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# CYATHIFORMINES A-D, NEW CHORISMATE-DERIVED METABOLITES FROM THE FUNGUS CLITOCYBE CYATHIFORMIS [*1]

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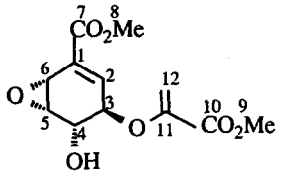
*Key Words: Basidiomycetae; Clitocybe; Chorismates; Structural Elucidation; Absolute Configuration.*

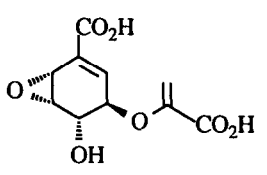
**Abstract:** *Cyathiformines A-D,* **1**, **4a**, **5** *and* **6a**, *have been isolated from the Basidiomycetous fungus Clitocybe cyathiformis. Structural determination was based on NMR studies and chemical evidence.*

## Introduction

During screening of the genus *Clitocybe (Basidiomycetae)* for secondary metabolites, we have reported the isolation from *C. illudens, C. elegans* and *C. candicans* of a large number of protoilludanic sesquiterpenes.[2-5] Further investigations led to the isolation from a strain of *C. cyathiformis* of an unexpected series of chorismic acid derivatives. This paper describes the isolation and characterisation of these metabolites, named cyathiformines A-D **1**, **4a**, **5** and **6a**.




**1**

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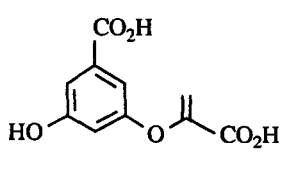


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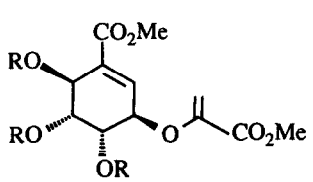
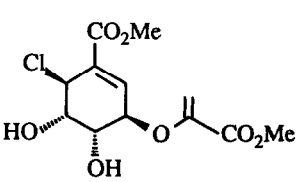
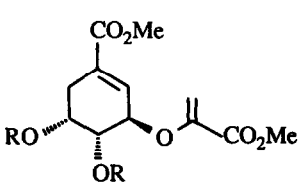



**4a** R=H
**4b** R=Ac




**5**




**6a** R=H
**6b** R=Ac

*Dedicated to Professor Max Viscontini on the occasion of his 80th birthday

## Results and Discussion

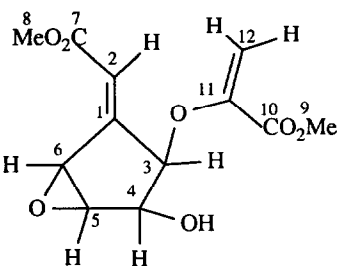
The crude oil obtained from the organic extracts of the solid cultures was purified through column and PLC chromatography, as described in the Experimental, to yield cyathiformines A-D. The IR spectra of all these metabolites **1**,**4a**,**5** and **6a** suggested the presence of α,β-unsaturated ester and hydroxy groups since they exhibited bands at *ca.* 1720 and 1625 and *ca.* 3400 $cm^{-1}$, respectively. Moreover, all these compounds contain a 1-methoxycarbonylvinyloxy unit linked at C-3 because in the NMR spectra (Table 1) H-3 presented a three-bond (C,H) coupling constant of *ca.* 3 Hz with C-11.

The first metabolite, cyathiformine A **1**, was obtained as white crystals, mp 88-90°C; CI mass spectra established its molecular formula as $C_{12}H_{14}O_7$. Accordingly, the $^{13}C$ and $^1H$ NMR spectra (Tables 1 and 2) confirmed that this compound contains 12 carbon and 14 hydrogen atoms, one of which belonging to an hydroxy group since it disappeared upon addition of $D_2O$. The $^{13}C$ resonances were attributed on the basis of chemical-shift criteria to 6 $sp^2$- and 6 $sp^3$-hybridized carbon atoms. Their multiplicities were deduced from DEPT experiments and the analysis of the fully $^1H$-coupled $^{13}C$ NMR spectrum while the correlations between the proton-bearing carbon atoms with specific proton resonances were established using an HETCOR experiment.

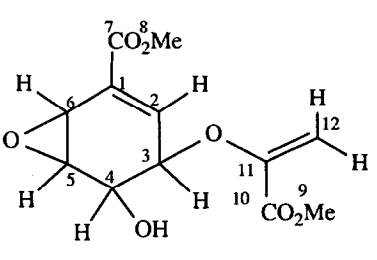
The $sp^2$ resonances were assigned to the carbons of two ester carbonyl groups (C-7 and C-10) and to those of trisubstituted and *gem*-disubstituted olefinic double bonds (C-1, C-2 and C-11, C-12) whereas four of the $sp^3$ resonances were indicative of oxygen-bearing methine (C-3 and C-4) and methyl (C-8 and C-9) carbons. The remaining two $sp^3$ resonances at $\delta_C$ 53.58 and 48.42, which correlate with the signals at $\delta_H$ 3.70 and 4.07, were assigned to the carbons of an oxirane ring (C-5 and C-6) on the basis of the one-bond (C,H) couplings [$^1J$ (CH) = 182.5 and 184.5 Hz].

The long-range (C,H) couplings observed between C-10 and $H_3$-9 [$^3J$ (CH) = 3.5 Hz], C-10 and $H_2$-12 [$^3J$ (CH) = 4 and 10.5 Hz] and C-7 and $H_3$-8 [$^3J$ (CH) = 3.5 Hz] allowed us to join C-9 to O-10, C-10 to C-11 and C-8 to O-7 while the (H,H) couplings observed between H-5 and H-6 and between H-3 and H-4 [$^3J$ (H,H) = 4.3 and 8.0 Hz, respectively], require that H-5 and H-6 are *cis* disposed[6] and that C-3 is linked to C-4.

The presence of a long-range (C,H) coupling of 3.5 Hz between H-3 and C-11 together with the absence of allylic couplings between H-3 or H-4 and $H_2$-12 indicated that C-3 and C-11 are connected *via* the O-3 atom. Finally, the long-range (C,H) couplings observed between H-2 and C-7 and the fact that H-3 and H-6, but not H-4 and H-5 which are coupled each other, presented vicinal or allylic couplings with H-2 indicated that C-3, C-6 and C-7 are linked to C-1 or C-2, and hence C-4 to C-5, to give structure A or B in which H-2 is spatially close to H-3 since a NOE was observed for H-3, but not for H-6, upon irradiation of H-2 (Table 3).




A




B

Cyathiformine A **1** showed an $^1H$ NMR spectrum very similar to that exhibited by the epoxide derivative **2** (Table 2) obtained from chorismic acid[7] and gave, as well as compound **2**, the 3-(1-carboxyvinyloxy)-5-hydroxybenzoic acid **3** by treatment with methanolic KOH (Experimental). These findings established the structure of cyathiformine A **1** as B and define the relative configuration at C-3,C-4,C-5 and C-6 as $R^*$,$R^*$,$S^*$, and $R^*$, having assumed that C-3 has the $R^*$-configuration.

The coupling constants of 8.0 and 1.3 Hz exhibited by H-3 and H-4 and H-4 and H-5 suggested that the cyclohexene ring preferentially adopts a conformation similar to that depicted in figure 1 in which H-3 and H-4 are pseudoaxially positionated and H-4 and H-5 form a dihedral angle of *ca.* 75°.

The second metabolite cyathiformine B **4a**, was obtained as an oil; CI mass spectroscopy indicated the formula $C_{12}H_{16}O_8$. The presence of three hydroxy groups (Table 2) was confirmed by the formation of the triacetate **4b**. This compound was also obtained from **1** upon treatment with pyridine-$Ac_2O$, this fact proving that compounds **1** and **4a** share a cyclohexene moiety and the relative configuration.

The magnitude of the vicinal couplings observed in compound **4a** between H-3 and H-4 and H-4 and H-5 [$^3J$ (H,H) = 7.9 and 2.4 Hz] requires that the cyclohexene ring preferentially assumes, as in *(3R,4R,5R)-4-epi*-shikimic acid,[8] the half-chair shown in figure 1 in which H-3 and H-4 and H-4 and H-5 are pseudoaxially and gauche disposed, respectively.

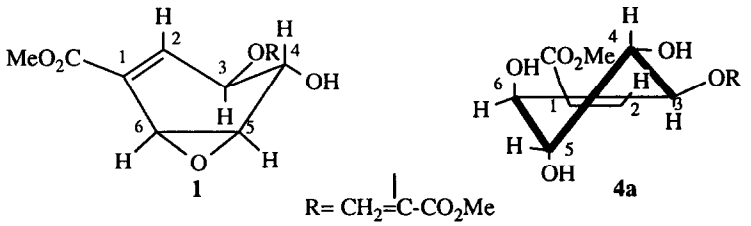



Fig.1 - Preferred conformations of cyathiformines A **1** and B **4a**

The small allylic coupling exhibited by H-2 and H-6 [$^4J$(H,H) <0.5 Hz] suggests that H-6 is α pseudoequatorially disposed thus forming a dihedral angle of *ca.* 20° with the C6-C1-C2 plane;[9] if H-6 were β axially disposed it would present an allylic coupling of *ca.*2 Hz because it would form a dihedral angle of *ca.* 110°. These data, together with the fact that the above reported opening of the epoxide ring in **1** should give a trans derivative[10], indicate the relative configuration at C-6 as $S^*$, it having determined that at C-5 as well as those at C-3 and C-4 as $R^*$.

The third metabolite, cyathiformine C **5**, was obtained as an oil; it presented molecular ions at *m/z* 306/308 with a ratio indicating the presence of one chlorine atom and corresponding to the formula $C_{12}H_{15}O_7Cl$. The $^1H$ NMR spectrum (Table 2) showed the presence of two hydroxy protons vicinally coupled to H-4 and H-5 [$^3J$ (HH)=4.0 and 5.5 Hz] which were therefore placed at C-4 and C-5. Moreover, comparison of the $^{13}C$ NMR data of cyathiformines B and C **4a** and **5** (Table 1) revealed that the two compounds share the same basic structure, the only significant difference being the presence of a chlorine atom in **5** in place of the 6-hydroxy group ($\delta_C$ 53.35 vs 68.09). Finally, the close similarity of the (H,H) couplings observed in the above compounds **4a** and **5** implies that **5** has the same relative configuration of **4a**, $3R^*$, $4R^*$, $5S^*$, $6S^*$, the change in the descriptor for the C-5 chiral centre being merely the result of the sequence rules of the Cahn-Ingold-Prelog system.

Table 1. $^{13}C$ NMR data for compounds 1, 4a, 5 and 6a in $CDCl_3$

| | 1 | | | | 4a | | |
|---|---|---|---|---|---|---|---|
| Carbon | $\delta_C$ | | $^1J(C,H)$ | $^{>1}J(C,H)$ | $\delta_C$ | | $^1J(C,H)$ |
| 1 | 129.48 | Sbrddd | | 4,3,3 | 131.76 | S | |
| 2 | 139.22 | Dbrdd | 168 | 5,4.5 | 135.40 | D | 164 |
| 3 | 77.12 | Dm | 148 | | 75.65 | D | 148 |
| 4 | 70.27 | Dm | 142.5 | | 68.57 | D | 146 |
| 5 | 53.58 | Dm | 182.5 | | 72.86 | D | 149 |
| 6 | 48.42 | Dm | 184.5 | | 68.09 | D | 149.5 |
| 7 | 164.67 | Sbrddq | | 6,3,3.5 | 166.48 | S | |
| 8 | 52.24 | Qs | 147.5 | | 52.18 | Q | 147.5 |
| 9 | 52.28 | Qs | 148 | | 52.65 | Q | 148 |
| 10 | 163.63 | Sddq | | 10.5,4,3.5 | 163.99 | S | |
| 11 | 149.31 | Sddd | | 3.5,3,3 | 149.20 | S | |
| 12 | 98.48 | DDs | 160<br>167 | | 97.60 | DD | 160.5<br>166 |

| | 5 | | | | 6a | | |
|---|---|---|---|---|---|---|---|
| Carbon | $\delta_C$ | | $^1J(C,H)$ | $^{>1}J(C,H)$ | $\delta_C$ | $^1J(C,H)$ | $^{>1}J(C,H)$ |
| 1 | 130.25 | Sdddd | | 5.5,5.5,5.5,2.5 | 130.01 | Sdddt | 2.5(H-2),4.5(H-3),6.5(H-5),7.5($H_2$-6) |
| 2 | 135.93 | Dbrdd | 165.5 | 5.5,4.5 | 132.24 | Dbrdt | 165.5 | 4.5(H-3),5($H_2$-6) |
| 3 | 76.45 | Dm | 146.5 | | 77.07 | Dm | 147.5 | |
| 4 | 67.78 | Dm | 145 | | 71.75 | Dm | 145 | |
| 5 | 73.72 | Dm | 153 | | 67.93 | Dm | 147.5 | |
| 6 | 53.35 | Dm | 159 | | 31.16 | Tm | 130.5 | |
| 7 | 164.70 | Sbrddq | | 5.5,2.5,3.5 | 166.55 | brdtq | | 6.5(H-2),3($H_2$-6),3.5($H_3$-8) |
| 8 | 52.31 | Qs | 147.5 | | 52.08 | Qs | 147.5 | |
| 9 | 52.40 | Qs | 148 | | 52.76 | Qs | 148 | |
| 10 | 164.10 | Sddq | | 10.6,4,3.5 | 164.18 | Sddq | | 4(H-12a),10.5(H-12b),3.5($H_3$-9) |
| 11 | 149.14 | Sddd | | 3.5,3.5,3 | 149.48 | Sddd | | 3(H-3),3(H-12a),3(H-12b) |
| 12 | 99.11 | DDs | 159.5<br>166.5 | | 99.33 | DDs | 160<br>166.5 | |

Table 2. $^1$H NMR data for compounds 1, 2, 4a, 4b, 5, 6a and 6b in $CDCl_3$

| | $\delta_H$ | | | | | | |
|---|---|---|---|---|---|---|---|
| Proton | 1 | 2[a] | 4a | 4b | 5 | 6a | 6b |
| 2 | 6.91 (6.82[b]) | (6.88) | 6.86 (6.79) | 7.14 (7.13) | 6.95 (6.89) | 6.81 (6.75) | 6.89 |
| 3 | 4.61 (4.58) | (4.62) | 4.78 (4.85) | 4.90 (5.12) | 4.87 (4.93) | 4.76 (4.79) | 4.84 |
| 4 | 4.25 (4.17) | (4.21) | 4.22 (4.16) | 5.42 (5.28) | 4.44 (4.29) | 3.95 (3.90) | 5.26 |
| 5 | 3.70 (3.63) | (3.65) | 4.20 (4.06) | 5.44 (5.42) | 4.39 (4.26) | 4.27 (4.09) | 5.43 |
| 6α | | | 4.60 (4.57) | 5.76 (5.71) | 4.88 (4.87) | 2.63 (2.49) | 2.62 |
| 6β | 4.07 (3.96) | (3.97) | | | | 2.63 (2.61) | 2.81 |
| 8 | 3.82 (3.78[c]) | | 3.77 (3.73[c]) | 3.78[c] (3.76[c]) | 3.83[c] (3.77[c]) | 3.74 (3.72[c]) | 3.80[c] |
| 9 | 3.82 (3.76[c]) | | 3.82 (3.77[c]) | 3.82[c] (3.77[c]) | 3.81[c] (3.76[c]) | 3.82 (3.75[c]) | 3.78[c] |
| 12a | 5.54 (5.45) | (5.51) | 5.54 (5.44) | 5.61 (5.56) | 5.58 (5.45) | 5.56 (5.43) | 5.59 |
| 12b | 4.79 (4.90) | (4.90) | 4.92 (4.98) | 4.94 (5.20) | 4.94 (5.02[d]) | 4.94 (4.96) | 5.00 |
| OR-4 | 4.45 (5.10) | | 4.55 (4.55) | 2.10[d] (2.08[d]) | 4.50 (4.69[d]) | 3.50 (4.40[d]) | 2.08[d] |
| OR-5 | | | 3.85 (4.55) | 2.10[d] (2.07[d]) | | 3.50 (4.10[d]) | 2.06[d] |
| OR-6 | | | 2.40 (4.55) | 2.03[d] (1.99[d]) | 4.50 (5.03[d]) | | |
| J(2,3) | 1.7 | 2.0 | 2.3 | 2.6 | 2.2 | 2.9 | 3.1 |
| J(2,6α) | | | <0.5 | <0.5 | <0.5 | 1.6 | 1.5 |
| J(2,6β) | 2.3 | 2.0 | | | | 2.1 | 2.2 |
| J(3,4) | 8.0 | 8.0 | 7.9 | 7.8 | 7.5 | 6.3 | 6.6 |
| J(3,6α) | | | 0.7 | 0.9 | <0.5 | 1.7 | 1.5 |
| J(3,6β) | <0.5 | e | | | | 2.4 | 2.2 |
| J(3,12b) | 0.7 | e | 0.7 | 0.6 | 0.7 | 0.7 | 0.7 |
| J(4,5) | 1.3 | 1.0 | 2.4 | 2.5 | 2.2 | 2.3 | 2.4 |
| J(5,6α) | | | 3.3 | 3.9 | 2.8 | 4.9 | 5.2 |
| J(5,6β) | 4.3 | 4.0 | | | | 4.5 | 4.6 |
| J(6α,6β) | | | | | | 18.3 | 18.7 |
| J(12a,12b) | 2.9 | 3.0 | 2.7 | 2.9 | 2.8 | 2.7 | 2.9 |

[a]See ref.4. [b]Values in parentheses are chemical shifts in acetone -$d_6$.
[c,d]Assignments within each column may be interchanged. [e]Not assigned.

The fourth metabolite, cyathiformine D **6a**, was isolated as an oil and analysed for $C_{12}H_{16}O_7$ ($M^+$, 272).Comparison of $^{13}C$ and $^1H$ NMR data of the compounds **4a**,**5** and **6a** (Tables 1 and 2) proved that cyathiformine D **6a** differs from the other two metabolites in that the cyclohexene ring contains an additional hydrogen atom at C-6 in place of the hydroxy or chlorine substituents. As expected, acetylation of **6a** gave the diacetate **6b** with the methine protons assigned to H-4 and H-5 resonating downfield of 1.31 and 1.16 ppm, respectively. Here again, the assignment of the $R^*$ relative configuration at C-3, C-4 and C-5 followed from the magnitude of the (H,H) couplings and NOE experiments (Table 3).

Table 3. Selected connectivities established by NOE difference experiments for compounds **1** and **6a**[a]

| Proton irradiated | Protons affected (%) in compound | |
|---|---|---|
| | **1** | **6a** |
| 2 | 3(2.5%),12b(1.5) | 3(3) , 12b(1.5) |
| 3 | 2(2) , 12b(3) | 2(3.5), 12b(3.5) |
| 4 | 5(4.5) | 5(4.5), 6β(1) |
| 5 | 4(3.5), 6(3.5) | 4(5), 6α(3.5), 6β(3.5) |
| 6β | 5(3.5) | 4(1.5) |
| 12a | 12b(17) | 12b(19.5) |
| 12b | 2(2), 3(7), 12a(16) | 2(3), 3(6), 12a(15) |
| OH | 3(1.5), 4(2.5), 5(1) | |

[a] NOEs obtained in [$^2H_6$]acetone for compound 1 and in [$^2H_6$]acetone+$D_2O$ for compound 6a.

The $[\alpha]_D$ values exhibited by cyathiformines A-D paralleled that reported for (3*R*,4*R*,5*R*)-4-*epi*-shikimic acid[8] (-104, -98, -76.5 and -92.8 *vs* -93) this fact indicating that the above-mentioned relative configurations for these compounds coincide with the absolute ones.

Cyathiformines A-D may be considered as derivatives of chorismic acid, the last common intermediate in the shikimate pathway. Along this way fungi and lower plants convert glucose-6-phosphate into a wide variety of primary and secondary metabolites as phenylalanine, tyrosine, tryptophan and isoprenoid quinones.[11] This is, to our knowledge, the first isolation of chorismate derivatives from higher fungi. Probably, the mushroom synthesizes these metabolites in a stabilized ester form as defensive substances or as potential sources of hydroxy or amino aromatic acids. In effect, many shikimate derivatives (*i.e.* salicylic acid, vanillic acid, 4-hydroxybenzoic acid and ferulic acid) have a rather interesting ecological function: they are allelopathic and inhibit the growth or germination of a wide range of plant species[12].

Finally, besides cyathiformines, the fungus produces a little amount of 4-hydroxybenzoic acid and of its methyl ester, thus confirming the above findings.

*C. cyathiformis* exhibits antifungal and antibacterial activity, but only cyathiformine A **1** is active against *Bacillus subtilis* and *Cladosporium cladosporioides*.

## Experimental

*General.* Optical rotations: $CHCl_3$; IR: neat; UV: EtOH; $^1H$ and $^{13}C$ NMR: 250.1 and 62.9 MHz, respectively, using $SiMe_4$ as internal standard. NOE difference spectra were obtained by subtracting alternatively right-off resonance-free induction decays (FIDs) from right-on resonance-induced FIDs. Analytical and preparative TLC: silica gel Merck $HF_{254}$. Column chromatography: silica gel (Merck, Kieselgel 0.063-0.02 mm).

*Production, extraction and purification of cyathiformines. Clitocybe cyathiformis* (CBS 149.50) was grown in 30 Roux flasks on PDA (Potato, dextrose, agar) for 21 days. Ethyl acetate extracts of the cultures (1.3 g) were evaporated, subjected to column chromatography on silica gel and eluted with a mixture of hexane-ethyl acetate (1:1) to yield in the following order of decreasing $R_f$, compounds **1** (60 mg), **5** (50 mg), **6a** (290 mg) and **4a** (50 mg).

*Cyathiformine A* **1**: crystals, mp 88-90°C (from ethanol); $[\alpha]_D$ -104 (c 0.1); IR, $\nu_{max}$ $cm^{-1}$: 3450, 1725, 1625; $^{13}C$ and $^1H$ NMR spectra: Tables 1 and 2; CIMS (isobutane), *m/z*: 271 $[MH]^+$. (Found: C,55.2; H,5.1. $C_{12}H_{14}O_7$ requires C,55.33; H,5.22).

*Cyathiformine B* **4a**: oil; $[\alpha]_D$ -98 (c 0.1); IR, $\nu_{max}$ $cm^{-1}$: 3400, 1720, 1625; $^{13}C$ and $^1H$ NMR spectra: Tables 1 and 2; CIMS (isobutane), *m/z*: 289 $[MH]^+$. (Found: C,49.9; H,5.5. $C_{12}H_{16}O_8$ requires C,50.00; H,5.60).

*Cyathiformine B triacetate* **4b**: Cyathiformine B **4a** (40 mg) was dissolved in dry pyridine (1 $cm^3$) and treated with $Ac_2O$ (2 $cm^3$) at room temperature for 24 hours. Standard work up followed by PLC on silica gel in hexane-AcOEt (2:1) gave the triacetate **4b** (30 mg): $^1H$ NMR spectrum: Table 2. Triacetate **4b** was also obtained by acetylation of cyathiformine A **1** under the same conditions.

*Cyathiformine C* **5**: oil; $[\alpha]_D$ -76.5 (c 0.12); IR, $\nu_{max}$ $cm^{-1}$: 3360, 1725, 1620; $^{13}C$ and $^1H$ NMR spectra: Tables 1 and 2; EIMS, *m/z*: 306/308 $[M]^+$. (Found: C,46.8; H,4.8; Cl,11.4. $C_{12}H_{15}O_7Cl$ requires C,46.99; H,4.92; Cl,11.56).

*Cyathiformine D* **6a**: oil; $[\alpha]_D$ -92.8 (c 0.1); IR, $\nu_{max}$ $cm^{-1}$: 3450, 1720, 1625; $^{13}C$ and $^1H$ NMR spectra: Tables 1 and 2; CIMS, *m/z*: 273 $[MH]^+$. (Found: C,52.8; H,5.7. $C_{12}H_{16}O_7$ requires C,52.94; H,5.92).

*Cyathiformine D diacetate* **6b**: Cyathiformine *D* **6a** (30 mg) was dissolved in dry pyridine (0.5 $cm^3$) and treated with $Ac_2O$ (1 $cm^3$) at 0°C for 24 hours. Standard work-up followed by PLC on silica gel (hexane-AcOEt, 2:1) gave **6b** (20 mg): EIMS, *m/z* 356 $[M]^+$, 255, 212, 152; $^1H$ NMR spectrum: Table 2.

*Aromatisation of cyathiformine A* **1**: Cyathiformine A was dissolved in methanol (5 $cm^3$) containing KOH (280 mg) and the solution was refluxed for 1 hour. After neutralisation, the solution was extracted with AcOEt. Standard work up gave 3-(1-carboxyvinyloxy)-5-hydroxybenzoic acid **3**. $^1H$ NMR (acetone-d6), δ: 8.75(broad signal, 2 x COOH + $H_2O$); 7.31, 7.17 and 6.76(3H, dd, J=2.2 and 1.3, 2.4 and 1.3, and 2.4 and 2.2 Hz, respectively, H-2, H-4 and/or H-6); 5.94(1H, d, J=1.9 Hz, H-12a) and 5.32(1H, d, J=1.9Hz, H-12b).

*Biological Tests.*- Antibacterial and antifungal activity were tested with paper disks (6 mm diam.), soaked with an EtOH solution of cyathiformines A-D (400, 200 and 100 μg) and placed in a suitable culture medium, cooled at 45°C, and poured into Petri dishes with *Bacillus cereus* (ATCC 10702), *Sarcina lutea* (DMS 348), *Escherichia coli* (IPV 287), *Bacillus subtilis* (ATCC 6633), *Cladosporium cladosporioides* (IPV F167), *Chlorella vulgaris* (Algae), as test micro-organisms. Cyathiformine A **1** exhibited activity at concentration of 100 μg/disks.

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References and Notes

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