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FINAL STRUCTURE OF CAULERPICIN, A TOXIN MIXTURE FROM THE GREEN ALGA *CAULERPA RACEMOSA*

PETER G. NIELSEN, JØRGEN S. CARLÉ and CARSTEN CHRISTOPHERSEN

Marine Chemistry Section, University of Copenhagen, The H. C. Ørsted Institute, Universitetsparken 5, DK 2100 Copenhagen, Denmark

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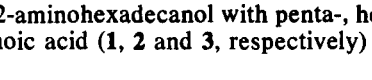
Key Word Index—*Caulerpa racemosa*; Chlorophyta; green alga; caulerpicin; *N*-acylsphinganines.

Abstract—Caulerpicin from the green alga *Caulerpa racemosa* is shown to consist of a mixture of ceramides derived from 2*S*, 3*R*-sphinganine with C_{18} (32%), C_{20}(2%), C_{22}(6%), C_{24}(35%) and C_{26}(25%) saturated fatty acid residues.

Through the work of Aguilar-Santos and Doty [1–3], caulerpicin is established as a health threat to certain populations depending to some extent on the productivity of tropical waters. The agent responsible for the adverse effect on humans is believed to be a secondary metabolite from *Caulerpa racemosa* (Forsskal) J. Agardh (Caulerpales) as well as other *Caulerpa* species [1–3], which are widely used in the Philippines as ingredients in salads. Caulerpicin has been shown, in certain instances, to be concentrated in the marine food chain [3]. Shrimps and crabs from the infested areas are known to be unacceptable as food for sensitized persons, even if caulerpicin could not be demonstrated in these crustaceans [3].

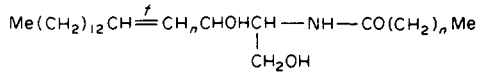
Aquilar-Santos and Doty tentatively proposed caulerpicin to consist of a mixture of three homologous amides of 2-aminohexadecanol with penta-, hexa- and heptacosanoic acid (**1**, **2** and **3**, respectively) [2]. The



1 *n* = 23
2 *n* = 24
3 *n* = 25

amino alcohol is readily accessible from hexadecanoic acid via α-bromohexadecanoic acid [4], α-aminohexadecanoic acid [5], and ethyl α-aminohexadecanoate [6, 7], which on lithium aluminium hydride reduction [8] gave a high yield of 2-aminohexadecanol. Compounds **2** and **3** were then obtained from the acid chloride [9] and amino alcohol [10]. Careful analysis of the spectral characteristics of the synthetic compounds revealed, however, that they are incompatible with the ones published for caulerpicin.

Recently a revised structure of caulerpicin has appeared [11]. This work is based on a mixture of compounds isolated from *C. racemosa* collected near Sri Lanka. The sample investigated was shown to be a mixture of ceramides (**4–7**), i.e. amides of sphingosine with C_{14}, C_{16}, C_{22} and C_{24} saturated acids containing also C_{22} and C_{24} monounsaturated analogues. This sample was not compared with the original caulerpicin.



4 *n* = 12
5 *n* = 14
6 *n* = 20
7 *n* = 22

A generous gift of the original sample of caulerpicin (32 mg) has enabled us to reinvestigate the natural product. Inspection of the ^{13}C NMR spectrum (67.889 MHz) of this material ruled out the possible identity of the Sri Lankan sample with Philippine caulerpicin. Apart from minor impurities, the only resonance in the sp^2 region was the amide carbonyl appearing at δ 173.8. Off resonance experiments identified signals at δ 74.1, 54.2 and 62.5 as originating from two methine and a methylene group, respectively, incompatible with the originally proposed structures.

After silylation the mixture could be resolved by GC allowing the mass spectra of the pure components to be obtained. According to GC analyses caulerpicin is a mixture of three main and two minor components. The mass spectra of these five compounds all exhibited similar fragmentation patterns characteristic of ceramides [12, 13] (Fig. 1). In contrast to the mass spectral data published for ceramides [13], $[M]^+$ or $[M-1]^+$ ions were observed at *m/z* 683 $[M]^+$, 714 $[M-1]^+$, 739 $[M]^+$, 767 $[M]^+$, and 795 $[M]^+$, respectively, for the components arranged in order of increasing R_f. The spectra are dominated by *m/z* 313

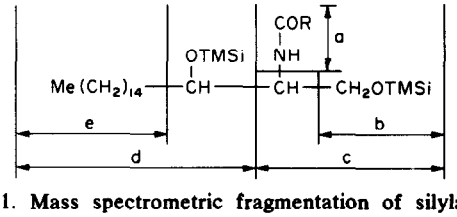
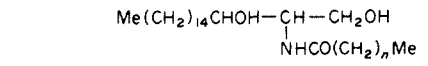


Fig. 1. Mass spectrometric fragmentation of silylated sphinganine derivatives.

representing $[M-c]^+$, being the most abundant ion above m/z 200. This fragment is indicative of the oxygen function at position three, also substantiated by the presence of m/z 217, representing $[M-(a+e+1)]^+$, in all spectra. Other prominent peaks in the high mass region correspond to ions $[M-(d+73)]^+$, formed by cleavage of the 2, 3 bond and migration of the TMSi group, and $[M-(d+83)]^+$ where fragment d and the TMSi group of position one are lost. Characteristic fragmentations leading to $[M-b]^+$, $[M-103]^+$, $[M-TMSiOH]^+$, $[M-90]^+$, and $[M-1-TMSiOH]^+$ appear in all spectra. Careful examination of the mass spectroscopic data revealed the presence of trace amounts of unsaturated ceramides as well. In view of the extreme sparsity of these compounds their structures have not been further pursued.

The data presented above define the structure of caulerpicin as a mixture of sphinganine derived ceramides with C_{18} (**8**, 32%), C_{20} (**9**, 2%), C_{22} (**10**, 6%), C_{24} (**11**, 35%), and C_{26} (**12**, 25%) saturated fatty acid



8 $n=16$ **11** $n=22$
9 $n=18$ **12** $n=24$
10 $n=20$

residues. The NMR spectroscopic evidence (^1H as well as ^{13}C) is fully compatible with this assignment. Especially ^{13}C NMR demonstrates that the hydrocarbon chains are unbranched since comparison with available data [14] and off resonance decoupling techniques allows assignment of all resonances observed (see Experimental).

To investigate further the sphinganine portion of the molecules a basic hydrolysis was performed [15]. In contrast to the acidic hydrolysis often used [16, 17], this procedure is known largely to maintain the stereochemistry of sphinganine derivatives [15]. A circular dicroism (CD) experiment on the hydrolysed product gave rise to a CD curve with a minimum at 218 nm ($\Delta\epsilon \sim -0.5$) and a maximum at 227.5 nm ($\Delta\epsilon \sim 0.3$) thus defining the sphinganine as either 2*S*, 3*R* [D(+)-erythro] or 2*R*, 3*R* [D(+)-threo] sphinganine [18], the former representing the stereoisomer usually encountered in nature. To distinguish between the two diastereomeric possibilities, a sample of erythro-*N*-palmitoylsphinganine was prepared [19], and the 67.889 MHz ^{13}C NMR recorded. The erythro isomer gave rise to a spectrum superimposable with that of the original sample. We therefore conclude that caulerpicin is a mixture of ceramides derived from 2*S*, 3*R*-sphinganine.

Whether the adverse effects repeatedly warned against [20–22] are actually attributable to the ceramides described above is still an open question. A possibility still exists that the toxic principle is a minor, as yet unknown, impurity in the ceramide mixture. Future pharmacological testing of synthetic ceramides should serve to clarify this intriguing question.

EXPERIMENTAL

Properties of caulerpicin. The ^{13}C (67.889 MHz) NMR spectrum of the original sample (31.3 mg in 400 μl $CDCl_3$) on comparison with an off-resonance decoupled spectrum of the same sample gave the following assignments (δ, multiplicity, assignment): 173.8, *s*, $\underline{C}$=O; 74.1, *d*, $\underline{C}$HOH; 62.5, *t*, $\underline{C}H_2OH$; 54.2, *d*, $\underline{C}$HNH; 36.9, *t*, $\underline{C}H_2$–CHOH–; 34.6, *t*, $\underline{C}H_2$–C=O; 32.0, *t*, $\underline{C}H_2$ third last carbon in the alkyl chains, 29.4–29.7, *t*, $\underline{C}H_2$ in the chains, 26.1 and 25.8, *t*, $\underline{C}H_2$–CH_2C=O; 22.7, *t*, last $\underline{C}H_2$ groups in the alkyl chains, 14.0, *q*, terminal $\underline{C}H_3$. The same sample was used to record the ^1H (270 MHz) NMR which shows the expected paraffinic signals at δ 0.88, ~1.26, 1.53, and 1.64. The two α-protons of the C=O group appear at δ 2.22, the four protons on carbons bearing a heteroatom appear between δ 3.69–3.87 (3H, *m*) and at 3.99 (1H, *dd*). The NH proton gives rise to a *br d* centered at δ 6.32. The above assignments were verified, whenever possible, by decoupling experiments. The IR spectrum (KBr) is dominated by bands assigned to the primary hydroxyl group (3400 and 1048 cm^{-1}) and the secondary amide function (3300, 1635, 1572 and 1325 cm^{-1}). Caulerpicin could not be separated by TLC giving spots with R_f 0.3 (HP Si gel, $CHCl_3$) and 0.5 (reverse phase, CH_2Cl_2). The GC expts on a silylated sample (1.1 mg caulerpicin reacted with 20 μl 1.5 mM trimethylsilylimidazole in pyridine, 60° for 6 hr) were carried out using a glass column (2 m, 2 mm i.d. packed with OV-1) flow rate 15 ml/min N_2, injection temp. 350°.

Hydrolysis. A mixture of caulerpicin (30 mg) and 50 ml KOH soln (N in 90% MeOH) was refluxed for 18 hr. Extraction with Et_2O (3 × 50 ml) followed by washing the Et_2O phase with H_2O (2 × 50 ml) and evaporation left a product which was dried by treatment with dry Et_2O (100 ml). A $CHCl_3$ soln was filtered and evaporated (yield 14.8 mg) which showed the characteristic sphinganine MS fragmentation pattern, This product was used directly for CD analyses.

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