17%) for $\text{C}_{21}\text{H}_{21}\text{Br}_2\text{NO}_2\text{S}_2$.

3.1.3. 6,8-Dibromo-2-(4'-ethylphenyl)-4-oxochroman-3-yl *N,N*-diethyldithiocarbamate (**4c**)

Colorless crystals, m.p. 145–146 °C, 0.400 g, 72%. IR (ATR, cm^{-1}) 2983, 2714, 1682, 1484, 1402, 1269, 1156, 809, 714, 632, 541. ^1H NMR (CDCl_3 , selected data for the major *anti* isomer) δ 7.96 (d, J = 2.4 Hz, 1H), 7.90 (d, J = 2.4 Hz, 1H), 7.41 (d, J = 8.0 Hz, 2H), 7.20 (d, J = 7.8 Hz, 2H), 6.02 (d, J = 7.0 Hz, 1H), 5.79 (d, J = 7.0 Hz, 1H), 3.98 (q, J = 7.6 Hz, 2H), 3.69 (q, J = 6.2 Hz, 2H), 2.64 (d, J = 7.6 Hz, 2H), 1.37–1.24 (m, 6H), 1.23 (m, 3H). ^{13}C NMR (CDCl_3 , selected data for the major *anti* isomer) δ 191.1, 186.3, 156.0, 145.0, 141.4, 133.0, 129.3, 128.1, 127.2, 123.2, 114.2, 113.1, 83.1, 58.1, 50.5, 47.3, 28.5, 15.3, 12.6, 11.4. MS (EI) m/z : 554.9 (M^+ , 18%) for $\text{C}_{22}\text{H}_{23}\text{Br}_2\text{NO}_2\text{S}_2$.

3.1.4. 6,8-Dibromo-2-(4'-methoxyphenyl)-4-oxochroman-3-yl *N,N*-diethyldithiocarbamate (**4d**)

Colorless crystals, m.p. 164–165 °C, 0.390 g, 70%. IR (ATR, cm^{-1}) 2964, 1685, 1471, 1439, 1244, 1213, 798, 723, 614, 564. ^1H NMR (CDCl_3 , selected data for the major *anti* isomer) δ 7.97 (d, J = 2.4 Hz, 1H), 7.89 (d, J = 2.4 Hz, 1H), 7.43 (d, J = 8.7 Hz, 2H), 6.90 (d, J = 8.8 Hz, 2H), 5.96 (d, J = 7.7 Hz, 1H), 5.81 (d, J = 7.7 Hz, 1H), 4.09–3.98 (m, 1H), 3.98–3.88 (m, 1H), 3.81 (s, 3H), 3.69 (q, J = 7.3 Hz, 2H), 1.24 (m, 6H). ^{13}C NMR (CDCl_3 , selected data for the major *anti* isomer) δ 191.1, 186.5, 160.0, 156.0, 141.4, 129.3, 128.7, 127.8, 123.1, 114.2, 113.9, 113.1, 82.9, 58.4, 55.3, 50.6, 47.3, 12.6, 11.4. MS (EI) m/z : 556.9 (M^+ , 14%) for $\text{C}_{21}\text{H}_{21}\text{Br}_2\text{NO}_3\text{S}_2$.

3.1.5. 2-*N,N*-Diethylamino-6,8-dibromo-4-phenyl-4*H*-1,3-dithiol[4,5-*c*]chromen-2-ylum tetrafluoroborate (**5a**); General Procedure

To a mixture of sulfuric acid (1 mL) and acetic acid (3 mL), flavanone **4a** (0.264 g, 0.5 mmol) was added and the resulting solution was heated to 70 °C for 30 min. The reaction mixture was then left to cool to room temperature and a solution of sodium tetrafluoroborate (0.3 g) in water (10 mL) was added dropwise, under vigorous stirring. The resulting precipitate was then filtered, washed thoroughly with water and recrystallized from ethanol, yielding the desired tetrafluoroborate **5a** in the form of

colorless crystals (0.23 g, 76%). M.p. 203–204 °C. IR (ATR, cm^{-1}) 1562, 1444, 1202, 1050, 721, 523. ^1H NMR ($\text{DMSO}-d_6$) δ 7.91 (d, $J = 2.2$ Hz, 1H), 7.78 (d, $J = 2.2$ Hz, 1H), 7.51 (dd, $J = 6.9$, 3.2 Hz, 2H), 7.47 (q, $J = 3.0$ Hz, 3H), 6.99 (s, 1H), 4.00–3.82 (m, 4H), 1.41 (t, $J = 7.2$ Hz, 3H), 1.33 (t, $J = 7.2$ Hz, 3H). ^{13}C NMR ($\text{DMSO}-d_6$) δ 184.9, 147.7, 136.9, 136.7, 130.6, 130.0, 129.6, 127.9, 127.1, 126.8, 119.7, 115.0, 112.4, 76.3, 54.8, 54.7, 10.8, 10.5. MS (EI) m/z : 509.9 (M^+-BF_4 , 6%) for $\text{C}_{20}\text{H}_{18}^{79}\text{Br}_2\text{NOS}_2]^+$.

3.1.6. 2-*N,N*-Diethylamino-6,8-dibromo-4-(4'-methylphenyl)-4*H*-1,3-dithiol[4,5-*c*]chromen-2-ylum tetrafluoroborate (**5b**)

Colorless crystals, m.p. 209–210 °C (0.274 g, 91%). IR (ATR, cm^{-1}) 2981, 1556, 1441, 1217, 1046, 741, 506. ^1H NMR ($\text{DMSO}-d_6$) δ 7.89 (d, $J = 2.2$ Hz, 1H), 7.77 (d, $J = 2.2$ Hz, 1H), 7.39 (d, $J = 8.0$ Hz, 2H), 7.27 (d, $J = 7.9$ Hz, 2H), 6.93 (s, 1H), 4.00–3.82 (m, 4H), 2.32 (s, 3H), 1.41 (t, $J = 7.2$ Hz, 3H), 1.33 (t, $J = 7.2$ Hz, 3H). ^{13}C NMR ($\text{DMSO}-d_6$) δ 184.9, 147.8, 140.4, 136.8, 133.7, 130.3, 130.1, 127.9, 127.0, 126.7, 119.7, 114.9, 112.4, 76.3, 54.8, 54.7, 21.3, 10.8, 10.5. MS (EI) m/z : 523.9 (M^+-BF_4 , 5%) for $\text{C}_{21}\text{H}_{20}^{79}\text{Br}_2\text{NOS}_2]^+$.

3.1.7. 2-*N,N*-Diethylamino-6,8-dibromo-4-(4'-ethylphenyl)-4*H*-1,3-dithiol[4,5-*c*]chromen-2-ylum tetrafluoroborate (**5c**)

Colorless crystals, m.p. 211–212 °C (0.26 g, 85%). IR (ATR, cm^{-1}) 2973, 1549, 1433, 1225, 1049, 757, 584, 530. ^1H NMR ($\text{DMSO}-d_6$) δ 7.89 (d, $J = 2.2$ Hz, 1H), 7.76 (d, $J = 2.2$ Hz, 1H), 7.40 (d, $J = 7.9$ Hz, 2H), 7.30 (d, $J = 8.0$ Hz, 2H), 6.92 (s, 1H), 3.98–3.80 (m, 4H), 2.62 (q, $J = 7.6$ Hz, 2H), 1.40 (t, $J = 7.2$ Hz, 3H), 1.32 (t, $J = 7.2$ Hz, 3H), 1.17 (t, $J = 7.6$ Hz, 3H). ^{13}C NMR ($\text{DMSO}-d_6$) δ 184.8, 147.7, 146.5, 136.8, 134.0, 130.2, 129.0, 128.0, 127.0, 126.7, 119.6, 114.9, 112.4, 76.3, 54.8, 54.7, 28.3, 15.8, 10.8, 10.5. MS (EI) m/z : 537.9 (M^+-BF_4 , 7%) for $\text{C}_{22}\text{H}_{22}^{79}\text{Br}_2\text{NOS}_2]^+$.

3.1.8. 2-*N,N*-Diethylamino-6,8-dibromo-4-(4'-methoxyphenyl)-4*H*-1,3-dithiol[4,5-*c*]chromen-2-ylum tetrafluoroborate (**5d**)

Colorless crystals, m.p. 219–220 °C (0.27 g, 79%). IR (ATR, cm^{-1}) 1550, 1493, 1251, 1230, 1178, 1046, 820, 708, 563, 518. ^1H NMR ($\text{DMSO}-d_6$) δ 7.89 (d, $J = 2.2$ Hz, 1H), 7.75 (d, $J = 2.2$ Hz, 1H), 7.43 (d, $J = 8.5$ Hz, 2H), 7.00 (d, $J = 8.5$ Hz, 2H), 6.88 (s, 1H), 3.98–3.81 (m, 4H), 3.77 (s, 3H), 1.40 (t, $J = 7.2$ Hz, 3H), 1.32 (t, $J = 7.2$ Hz, 3H). ^{13}C NMR ($\text{DMSO}-d_6$) δ 184.8, 161.0, 147.8, 136.8, 130.5, 129.8, 128.5, 127.0, 126.7, 119.7, 114.9, 114.8, 112.4, 76.2, 55.8, 54.8, 54.6, 10.8, 10.5. MS (EI) m/z : 539.9 (M^+-BF_4 , 8%) for $\text{C}_{21}\text{H}_{20}^{79}\text{Br}_2\text{NO}_2\text{S}_2]^+$.

3.2. Bacterial Susceptibility Assays

3.2.1. Strains and Culture Conditions

The strains selected for antimicrobial testing included *Staphylococcus aureus* ATCC 25923 (methicillin-sensitive, MSSA) and *Escherichia coli* ATCC 25922, both obtained from the culture collection of the Microbiology Laboratory, Alexandru Ioan Cuza University of Iași. Additionally, the clinical isolate *S. aureus* medbio1-2012 (methicillin-resistant, MRSA; resistant to cefoxitin, clindamycin, erythromycin, moxifloxacin, penicillin, and tetracycline) was provided by PhD Simona Matiut from Praxis Clinical Laboratory, Iași, Romania; *Acinetobacter baumannii* medbio3-2013 (resistant to cefotaxime and ceftazidime) was isolated from a local wastewater treatment plant and identified via MALDI-TOF/MS.

Strains were maintained as glycerol stocks at -80 °C. Prior to testing, frozen cultures were streaked onto Mueller–Hinton agar (MHA; Accumix, Belgium) and incubated overnight at 37 °C under aerobic conditions to ensure viability and purity. A single, morphologically typical colony from each strain was used to inoculate 10 mL of Mueller–

Hinton broth (MHB; Roth, Germany). Pre-cultures were incubated for 24 h at 37 °C with agitation at 190 rpm to obtain inoculum for subsequent assays.

3.2.2. Minimum Inhibitory and Bactericidal Concentration Assays

Minimum inhibitory concentration (MIC) values were determined using the broth microdilution method using 96-well microplates, following CLSI guidelines, as we previously described [14]. Serial two-fold dilutions of each flavonoid were prepared in Mueller–Hinton broth (MHB) with dimethyl sulfoxide (DMSO, Roth, Germany) as solvent, yielding final concentrations ranging from 0.12 to 250 µg/mL. Final cell density in the well was approximately 1×10^5 CFU (colony-forming units)/mL for all strains tested. Growth controls consisted of inoculated MHB, and solvent controls included DMSO at concentrations up to 24.87% (v/v). No inhibitory effect of DMSO was observed at concentrations up to 3.10%, corresponding to the highest MIC value recorded (31.25 µg/mL). Reference antibiotics (ampicillin, chloramphenicol, kanamycin, and gentamicin) were included as positive controls. After incubation at 37 °C for 24 h, MIC values were determined using a resazurin assay, based on visual evaluation of the distinct color change (blue to pink) indicating bacterial growth. MIC values were determined in triplicate and are reported as modal values. Variability between replicates did not exceed one two-fold dilution.

To determine the minimum bactericidal concentration (MBC), 5 µL aliquots from wells exhibiting no visible growth were serially diluted and subsequently plated onto Mueller–Hinton agar. The MBC was recorded as the lowest concentration at which no colony formation occurred after incubation at 37 °C. All assays were performed in biological triplicate.

3.3. Cytotoxicity Assay

The cytotoxicity of tricyclic flavonoids **5a–d** was evaluated using human dermal fibroblasts in culture, taken from an adult (HDF-Ad). HDF-Ad cells were cultured in dishes treated for cell cultures with complete MEM Eagle alpha culture medium containing 10% fetal bovine serum and 1% antibiotic-antimycotic mixture at 37 °C in a humidified atmosphere with 5% CO₂. TrypLE™ Express Dissociation Reagent was used for cell passage, when the cells reached 80% confluence, to avoid contact inhibition. Solutions of **5a–d** were investigated by first preparing stock solutions in DMSO, followed by diluting them 100-fold in the culture medium, so that the final percentage of DMSO was 1% in the cell plate. The control group also contained 1% DMSO. To investigate the cytotoxicity of **5a–d** we used the CellTiter 96® AQueous One Solution (MTS) assay, according to the manufacturer's instructions. Briefly, cells were seeded in 96-well plates at a density of 5×10^3 wells in 100 µL complete culture medium/well and incubated overnight. After 24 h, the culture medium was replaced with test solutions at different concentrations, and the plates were incubated for 48 h. After this incubation period, 20 µL MTS was added in each well, and the plates were incubated for 3 h. Finally, the absorbance at 490 nm was measured with a microplate reader. The relative cell viability is expressed as a percentage of the control group viability. Graphical data were expressed as means ± standard deviation (SD).

4. Conclusions

The synthetic tricyclic flavonoids **5a–d** exhibited strong antibacterial activity against selected WHO priority pathogens, including *Staphylococcus aureus* and *Acinetobacter baumannii*, surpassing the efficacy of both natural and synthetic flavonoids and several conventional antibiotics. MIC and MBC values against *S. aureus* ATCC 25923 as low as 0.12 µg/mL and 1.9 µg/mL, respectively, have been recorded for **5b** and **5c**. The tested derivatives were found to induce little to no cytotoxicity on HDF-Ad cells at concentrations up

to 7.8 µg/mL, a value at which most of them are active against the four tested bacterial strains, further confirming their potential use as antibacterial agents. These findings underscore the promise of tricyclic flavonoid scaffolds as lead compounds for the development of new therapeutic agents targeting multidrug-resistant bacterial infections. Nevertheless, further investigations are needed to evaluate their antimicrobial spectrum against a broader panel of multidrug-resistant clinical isolates and to assess the potential for resistance development against these novel flavonoids.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ph19020259/s1>, Table S1: Elemental analysis data; Copies of ¹³C NMR and ¹H NMR spectra.

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