Antibacterial Oligomeric Polyphenols from the Green Alga Cladophora socialis

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Supporting Information

ABSTRACT: A series of oligomeric phenols including the known natural product 3,4,3,4-tetrahydroxy-1,1′-biphenyl (3), the previously synthesized 2,3,8,9-tetrahydroxybenzo[c]-chromen-6-one (4), and eight new related natural products, cladophorols B–I (5–12), were isolated from the Fijian green alga Cladophora socialis and identified by a combination of NMR spectroscopy, mass spectrometric analysis, and computational modeling using DFT calculations. J-resolved spectroscopy and line width reduction by picric acid addition aided in resolving the heavily overlapped aromatic signals. A panel of Gram-positive and Gram-negative pathogens used to evaluate pharmacological potential led to the determination that cladophorol C (6) exhibits potent antibiotic activity selective toward methicillin-resistant Staphylococcus aureus (MRSA) with an MIC of 1.4 μg/mL. Cladophorols B (5) and D–H (7–11) had more modest but also selective antibiotic potency. Activities of cladophorols A–I (4–12) were also assessed against the asexual blood stages of Plasmodium falciparum and revealed cladophorols A (4) and B (5) to have modest activity with EC₅₀ values of 0.7 and 1.9 μg/mL, respectively.

INTRODUCTION

Aromatic systems are ubiquitous within natural products, reflecting the availability of their biosynthetic precursors in plants, algae, and microorganisms via the shikimate and polyketide pathways and the varied biological effects of such functional groups. When incorporated as building blocks in vascular plants, aromatic polymers such as lignin provide structural support that ultimately enabled plants to occupy terrestrial ecosystems leading to the evolution of trees. Aromatic natural products conferring structural rigidity were unknown in the marine realm until lignin was reported in the coraline red alga Calliarthron chelosporioides (phylum Rhodophyta). This startling discovery suggested that an ancestor of green and red algae acquired the capacity to polymerize phenolic monomers but that this feature was silenced in most evolving lineages. Other red algae produce a variety of structurally simple, often halogenated phenolics of mostly unknown biological function, although more complex, aromatic natural products of mixed shikimate and isoprenoid biosynthetic origin have also been observed.

Many members of the brown algae (phylum Ochrophyta), which evolutionarily diverged just before land plants, produce polymeric, acetylenic, phenolic compounds (phlorotannins) constituting 1–20% of dry mass. These oligomers of phloroglucinol protect tissues against UV radiation and herbivory and may contribute to structural support. In contrast, significantly fewer phenolic natural products have been reported in green algae (phylum Chlorophyta). Brominated polyphenolic compounds including the feeding deterrent atravinvilleol and the condensation product of two molecules of atravinvilleol, rawsonol, are produced by green algae belonging to the genus Atravinvillea. Another example of phenolic compounds from green algae are two vanillic acid analogues from an Australian Cladophora socialis that have been found to disrupt insulin cell signaling.

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The present study describes a series of related oligomeric phenols, cladophorols A–I (4–12), from the green alga *C. socialis* that attracted our attention for its antibiotic activity against methicillin-resistant *Staphylococcus aureus* (MRSA). Analysis of mass spectrometric and NMR spectroscopic data enabled structure elucidation despite the high molecular weights and repeating structural patterns of members of this natural product family. We used J-resolved NMR spectroscopy to reduce signal overlap in the 1H and 13C NMR spectra and employed picric acid line width-narrowing to reveal the exact linkage pattern of the subunits. In addition, we propose a structural revision of known vanillic acid analogues (1–2) based on a combination of spectroscopic and computational approaches.

### RESULTS AND DISCUSSION

**Collection and Isolation.** Offshore of Vanua Levu Island in Fiji, *C. socialis*, identified by morphological features and nuclear small-subunit 18S rRNA analyses (Figure S1), was collected floating at the ocean surface. The aqueous methanol extract was partitioned using a sequence of biphasic organic solvents and water. Following evaluation of MRSA activity, the ethyl acetate-soluble fraction was chromatographed on silica gel followed by repeated reversed-phase semipreparative HPLC, yielding the known compound 3,4,3′,4′-tetrahydroxy-1,1′-biphenyl (3), previously synthesized 2,3,8,9-tetrahydroxybenzo[c]chromen-6-one (4), and eight new phenolic natural products (5–12) (Figure 1).

**Characterization of 4.** HRESIMS was used to assign the molecular formula of 4 as C_{13}H_{8}O_{6}. The 1H NMR spectrum exhibited only four singlets at δH 7.58, 7.33, 7.32, and 6.73 (Table 1), attributed to two aromatic rings based on HSQC correlations with δC 115.4, 108.3, 107.4, and 104.4, respectively, and the absence of coupling between the protons.

| position | δC | δH   | δC | δH   |
|----------|----|------|----|------|
| Benzo[c]chromone | 1 | 107.4 (d) | 7.32 s | 108.2 (d) | 8.37 d (2.8) |
| 2        | 144.4 (s) | 155.6 (s) | 146.0 (s) | 136.3 (s) |
| 3        | 148.6 (s) | 106.9 (d) | 6.69 d (2.8) |
| 4        | 104.4 (d) | 6.73 s | 146.6 (s) |
| 4a       | 146.0 (s) | 136.3 (s) |
| 5        | 164.0 (s) | 163.4 (s) |
| 6        | 112.9 (s) | 114.5 (s) |
| 7        | 115.4 (d) | 7.58 s | 123.9 (d) | 7.84 d (8.5) |
| 8        | 147.3 (s) | 116.4 (d) | 7.08 d (8.5) |
| 9        | 154.8 (s) | 152.7 (s) |
| 10       | 108.3 (d) | 7.33 s | 144.3 (s) |
| 10a      | 131.7 (s) | 123.1 (s) |
| 10b      | 111.4 (s) | 120.7 (s) |
| Biphenyl 1A | 139.6 (s) |
| 2A       | 1160.0 (d) | 7.14 d (2.2) |
| 3A       | 150.1 (s) |
| 4A       | 144.0 (s) |
| 5A       | 122.1 (d) | 6.98 d (8.3) |
| 6A       | 119.1 (d) | 7.02 dd (8.3, 2.2) |
| 6B       | 115.4 (d) | 7.04 d (2.2) |
| 7B       | 146.5 (s) |
| 8B       | 145.9 (s) |
| 9B       | 116.6 (d) | 6.82 d (8.2) |
| 10B      | 119.2 (d) | 6.93 dd (8.2, 2.2) |

Correlations observed in HMBC from the signal at δH 6.73 (H-4) to δC 111.4 (C-10b), 144.4 (C-2), 146.0 (C-4a), and 148.6 (C-3) established the first ring. The second ring was determined from HMBC correlations from δH 7.58 (H-7) to δC 131.7 (C-10a), 147.3 (C-8), 154.8 (C-9), and 164.0 (C-6). Direct linkage between the two aromatic rings was established by HMBC correlations from δH 7.32 (H-1) to C-10a and from δH 7.33 (H-10) to C-10b. With connectivity of the carbonyl functionality and the two aromatic rings established, a third

![Figure 1. Structures of compounds 3–12 from *C. socialis*.](image-url)
ring was necessary to account for the 10 degrees of unsaturation calculated for this compound.

Since the $^1$H and $^{13}$C NMR chemical shifts of 4 were similar to those reported for 1, the structure depicted in Figure 2a was considered. Although it did not conflict with any of the observed NMR spectroscopic features, full consideration of all plausible structures and evaluation of this data to determine the best fit led to a more plausible alternative (Figure 2b). An HMBC spectrum recorded in DMSO-$d_6$ with picric acid, which reduced the line widths of the phenolic hydroxy region, revealed four exchangeable protons at $\delta_{H}$ 10.41, 9.89, 9.74, and 9.23, each correlating with three carbons (Figure S13). In the originally considered structure (Figure 2a), only three hydroxyl groups were within this range; the carboxylic acid group is expected to shift the fourth labile proton downfield. Additionally, HMBC signals from $\delta_{H}$ 9.89 to C-7, C-8, and C-9 indicated an alcohol group at C-8. Since none of the labile protons correlated with carboxyl C-6, we concluded that it was part of the third ring. Compound 4 was thus identified as 2,3,8,9-tetrahydroxybenzo[c]chromen-6-one and named cladophorol A. This compound was previously reported as a synthetic analogue of ellagic acid created to improve potency for DNA gyrase inhibition, but the spectroscopic data were not disclosed at that time.\(^{18}\)

**Characterization of 5.** Compound 5 was obtained as a pale red amorphous solid with a molecular formula of C$_{20}$H$_{16}$O$_{9}$ deduced by HRESIMS. The $^1$H and COSY NMR spectra exhibited resonances accounting for four aromatic protons correlated with carbonyl C-6, we concluded that it was 4,9,10-trihydroxy-2-[(3,3,4′-trihydroxy-1,1′-biphenyl)-4-yl]oxy]benzo[c]-chromen-6-one and named cladophorol B.

**Structure Revision of 1.** Since 4 and 5 were well characterized, the validity of the structure previously reported for 1 was questioned.\(^{16}\) When recorded in DMSO-$d_6$ the carboxyl carbons of 4 and 5, which are part of a lactone ring, produced NMR signals at $\delta_{C}$ 164.5 and 160.3, respectively. The carboxyl carbon of 1 was reported at $\delta_{C}$ 161.1, a value upfield of expectations for a free carboxylic acid.\(^{16}\) DFT calculations of the $^1$H and $^{13}$C chemical shift values were performed for the published structure 1 and for two other hypothetical structures (13 and 14) having a benzo[c]-chromen-6-one unit (Figure 3).\(^{19,20}\)

Indeed, the mPW1PW91/6-311+G(d,p) $^{13}$C chemical shift values did not match the experimental values for the originally published structure 1 with an rmsd of 3.3 ppm (Table 2). The largest discrepancies were due to C-4a and C-6 which are involved in the lactone ring. The hypothetical structures 13 and 14 showed smaller deviations for $\delta_{C}$, with rmsd of 1.7 and 1.8 ppm, respectively, but only 13 exhibited a small $\delta_{H}$ rmsd (0.08 ppm). When DP4+ probabilities were calculated, structure 13 scored 100% when considering chemical shift data for both $^1$H and $^{13}$C. For this reason, 1 should be revised to 13 and named 2,8,9-trihydroxy-4-[(3,3,4′-trihydroxy[1,1′-biphenyl]-4-yl]oxy]benzo[c]-chromen-6-one.

**Characterization of 6.** Compound 6 was isolated as a pale red solid with a molecular formula of C$_{26}$H$_{18}$O$_{8}$ based on an [M − H]$^-$ pseudomolecular ion in the HRESIMS at m/z 973.1967. The $^1$H NMR spectrum was difficult to interpret due to severe signal overlap in the aromatic region. J-resolved 2D NMR spectroscopy aided the interpretation and

![Figure 2. Possible structures (a and b) for cladophorol A (4) based on selected observed HMBC correlations indicated by arrows.](Image 132x607 to 229x694)

![Figure 3. Original and hypothetical structures proposed for 1, supporting realignment of previously published 1 as 13.](Image 370x299 to 519x518)
allowed assignment of all chemical shifts and coupling constants (Table 3). Using DQF-COSY and J-resolved spectroscopy, seven AMX aromatic spin systems resulting from 1,2,4-trihydroxyphenyl groups were observed as doublets at $\delta^H 6.67 - 6.91$ and 6.42 - 6.57 and doublet of doublets at $\delta^H 6.27 - 6.47$. Interestingly, within each of these ranges, a gradient of proton chemical shifts was noted whereby each set of signals from a given ring appeared in a predictable order. This feature was highlighted by simulating each set of protons using the extracted $\delta^C$ and $J$ from NMR spectra (Figure 4). When comparing proton signals for H-3, H-5, and H-6, the two signals corresponding to rings Y and Z appeared to be outliers. Most of the carbon signals were resolved in the $^{13}$C NMR spectrum, although HSQC and HMBC spectra showed large clusters of correlations between protons of rings D–G and carbons averaged at 157.5 ($\delta^C$ 151.2 (C-2D–G), 139.6 (C-1D–G), 123.1 (C-6D–G), 108.8 (C-5D–G), and 106.4 (C-3D–G)). This prevented a precise assignment of signals but also indicated these rings were similar. Two other spin systems unrelated to the gradient of chemical shifts showed HMBC correlations from H-2A to $\delta^C$ 139.9 (C-1B) and from H-2B and H-6B to $\delta^C$ 133.9 (C-1A) allowing assignment of these two spin systems to a biphenyl unit (Figure S2). Given all these data, Table 2. Experimental and Calculated NMR Chemical Shifts for 1, 13, and 14

| position | exp.\textsuperscript{16} | calc. | $\Delta$ | calc. | $\Delta$ | calc. | $\Delta$ |
|----------|-----------------|------|--------|------|--------|------|--------|
| H-1      | 6.92            | 7.00 | −0.08  | 7.01 | −0.09  | 6.71 | +0.21  |
| H-3      | 6.55            | 6.61 | +0.06  | 6.47 | +0.08  | 6.83 | −0.28  |
| H-7      | 7.55            | 7.63 | −0.08  | 7.47 | +0.08  | 7.59 | −0.04  |
| H-10     | 7.38            | 7.22 | +0.16  | 7.35 | +0.03  | 7.10 | +0.28  |
| H-2\textsuperscript{a} | 6.96  | 6.92 | +0.04  | 6.92 | +0.04  | 7.02 | −0.06  |
| H-5\textsuperscript{a} | 6.80  | 6.88 | −0.08  | 6.89 | −0.09  | 6.84 | −0.04  |
| H-6\textsuperscript{a} | 6.87  | 6.99 | −0.12  | 7.04 | −0.17  | 7.01 | −0.14  |
| H-2\textsuperscript{b} | 7.14  | 7.11 | +0.03  | 7.15 | −0.01  | 7.12 | +0.02  |
| H-5\textsuperscript{b} | 7.00  | 6.94 | +0.06  | 6.97 | +0.03  | 6.99 | +0.01  |
| H-6\textsuperscript{b} | 7.04  | 6.89 | +0.15  | 6.96 | +0.08  | 6.99 | +0.05  |

$^a$δ values calculated at the mPW1PW91/6-311+G(d,p)//B3LYP/6-31G(d) level of theory performed with the polarizable continuum model (PCM) using DMSO. Red color highlights large differences in calculated values: |ΔδH| > 0.2, |ΔδC| > 3.0. $^b$Root-mean-square deviation. $^c$Maximum absolute error. $^d$DP4+ probability.21

TABLE 2. Experimental and Calculated NMR Chemical Shifts for 1, 13, and 14

| position | exp.\textsuperscript{16} | calc. | $\Delta$ | calc. | $\Delta$ | calc. | $\Delta$ |
|----------|-----------------|------|--------|------|--------|------|--------|
| $\delta^C$ |      |      |        |      |        |      |        |
| C-1      | 98.7            | 102.1 | −3.4  | 100.2 | −1.5  | 97.7 | +1.0  |
| C-2      | 154.2           | 150.3 | +3.9  | 153.5 | +0.7  | 155.6 | −1.4  |
| C-3      | 104.2           | 106.4 | −2.2  | 103.6 | +0.6  | 104.8 | −0.6  |
| C-4      | 145.9           | 140.5 | +5.4  | 146.3 | −0.4  | 145.5 | +0.4  |
| C-4a     | 134.0           | 140.1 | −6.1  | 135.9 | −1.9  | 134.3 | −0.3  |
| C-6      | 161.1           | 166.7 | −5.6  | 160.7 | +0.4  | 159.5 | +1.6  |
| C-6a     | 112.8           | 108.3 | +4.5  | 112.2 | +0.6  | 113.0 | −0.2  |
| C-7      | 114.0           | 113.2 | +0.8  | 113.3 | +0.7  | 114.6 | −0.6  |
| C-8      | 146.9           | 145.4 | +1.5  | 144.2 | +2.7  | 144.4 | +2.5  |
| C-9      | 152.6           | 155.8 | −3.2  | 151.6 | +1.0  | 150.8 | +1.8  |
| C-10     | 107.9           | 108.3 | −0.4  | 107.1 | +0.8  | 107.0 | +0.9  |
| C-10a    | 128.1           | 130.7 | −2.6  | 129.5 | −1.4  | 129.6 | −1.5  |
| C-10b    | 118.6           | 124.4 | −5.8  | 119.8 | −1.2  | 118.4 | +0.2  |
| C-1\textsuperscript{a} | 130.6 | 131.5 | −0.9  | 133.8 | −3.2  | 133.7 | −3.1  |
| C-2\textsuperscript{a} | 113.7 | 112.4 | +1.3  | 112.3 | +1.4  | 112.1 | +1.6  |
| C-3\textsuperscript{a} | 145.5 | 140.5 | +5.0  | 143.8 | +1.7  | 143.7 | +1.8  |
| C-4\textsuperscript{c} | 144.6 | 141.7 | +2.9  | 144.0 | +0.6  | 143.6 | +1.0  |
| C-5\textsuperscript{c} | 155.5 | 146.8 | +1.1  | 113.6 | +1.9  | 114.0 | +1.5  |
| C-6\textsuperscript{d} | 116.9 | 119.7 | −2.8  | 119.7 | −2.8  | 119.2 | −2.3  |
| C-1\textsuperscript{b} | 137.8 | 137.9 | −0.1  | 139.9 | −2.1  | 140.6 | −2.8  |
| C-2\textsuperscript{b} | 114.6 | 114.1 | +0.5  | 113.4 | +1.2  | 113.8 | +0.8  |
| C-3\textsuperscript{b} | 148.7 | 145.7 | +3.0  | 149.4 | −0.7  | 150.1 | −1.4  |
| C-4\textsuperscript{b} | 141.1 | 138.9 | +2.2  | 141.2 | −0.1  | 140.6 | +0.5  |
| C-5\textsuperscript{b} | 116.8 | 118.9 | −2.1  | 119.1 | −2.3  | 120.9 | −4.1  |
| C-6\textsuperscript{b} | 121.3 | 118.1 | +3.2  | 118.1 | +3.2  | 118.6 | +2.7  |

$^e$RMSE\textsuperscript{b} | 3.3 | 1.7 | 1.8 |
| $\Delta$ | 6.1 | 3.2 | 4.1 |
| $\delta^C$ | 96.9% | 3.2% | 100.0% | 0.0% |
Two structures were hypothesized, differing by the position of the free alcohol groups on each hydroxyphenyl (Figure 5). Two model structures, 15a and 15b, containing a biphenyl unit and three 1,2,4-trihydroxyphenyl rings (Figure 5), were computationally tested to determine which arrangement was more probable. Their 1H and 13C NMR chemical shifts were predicted (Tables S26 and S27) following DFT computation at the mPW1PW91/6-311+G(d,p) level of theory and compared to the corresponding position of 6. The rmsd and MAE values calculated for both 1H and 13C were lower when 15a was considered, indicating that 6a is the correct structure. Based on these results, 6 was identified as α-hydro-ω-[3,4-dihydroxyphenyl]octa[oxy(2-hydroxyphen-4-yl)] and named cladophorol C.

Characterization of 7. The molecular formula for 7 was established as C57H43O20 based on HRESIMS, accounting for one additional dihydroxyphenyl moiety. The 1H and 13C NMR spectra almost perfectly superimposed those of 6, the only differences being three additional proton signals at δH 6.86 (d, J = 8.8 Hz), 6.50 (d, J = 2.9 Hz), and 6.40 (dd, J = 8.8 Hz) and six additional carbon signals at δC 157.6, 151.2, 151.2, 139.5, 123.1, 108.7, and 106.5. As a result, 7 was identified as α-hydro-ω-[3,4-dihydroxyphenyl]nona[oxy(2-hydroxyphen-4-yl)] and named cladophorol D.

Characterization of 8. Compound 8 was isolated as a reddish solid with the molecular formula C67H44O23 based on an [M – H]− HRESIMS peak at m/z 1215.2188. The 1H and 13C NMR spectra strongly resembled those of 6 and 7.

### Table 3. 1H (800 MHz) and 13C (200 MHz) NMR Spectroscopic Data of Cladophorols C and D (6–7) in CD3OD

| Position   | δC  | δH  | Position   | δC  | δH  |
|------------|-----|-----|------------|-----|-----|
| Rings A–B (Biphenyl) |     |     | Rings A–B (Biphenyl) |     |     |
| 1a         | 134.0 |     | 2a         | 114.9 | 7.01 (d, 2.2) |
| 2a         | 146.5 |     | 3a         | 146.0 |     |
| 4a         | 116.6 | 6.81 (d, 8.2) | 5a | 119.3 | 6.91 (dd, 8.2, 2.2) |
| 5a         | 139.9 |     | 6a         | 116.1 | 7.11 (d, 2.2) |
| 1b         | 150.2 |     | 2b         | 143.9 |     |
| 5b         | 122.3 | 6.94 (d, 8.3) | 6b | 119.2 | 7.00 (dd, 8.3, 2.2) |
| Ring C     |     |     |            |     |     |
| 1          | 139.8 |     | 2          | 151.3 |     |
| 3          | 107.0 | 6.57 (d, 2.9) | 4  | 157.0 |     |
| 5          | 109.4 | 6.47 (dd, 8.2, 2.9) | 6  | 123.2 | 6.90 (d, 8.2) |
| Middle Rings |     |     |            |     |     |
| 1          | 139.7, 139.5, 139.5, 139.5 |     | 2  | 151.2, 151.2, 151.2, 151.2 | 139.8, 139.5, 139.5, 139.5 |
| 3          | 106.5, 106.3, 106.3, 106.3 | 6.53, 6.51, 6.50, 6.49, (all d, 2.9) | 4  | 157.8, 157.5, 157.5, 157.5 | 157.8, 157.5, 157.5, 157.5 |
| 5          | 108.8, 108.7, 108.7, 108.7 | 6.42, 6.41, 6.40, 6.39 (all dd, 8.3, 2.9) | 6  | 123.1 (2x), 123.1, 122.9 | 6.88, 6.87, 6.86, 6.84 (all d, 8.8) |
| Ring Y     |     |     |            |     |     |
| 1          | 141.0 |     | 2          | 150.6 |     |
| 3          | 106.3, 106.3 | 6.48 (d, 2.9) | 4  | 156.7 |     |
| 5          | 108.6 | 6.34 (dd, 8.8, 2.9) | 6  | 121.7 | 6.74 (d, 8.8) |
| Ring Z     |     |     |            |     |     |
| 1          | 141.7 |     | 2          | 147.0 |     |
| 3          | 106.6 | 6.42 (d, 2.9) | 4  | 152.9 |     |
| 5          | 109.1 | 6.27 (dd, 8.6, 2.9) | 6  | 116.4 | 6.67 (d, 8.6) |
| 6          | 121.7 | 6.74 (d, 8.8) | 7          | 141.0 |     |

“Rings Y and Z are the last two rings of the oligomer. 13C signal can be interchanged with one of the corresponding signals of rings D–G.”
suggesting that 8 was also an oligomeric phenolic with a terminal biphenyl. A limited spread of the signals for the repeating phenol units, as illustrated by the $^1$H spectral comparison of H-6 (Figure 6), suggested an end-capping of the oligomer with a different moiety. A carbonyl, indicated by a peak on the $^{13}$C NMR spectrum at $\delta_C$ 163.4, and 12 other distinct $^{13}$C NMR signals, agreed with a benzo[c]chromen-6-one unit (Table 4). The substitution pattern was determined as 2,4,8,9-tetrahydroxy from the presence of two distinct singlets at $\delta_H$ 7.63 and 7.32 and two doublets at $\delta_H$ 6.96 and 6.55 (both $J = 2.7$ Hz) in the $^1$H NMR spectrum (Table 5). This assignment was corroborated by HMBC correlations from H-1 and H-3 to $\delta_C$ 136.2 (C-4a) and from H-1 to $\delta_C$ 130.7 (C-10a) (Figure S2). This benzo[c]chromen-6-one group matched revised structures 13 and 14; however, the exact point of attachment of the oligomer needed to be established to determine which configuration was more probable for 8. A comparison of $^{13}$C NMR values of 8 recorded in DMSO-$d_6$ and those reported for 13 and 14 (Table S1) showed the greatest differences at C-2, C-4, and C-6, suggesting the oligomer was attached to C-2 as in 14 instead of C-4. When the calculated values for 13 and 14 were considered, a 94% DP+ value favoring 14 confirmed this hypothesis. For this reason, 8 was identified as $\alpha$-[(4,8,9-trihydroxy-2-benzo[c]chromen-6-on-2-yl)oxy]-$\omega$-[3,4-dihydroxyphenyl]octa[ oxy(2-hydroxyphen-4-yl)] and named cladophorol E.

Characterization of 9–12. Compounds 9–12 each exhibited $^1$H and $^{13}$C NMR spectra similar to that of 8 (Tables 4 and 5). Proton integrations in the three overlapping regions ($\delta_H$ 6.85–6.98, 6.49–6.56, and 6.39–6.45) indicated three fewer protons for 9 and 3, 6, and 9 additional protons for 10–12. This suggested 9–12 were members of a series with 8, which was further supported by high-resolution mass spectrometry with [M – H]$^-$ ions at $m/z$ 1107.1973, 1323.2394, 1431.2617, and 1539.2797 for 9–12, respectively. Since 10 was more abundant than the other members of the series, its NMR spectra in DMSO-$d_6$ doped with picric acid were recorded (Figure S70). Three labile protons at $\delta_H$ 10.52, 10.16, and 10.11 correlated with $\delta_C$ 153.1 (C-9), 147.4 (C-8), 146.1 (C-4), 134.4 (C-4a), 114.5 (C-7), 107.9 (C-10), and 103.7 (C-3), indicating a substituted oxygen attached to C-2 as determined previously from the comparison of chemical shifts of 8 with experimental and calculated values of 13 and 14.
Table 4. $^{13}$C (200 MHz) NMR Spectroscopic Data of Cladophorols E–I (8–12) in CD$_3$OD

| position | 8 | 9 | 10 | 11 | 12 |
|----------|---|---|----|----|----|
| Rings A–B (Biphenyl) | | | | | |
| 1$^a$ | 139.8, 139.6, 139.5, 139.4, 139.2 | 139.8, 139.6, 139.5, 139.4, 139.2 | 139.8, 139.6, 139.5, 139.4, 139.2 | 139.8, 139.6, 139.4, 139.2 | 139.8, 139.6, 139.4, 139.2 |
| 2$^a$ | 139.9 | 139.9 | 139.9 | 139.9 | 139.9 |
| 3$^a$ | 139.9 | 139.9 | 139.9 | 139.9 | 139.9 |
| 4$^a$ | 139.9 | 139.9 | 139.9 | 139.9 | 139.9 |
| 5$^a$ | 139.9 | 139.9 | 139.9 | 139.9 | 139.9 |
| 6$^a$ | 139.9 | 139.9 | 139.9 | 139.9 | 139.9 |
| 7$^a$ | 139.9 | 139.9 | 139.9 | 139.9 | 139.9 |
| 8 | 139.9 | 139.9 | 139.9 | 139.9 | 139.9 |
| 9 | 139.9 | 139.9 | 139.9 | 139.9 | 139.9 |
| 10 | 139.9 | 139.9 | 139.9 | 139.9 | 139.9 |
| Middle Rings | | | | | |
| 1 | 139.8, 139.6, 139.5, 139.4, 139.2 | 139.8, 139.6, 139.5, 139.4, 139.2 | 139.8, 139.6, 139.5, 139.4, 139.2 | 139.8, 139.6, 139.4, 139.2 | 139.8, 139.6, 139.4, 139.2 |
| 2 | 139.9 | 139.9 | 139.9 | 139.9 | 139.9 |
| 3 | 139.9 | 139.9 | 139.9 | 139.9 | 139.9 |
| 4 | 139.9 | 139.9 | 139.9 | 139.9 | 139.9 |
| 5 | 139.9 | 139.9 | 139.9 | 139.9 | 139.9 |
| 6 | 139.9 | 139.9 | 139.9 | 139.9 | 139.9 |
| 7 | 139.9 | 139.9 | 139.9 | 139.9 | 139.9 |
| 8 | 139.9 | 139.9 | 139.9 | 139.9 | 139.9 |
| 9 | 139.9 | 139.9 | 139.9 | 139.9 | 139.9 |
| 10 | 139.9 | 139.9 | 139.9 | 139.9 | 139.9 |
| Benzo[c]chromone | | | | | |
| 1 | 100.4 | 100.4 | 100.4 | 100.4 | 100.4 |
| 2 | 156.9 | 156.9 | 156.9 | 156.9 | 156.9 |
| 3 | 105.8 | 105.8 | 105.8 | 105.8 | 105.8 |
| 4 | 147.5 | 147.5 | 147.5 | 147.5 | 147.5 |
| 4a | 136.2 | 136.2 | 136.2 | 136.2 | 136.2 |
| 5 | 162.7 | 162.7 | 162.7 | 162.7 | 162.7 |
| 6 | 114.5 | 114.5 | 114.5 | 114.5 | 114.5 |
| 7 | 115.6 | 115.6 | 115.6 | 115.6 | 115.6 |
| 8 | 148.6 | 148.6 | 148.6 | 148.6 | 148.6 |
| 9 | 154.6 | 154.6 | 154.6 | 154.6 | 154.6 |
| 10 | 108.6 | 108.6 | 108.6 | 108.6 | 108.6 |
| 10a | 108.6 | 108.6 | 108.6 | 108.6 | 108.6 |
| 10b | 120.7 | 120.7 | 120.7 | 120.7 | 120.7 |

(Figure S2). Altogether, these data supported the assignment of these natural products as α-[4,8,9-trihydroxy-2-benzo[c]-chromen-6-ono-2-yloxy]-ω-[3,4-dihydroxyphenyl]hepta[oxy-(2-hydroxyphen-4-yl)] (9), α-[4,8,9-trihydroxy-2-benzo[c]-chromen-6-ono-2-yloxy]-ω-[3,4-dihydroxyphenyl]nons[oxy-(2-hydroxyphen-4-yl)] (10), α-[4,8,9-trihydroxy-2-benzo[c]-chromen-6-ono-2-yloxy]-ω-[3,4-dihydroxyphenyl]deca[xy-(2-hydroxyphen-4-yl)] (11), and α-[4,8,9-trihydroxy-2-benzo[c]-chromen-6-ono-2-yloxy]-ω-[3,4-dihydroxyphenyl]undeca[xy-(2-hydroxyphen-4-yl)] (12) and named cladophorols F–I.

Pharmacological Assays. The antimicrobial activities of all isolated natural products (3–12) were assessed against multiple drug-resistant (MDR) Gram-positive and Gram-negative human pathogens as well as amphotericin-resistant Candida albicans (Table 6, Figure S3). Cladophorol C (6) was found to be very potent only against MRSA with a minimal inhibitory concentration (MIC) value of 1.4 μg/mL. The bioactivity is negatively correlated by the presence of a benzo[c]chromen-6-one unit, as shown by the weaker MIC values for 5 and 8–12 (13–49 μg/mL) relative to 6 and 7 (1.4–9 μg/mL). The total number of phenol rings is also an important factor since the most potent compounds in both series (6–7 vs 8–12) have 9–10 rings and the activity weakens as this number increases. Surprisingly, all of the other cell lines, including the other Gram-positive bacterium, VREF, were much less sensitive to this family of natural products. The bioactivities of 4–12 were also evaluated in an assay using intraerythrocytic Plasmodium falciparum. Only 4 and 5 inhibited the parasite with half-maximal effective concentration (EC$_{50}$) values of 0.7 and 1.9 μg/mL, respectively. In addition, the cytotoxicities of 3–12 were evaluated using immortalized human keratinocytes (HaCaT) and human kidney cells (HEK293T). Although compounds 5–11 exhibited weak toxicity (<25%) at their respective maximum concentrations against HaCaT (Figure 7) and 4 and 5 had weak toxicity toward HEK293T (half maximal cytotoxicity concentration (CC$_{50}$) of 9.4 and 4.5 μg/mL, respectively), no toxicity was observed at the MRSA MICs for 5–11 or the P. falciparum EC$_{50}$ values for 4 and 5.
EXPERIMENTAL DETAILS

General Experimental Procedures. NMR spectra (1H, HMBC, HSQC, DQF-COSY, and J-resolved) were recorded on an 18.8 T (800 MHz for 1H and 200 MHz for 13C) Bruker Avance III HD 800 instrument equipped with a 5 mm triple resonance broadband cryoprobe. All spectra were acquired in CD3OD or DMSO-d6, and chemical shifts were reported in ppm (δ) relative to the residual solvent peaks (δH 3.31 and δC 49.00 for CD3OD, δH 2.50 and δC 39.52 for DMSO-d6). All spectra were processed using MestReNova 11.0. HPLC separations were performed with a Waters 1525 binary pump and a Waters 2487 dual wavelength absorbance detector set at 260 nm, using two different columns: a 9.4 × 250 mm, C18 silica reversed-phase (Zorbax stable-bond, 5 μm particle size), and a 4.6 × 250 mm phenylhexyl phase (Phenomenex Luna, 5 μm particle size). High-resolution mass spectrometry was conducted on an Orbitrap spectrometer in negative ion mode. The masses of isolated compounds were estimated by qNMR using a capillary filled with benzene-d6 and calibrated against caffeine.23

Specimen Collection. Green alga (collection G-1240) was harvested as floating clumps on Dec 3, 2015, at the ocean surface near Titi Island, Viti Levu, Fiji (16°16′26" S, 179°26′02" E). This collection was identified as Cladophora socialis Kützing by morphological and 18S rRNA phylogenetic analyses. Voucher specimens were preserved in aqueous formaldehyde and stored at the University of South Pacific. The collection was stored at −80 °C until extraction.

Species Identification. The collected green alga (G-1240) was identified by comparing its morphological traits with that of previously described Cladophora species and by sequence analysis of nuclear, small subunit (SSU) rRNA (18S rRNA). Genomic DNA from an ethanol-preserved algal specimen was extracted using the innuPREP plant DNA kit (Analytik Jena, Germany) according to the manufacturer’s protocol. The three overlapping 18S rRNA gene fragments from genomic DNA were amplified via the polymerase chain reaction (PCR) in three separate reactions using universal primer pairs, NS3/NS4, NS5/NS6, and NS7/NS8.25 Each PCR amplification was performed in a 25 µL reaction volume consisting of

Table 5. 1H (800 MHz) NMR Spectroscopic Data of Cladophorols E–I (8–12) in CD3OD

| position | 8  | 9  | 10 | 11 | 12 |
|----------|----|----|----|----|----|
| Rings A–B (Biphenyl) |    |    |    |    |    |
| 2a       | 7.01 (d, 2.2) | 7.02 (d, 2.2) | 7.02 (d, 2.2) | 7.01 (d, 2.2) | 7.01 (d, 2.2) |
| 5b       | 6.81 (d, 8.2) | 6.81 (d, 8.2) | 6.81 (d, 8.2) | 6.81 (d, 8.2) | 6.81 (d, 8.2) |
| 6b       | 6.91 (dd, 8.2, 2.4) | 6.91 (dd, 8.2, 2.2) | 6.91 (dd, 8.2, 2.2) | 6.91 (dd, 8.2, 2.2) | 6.91 (dd, 8.2, 2.2) |
| 7b       | 7.11 (d, 2.2) | 7.11 (d, 2.2) | 7.11 (d, 2.2) | 7.11 (d, 2.2) | 7.11 (d, 2.2) |
| 9b       | 6.94 (d, 8.3) | 6.94 (d, 8.3) | 6.94 (d, 8.3) | 6.94 (d, 8.3) | 6.94 (d, 8.3) |
| 6b       | 7.00 (dd, 8.4, 2.2) | 7.00 (dd, 8.3, 2.2) | 7.00 (dd, 8.3, 2.2) | 7.00 (dd, 8.3, 2.2) | 7.00 (dd, 8.3, 2.2) |
| Middle Rings |    |    |    |    |    |
| 3        | 6.58, 6.54, 6.53, 6.52, 6.51, 6.51, 6.51 (all d, 2.9) | 6.58, 6.54, 6.53, 6.52, 6.51, 6.51, 6.51 (all d, 2.9) | 6.58, 6.54, 6.53, 6.52, 6.51, 6.51, 6.51 (all d, 2.9) | 6.58, 6.54, 6.53, 6.52, 6.51, 6.51, 6.51, 6.51 (all d, 2.9) |
| 5        | 6.47, 6.44, 6.42, 6.42, 6.42, 6.40, 6.40 (all dd, 8.8, 2.9) | 6.47, 6.44, 6.42, 6.42, 6.41, 6.40, 6.40, 6.40 (all dd, 8.8, 2.9) | 6.47, 6.44, 6.42, 6.42, 6.41, 6.40, 6.40, 6.40 (all dd, 8.8, 2.9) | 6.47, 6.44, 6.42, 6.42, 6.40, 6.40, 6.40, 6.40 (all dd, 8.8, 2.9) |
| 6        | 6.92, 6.91, 6.90, 6.88, 6.87, 6.87 (2× all d, 8.8) | 6.92, 6.91, 6.90, 6.88, 6.87, 6.87 (2× all d, 8.8) | 6.92, 6.91, 6.90, 6.88, 6.87, 6.87 (2× all d, 8.8) | 6.92, 6.91, 6.90, 6.88, 6.87, 6.87 (2× all d, 8.8) |
| Benzo(c)chromone |    |    |    |    |    |
| 1        | 6.96 (d, 2.7) | 6.96 (d, 2.7) | 6.96 (d, 2.7) | 6.96 (d, 2.7) | 6.96 (d, 2.7) |
| 3        | 6.55 (d, 2.7) | 6.55 (d, 2.7) | 6.55 (d, 2.7) | 6.55 (d, 2.7) | 6.55 (d, 2.7) |
| 7        | 7.63 (s) | 7.63 (s) | 7.64 (s) | 7.63 (s) | 7.63 (s) |
| 10       | 7.32 (s) | 7.32 (s) | 7.33 (s) | 7.32 (s) | 7.33 (s) |

Table 6. Pharmacological Activitiesa of 3,4,3′,4′-Tetrahydroxy-1,1′-biphenyl (3) and Cladophorols A–I (4–12)

| compd | no. of ringsb | MRSA | VREF | Gram− | WTCA | ARCA | P. falciparumd |
|-------|--------------|------|------|-------|------|------|---------------|
| 3     | 2            | >50  | >50  | >50   | >50  | >50  | NT            |
| 4     | 2            | >50  | >50  | >50   | >50  | >50  | 0.7 ± 0.1     |
| 5     | 4            | 13 ± 2 | >50 | >50   | >50  | >50  | 1.9 ± 0.2     |
| 6     | 9            | 1.4 ± 0.3 | >50 | >50   | >50  | >50  | >2            |
| 7     | 10           | 9 ± 2 | >50  | >50   | >50  | >50  | >2            |
| 8     | 11           | 25 ± 3 | >50 | >50   | >50  | >50  | >2            |
| 9     | 10           | 14 ± 3 | >50 | >50   | >50  | >50  | >2            |
| 10    | 12           | 31 ± 3 | >50 | >50   | >50  | >50  | >2            |
| 11    | 13           | 49 ± 8 | >50 | >50   | >50  | >50  | >2            |
| 12    | 14           | >50  | >50  | >50   | >50  | >50  | >2            |

The values are expressed as a 95% confidence interval. bTotal number of phenyl rings. cMinimal inhibitory concentration (μg/mL). Cell lines are MRSA = methicillin-resistant Staphylococcus aureus; VREF = vancomycin-resistant Enterococcus faecium; Gram−: [Escherichia coli; multiple drug-resistant (MDR) E. coli; MDR Klebsiella pneumonia; MDR K. pneumonia; MDR Acinetobacter baumannii; A. baumannii; MDR Enterobacter aerogenes; MDR Enterobacter cloacae; wild type Pseudomonas aeruginosa]; WTCA = wild type Candida albicans; ARCA = amphotericin B-resistant C. albicans. Details for the positive controls are provided in the Experimental Section. dHalf-maximal effective concentration (μg/mL). eTested only against E. coli.
5–50 ng of purified genomic DNA; 200 μM of each of the dNTPs; 1 μM of each of the oligonucleotide primer; 1.0 U Taq DNA polymerase, and 1× standard PCR reaction buffer (NEB, Ipswich, MA). All PCR amplifications were performed in a GeneAmp PCR system 2700 (Applied Biosystems, Foster City, CA) thermocycler using the following temperature cycling parameters: initial denaturation at 94 °C for 5 min followed by a total of 40 cycles of amplification in which each cycle consisted of denaturation at 94 °C for 40 s, primer annealing at 50 °C for 40 s and primer extension at 72 °C for 1 min. After amplification, final extension of the incompletely synthesized DNA was carried out at 72 °C for 7 min. The PCR fragments were analyzed by agarose gel electrophoresis (1% wt/vol).

Further, the 18S rRNA sequence of G-1240 was compared with that of other known species of the genus Cladophora (Table 5; 13C NMR data, see Table 4; HRMS (ESI) data, see Table 3). The assembled G-1240 18S rRNA sequence (1334 bp) was submitted to GenBank (accession no. MK127549). The sequence similarity of the assembled contig of G-1240 18S rRNA to other known Cladophora spp. was determined by comparing it with the nonredundant nucleotide database (NCBI) using the blastn program. The top ranked matches according to E values and maximum scores revealed high similarity to C. socialis with 96% identity.

Figure 7. Cytotoxicity of 3–12 against human keratinocytes (HaCaT). The parenthetical values provide the relative cytotoxicities (%) at the maximum concentration tested (60 μg/mL for 9, 50 μg/mL for 5–8, 10, 11, 45 μg/mL for 12, and 20 μg/mL for 6 and 7).

Isolation of 3–12. Frozen material (659 g) was thawed and broken into small pieces (~1 cm) before being agitated in 60% aqueous MeOH (1:3 L) for 3 days. The mixture was filtered with a metal wire mesh, and the residues were extracted four more times with pure MeOH (2 × 1 L) and CHCl3/MeOH 1:1 (2 × 1 L). The solutions were pooled, filtered, and evaporated under reduced pressure to yield a brown gummy extract (33.4 g). The residue was dissolved in MeOH/H2O 9:1 (500 mL) and partitioned with hexanes (4 × 250 mL, 1.56 g). The amount of water in the MeOH/H2O phase was increased to a 3:2 ratio, and the resulting solution was partitioned with CHCl3 (3 × 500 mL, 0.75 g). The methanol was removed from the MeOH/H2O phase by vacuum evaporation, and the aqueous phase was extracted with EtOAc (3 × 250 mL, 2.05 g). The EtOAc fraction was subjected to silica gel column chromatography eluting with a gradient of CH3Cl/MeOH from 49:1 to 1:2 to yield nine fractions (3.1–3.9). Fraction 3.5 (97.4 mg) was separated by reverse-phase semipreparative HPLC eluting with H2O + 0.05% TFA and MeCN (25 to 75% gradient, in 25 min). In addition to pure 3 (tR = 3.8 min, 0.50 mg), 4 (tR = 4.9 min, 0.68 mg), and 5 (tR = 8.7 min, 3.4 mg), eight other fractions were collected (3.5.1–3.5.8).

Fraction 3.5.1 was subjected to HPLC using a phenylhexyl column and eluting with H2O + 0.05% TFA and MeOH (75 to 85% gradient, in 15 min) to yield 6 (tR = 7.9 min, 0.057 mg) and 11 (tR = 12.8 min, 0.62 mg). Fraction 3.5.2 was separated by HPLC with a phenylhexyl column eluting with H2O + 0.05% TFA and MeOH (75 to 85% gradient, in 15 min) to obtain 9 (tR = 9.5 min, 0.10 mg) and 12 (tR = 12.8 min, 0.027 mg). The same conditions were used to purify fraction 3.5.3 yielding 7 (tR = 9.0 min, 0.32 mg). Fraction 3.5.4 was purified by HPLC using a phenylhexyl column and eluting with H2O + 0.05% TFA and MeOH (20 to 82.5% gradient, in 7.5 min) to obtain 8 (tR = 6.5 min, 0.60 mg). Fraction 3.5.6 was subjected to HPLC using a phenylhexyl column and eluting with H2O + 0.05% TFA and MeOH (20 to 82.5% gradient, in 7.5 min) to yield 10 (tR = 6.4 min, 2.3 mg).
inhibition of growth (MIC90).

Additional antibacterial assays were performed using MDR Klebsiella pneumonia (MDRKP1, CDC0087), MDR K. pneumonia (MDRPK2, CDC0003), MDR Acinetobacter baumannii (MDRAB1, CDC0280), MDR A. baumannii (MDRAB2, CDC0033), MDR Enterobacter aerogenes (MDREA, CDC0018), MDR Enterobacter cloacae (MDRECL, CDC0050), and wild type Pseudomonas aeruginosa (WTPA, PAO1). The bacterial cultures were grown in tryptic soy broth (TSB). Minimum inhibitory concentrations were evaluated with cation-adjusted Mueller Hinton broth (CAMHB) following Clinical and Laboratory Standards Institute (CLSI) methods. Overnight cultures were standardized by optical density (OD) to 5 × 10^5 CFU/mL, and this was confirmed by plate counts of colonies. MIC values was assigned as described; this was determined by reading at an OD600 nm in a Cytation 3 multimode plate reader after 18–22 h incubation. Gentamicin was used as a positive control.

The human keratinocyte cell line (HaCaT) was maintained, and cytotoxicity of isolated compounds were assessed using LDH cytotoxicity assay as previously described. Antimalarial activity was assessed using a standard parasite proliferation assay with asexual blood-stage P. falciparum and SYBR Green detection. Screening media (complete media supplemented with 0.05% Albumax II but without human serum) and fresh O’ erythrocytes (TSRI Normal Blood Donation) were used for cultures of P. falciparum strain Dd2L (gift from Dr. David Fidock, Columbia University). A Labcyte ECHO acoustic liquid handler transferred compounds into assay plates. The plates were inoculated (Multi-Flo; BioTek, VT) with parasitized erythrocytes and fresh erythrocytes prepared in screening media, resulting in a final parasitemia of 0.3% and hemocrit of 2.5%. A chamber with a low oxygen gas mixture at 37 °C was used to culture the assay plates for 72 h with daily gas exchanges. SYBR Green lysis buffer was added to the wells after incubation using a Multi-Flo liquid dispenser, and plates were incubated at room temperature for an additional 24 h to achieve optimal development of the fluorescence signal which was read by an Envision Multimode Reader (PerkinElmer, MA). The positive controls used were atovapone (EC_{50} = 0.0006 μM) and artesiminin (EC_{50} = 0.03 μM).

**ASSOCIATED CONTENT**

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.joc.8b03218.

Phylogenetic relationship of G-1240 with other species of the genus Cladophora; key HMBC correlations for compounds 5, 6, 8, and 10; dose–response curves of 3–12 μM for multiple cell lines; spectroscopic data (NMR, MS, UV) for compounds 3–12; DFT energies and geometries for compounds 1, 13, 14, 15a, and 15b; NMR spectroscopic data comparison between 6 and 15a/15b (PDF) NMR FIDs for all isolated compounds (ZIP)

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**Notes**

The authors declare no competing financial interest.

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**DEDICATION**

This paper is dedicated to William Aalbersberg, in memoriam, for his active mentoring of junior scientists, for his engaged international collaborations, and for his advocacy on behalf of Fijian science and society.

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