# Kalkipyrone, a Toxic γ-Pyrone from an Assemblage of the Marine Cyanobacteria *Lyngbya majuscula* and *Tolypothrix* sp.

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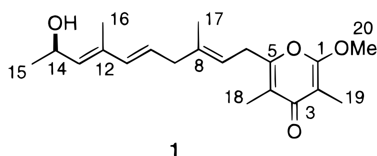
Kalkipyrone, a novel α-methoxy-β,β′-dimethyl-γ-pyrone possessing an alkyl side chain, was isolated from an assemblage of the marine cyanobacteria *Lyngbya majuscula* and *Tolypothrix sp.* Its structure, including stereochemistry, was determined by NMR, UV, and IR analysis and by GC−MS of the natural product and key derivatives. Kalkipyrone is toxic to brine shrimp ($LD_{50}$ 1 μg/mL) and gold fish ($LD_{50}$ 2 μg/mL) and is structurally related to the actinopyrones that were previously isolated from *Streptomyces* spp.

Cyanobacteria are an important source of structurally new and biologically active natural products for drug discovery efforts.[1,2] In our continuing efforts to describe the unique organic chemistry of marine cyanobacteria, we were prompted to undertake a detailed study of the extract of an assemblage of *Lyngbya majuscula* and *Tolypothrix sp.* from Curaçao on the basis of an unusual thin layer chromatogram and brine shrimp toxicity (80% toxicity at 100 ppm). Repeated chromatography of this extract yielded a novel γ-pyrone derivative, kalkipyrone (**1**), as the principle bioactive component. Kalkipyrone is the first γ-pyrone-containing natural product isolated from a cyanobacterium, and it displays potent brine shrimp ($LD_{50}$ 1 μg/mL) and gold fish ($LD_{50}$ 2 μg/mL) toxicity.




Kalkipyrone (**1**), a colorless and somewhat unstable oil, was shown by HREIMS to have a $C_{20}H_{28}O_4$ (*m/z* 332.1993) molecular formula, indicating 7 degrees of unsaturation. $^{13}C$ NMR analysis in $C_6D_6$ (but not $CDCl_3$) revealed all 20 carbon resonances (Table 1), including one carbonyl and 10 olefinic carbons, accounting for 6 of these 7 degrees of unsaturation. Hence, kalkipyrone possessed a single ring. By IR and UV spectroscopy, kalkipyrone possessed hydroxyl (υ 3374, br) and dienone (υ 1665; $\lambda_{max}$ = 238 nm, ε = 33 000) absorption bands.

Three key partial structures (**a**, **b**, and **c**; Figure 1) of kalkipyrone were deduced from $^1H-^1H$ COSY analysis. The C13−C15 portion (partial structure **a**) was defined by observing coupling between an oxygenated methine (H14) and both a methyl group ($H_315$) and a vinyl proton (H13). The C14 substituent was shown to be a hydroxyl group by IR and by reaction chemistry associated with determination of the C14 stereochemistry. An aliquot of kalkipyrone was converted to the

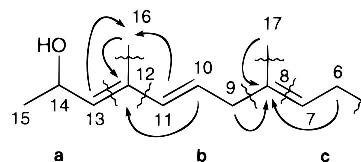



**Figure 1.** Partial structures **a**−**c** of kalkipyrone (**1**) (arrows indicate HMBC correlations).

corresponding (−)-menthoxycarbonyl (MC) derivative, oxidatively ozonolyzed to release the C13−C15 fragment, and then methylated to the corresponding methyl ester.[3] This derivatized lactate fragment was analyzed by GC−MS under optimized conditions and in comparison with standards and showed that it was partially racemic (84% *R*, 16% *S*).[4]

Subunit **b** possessed a trans double bond (H10−H11, *J* = 15.6 Hz) adjacent to an isolated methylene group ($H_29$). Partial structure **c** was composed of a vinyl proton (H7) next to another isolated methylene group ($H_26$). The $^1H$ NMR of kalkipyrone also showed five singlet methyl groups; four vinylic (δ 1.47, 1.60, 1.98, 2.10) and one as part of a methoxy group (δ 3.20).

Long-range heteronuclear $^{13}C-^1H$ coupling (HMBC) together with NOESY information readily allowed connection of the spin systems and several of the methyl groups of kalkipyrone (Figure 1, Table 1). Coupling from two vinyl protons, H13 (δ 5.44) and H11 (δ 6.05), and the C16 vinyl methyl group carbon (δ 13.1), connected partial fragments **a** and **b**. This connection was confirmed by observing the NOE between the $H_316$ methyl and the H10 vinyl proton. Finally, identification of C12 at δ 133.8 was through HMBC correlations between C12 and H16 (δ 1.60) and H10 (δ 5.53).

A similar connection between fragments **b** and **c** was made by observing HMBC correlations between C8 (δ 137.9) and both sets of methylene protons, $H_26$ (δ 2.85) and $H_29$ (δ 2.66). The C8 quaternary olefinic carbon was also shown to possess a methyl group substituent by observing HMBC correlations between it and a vinyl methyl group at δ 1.47 ($H_317$). The configurations of the two trisubstituted olefinic bonds were both determined to be *E* by the distinctively highfield chemical shifts of the C16 (δ 13.1) and C17 (δ 16.7) methyl groups.[5−7] The geometry of the C7−C8 olefin and

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**Table 1.** $^{1}$H, $^{13}$C, HMBC, and NOESY Data of Kalkipyrone (**1**)

| C no. | $^{13}$C CDCl$_3$$^{a,b}$ ($\delta$) | $^{1}$H CDCl$_3$ ($\delta$ mult, $J$ in Hz) | $^{13}$C C$_6$D$_6$$^{c}$ ($\delta$) | $^{1}$H C$_6$D$_6$ ($\delta$) | NOESY$^{d}$ correlations | HMBC correlations$^{a}$ H → C |
|---|---|---|---|---|---|---|
| 1 | 162.2 | | 162.3 | | | |
| 2 | 99.4 | | 100.1 | | | |
| 3 | 181.1 | | 180.7 | | | |
| 4 | 118.1 | | 118.1 | | | |
| 5 | 157.0 | | 156.8 | | | |
| 6 | 30.0 | 3.30 d 7.2 | 30.3 | 2.85 | 7, 17, 18 | 5, 7, 8 |
| 7 | 118.1 | 5.25 bt 7.2 | 119.2 | 5.12 | 6 | 6, 9, 17 |
| 8 | 137.8 | | 137.9 | | | |
| 9 | 42.8 | 2.80 d 7.2 | 43.5 | 2.66 | 10, 11 | 7, 8, 10 |
| 10 | 127.0 | 5.61 dd 7.2, 15.6 | 126.8 | 5.53 | 9, 11, 16 | 8, 9, 11, 12 |
| 11 | 136.0 | 6.04 d 15.6 | 137.2 | 6.05 | 9, 10 | 9, 12, 13, 16 |
| 12 | 134.3 | | 133.8 | | | |
| 13 | 134.3 | 5.40 d 8.4 | 136.4 | 5.44 | 14 | 11, 15, 16 |
| 14 | 64.6 | 4.69 dt 6.4, 8.4 | 64.8 | 4.47 | 13, 15 | 12, 13, 15 |
| 15 | 23.5 | 1.27 d 6.4 | 24.2 | 1.15 | 14 | 13, 14 |
| 16 | 12.7 | 1.78 s | 13.1 | 1.60 | 10 | 12, 13 |
| 17 | 16.5 | 1.72 s | 16.7 | 1.47 | 6 | 7, 8, 9 |
| 18 | 9.8 | 1.95 s | 10.5 | 1.98 | 6 | 3, 4, 5 |
| 19 | 6.8 | 1.83 s | 7.7 | 2.10 | | 1, 2, 3 |
| 20 | 55.2 | 3.91 s | 55.0 | 3.20 | | 1 |

$^{a}$ Spectra in CDCl$_3$ recorded on a Bruker AM-400 spectrometer ($^{1}$H spectra referenced to TMS at 0.0 ppm; $^{13}$C spectra referenced to the centerline of CDCl$_3$ at 77.0 ppm). $^{b}$ Assignments in accord with a DEPT 135 experiment. $^{c}$ Spectra in C$_6$D$_6$ recorded on a Bruker DRX-600 spectrometer ($^{1}$H spectra referenced to C$_6$D$_6$ at 7.16 ppm; $^{13}$C spectra referenced to the centerline of C$_6$D$_6$ at 128.39 ppm). $^{d}$ NOESY recorded in CDCl$_3$ on a Bruker AC-300 spectrometer.

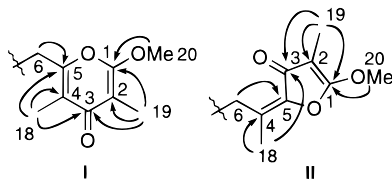


**Figure 2.** Possible ring substructures (**I** and **II**) of kalkipyrone (arrows indicate key HMBC correlations).

connection of substructures was confirmed by observing NOE between H$_2$6 and H17.

Comparison of the molecular formula of kalkipyrone with that for the assembled fragments **a**, **b**, and **c** indicated that a group composed of $C_8H_9O_3$ was attached to fragment **c**. As these nine protons were present as three sharp methyl singlets, $^{1}$H NMR-based structural information was quite limited. By HMBC, a conjugated carbonyl (C3, $\delta$ 180.7) was correlated to a vinyl methyl group (H$_3$19, $\delta$ 2.10); in turn, this vinyl methyl group showed connections to a polarized olefin (C1, $\delta$ 162.3 and C2, $\delta$ 100.1). The deshielded olefinic carbon (C1) also showed an HMBC correlation to a methoxy group methyl (H$_3$20, $\delta$ 3.20). A second vinyl methyl group (H$_3$-18, $\delta$ 1.98) was also correlated to the C3 conjugated carbonyl as well as to a second polarized olefin (C4, $\delta$ 118.1 and C5, $\delta$ 156.8). Connection of this latter system to fragment **c** was possible through an HMBC correlation from the H$_2$6 methylene to this deshielded olefinic carbon atom (C5). Two different ring substructures were conceivable from these data. A $\gamma$-pyrone ring (**I**) was indicated if the C3-to-H$_3$18 correlation represented a three-bond coupling, whereas a five-membered ring with an exocyclic double bond (**II**, Figure 2) was likely if this same correlation were due to a four-bond coupling.

The above five-membered ring substructure (**II**) is closely related to that found in the marine sponge metabolites variabilin[8] and the ircinins[9] from *Ircinia* spp. and the fungal metabolites carolic acid and terrestic acid.[10] Alternatively, the $\gamma$-pyrone (**I**) ring is found in the metabolites of several marine mollusks: the tridachiapyrones from *Tridachia*,[11] the peroniatriols from *Peronia*,[12] and the cyercenes from *Cyerce*.[13] Additionally, several *Streptomyces* spp. produce bioactive metabolites with the $\gamma$-pyrone functionality, including spectinabilin,[14] aureothin,[15] the actinopyrones,[16,17] and an actinopyrone analogue.[18] Data comparisons with these literature examples clearly defined kalkipyrone as the α-methoxy-$\beta$,$\beta'$-dimethyl-$\gamma$-pyrone (**I**). Particularly convincing was comparison of IR and $^{1}$H and $^{13}$C NMR data for the ring portion of the actinopyrones, structures ultimately linked to an X-ray crystal structure.[17,19] On the basis of HMBC data (assuming no four-bond correlations) and these favorable data comparisons with the actinopyrones, the structure of kalkipyrone (**1**) is assembled by joining together the alkyl chain (partial structures **a**, **b**, and **c**) and an α-methoxy-$\beta$,$\beta'$-dimethyl-$\gamma$-pyrone (**I**) moiety.

Attempts to determine whether *L. majuscula* or *Tolypothrix sp.* produce kalkipyrone were inconclusive. Two laboratory cultures of *L. majuscula* isolated from the original mixture were extracted and analyzed by GC−EIMS using conditions developed with authentic kalkipyrone (see Experimental Section). Kalkipyrone was not detected in either sample.

Kalkipyrone possessed potent brine shrimp toxicity (LD$_{50}$ 1 $\mu$g/mL) and ichthyotoxicity to goldfish (LD$_{50}$ 2 $\mu$g/mL). We hypothesize that this metabolite may function in nature to protect the alga from predation by crustacea and herbivorous fish. Kalkipyrone was also evaluated in the NCI's 60 human-tumor cell line in vitro assay, but was found only modestly inhibitory to several renal and melanoma cell lines.

The structural similarity of kalkipyrone to the actinopyrones from *Streptomyces* is striking. Both *Tolypothrix sp.* and *L. majuscula* have previously been reported to produce compounds identical to *Streptomyces* metabolites, such as tubercidin, toyocamycin,[20,21] and lyngbyatoxin A (teleocidin A-1).[22] The occurrence of metabolites produced in common raises interesting

questions regarding the relationship between these cyanobacteria and *Streptomyces*.

## Experimental Section

**General Experimental Procedures.** UV spectra were obtained using a Hewlett−Packard 8452A diode array spectrophotometer. IR spectra were run on a Nicolet 510 Fourier transform IR (FTIR) spectrophotometer. LRMS were obtained on a Varian MAT CH7 spectrometer; GC−MS on a Hewlett−Packard 5890 Series II GC connected to a Hewlett−Packard 5971 mass spectrometer; and HREIMS, on a Kratos MS 50 TC. For details of NMR instrumentation and solvents, see Table 1. Merck aluminum-backed TLC sheets (Si gel 60 $F_{254}$) were utilized for TLC analysis. Reversed-phase C-18 Si (Analytichem Bond Elut) was utilized for column chromatography, and TLC-grade (10−40 $\mu$m) Si gel was used for vacuum chromatography. All solvents were glass distilled prior to use.

**Collection.** Of an assemblage of *Tolypothrix sp.* and *L. majuscula* 0.5 L was collected from the splash zone at Playa Kalki, Curaçao, in May 1996, preserved in isopropyl alcohol, and stored at −20 °C. A voucher specimen is housed at Oregon State University and is available from WHG as collection number NAK-5 May 96−1.

**Extraction and Isolation.** A total of 80 g (dry wt) of the cyanobacterium was homogenized in $CH_2Cl_2$−MeOH (2:1) and filtered. The cellular residue was extracted with warm $CH_2Cl_2$−MeOH (2:1; 3 ×) and the extracts combined (550 mg dark oil). Vacuum Si gel chromatography of 540 mg (hexane−EtOAc−MeOH gradient) yielded several fractions (10% EtOAc−hexane) containing UV-active, charring compounds (50% $H_2SO_4$) by TLC. These fractions were combined (30.5 mg) and further fractionated over reversed-phase C-18 Si ($H_2O$−MeOH gradient), to give 7.3 mg of kalkipyrone (**1**, eluting with 60−80% MeOH−$H_2O$) as a colorless oil. A further 8.5 mg of **1** was subsequently isolated from side fractions obtained in the first two levels of chromatography.

**Kalkipyrone (1):** $[\alpha]^{20}_D$ +8.2° (*c* 0.22, $CHCl_3$); UV (EtOH) $\lambda_{max}$ nm (log $\epsilon$) 204 (4.3), 238 (4.5); IR (film) $\upsilon_{max}$ 3374 (br), 2960, 2925, 1665, 1579, 1327, 1165 $cm^{-1}$; HREIMS *m/z* obs (rel int, mmu dev) $[M]^+$ 332.1993 (12, 0.6) $C_{20}H_{28}O_4$, $[M - CH_3]^+$ 317.1762 (25, 1.0), $[M - H_2O]^+$ 314.1907 (52, 2.5), $[M - C_2H_3O]^+$ 289.1811 (55, 0.7), $[M - C_6H_9O]^+$ 235.1334 (22, 0.0), $[M - C_7H_{11}O]^+$ 221.1181 (74, 0.3), $[M - C_7H_{12}O_2]^+$ 204.1160 (41, 1.0), $[M - C_8H_{15}O_2]^+$ 189.0923 (37, 0.7), $[M - C_{11}H_{16}O]^+$ 168.0788 (100, 0.1), $[M - C_9H_{15}O_4]^+$ 145.0992 (62, −2.5); for $^1H$ and $^{13}C$ NMR data, see Table 1.

**Determination of the Absolute Stereochemistry at C14 of Kalkipyrone (1).** An aliquot of kalkipyrone (0.2 mg) was reacted with 20 $\mu$L of a 1-$\mu$mol/$\mu$L (−)-menthyl chloroformate solution (benzene), in 120 $\mu$L dry benzene and 20 $\mu$L dry pyridine for 2 h at room temperature. The sample was dried under nitrogen and the residue resuspended in 100% hexane. The reaction products were purified utilizing a 1.2 cm × 1 cm NP Si column with an EtOAc−hexane gradient. The (−)-menthyl chloroformate products eluting with 20−40% EtOAc−hexane were suspended in $CH_2Cl_2$ and ozonized for 2 min at −8 °C followed by 10 min at room temperature. The reaction products were then treated with peracetic acid overnight at 50 °C. The reaction mixture was dried under nitrogen and resuspended in MeOH, and the methyl ester was generated by treatment with ethereal $CH_2N_2$. The resulting products were examined by GC−EIMS (11.5 m of HP Ultra-1, 100−210 °C at 3.0 °C/min, then isothermal for 5 min) and compared with the corresponding authentic standards; 14.81 min for *(S)* and 15.07 min for *(R)*. Integration of these peaks gave 84% *R* and 16% *S*.

**Brine Shrimp Toxicity Assay.** In a method slightly modified from the original description,[23] about 15 newly hatched brine shrimp *(Artemia salina)* in ca. 0.5 mL seawater were added to each well containing different concentrations of sample in 50 $\mu$L EtOH and 4.5 mL artificial seawater to make a total volume of ca. 5 mL. Samples and controls were run in duplicate. After 24 h at 28 °C, the brine shrimp were observed and counted with a dissecting light microscope. The percentage of live shrimp versus total shrimp was used to determine $LD_{50}$ values.

**Ichthyotoxicity Assay.** A modification of a previously described method was used to assess toxicity to the goldfish *Carassius auratus*.[24] Dilutions of samples in 40 $\mu$L EtOH were added to 40 mL distilled $H_2O$ in a 50-mL beaker and mixed. A single goldfish was added and observed for 1 h. Samples and controls were run in duplicate.

**GC−EIMS Analysis of Cultured *L. majuscula* Cells.** Two laboratory cultures of *L. majuscula* isolated from the original collection of NAK-5 May 96−1 were extracted (<1 g each) with $CH_2Cl_2$−MeOH (2:1, 3 ×), dried under nitrogen, resuspended in 10% (v/v) EtOAc−hexane, filtered, and analyzed by GC−EIMS (11.5 m of HP Ultra-1, 70−250 °C at 15.0 °C/min, then isothermal for 15 min). Peaks indicative of kalkipyrone ($t_R$ 12.29, 12.97 min, *m/z* obs 332, 314, 189, 221, 168, 145) were not apparent in extracts of the cultured specimens. Thermal isomerization (*E* to *Z*) of an olefinic bond in the alkyl chain likely accounts for the occurrence of more than one GC peak for authentic kalkipyrone (**1**).

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