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Note

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Biological Evaluation of Hyperforin and Its Hydrogenated Analogue on Bacterial Growth and Biofilm Production

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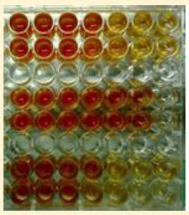
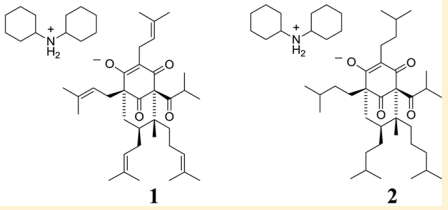
ABSTRACT: Bacterial biofilms are organized communities of microorganisms, embedded in a self-produced matrix, growing on a biotic surface and resistant to many antimicrobial agents when associated with a medical device. These biofilms require the development of new strategies for the prevention and treatment of infectious disease, including the potential use of natural products. One interesting natural product example is *Hypericum*, a plant genus that contains species known to have antimicrobial properties. The major constituent of *Hypericum perforatum* is an unstable compound named hyperforin (**1**); for this reason it was not believed to play a significant role in the pharmacological effects. In this investigation a hydrogenated hyperforin analogue (**2**) was tested on several ATCC and clinical isolate strains, in their planktonic and biofilm form (*Staphylococcus aureus*, MRSA, and *Enterococcus faecalis*). Compound **2** was effective against planktonic and biofilm cultures, probably due to higher stability, showing the percentage of cells killed in the range from 45% to 52%. These results are noteworthy from the point of view of future development of these polyprenylated phloroglucinols as potential antibiotics.





1 **2**

Despite significant progress made in microbiology and the control of microorganisms, sporadic incidents of epidemics due to drug-resistant microorganisms represent an enormous threat to global public health. In recent years, the use of medical implants such as catheters, pacemakers, prosthetic heart valves, and joint replacements has increased dramatically. These devices can become colonized by microorganisms that form a biofilm consisting of a mono- or multilayer of cells embedded within a matrix of extracellular polymeric material.[1−7] Release of microorganisms from the biofilm may initiate an acute disseminated infection. Implant-associated infections are difficult to resolve, because biofilm microorganisms are resistant to both host defense mechanisms and antibiotic therapy.[1,2,6] Bacterial biofilms have received increasing attention over the past decade,[1,7] and a number of model systems have been devised for studying the colonization of various solid surfaces by bacteria. In vitro investigations with pathogenic bacteria have shown that biofilm bacteria have a substantially reduced sensitivity to clinically important antibiotics compared with cells of the same organism in the dispersed form.[2] Although the majority of implant infections are caused by Gram-positive bacteria, notably staphylococci, infections due to Gram-negative bacteria and fungi tend to be more serious.[3] New drugs are needed to combat these pathogens. Antimicrobial compounds of plant origin have enormous therapeutic potential, not only because they mitigate infectious diseases but since they may also lack adverse side effects associated with antimicrobial agents. One interesting example is *Hypericum*, a plant genus that biosynthesizes secondary metabolites known to have antimicrobial properties.[8]

St. John's Wort (*Hypericum perforatum* L.) (Hypericaceae) extracts have been used in European folk medicine for centuries in a variety of indications, including skin injuries and burns. A petroleum ether extract of the aerial parts of *H. perforatum* was reported to be active against Gram-positive bacteria.[9] A major constituent of lipophilic extracts of *H. perforatum* is the prenylated acylphloroglucinol hyperforin, accompanied by 10% of its homologue adhyperforin and a number of oxidized acylphloroglucinols derived from the parent compounds.[10] In fact, hyperforin is unstable, in particular when exposed to light and air since the enolized β-dicarbonyl system and the prenyl groups are involved readily in oxidative reactions.[10,11] For this reason, hyperforin was not believed to play a significant role in the pharmacological effects of the extracts. However, it was recently shown that the extraction of the plant material under controlled conditions results in extracts containing 1−5% hyperforin, which can be maintained, if appropriately stored, for prolonged periods.[12] An *H. perforatum* extract obtained by supercritical extraction of the dried herb with carbon dioxide contains a much higher proportion of hyperforin (more than 20%), which does not deteriorate even after prolonged storage. From such an extract, pure hyperforin can be isolated through

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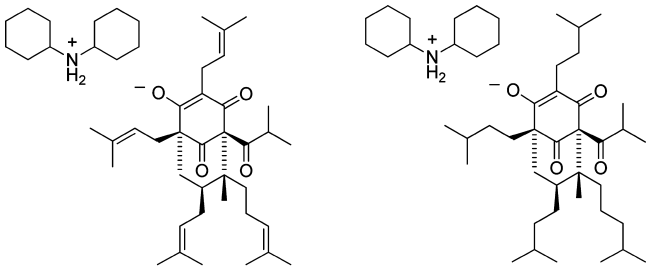
previous treatment with dicyclohexylamine and crystallization of the dicyclohexylamonium salt (DCHA) salt (**1**). In this salt form, hyperforin demonstrates good stability so that biological assays can be readily performed. Additionally, a slight increase in water solubility is also observed.[13−16a,b]

Hyperforin can be hydrogenated in quantitative yields to give the corresponding octahydro derivative (**2**). This shows an increase in lipophilicity and a considerably higher stability due to the absence of the double bonds.



1 **2**

Compounds **1** and **2** were tested for inhibition of bacterial growth and biofilm formation against a panel of clinically relevant pathogens, comprising the American Type Culture Collection (ATCC) strains *Staphylococcus aureus* ATCC 29213, methicillin-resistant *Staphylococcus aureus* ATCC 43300 (MRSA), *Enterococcus faecalis* ATCC 29212, and *Escherichia coli* ATCC 25922, and a clinical isolate of methicillin-resistant *Staphylococcus aureus* (*Staphylococcus aureus* Ig5). Growth inhibitory properties against planktonic strains were tested for the ability to prevent biofilm formation. Minimum biofilm inhibitory concentration (MBIC), which represents the lowest concentration of an antimicrobial agent resulting in no detectable biofilm growth, was determined against biofilms formed on 96-well microtiter plates using the XTT reduction assay. Reduction of the tetrazolium salt (XTT) is a commonly used method for determining microbial cell viability. It measures metabolic activity and requires the addition of an electron coupling reagent. The viability of all strains was decreased by octahydrohyperforin DCHA salt (**2**) in the biofilm assay compared to the untreated control. Interestingly, for hyperforin DCHA salt (**1**) there was no significant difference in the reduction of XTT in biofilm cells.

Compounds **1** and **2** demonstrated antimicrobial activity against the Gram-positive bacteria tested (*S. aureus*, MRSA, *E. faecalis*), but not against the Gram-negative bacterium (*E. coli*), in agreement with previous studies.[17] The minimum inhibitory concentration (MIC) was defined as the lowest concentration of antimicrobial agent that inhibited visible bacterial growth after overnight incubation. The MIC values of **2** were 4-fold higher than those for **1** for all sensitive strains tested, as reported in Table 1. The MIC values obtained for **1** were comparable to the results obtained by other investigators.[17] Five percent (v/v) DMSO and ethanol were used in the dilution of the drugs and did not show any antimicrobial activity against the planktonic bacteria (data not shown). Each experiment was performed in triplicate.

Antimicrobial activity testing for biofilm was evaluated in a 96-well plate assay, as described above, and the results are shown in Table 2 and in Figures 1 and 2. The converted amount of XTT is a measure of the metabolic activity of the microorganisms in the biofilm. In Table 2, the efficacy of compounds **1** and **2** is expressed as a percentage reduction of

Table 1. Minimum Inhibitory Concentration (MIC) Results of Compounds 1 and 2 and Norfloxacin (Nor) against Bacterial Strains Tested in Suspension

	MIC (μg/mL)		
bacterial strain	**1**	**2**	Nor
S. aureus ATCC 29213	1	4	2
S. aureus ATCC 25923	1	4	2
S. aureus ATCC 43300	1	4	4
S. aureus EMRSA-15	2		0.5
S. aureus SA1199B	2	4	32
S. aureus XU212	0.5	1	4
S. aureus RN4220		1	1
S. aureus Ig5	1	4	2
E. faecalis ATCC 29212	1	4	4
E. coli ATCC 25922	R[a]	R[a]	0.12

[a]R = resistant.

Table 2. Minimum Biofilm Inhibitory Concentration (MBIC) Results in μg/mL and Percentage Reduction of Microbial Cell Viability of Octahydrohyperforin DCHA and Hyperforin DCHA against Bacterial Strains Tested in Biofilms

	1		**2**	
	MBIC		MBIC	
bacterial strain	μg/mL	%	μg/mL	%
S. aureus ATCC 29213	25	45	150	52
S. aureus ATCC 43300	25	21	37.5	47
E. faecalis ATCC 29212	25	34	37.5	45
S. aureus Ig5	25	33	37.5	52

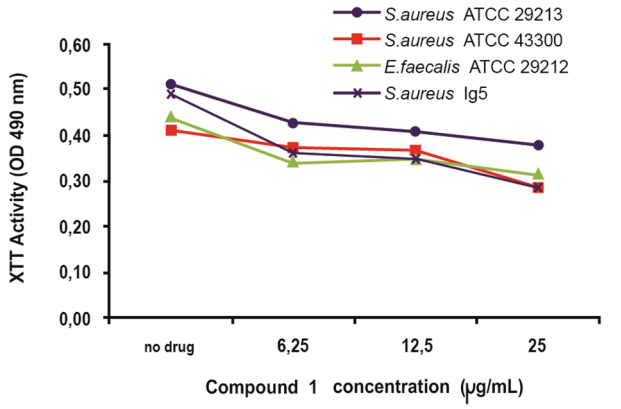


Figure 1. Metabolic activities of biofilms exposed to different concentrations of compound **1**. Metabolic activity is expressed as the optical density of treated biofilms compared to that for untreated biofilms (control).

the microorganisms in the biofilm as compared to an untreated biofilm. In this assay, PEG-200 was used as alternative solvent for **2** to promote its passage through the matrix of the biofilm. The results showed that for strains *S. aureus* ATCC 29213 and *E. faecalis* ATCC 29212 a percentage reduction of 52% at 150 μg/mL and a percentage reduction of 45% at 37.5 μg/mL were observed. *S. aureus* ATCC 43300 showed a reduction of 47% at 37.5 μg/mL, and the isolated strain *S. aureus* Ig5 (MRSA) was reduced to 45% at 150 μg/mL. Despite the fact that *S. aureus* Ig5 (a clinical isolate of MRSA) and *S. aureus* ATCC 43300 (MRSA) showed the same MIC value of 4 μg/mL, their MBIC

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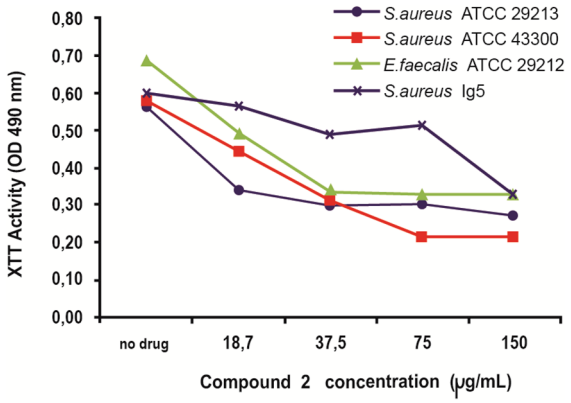

Figure 2. Metabolic activities of biofilms exposed to different concentrations of compound **2**. Metabolic activity is expressed as the optical density of treated biofilms compared to that for untreated biofilms (control).

values were different. To obtain the same percentage reduction for *S. aureus* Ig5, it was necessary to increase the concentration of **2** four times. These results clearly showed that clinical isolates, such as *S. aureus* Ig5, are less sensitive compared to their ATCC counterpart strains.

Compound **1**, at the highest test concentration used of 25 μg/mL, showed moderate activity for *S. aureus* ATCC 29213, with a 45% reduction, and demonstrated low activity for the other strains, with the lowest percentage reduction being 50%. The MIC values of **1** and **2** were similar and in the range 0.5−4 μg/mL (Table 1). Notably, the reduction of the prenyl side chains of hyperforin gave only a moderate increase in MIC and only a slight reduction of biological activity, which is somewhat surprising given the difference in chemical reactivity between the two compounds. Of clinical interest, both compounds displayed good potency against the tetracycline-resistant *S. aureus* strain (XU212), which is also a MRSA strain.

The direct antibacterial activities of compounds **1** and **2** are noteworthy, particularly given that they were found active toward multidrug-effluxing and MRSA strains at low MIC values (Table 1). Once stability issues are resolved, it is entirely plausible that these agents could be formulated into topical products to facilitate in the decolonization of mupirocin- or fusidic acid-resistant MRSA strains, which continues to be a pressing clinical issue.

The activities of **1** and **2** against biofilms also may be advantageous. Biofilms are especially problematic since they exhibit increased resistance to antibiotics. Using a variety of mechanisms, bacteria in biofilms can be 10 to 1000 times more resistant than their planktonic counterparts.[18] Along with the reduced antibiotic penetration in biofilms, these exopolysaccharide structures limit oxygen and nutrient diffusion and allow for the development of persister cells within the matrix, which results in slow- or nongrowing bacteria that reduce the antibacterial effects of many antibiotics.[19] Due to the high failure rate of commonly used antibiotics, it is reasonable to consider alternative treatments, and this study offers evidence that a natural compound can be a feasible candidate.

The aim of this study was to assess the antimicrobial efficacy of **1** and **2** against common microorganisms involved in infectious diseases and to determine the antimicrobial activity against planktonic and biofilm cells. In fact, previous investigations have demonstrated the efficacy of *Hypericum* constituents against *S. aureus* ATCC and MRSA biofilms.[20] In the present study, the XTT assay was performed to evaluate biofilm formation by four strains. The XTT assay indirectly measures microbial activity; it is reduced by dehydrogenase enzymes, present in the electron transport system, to a water-soluble formazan dye. The XTT assay shows an excellent correlation with other biofilm susceptibility methods, such as the AlamarBlue assay and viable counts, and offers the benefits of simplicity, relative cost, compatibility, and, most importantly, high reproducibility and a lack of toxicity.

As shown in Table 3, compound **1** was more effective against planktonic cells compared to **2**, with the MIC value of **1** being

Table 3. Minimum Inhibitory Concentration (MIC) Results in μg/mL against Bacterial Strains in Suspension and Minimum Biofilm Inhibitory Concentration (MBIC) Results in μg/mL and Percentage Reduction of Microbial Cell Viability of Compounds 1 and 2 against Bacterial Strains Tested in Biofilms

	1			2		
	MIC	MBIC		MIC	MBIC	
bacterial strain	μg/mL	μg/mL	%	μg/mL	μg/mL	%
S. aureus ATCC 29213	1	25	45	4	150	52
S. aureus ATCC 43300	1	25	21	4	37.5	47
E. faecalis ATCC 29212	1	25	34	4	37.5	45
S. aureus Ig5	1	25	33	4	37.5	52
E. coli ATCC 25922	R[a]			R		

[a]R = resistant.

4-fold greater than that of **2**. In clinical terms, **1** may have use for the treatment of Gram-positive bacterial infections, but it showed only weak utility to treat biofilm-associated infections. When cells were grown as surface-attached biofilms in order to mimic a device-related infection, **1** lacked the ability to inhibit cells within the biofilms. This may suggest that this agent, although effective against bacteria in suspension, for example in bloodstream infections, may not be the most suitable substance for treating biofilm-mediated device-related infections. These observations are probably related to the facile degradation of hyperforin in oxygenated conditions and correlate with insights made in the traditional uses of *Hypericum* to treat topical infections.

Compound **2** was effective against planktonic and biofilm cultures, probably due to its higher stability than **1**. In biofilms treated with **2**, the percentage of cells killed ranged from 45% to 52%. The obtained results suggested that this compound may be used in the development of an antibiofilm drug. This is noteworthy from the point of view of the potential development of polyprenylated phloroglucinols as antibiotics. Octahydrohyperforin **2** is readily obtainable by hydrogenation of a crude *H. perforatum* lipophilic extract without the need to isolate the parent compound (hyperforin, **1**). The stability of octahydrohyperforin to oxygen, light, and temperature also indicates that this agent could have topical utility.

EXPERIMENTAL SECTION

Test Compounds. Hyperforin DCHA (**1**) was a generous gift from Indena SpA, Milan, Italy. Octahydrohyperforin DCHA (**2**) was obtained from **1** as reported in L. Verotta et al.[21] Compound purity was >95% (Indena Drug Master File).

Bacterial Strains and Culture Conditions. The bacteria used in this study were *Staphylococcus aureus* ATCC 29213, *Staphylococcus*

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aureus ATCC 43300, *Enterococcus faecalis* ATCC 29212, and *Staphylococcus aureus* Ig5 (clinical MRSA isolate). A standard *S. aureus* strain (ATCC 25923) and a clinical isolate (XU212), which possesses the TetK efflux pump and is also a MRSA strain, were obtained from Dr. E. Udo.[22] Strain RN4220, which has the MsrA macrolide efflux pump, was provided by Dr. J. H. Cove.[23] EMRSA-15[24] was obtained from Dr. P. Stapleton, UCL School of Pharmacy. Strain SA1199B, which overexpresses the NorA MDR efflux pump, was a gift of Professor G. W. Kaatz.[25] Norfloxacin was obtained from Sigma Chemical Co. Mueller-Hinton broth (MHB; Oxoid) was adjusted to contain 20 mg/L Ca^{2+} and 10 mg/L Mg^{2+}. All the strains were stored at −70 °C in nutrient broth (Muller-Hinton II broth, MHBII, Becton Dickinson, France) containing 15% glycerol, until used. Cultures were grown on MHBII from 100 μL of frozen culture and incubated aerobically for approximately 4 h at 37 °C.

Antibacterial Agents. Compound **1** was freshly prepared in dimethyl sulfoxide (DMSO) and diluted in CaMHB before use, and **2** was dissolved in absolute ethanol for planktonic susceptibility tests and in sterile polyethylene glycol-200 for biofilm susceptibility tests.

Planktonic Susceptibility Testing. The minimum inhibitory concentration was determined for each strain in planktonic culture according to the Clinical and Laboratory Standards Institute (CLSI, M7-A6, 2003) as previously reported.[26] Serial 2-fold dilutions of **1** and **2** were prepared in 96-well microtiter plates with concentrations ranging, respectively, from 16 to 0.25 μg/mL and from 256 to 0.25 μg/mL. CaMHB was used to perform all dilutions. To ensure that the solvents themselves had no antibacterial activity, a control test was carried out by using them at their maximum concentration in the medium. *S. aureus* ATCC 25953 was included as a control strain in each test, and all results were within the published data.[17] All MIC tests were performed in triplicate.

Minimum Inhibitory Concentration Assay Determination. Overnight cultures of each strain were made up in 0.9% saline to an inoculum density of 5×10^5 colony-forming units (cfu) by comparison with a MacFarland standard. Norfloxacin was dissolved in DMSO and then diluted in MHB to give a starting concentration of 512 μg/mL. Using Nunc 96-well microtiter plates, 125 μL of MHB was dispensed into wells 1−11. Then, 125 μL of the test compound or the appropriate antibiotic was dispensed into well 1 and serially diluted across the plate, leaving well 11 empty for the growth control. The final volume was dispensed into well 12, which being free of MHB or inoculum served as the sterile control. Finally, the bacterial inoculum (125 μL) was added to wells 1−11, and the plate was incubated at 37 °C for 18 h. A DMSO control (3.125%) was also included. All MICs were determined in duplicate. The MIC was determined as the lowest concentration at which no growth was observed. A methanol solution (5 mg/mL) of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliium bromide (MTT; Lancaster, UK) was used to detect bacterial growth by a color change from yellow to blue.

Culturing of Biofilms. Biofilms were developed according to the method of Stepanović et al.,[27] with some minor modifications. Strains were streaked from frozen cultures and grown overnight at 37 °C on a Tryptic soy agar plate with 2% glucose.[27] A number of colonies were suspended in Tryptic soy broth (TSB, Merck KGaA, Germany) with 2% glucose to a density of approximately 10^5 cfu/mL. Briefly, 200 μL of each bacterial suspension was transferred in a 96-well, flat-bottom, tissue culture-treated microtiter plate (IWAKI, Tokio, Japan), to promote biofilm formation; the plates were incubated aerobically on a horizontal shaker for 4 h at 37 °C. After the suspension was gently drawn off and replaced by sterile medium, the plate was incubated for 24 h at 37 °C to obtain mature biofilms.[28] Each strain was tested in triplicate.

Treatment with Antimicrobial Compound. Following incubation, the plate was rinsed twice with phosphate-buffered saline (PBS) to remove loosely attached planktonic cells, and a serial dilution of the antimicrobial compound was added to each well. TSB with 2% glucose was used to perform all dilutions. Compound **1** was tested at a concentration ranging from 150 to 18.7 μg/mL, while **2** was evaluated from 25 to 6.25 μg/mL. Biofilms were incubated for 18 h at 37 °C on a horizontal shaker, and control antibiotic-free biofilms were included in each experiment.

Quantification of Viable Cells in a Biofilm Using the XTT Assay. The XTT assay was used to quantify the number of viable cells in each of the wells following antimicrobial compound treatment in comparison with biofilms formed in the presence of TSB (control). This method has been used extensively for the quantification of viable bacterial cells in a biofilm.[29] It measures the reduction of a tetrazolium salt (2,3-bis[2-methoxyloxy-4-nitro-5-sulfophenyl]-2*H*-tetrazolium-5-carboxanilide, Sigma-Aldrich, St. Louis, MO, USA) by metabolically active cells to a colored, water-soluble formazan derivative that can be easily quantified colorimetrically. Briefly, an XTT solution (1 mg/mL) was prepared in PBS and sterilized through a 0.22 μm pore size filter. Menadione solution (Fluka, Newport News, VA, USA) (0.4 mM) was prepared in DMSO before each assay. Following antimicrobial compound exposure, the plate was rinsed with PBS to remove loosely attached cells and dried in an inverted position at 37 °C for 20 min, and then 180 μL of PBS and 20 μL of the XTT−menadione solution (12.5 times the volume of the XTT solution was mixed with 1 volume of the menadione solution) were added to each well. The plate was incubated for 2 h at 37 °C on a horizontal shaker in the dark.[30] Reduction of XTT (oxidative activity) was then measured at 492 nm using a Perkin-Elmer Wallac Victor3 microplate reader.

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Notes

The authors declare no competing financial interest.

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