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Hydnocarpin-Type Flavonolignans: Semisynthesis and Inhibitory Effects on *Staphylococcus aureus* Biofilm Formation

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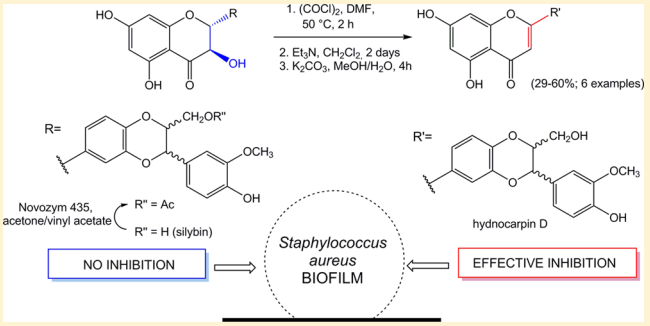
Supporting Information

ABSTRACT: A new, efficient, and general semisynthesis of hydnocarpin-type flavonolignans was developed and optimized, enabling gram-scale production of hydnocarpin D (**2**). Moreover, the syntheses of optically pure hydnocarpin isomers [(10*R*,11*R*)-hydnocarpin (**1a**), (10*R*,11*R*)-hydnocarpin D (**2a**), and (10*S*,11*S*)-hydnocarpin D (**2b**)], as well as the synthesis of isohydnocarpin (**8**), were achieved for the first time utilizing this new method. The synthesis is based on the two-step transformation of the readily available flavonolignans from milk thistle (*Silybum marianum*), accessible by isolation from the commercial extract silymarin. The first step relies on the regioselective formylation of the C-3 hydroxy group of the dihydroflavonol-type precursor using the Vilsmeier−Haack reagent, followed by formic acid elimination by triethylamine in the second step. The synthesized compounds were effective inhibitors of *Staphylococcus aureus* biofilm formation, with (10*S*,11*S*)-hydnocarpin D (**2b**) being the most potent inhibitor. Furthermore, the effect of glucose on biofilm formation was tested, and glucose decreased the biofilm inhibitory activity of **2b**. Moreover, **2b** increased the susceptibility of *Staph. aureus* to enrofloxacin.



3-Deoxy-2,3-dehydroflavonolignans, also called hydnocarpin-type flavonolignans (henceforth referred to as hydnocarpins), are a relatively small group of natural flavonoids named according to the first member, hydnocarpin (**1**), from *Hydnocarpus wightiana*,[1] which is structurally related to the flavonolignan isosilybin (**11**) contained in *Silybum marianum* extract (Figure 1). Currently, nine representatives of this group of flavonolignans have been isolated (Figure 1): hydnocarpin (**1**),[1,2] its regioisomer hydnocarpin D (**2**; D in the name indicates "D-ring down"),[3] sinaiticin (**3**),[4] 5′-methoxyhydnocarpin D (**4**),[5] 5″-methoxyhydnocarpin D (**5**),[6] palstatin (**6**),[3] hydnowightin (**7**),[7] isohydnocarpin (**8**),[8] and neohydnocarpin (**9**).[7] Moreover, several regioisomers of these natural compounds were obtained by chemical synthesis, e.g., regioisomers of **3** and **4**.[9]

In contrast to the related flavonolignans from *S. marianum*, specifically silybin (**10**), isosilybin (**11**), and silychristin (**12**) (Figure 1), knowledge of the biological activity of hydnocarpins is rather limited due to their poor availability. These compounds are obtained either by complicated isolation from a few tropical plants (usually in minute amounts) or by inefficient synthesis yielding mostly regioisomeric mixtures.[9]

Although biological studies of the hydnocarpins are sporadic (ca. 12 papers published), they show that hydnocarpins are not only effective antioxidants[10] but also promising anticancer compounds. The anticancer efficiency of **1** has been reported in numerous cancer cell lines (ED_{50} < 10 μM).[4,7] In another study, (−)-hydnocarpin (**1b**) (Figure 2) was inactive (ED_{50} > 10 μM) against the MCF-7 human breast cancer cell line, but it potentiated the cytotoxic effects of bruceantin and bruceine A by 7- and 10-fold, respectively.[11] Moreover, **2** was the most potent inhibitor of *Staphylococcus aureus* multidrug resistance efflux protein Nor A in the presence of subinhibitory quantities of the alkaloid berberine, whereas **4** and **1** exhibited 10 and 30 times lower activity, respectively.[12]

Previous studies on the antimicrobial activities of hydnocarpins reported moderate or low effects; however, these studies did not focus on microbial biofilms. Bacterial biofilms and the emergence of multiple drug resistance have become a major threat for the current medical treatment of nosocomial infections. In fact, approximately 65−80% of microbial infections in the developed countries are associated with biofilms.[13] The treatment of microbial biofilms is still a challenge, as even the most potent antibiotics have little effect on the viability of microorganisms forming biofilms, especially during late-stage chronic infections.[14,15] *Staphylococcus aureus*, one of the most important human pathogens that causes a wide range of infections and is

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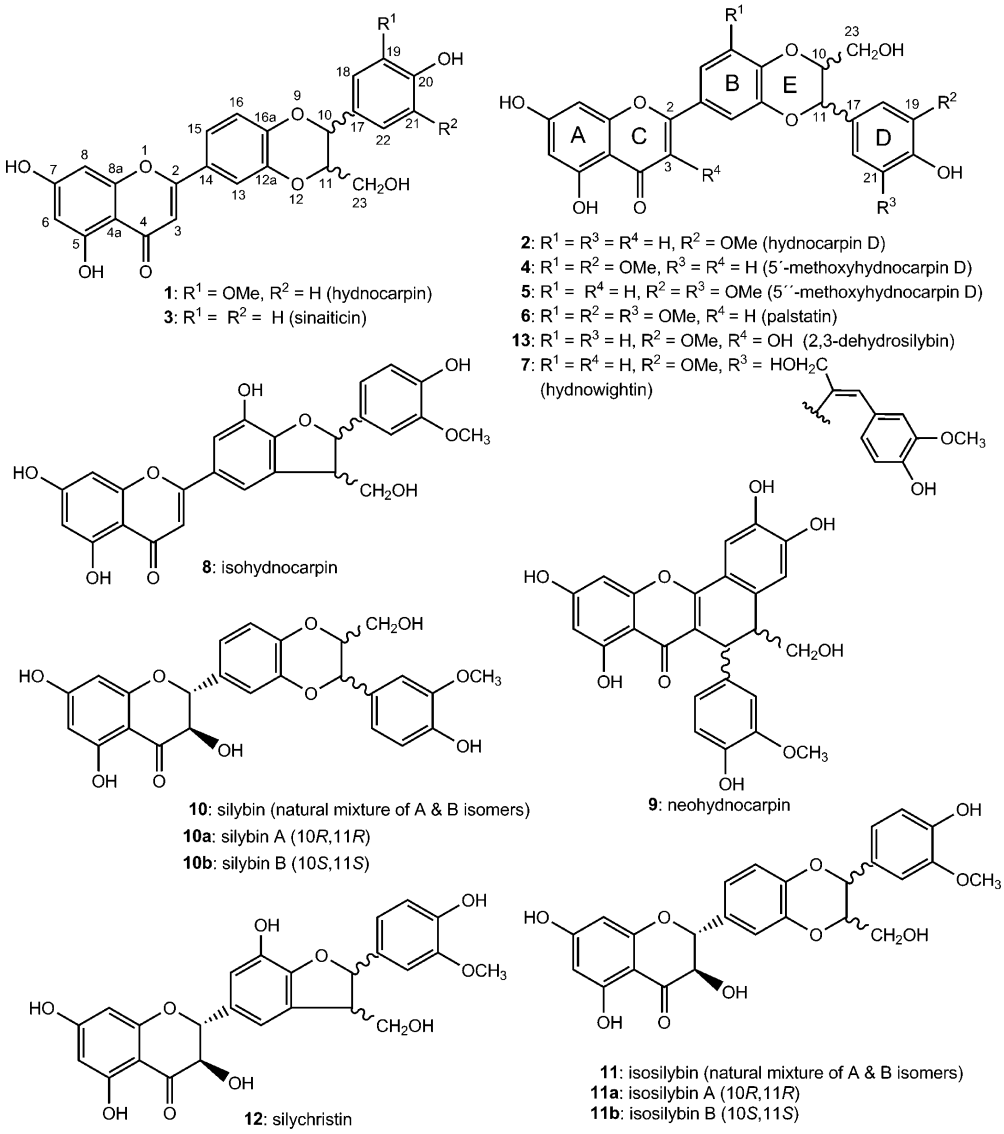

Figure 1. Naturally occurring hydnocarpins (**1**–**9**) and related flavonolignans from *S. marianum* (**10**–**13**).

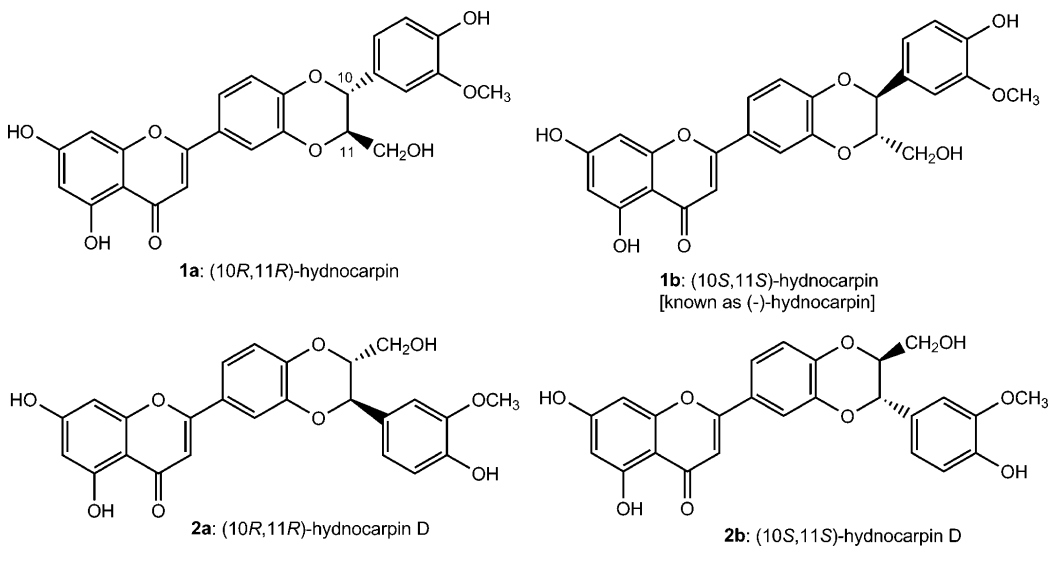

Figure 2. Synthesized enantiomers of hydnocarpin (**1**) and hydnocarpin D (**2**) (except **1b**).

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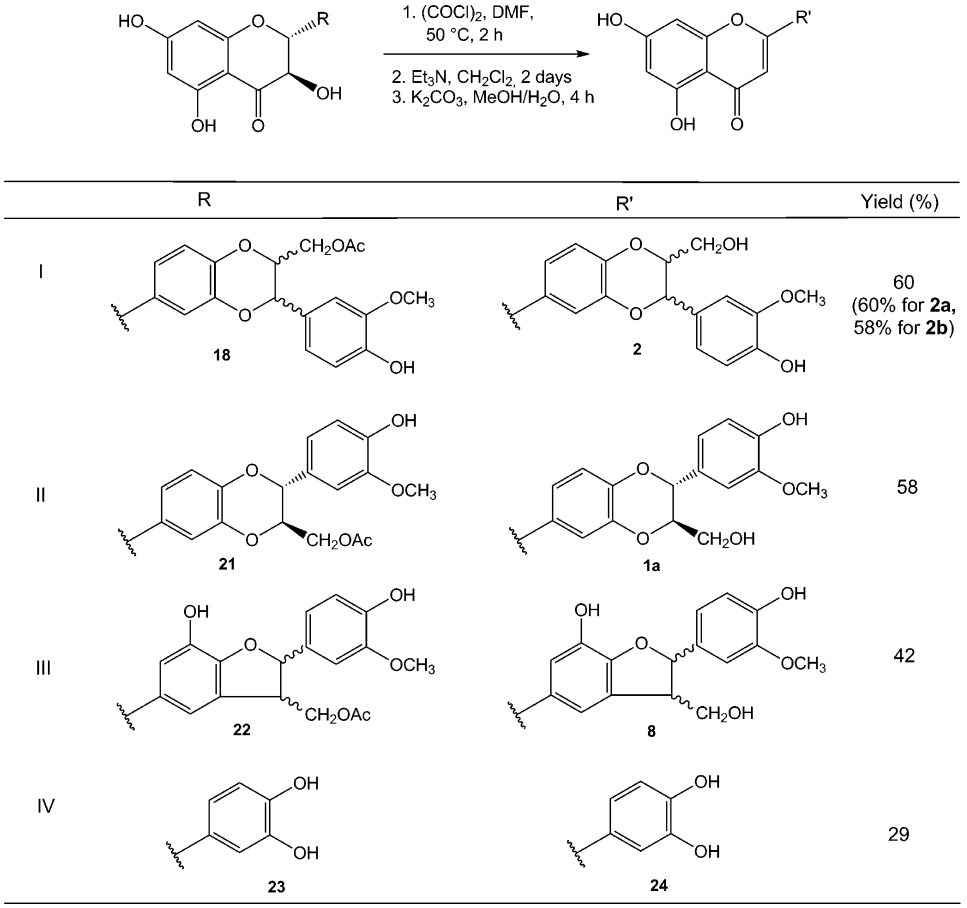

Figure 3. Preparation of flavones from corresponding 3-hydroxyflavanones.

responsible for thousands of deaths, is regularly found in biofilms from catheters, implanted devices, and wounds, and its virulence is frequently associated with biofilm formation.[16−19] Compounds that inhibit biofilm formation may facilitate treatment and prevention of staphylococci-associated infections.[20]

The objective of this work is to develop a new, efficient, and general method for the semisynthesis of hydnocarpins from related and readily available flavonolignans present in *S. marianum*, specifically **10**, **11**, and **12**. Additionally, the evaluation of the inhibitory effect of the synthesized hydnocarpins on biofilm formation of *Staph. aureus* is presented and compared to the antibiofilm effect of the starting compounds and the structurally related flavonoid 2,3-dehydrosilybin (**13**) (Figure 1).

RESULTS AND DISCUSSION

Chemistry. The first syntheses of hydnocarpin (**1**) were accomplished by an oxidative coupling reaction of the flavonoid luteolin (**24**) (Figure 3) with coniferyl alcohol catalyzed by either horseradish peroxidase or Ag_2CO_3.[9] Both coupling reactions afforded regioisomeric mixtures of **1** and **2** (**1**:**2** ratio of 3:2 and 1:9, respectively). These reactions were also used for the preparation of hydnocarpins **3** and **5** (Figure 1) with similar regioselectivities and yields.[12] Aside from the low regioselectivity of both coupling reactions, drawbacks of this type of synthesis are the relatively low yield (less than 40% of the mixture of **1** and **2**) and the requisite use of a rather expensive starting material, luteolin (**24**). A second approach to the synthesis of flavonolignan **4** but also applicable to the synthesis of **1** and **2** was based on the aldol condensation of MOM-protected acetophenone **14** with aldehyde **15** (Scheme 1).[9]

This approach afforded a single regioisomer (in the form of the racemic mixture); however, it represents multistep synthesis with moderate overall yield. Moreover, starting aldehyde **15** is not commercially available, which further complicates synthesis of the target compound.

To overcome the drawbacks of the previous syntheses of these rare natural compounds, we used their "hydrated" analogues, **10**, **11**, and **12** (Figure 1), which are readily available flavonolignans from the extract of *S. marianum* seeds, for transformation into the corresponding hydnocarpins. To achieve this aim, we exploited a reaction that we developed during our attempts to introduce a formyl group into **10** using the Vilsmeier−Haack reaction. Reacting **10** with $(COCl)_2$ in DMF resulted in a complex mixture of products that was difficult to separate. However, using 23-*O*-acetylsilybin (**18**)[21] in the Vilsmeier−Haack reaction resulted in a single product, **19** (Scheme 2), in excellent yield (88%). The use of $POCl_3$ instead of $(COCl)_2$ for generation of the Vilsmeier−Haack reagent was assessed as well, but the yield of **19** was substantially lower in this case.

The elimination of formic acid from **19** was assessed using three different bases (Table 1), where Et_3N was the most effective. Because the use of anhydrous conditions did not lead to simultaneous deacetylation, 23-*O*-acetylhydnocarpin D (**20**) was ultimately treated with K_2CO_3 in MeOH/H_2O (9:1), which resulted in the formation of **2** in ca. 60% overall yield (Scheme 2, Figure 3).

The same procedure was applied for the semisyntheses of optically pure isomers of hydnocarpins from corresponding stereochemically pure flavonolignans from *S. marianum*, which

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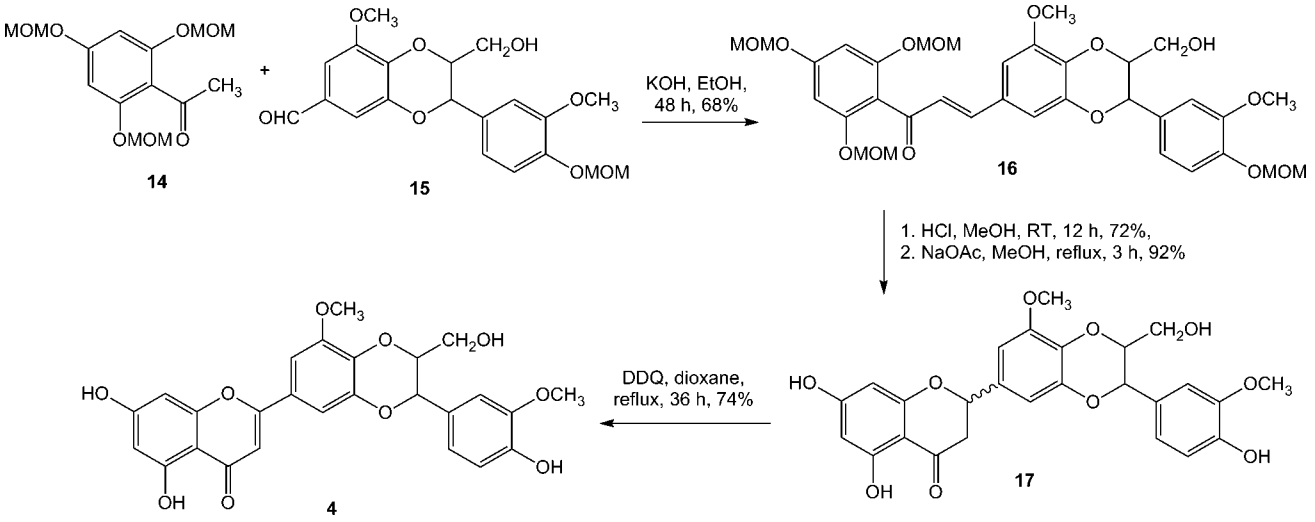
Scheme 1. Reported[9] Synthesis of Hydnocarpin 4



Scheme 2. Synthesis of Hydnocarpin D (2)

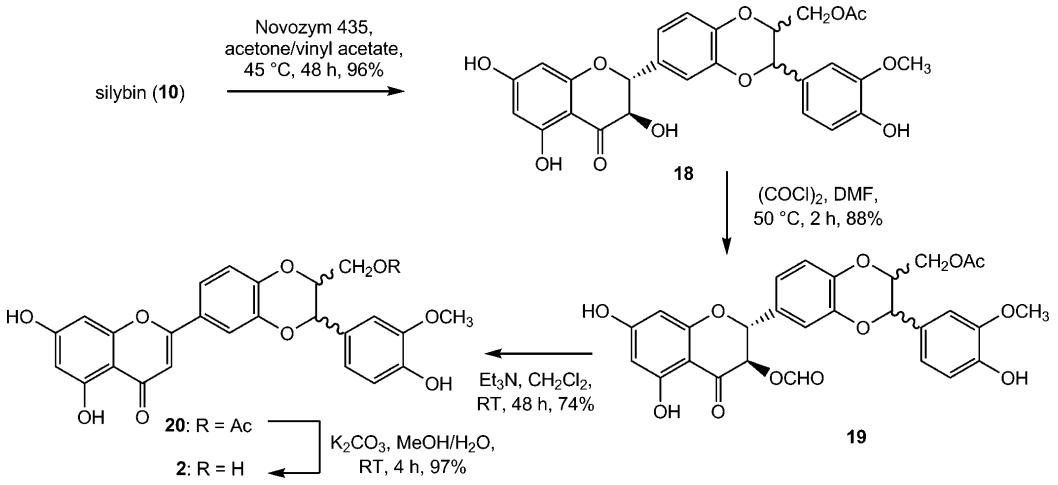


Table 1. Bases Tested for Elimination of HCO_2H from 19

entry	base[a]	yield of **20** [%]	note
1	Et_3N	74	isolated yield
2	DIPEA	20	low conversion (ca. 20%)
3	DBU	n.d.	elimination is accompanied by decomposition

[a]Procedure: compound **19**, base (10 equiv), CH_2Cl_2, 48 h; n.d., not determined.

were obtained by using recently developed enzymatic separation methods.[21−23] Utilizing this procedure, (10*R*,11*R*)-hydnocarpin (**1a**) from **11a**, (10*R*,11*R*)-hydnocarpin D (**2a**) from **10a**, and (10*S*,11*S*)-hydnocarpin D (**2b**) from **10b** were prepared (Figure 2). Because the description of optically pure compounds according to their specific optical rotations $[\alpha]_D^T$ is rather impractical for comparison of their structures and may be rather confusing ($[\alpha]_D^T$ of **1a** and **2a** are opposite, in spite of having the same absolute configuration), we strictly use the *R*/*S* notation for these isomers. Determination of their absolute configurations was based on ECD, NMR, and optical rotation measurements as well as comparison of these data with those of closely related enantiomers of **13**.[24]

In addition to the enantiomers of **1** and **2**, isohydnocarpin (**8**) was synthesized for the first time (Figure 3), starting from **12**. The silymarin dihydroflavonol (3-hydroxyflavanone), (2*R*,3*R*)-taxifolin (**23**) [(+)-taxifolin], was subjected to formylation and subsequent elimination to verify the applicability of this procedure for structurally related flavonoids as well. As a result, the flavone luteolin (**24**) was obtained in 29% yield (Figure 3). Notably, the new procedure did not require separation after each individual step, and only the final compound had to be purified by silica gel column chromatography, then typically recrystallized from MeOH or MeOH/H_2O.

However, the regioselective acetylation of the 23-OH function prior to formylation was problematic with isosilybin A (**11a**) and especially silychristin (**12**), as enzymatic transesterification either proceeded with moderate conversion for **11a** or did not proceed at all for **12**. Therefore, an alternative acetylation method had to be developed for **12** ($Ac_2O/BF_3{\cdot}OEt_2$, THF), which resulted in the formation of acetate **22** in moderate yield only. To overcome the problem of the selective protection of the primary 23-OH function in the corresponding flavonolignans, we also applied a simpler procedure that consisted of nonselective per-acylation of compound **10** and subsequent elimination by the aforementioned method (Et_3N, CH_2Cl_2). However, these attempts failed to give elimination products at C-2 and C-3, and only degradation was observed.

S. marianum extract is produced in large quantities for its application in the pharmaceutical industry, and the separation

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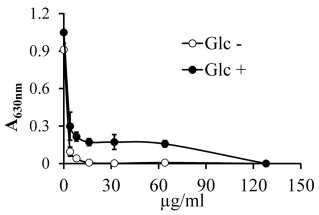
of **10** and other constituents is well described, affording gram quantities of each starting compound (from 200 g of the dried *S. marianum* extract, ca. 80 g of **10**, 3 g of **11a**, 30 g of **12**, and 2 g of **23** can be obtained[22]), including stereochemically pure isomers of silybin **10a** and **10b**.[21,23] Therefore, the method described above for the semisyntheses of hydnocarpins and luteolin represents a feasible way to synthesize sufficient amounts of these rare compounds for more detailed biological testing and for the synthesis of their derivatives.

Effect of Flavonolignans on *Staph. aureus* Biofilm Formation. The effect of flavonolignans on *Staph. aureus* 8325-4 growth and biofilm formation in a static microtiter plate was investigated. Although there was little or no effect of the compounds on bacterial growth, all flavonolignans inhibited biofilm formation. The efficiency of inhibition strongly depended on the structure of the compound (Figure 4A). Isosilybin

A

flavonolignan	EC_{50}	Minimal antibiofilm concentration (μg/ml)
(10*S*,11*S*)-hydnocarpin D (**2b**)	2	8-16
hydnocarpin D (**2**)	4	32
(10*R*,11*R*)-hydnocarpin D (**2a**)	16	64
(10*R*,11*R*)-hydnocarpin (**1a**)	16-32	64
2,3-dehydrosilybin (**13**)	16-32	64
isosilybin A (**11a**)	32-64	124
isohydnocarpin (**8**)	64	>124
silybin (**10**)	64	>124

B



C

(10*S*,11*S*)-hydnocarpin D (**2b**)	MIC (μg/ml)		
	ATCC29213	8325-4	N315
0 μg/ml	1	0.5	2-4
1 μg/ml	0.25	0.125	1
8 μg/ml	0.0625-0.125	0.0625	0.5
32 μg/ml	0.0625	0.0625	0.5

Figure 4. Inhibitory effect of flavonolignans on biofilm formation by the *Staph. aureus* strain 8325-4. (A) EC_{50} and minimal antibiofilm concentration of flavonoids required for antibiofilm activity in the absence of glucose. (B) Effect of glucose on inhibition of biofilm formation by **2b**. The graphic demonstrates the mean values obtained from three independent experiments with their statistical deviations. (C) Minimal inhibitory concentration of enrofloxacin in the presence of **2b** for *Staph. aureus* strains ATCC29213, 8325-4, and N315.

A (**11a**), isohydnocarpin (**8**), and silybin (**10**) were the least efficient inhibitors. (10*R*,11*R*)-Hydnocarpin (**1a**), 2,3-dehydrosilybin (**13**), and (10*R*,11*R*)-hydnocarpin D (**2a**) showed moderate inhibition of biofilm formation. The most effective compound was (10*S*,11*S*)-hydnocarpin D (**2b**), followed by racemic hydnocarpin D (**2**). On the basis of the effects of the tested compounds on the inhibition of *Staph. aureus* biofilm formation, it is possible to propose important structural motifs that should be present in the structure to achieve significant inhibitory activity. The most important feature is the presence of a 2,3-double bond (cf. compounds **1a** and **2** vs their saturated analogues **11a** and **10**, respectively, or compounds **13** and **10**). However, the presence of a dihydrobenzofuran instead of a dihydrobenzodioxine moiety (cf. **8** vs **1a**, **2**, and **13**) leads to an almost complete loss of inhibitory activity despite the presence of the 2,3-double bond. 2,3-Dehydrosilybin (**13**) is the 3-OH analogue of hydnocarpin D (**2**) and demonstrates that the presence of a 3-OH group of flavonolignans decreases the biofilm inhibitory activity of the compound. Comparing the activities of racemic **2** and its enantiomers **2a** and **2b** demonstrated that the configurations at C-9 and C-10 are important for influencing inhibition of biofilm formation. (10*S*,11*S*)-Hydnocarpin D (**2b**) was the most active compound from our series of flavonolignans, while its enantiomer **2a** (10*R*,11*R*) possessed significantly lower activity. The racemic mixture of both compounds, hydnocarpin D (**2**), exhibited the approximate median activity of the two enantiomers; that is, it was a better inhibitor than **2a** but worse than **2b**. In contrast, the regiochemistry at C-10 and C-11 seems to be less important, as the activities of (10*R*,11*R*)-hydnocarpin (**1a**) and (10*R*,11*R*)-hydnocarpin D (**2a**) were practically identical.

In addition to the effects of the tested compounds on biofilm inhibition, their ability to disrupt preformed biofilms was also evaluated. However, we did not observe any significant effects of the tested compounds in this case.

Glucose Affects the (10*S*,11*S*)-Hydnocarpin D Inhibition of *Staph. aureus* Biofilm Formation. The effects of the most potent inhibitor from the series, (10*S*,11*S*)-hydnocarpin D (**2b**), on biofilm formation in the presence of 1% glucose, which is frequently added to growth media as a biofilm formation enhancer, were assessed. Glucose positively regulates the two main mechanisms of biofilm formation in *Staph. aureus*: *icaADBC*-dependent and *icaADBC*-independent. Although the *icaADBC* operon, required for the synthesis of polymeric *N*-acetylglucosamine, is considered the main factor for biofilm formation,[25] glucose predominantly influences the *icaADBC*-independent mechanism of biofilm formation.[26,27] When glucose was added to the growth medium, biofilm formation was inhibited by 80% at a 8 mM concentration of (10*S*,11*S*)-hydnocarpin D (**2b**), while complete inhibition of biofilm formation occurred only at 128 mM of **2b** (Figure 4B). Two-level inhibition may suggest that **2b** efficiently inhibits the *icaADBC*-dependent biofilm formation pathway but has a moderate effect on the *icaADBC*-independent mechanism of biofilm formation, which is induced after the addition of the glucose.

***Staph. aureus* Susceptibility to Enrofloxacin Is Increased by (10*S*,11*S*)-Hydnocarpin D.** The antibiofilm activity of the flavonolignans may be associated with inhibition of the cell transport system, since hydnocarpin D (**2**) has been shown to inhibit NorA transporter,[12] responsible for *Staph. aureus* quinolone resistance and important for bacterial intracellular invasion.[28] Indeed, addition of **2b** increased susceptibility to enrofloxacin of the *Staph. aureus* 8325−4 strain, as well as two other tested *Staph. aureus* strains: ATCC29213, a control strain for MIC measurements, and N315, a methicillin-resistant strain (Figure 4C). Although this result suggests that the NorA transporter may be affected by (10*S*,11*S*)-hydnocarpin D (**2b**), further investigation is required to prove inhibition of the NorA transporter.

In conclusion, the chemoselective *O*-formylation of the 3-OH group of the flavonolignans from *S. marianum*, i.e., isosilybin A (**11a**), silybin (**10**), and silychristin (**12**), by the Vilsmeier−Haack reaction followed by the elimination of formic acid using Et_3N represents a straightforward way to prepare three members of the hydnocarpin-type flavonolignans, compounds **1a**, **2**, and **8**. This convenient procedure enables biological studies of these rare and neglected natural compounds, including their optically pure isomers, which are not accessible by isolation or by using reported synthetic methods.

An SAR study of the series of flavonolignans resulted in determination of the basic structural features important for effective inhibition of *Staph. aureus* biofilm formation: the presence of a 2,3-double bond together with a dihydrobenzodioxine moiety

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Table 2. NMR Spectroscopic Data (700.13 MHz, DMSO-d_6) for Compounds 19 and 21

	19		21	
position	δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)
2	79.64, CH; 79.58, CH	5.551, d (11.8)	82.52, CH	5.102, d (11.2)
3	71.69, CH; 71.64, CH	6.106, dd (0.8, 11.8); 6.094, dd (0.8, 11.8)	71.52, CH	4.608, dd (6.2, 11.2)
4	190.77, C; 190.76, C		197.72, C	
4a	100.57, C		100.51, C	
5	163.24, C		163.35, C	
6	96.57, CH	5.974, d (2.1)	96.13, CH	5.923, d (2.1)
7	167.46, C		166.89, C	
8	95.50, CH	5.935, d (2.1); 5.933, d (2.1)	95.10, CH	5.884, d (2.1)
8a	162.37, C; 162.35, C		162.49, C	
10	75.07, CH; 75.06, CH	4.555, ddd (2.7, 5.1, 9.3); 4.535, ddd (2.7, 5.2, 9.3)	75.92, CH	4.940, d (8.0)
11	75.86, CH; 75.84, CH	4.951, d (8.0); 4.941, d (8.0)	74.97, CH	4.498, ddd (2.6, 5.2, 8.0)
12a	143.54, C; 143.50, C		142.39, C	
13	116.82, CH	7.203, d (2.0); 7.193, d (2.0)	116.55, CH	7.142, d (2.0)
14	128.55, C		130.67, C	
15	121.61, CH; 121.52, CH	7.079, dd (2.0, 8.6); 7.074, dd (2.1, 8.6)	121.38, CH	7.018, dd (2.0, 8.3)
16	116.82, CH	7.027, d (8.3)	116.62, CH	6.964, d (8.3)
16a	143.70, C; 143.69, C		143.90, C	
17	126.51, C; 126.50, C		126.60, C	
18	111.77, CH	7.030, d (1.8); 7.025, d (1.6)	111.79, CH	7.019, d (2.0)
19	147.80, C		147.77, C	
20	147.37, C; 147.36, C		147.35, C	
21	115.49, CH	6.810, d(8.0); 6.809, d (8.1)	115.52, CH	6.816, d (8.0)
22	120.68, CH; 120.64, CH	6.870, dd (2.0, 8.4); 6.868, dd (2.0, 8.4)	120.59, CH	6.867, dd (2.0, 8.0)
23	62.58, CH_2	4.087, dd (2.6, 12.4); 3.929, dd (5.1, 12.4); 3.925, dd (5.0, 12.4)	62.64, CH_2	4.085, dd (2.6, 12.4); 3.932, dd (5.2, 12.4)
19-OMe	55.74, CH_3; 55.72, CH_3	3.777, s; 3.773, s	55.74, CH_3	3.777, s
3-OC(═O)H	160.71, CH; 160.70, CH	8.325, d (0.8); 8.321, d (0.8)		
23-CO	170.05, C		170.09, C	
23-OAc	20.456, CH_3	2.026, s; 2.025, s	20.48, CH_3	2.030, s
3-OH				5.796, d (6.3)
5-OH		11.353, s		11.884, s
7-OH		11.005, s		10.827, s
20-OH		9.179, s		9.185, s

and the absence of a 3-OH group. (10*S*,11*S*)-Hydnocarpin D (**2b**) was the most effective inhibitor of *Staph. aureus* biofilm formation from this series. This compound was also an effective chemosensitizer of *Staph. aureus* to quinolones. Additionally, testing the biofilm inhibitory activity of **2b** in the presence and/or absence of glucose suggested that **2b** most likely effectively inhibits the *icaADBC*-dependent biofilm formation pathway but has only a moderate effect on the *icaADBC*-independent mechanism.

This is the first report regarding the effect of flavonolignans on biofilm formation. Moreover, the preparation and biological studies of both enantiomers of **2** are quite unique.

EXPERIMENTAL SECTION

General Experimental Procedures. Melting points (mp) were recorded on a Kofler hot-stage microscope and are uncorrected. Optical rotations were measured in MeOH (UV/vis purity) using an Autopol IV polarimeter (Rudolph Research Analytical, NJ, USA). The ECD spectra were recorded on a Jasco-815 spectrometer (Japan) in MeOH.

NMR spectra were recorded on a Bruker Avance III 400 MHz spectrometer (400.00 MHz for ^{1}H and 100.58 MHz for ^{13}C), a Bruker Avance III 600 MHz spectrometer (600.23 MHz for ^{1}H and 150.93 MHz for ^{13}C), and a Bruker Avance III 700 MHz spectrometer (700.13 MHz for ^{1}H and 176.05 MHz for ^{13}C) in DMSO-d_6 (99.8 atom % D, VWR Chemicals, Leuven, Belgium) at 30 °C. Residual solvent signals were used as an internal standard (δ_H 2.500, δ_C 39.60). The ^{1}H and ^{13}C NMR, *J*-resolved, COSY, HSQC, HSQC-TOCSY, HMBC, band-selective HMBC, and 1D TOCSY experiments were performed using the manufacturer's software. ^{1}H and ^{13}C NMR spectra were zero filled to 4-fold data points and multiplied by a window function before Fourier transformation. The two-parameter double-exponential Lorentz−Gauss function was applied for ^{1}H to improve resolution, and line broadening (1 Hz) was applied to get better ^{13}C signal-to-noise ratio. Chemical shift

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values are presented in the δ-scale with digital resolution justifying the reported values to three (δ_H) or two (δ_C) decimal places.

Mass spectra were measured on a MALDI-TOF/TOF ultraFLEX III mass spectrometer (Bruker-Daltonics, Bremen, Germany). Positive mass spectra were externally calibrated using the monoisotopic $[M + H]^+$/$[M - H]^-$ ions of the PepMixII calibrant (Bruker-Daltonics, Bremen).

HR-MS were measured on a commercial 12T solariX FTMS instrument (Bruker Daltonics, Billerica MA, USA) equipped with an ESI/MALDI ion source and ParaCell. The analyses were performed using electrospray ionization (ESI), and the spectra were acquired in positive ion mode. The instrument was externally calibrated using singly charged NaTFA clusters, resulting in sub-ppm accuracy. The spectra were apodized using sine apodization with one zero filling. Data were processed with the software Data Analysis 4.2, and the possible elemental compositions were calculated using Smart Formula calculations.

The HPLC system consisted of a pump equipped with a 600E system controller, a 717 autosampler, and 2487 dual UV detector, and the data were processed using Empower 2 software (Waters, Milford, MA, USA). Water-containing mobile phases were filtered through a 0.22 μM GS filter (Millipore, Billerica, MA, USA) and degassed in an ultrasonic bath for 10 min before use. A Gemini 5 μm C_{18} column (250 × 4.6 mm; Phenomenex, CA, USA) with a guard column was used for analysis. The mobile phase consisted of 5% MeOH in H_2O with TFA (1%) (A) and MeOH with 1% TFA (B). Gradient elution started at 30% B (0 min), increasing linearly to 100% B over 20 min at a flow rate of 1.0 mL/min. UV detection was performed at 340 nm. The purity of the tested compounds that was determined under HPLC conditions and instrumentation mentioned above exceeded 95% in all cases.

The reactions were monitored by TLC on F_{254} silica gel (Merck, Germany), and the spots were visualized with UV light and by charring with an ethanolic solution of H_2SO_4 (5% v/v).

Materials. Silymarin (denoted as "milk thistle extract 60% silymarin (HPLC)") was purchased from Naturalin International Co. Ltd., China. Silybin (**10**), isosilybin A (**11a**), silychristin (**12**), and taxifolin (**23**) were isolated by the previously reported method.[22] Silybin stereoisomers **10a** and **10b** were separated by lipase-catalyzed discrimination.[21,23] 23-*O*-Acetylsilybin (**18**), 23-*O*-acetylsilybin A (**18a**), and 23-*O*-acetylsilybin B (**18b**) were prepared by enzymatic transacetylation as described previously.[21,23] All other reagents and chemicals were of analytical grade from Sigma-Aldrich and were used without further purification.

Staph. aureus 8325-4 was kindly provided by Dorte Free. *Staph. aureus* N315 was a gift from Dr. Malcolm Horsburgh.

*23-O-Acetylisosilybin A (**21**).* Isosilybin A (**11a**) (1 g, 2.1 mmol) was dissolved in a mixture of acetone/vinyl acetate (150 mL, 9:1, v/v), Novozym 435 (1 g, ≥10 000 U/g, 100% w/w) was added, and the mixture was shaken at 45 °C and 650 rpm for 48 h. Immobilized enzyme was removed by filtration and washed with acetone, and the combined filtrates were evaporated. The crude evaporated residue was purified by column chromatography ($CHCl_3$/acetone/HCO_2H, 90:10:1), yielding **21** (0.76 g, 70% yield). For ^1H and ^{13}C NMR data, see Table 2; ESIMS m/z 547 $[M + Na]^+$.

*23-O-Acetylsilychristin (**22**).* To a cooled solution (0 °C) of silychristin (**12**; 1.5 g, 3.1 mmol) in dry THF (75 mL) was added Ac_2O (0.5 mL, 5.6 mmol) and then $BF_3{\cdot}OEt_2$ (1.5 mL, 6.1 mmol, 50% solution in Et_2O), and the reaction mixture was stirred at 0 °C for 1 h. The reaction mixture was diluted with an ice-cold saturated solution of $NaHCO_3$ (100 mL), extracted with EtOAc (2 × 75 mL), dried over anhydrous Na_2SO_4, and evaporated to give a viscous oil. The oil was dissolved in acetone (10 mL), and *n*-hexane (100 mL) was added. The solution was briefly shaken and carefully decanted. The remaining oily solid was washed once again with *n*-hexane (100 mL), the solvent was decanted, and the remaining precipitate was dissolved in acetone and evaporated under reduced pressure to afford a solid residue. This residue provided, after chromatography (first $CHCl_3$/acetone/HCO_2H, 95:5:1–90:10:1; second EtOAc/hexanes with 1% HCO_2H 1:2–1:1), title compound **22** (0.798 g, 49%) as a white, amorphous solid: ^1H NMR (DMSO-d_6, 700.13 MHz) δ 1.975 (3H, s, 22-Ac), 3.764 (1H, dddd, J = 0.5, 5.8, 7.4, 7.7 Hz, H-11), 3.769 (3H, s, 18-OMe), 4.250 (1H, dd, J = 7.4, 11.0 Hz, H-22u), 4.375 (1H, dd, J = 5.8, 11.0 Hz, H-22d), 4.529 (1H, dd, J = 6.2, 11.3 Hz, H-3), 5.031 (1H, d, J = 11.3 Hz, H-2), 5.441 (1H, d, J = 7.7 Hz, H-10), 5.760 (1H, d, J = 6.2 Hz, 3-OH), 5.870 (1H, d, J = 2.1 Hz, H-8), 5.915 (1H, d, J = 2.1 Hz, H-6), 6.781 (1H, d, J = 8.0 Hz, H-20), 6.823 (1H, dd, J = 2.0, 8.0 Hz, H-21), 6.867 (1H, d, J = 1.7 Hz, H-14), 6.894 (1H, dd, J = 0.5, 1.7 Hz, H-12), 6.985 (1H, d, J = 2.0 Hz, H-17), 9.039 (1H, s, 19-OH), 9.406 (1H, s, 15-OH), 10.799 (1H, s, 7-OH), 11.888 (1H, s, 12-OH); ^{13}C NMR (DMSO-d_6, 176.05 MHz) δ 20.62 (CH_3, 22-$COCH_3$), 49.56 (CH, C-11), 55.72 (CH_3, 18-OMe), 65.11 (CH_2, C-22), 71.74 (CH, C-3), 83.14 (C, C-2), 87.49 (CH, C-10), 95.03 (CH, C-8), 96.07 (CH, C-6), 100.51 (C, C-4a), 110.62 (CH, C-17), 115.10 (CH, C-12), 115.37 (CH, C-20), 116.25 (CH, C-14), 119.07 (CH, C-21), 127.76 (C, C-11a), 130.42 (C, C-13), 131.45 (C, C-16), 140.94 (C, C-15), 146.69 (C, C-19), 147.07 (C, C-15a), 147.64 (C, C-18), 162.55 (C, C-8a), 163.34 (C, C-5), 166.82 (C, C-7), 170.38 (C, 22-CO), 197.76 (C, C-4); ESIMS m/z 547 $[M + Na]^+$.

Table 3. ^1H NMR Spectroscopic Data (700.13 MHz, DMSO-d_6) for Hydnocarpins 1a, 2a, 2b, 2, and 20

	1a	2a	2b	2	20
position	δ_H (J in Hz)	δ_H (J in Hz)	δ_H (J in Hz)	δ_H (J in Hz)	δ_H (J in Hz)
3	6.862, s	6.868, s	6.853, s	6.844, s	6.857, s
6	6.204, d (2.0)	6.193, d (2.1)	6.193, d (2.1)	6.190, d (1.9)	6.192, d (2.1)
8	6.517, d (2.0)	6.505, d (2.1)	6.500, d (2.1)	6.495, d (1.9)	6.497, d (2.1)
10	5.020, d (7.8)	4.972, d (8.0)	4.972, d (8.0)	4.300, ddd (2.8, 4.5, 8.0)	4.632, ddd (2.6, 5.1, 8.0)
11	4.265, ddd (2.5, 4.6, 7.8)	4.312, ddd (2.5, 4.5, 8.0)	4.306, ddd (2.6, 4.6, 8.0)	4.967, d (8.0)	5.000, d (8.0)
13	7.656, d (2.1)	7.666, d (2.2)	7.655, d (2.3)	7.647, d (2.0)	7.680, d (2.2)
15	7.592, dd (2.1, 8.5)	7.629, dd (2.2, 8.6)	7.620, dd (2.3, 8.6)	7.613, dd (2.0, 8.5)	7.629, dd (2.2, 8.6)
16	7.078, d (8.5)	7.120, d (8.6)	7.114, d (8.6)	7.108, d (8.5)	7.139, d (8.6)
18	7.039, d (1.7)	7.048, d (2.0)	7.048, d (2.0)	7.047, d (1.3)	7.057, d (2.0)
21	6.815, d (8.1)	6.819, d (8.0)	6.821, d (8.0)	6.822, d (8.0)	6.829, d (8.0)
22	6.883, dd (1.7, 8.1)	6.889, dd (2.0, 8.0)	6.891, dd (2.0, 8.0)	6.891, dd (1.3, 8.0)	6.891, dd (2.0, 8.0)
23	3.588, ddd (2.5, 4.9, 12.3)	3.575, dm (12.2)	3.579, ddd (2.6, 4.8, 12.4)	3.579, ddd (2.8, 3.5, 12.2)	4.114, dd (2.6, 12.5)
	3.384, ddd (4.6, 5.6, 12.3)	3.367, dm (12.2)	3.373, ddd (4.6, 6.1, 12.4)	3.373, ddd (4.5, 5.7, 12.2)	3.967, dd (5.1, 12.5)
5-OH	12.903, s	12.902, s	12.895, s	12.893, s	12.878, s
7-OH	10.835, s	10.890, s	10.827, s	10.828, s	10.829, s
19-OMe	3.786, s	3.787, s	3.790, s	3.791, s	3.787, s
20-OH	9.148, s	9.176, s	9.151, s	9.154, s	9.215, s
23-OH	4.959, dd (4.9, 5.6)	5.000, br s	4.982, dd (4.8, 6.1)	4.984, dd (3.8, 5.2)	
23-Ac					2.037, s

General Procedure A. Regioselective Formylation. Dry DMF (20 mL) was cooled to 0 °C by ice, and then $(COCl)_2$ (4 mL, 8 mmol, 2 M solution in CH_2Cl_2) was added dropwise (***caution**: gas evolution occurs violently—the release of gas has to be ensured*), to form a white precipitate of the Vilsmeier–Haack reagent. The resulting mixture was stirred at 0 °C for 10 min; then a solution of corresponding 3-hydroxyflavonoid (3.8 mmol) in dry DMF (15 mL) was added dropwise, and the mixture was briefly stirred at 0 °C. The reaction mixture was warmed to 50 °C and maintained at this temperature for 2 h (the color of the solution typically changed from slightly yellow to purple-red within ca. 30 min of heating). The reaction mixture was cooled to room temperature, diluted in ice-cold H_2O, and extracted with EtOAc (2 × 75 mL). The combined organic phases were washed with a saturated NaCl solution and H_2O, dried over anhydrous Na_2SO_4, and evaporated to dryness to afford the crude 3-*O*-formyl derivative, which was used in the next synthetic step without further purification.

General Procedure B. Elimination of Formic Acid. To a solution of the corresponding 3-formate obtained following general procedure

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Table 4. ^{13}C NMR Spectroscopic Data (176.05 MHz, DMSO-d_6) for Hydnocarpins 1a, 2a, 2b, 2, and 20

	1a		2a		2b		2		20	
position	δ_C	type	δ_C	type	δ_C	type	δ_C	type	δ_C	type
2	162.98	C	162.99	C	162.98	C	162.97	C	162.83	C
3	103.99	CH	103.93	CH	103.92	CH	103.92	CH	104.10	CH
4	181.82	C	181.84	C	181.81	C	181.81	C	181.84	C
4a	103.87	C	103.83	C	103.84	C	103.85	C	103.88	C
5	161.49	C	161.48	C	161.47	C	161.48	C	161.50	C
6	98.97	CH	98.98	CH	98.95	CH	98.95	CH	98.99	CH
7	164.31	C	164.37	C	164.28	C	164.29	C	164.35	C
8	94.14	CH	94.15	CH	94.11	CH	94.12	CH	94.15	CH
8a	157.40	C	157.41	C	157.38	C	157.38	C	157.41	C
10	76.43	CH	75.97	CH	75.95	CH	78.63	CH	75.51	CH
11	78.08	CH	78.62	CH	78.62	CH	75.96	CH	75.96	CH
12a	143.71	C	144.05	C	144.03	C	144.03	C	144.02	C
13	114.87	CH	115.12	CH	115.09	CH	115.08	CH	115.26	CH
14	123.76	C	123.51	C	123.49	C	123.49	C	123.96	C
15	119.95	CH	120.20	CH	120.17	CH	120.17	CH	120.33	CH
16	117.56	CH	117.43	CH	117.40	CH	117.40	CH	117.54	CH
16a	147.15	C	146.94	C	146.92	C	146.92	C	146.24	C
17	127.03	C	127.09	C	127.09	C	127.10	C	126.26	C
18	111.90	CH	111.85	CH	111.88	CH	111.89	CH	111.85	CH
19	147.72	C	147.72	C	147.71	C	147.72	C	147.87	C
20	147.24	C	147.22	C	147.21	C	147.22	C	147.51	C
21	115.43	CH	115.40	CH	115.40	CH	115.41	CH	115.55	CH
22	120.66	CH	120.74	CH	120.72	CH	120.72	CH	120.79	CH
23	60.14	CH_2	60.09	CH_2	60.08	CH_2	60.09	CH_2	62.51	CH_2
19-OMe	55.81	CH_3	55.78	CH_3	55.79	CH_3	55.80	CH_3	55.78	CH_3
23-CO									170.10	C
23-Ac									20.48	CH_3

A in CH_2Cl_2 (70 mL) was added Et_3N (5 mL), and the solution was stirred at room temperature for 2 days. The solvents were evaporated to dryness by coevaporation with toluene, affording crude 3-deoxy-2,3-dehydroflavonoid, which was subjected to basic hydrolysis without purification.

*23-O-Acetyl-3-O-formylsilybin (**19**).* The title compound was prepared according to general procedure A, starting from **18** (2 g, 3.8 mmol). Column chromatography (hexanes/EtOAc, 2:1−1:1, v/v) of the crude reaction mixture afforded pure **19** (1.849 g, 88%) as a white, amorphous solid. For ^{1}H and ^{13}C NMR data, see Table 2; ESIMS m/z 575 $[M + Na]^+$.

*23-O-Acetylhydnocarpin D (**20**).* The title compound was prepared according to general procedure B, starting from **19** (1.82 g, 3.3 mmol). Column chromatography ($CHCl_3$/acetone/HCO_2H, 90:10:1) of the crude reaction mixture afforded **20** (1.23 g, 74%) as a yellowish, amorphous solid. For ^{1}H and ^{13}C NMR data, see Tables 3 and 4; ESIMS m/z 507 $[M + H]^+$.

*Hydnocarpin D (**2**).* Compound **20** (1.2 g, 2.4 mmol) was dissolved in a mixture of MeOH/H_2O (22 mL, 10:1 v/v), K_2CO_3 (1.2 g) was added, and the mixture was stirred at room temperature for 4 h. The solution was diluted with ice-cold H_2O and acidified with HCl, and the precipitate was filtered off, washed with H_2O, and dried. The crude product was purified by silica gel column chromatography ($CHCl_3$/acetone/HCO_2H, 80:20:1), affording nearly pure title compound. This compound was recrystallized from MeOH/H_2O (9:1 v/v) to yield pure **2** (1.064 g, 97%) as a slightly yellowish, microcrystalline solid: mp 186−188 °C. For ^{1}H and ^{13}C NMR data, see Tables 3 and 4; HRESIMS m/z 465.1187 $[M + H]^+$ (calcd for $C_{25}H_{21}O_9$, 465.1180).

*(10R,11R)-Hydnocarpin D (**2a**).* Compound **2a** (0.531 g, 60%) was prepared analogously to **2**, starting from **18a** (1 g, 1.907 mmol). The final recrystallization of **2a** was performed in MeOH/H_2O (9:1): mp 190−192 °C; $[\alpha]_D^{25}$ −48 (*c* 0.2, MeOH); ECD spectrum, see Supporting Information (Figures S21 and S22); for ^{1}H and ^{13}C NMR data, see Tables 3 and 4; HRESIMS m/z 465.1188 $[M + H]^+$ (calcd for $C_{25}H_{21}O_9$, 465.1180).

*(10S,11S)-Hydnocarpin D (**2b**).* Compound **2b** (0.514 g, 58%) was prepared from **18b** (1 g, 1.9 mmol) by the procedure described for **2**. The final recrystallization of **2b** was performed in MeOH/H_2O (9:1): mp 182−184 °C; $[\alpha]_D^{25}$ +46 (*c* 0.2, MeOH); ECD spectrum, see Supporting Information (Figure S22); for ^{1}H and ^{13}C NMR data, see Tables 3 and 4; HRESIMS m/z 465.1188 $[M + H]^+$ (calcd for $C_{25}H_{21}O_9$, 465.1180).

*(10R,11R)-Hydnocarpin (**1a**).* The solid residue obtained from general procedures A and B, from acetate **21** (0.76 g, 1.449 mmol), was dissolved in a mixture of MeOH/H_2O (11 mL, 10:1 v/v), K_2CO_3 (0.76 g) was added, and the mixture was stirred at room temperature for 4 h. The solution was diluted with ice-cold H_2O and acidified with HCl, and the precipitate was filtered off, washed with H_2O, and dried. The crude product was purified by silica gel column chromatography ($CHCl_3$/acetone/HCO_2H, 80:20:1), affording nearly pure title compound, which was recrystallized from MeOH/H_2O (9:1 v/v) to provide pure **1a** (0.39 g, 58%) as a slightly yellowish, microcrystalline solid: mp 256−258 °C; $[\alpha]_D^{25}$ +39 (*c* 0.1, MeOH); ECD spectrum, see Supporting Information (Figure S21); for ^{1}H and ^{13}C NMR data, see Tables 3 and 4; HRESIMS m/z 465.1186 $[M + H]^+$ (calcd for $C_{25}H_{21}O_9$, 465.1180).

*Isohydnocarpin (**8**).* Compound **8** was prepared analogously to compound **2**, starting from acetate **22** (1.7 g, 3.2 mmol). Recrystallization (MeOH) yielded pure title compound (0.63 g, 42%) as a yellowish, microcrystalline solid: mp 237−239 °C; ^{1}H NMR (DMSO-d_6, 700.13 MHz) δ 3.552 (1H, ddd, J = 5.0, 6.4, 6.7 Hz, H-11), 3.718 (1H, dd, J = 6.4, 10.5 Hz, H-22u), 3.757 (3H, s, 18-OMe), 3.786 (1H, dd, J = 5.0, 10.5 Hz, H-22d), 5.066 (1H, br s, 22-OH), 5.597 (1H, d, J = 6.7 Hz, H-10), 6.194 (1H, d, J = 2.0 Hz, H-6), 6.447 (1H, d, J = 2.0 Hz, H-8), 6.703 (1H, s, H-3), 6.775 (1H, d, J = 8.1 Hz, H-20), 6.813 (1H, dd, J = 2.0, 8.1 Hz, H-21), 6.965 (1H, d, J = 2.0 Hz, H-17), 7.362 (1H, d, J = 1.8 Hz, H-14), 7.510 (1H, m, H-12), 9.045 (1H, s, 19-OH), 9.728 (1H, s, 15-OH), 10.837 (1H, s, 7-OH), 12.952 (1H, s, 12-OH); ^{13}C NMR (DMSO-d_6, 176.05 MHz) δ 52.75 (CH, C-11), 55.75 (CH_3, 18-OMe), 62.66 (CH_2, C-22), 88.02 (CH, C-10), 93.92 (CH, C-8), 98.93 (CH, C-6), 103.31 (CH, C-3), 103.76 (C, C-4a), 110.60 (CH, C-17), 114.26 (CH, C-14),

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114.71 (CH, C-12), 115.45 (CH, C-20), 118.88 (CH, C-21), 123.50 (C, C-13), 130.67 (C, C-11a), 131.91 (C, C-16), 141.56 (C, C-15), 146.67 (C, C-19), 147.67 (C, C-18), 150.63 (C, C-15a), 157.38 (C, C-8a), 161.54 (C, C-5), 163.93 (C, C-2), 164.31 (C, C-7), 181.69 (C, C-4); HRESIMS m/z 465.1183 $[M + H]^+$ (calcd for $C_{25}H_{21}O_9$, 465.1183).

*Luteolin (**24**).* The solid residue obtained according to general procedures A and B, starting from compound **23** (0.2 g, 0.7 mmol), was purified by silica gel column chromatography ($CHCl_3$/acetone/HCO_2H, 80:20:1), affording nearly pure title compound, which was recrystallized from MeOH/H_2O (9:1) to give pure **24** (55 mg, 29%) as a yellow, microcrystalline solid: mp 326−328 °C (reported[29] mp 326 °C); ^{1}H NMR (DMSO-d_6, 700.13 MHz) δ 6.187 (1H, d, J = 2.1 Hz, H-8), 6.441 (1H, d, J = 2.1 Hz, H-6), 6.664 (1H, s, H-3), 6.887 (1H, d, J = 8.3 Hz, H-13), 7.392 (1H, d, J = 2.3 Hz, H-10), 7.412 (1H, dd, J = 2.3, 8.3 Hz, H-14), 9.902, 9.391 (2H, br s, 11-OH, 12-OH), 10.814 (1H, br s, 7-OH), 12.969 (1H, s, 5-OH); ^{13}C NMR (DMSO-d_6, 176.05 MHz) δ 93.90 (CH, C-8), 98.89 (CH, C-6), 102.94 (CH, C-3), 103.77 (C, C-4a), 113.44 (CH, C-10), 116.08 (CH, C-13), 119.06 (CH, C-14), 121.58 (C, C-9), 145.80 (C, C-11), 149.76 (C, C-12), 157.36 (C, C-8a), 161.55 (C, C-5), 163.96 (C, C-2), 164.19 (C, C-7), 181.72 (C, C-4); HRESIMS m/z 287.0553 $[M + H]^+$ (calcd for $C_{15}H_{11}O_6$, 287.0550).

Preparation of Monosodium Salts of Compounds. To improve water solubility of the tested compounds, their monosodium salts were prepared. The corresponding flavonolignan (1 equiv) was dissolved in a 1 M methanolic solution of NaOH (1 equiv, MeOH/H_2O, 95:5 v/v), evaporated to dryness, redissolved in MeOH, and evaporated to dryness again by coevaporation with toluene, affording the monosodium salt of the compound.

Biofilm Assay. Biofilm formation was determined under static conditions by a microtiter plate assay;[30] however, instead of a 24-well plate, a 96-well plate was used. *Staph. aureus* 8325-4, pregrown overnight on a brain heart infusion (BHI) agar plate, was resuspended in 0.9% NaCl to McF = 0.5, and 5 μL of resuspended cells was inoculated into each well of the 96-well plate containing 100 μL of tryptic soy (TS) medium supplemented with the tested compounds (their monosodium salts) at concentrations of 0−124 mM. In the experiment with glucose, 1% glucose was added to the TS medium. The plate was incubated for 24 h at 37 °C, and the wells were then gently rinsed three times with water to remove nonadherent cells. The cells that remained attached to the wells were stained with 50 μL of 1% crystal violet for 15 min, then rinsed gently with water five times and air-dried. Crystal violet was recovered from the stained cells by adding 100 μL of a 96% ethanol solution. The optical density of the plate was measured at $A_{630\ nm}$ using a BioTek Synergy HT spectrophotometer. Every experiment was performed at least in triplicate.

Minimum Inhibitory Concentration (MIC). The MIC values were determined by the standard microbroth dilution method[31] in Mueller-Hinton broth. Experiments were performed with two biological replicates, and each biological replicate was performed in triplicate.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.5b00430.

^{1}H and ^{13}C NMR spectra of compounds **1a**, **2a**, **2b**, **2**, **8**, **19**, **20**, **21**, **22**, and **24** and ECD spectra of compounds **1a**, **2a**, and **2b** (PDF)

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Notes

The authors declare no competing financial interest.

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