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Nostoclide I and II, Extracellular Metabolites from a Symbiotic Cyanobacterium, *Nostoc* sp., from the Lichen *Peltigera canina*.

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Abstract: Two chlorine-containing metabolites, nostoclide I and II have been isolated from the culture of a symbiotic blue-green alga, *Nostoc* sp., in *Peltigera canina*, a common lichen. Their structures were determined by spectroscopic data and x-ray diffraction.

The lichens are unique biological forms, which are composed of blue-green or green algae and fungi. Chemically, they are also interesting entities, and a great number of metabolites have been isolated from the symbiotic organisms.[1] Those compounds, which are often called "lichen substances", are generally considered to be the metabolites of the fungal part of the symbiotic organisms. In fact, most of the representative lichen substances such as depsides and depsidones are closely related to common fungal acetogenins such as orsellinic acid. The algal parts are said to produce mostly polysaccharides such as lichenins. In this paper, however, we report the production of interesting metabolites by a blue-green alga or cyanobacterium isolated from the common lichen, *Peltigera canina* (L.) Wild (dog lichen).

The algal cells[2] were grown in ASM-1 medium[3] under fluorescent lighting and aeration. After three weeks, the algal cells were removed by centrifugation. The supernatant (84 L), which showed pH 9.2 due to the extreme depletion of carbon dioxide in the medium, was acidified to pH 3.6 with HCl. In this process, the color of the solution turned from yellow to colorless or opaque. The solution was passed through XAD-2 resin and the resin was extracted with methanol. The methanol extract was flash-chromatographed on silica gel using hexane-CH_2Cl_2. Two crystalline compounds (named nostoclides), **1** and **2** were obtained in pure form by this chromatography. The yields of the compounds were 235 mg and 8 mg respectively. The extract of the cells (15 g in dry weight) gave much smaller amounts of **1** and **2** (17.3 mg and 7.1 mg respectively) after the same process.

The compound **1** was obtained as prisms, mp. 186-187°, from MeOH. The high resolution EI mass spectrum gave a molecular formula, $C_{21}H_{18}Cl_2O_3$ (obsd. m/z 388.0668; calcd. m/z 388.0633). The presence of two chlorine atoms was evident from a 9:6:1 isotopic cluster of molecular isons: m/z 388.0633, 390.0603, and 392.05741. It has intensive UV absorptions, λ_{max} (MeOH) nm (ε): 349.9 (32,900), 250 (14,700), which shift to 400 (18,300), 262.5 (10,200) by addition of NaOH solution, indicating the presence of an ionizable extended conjugated system.

The NMR spectra (^1H, ^{13}C, HETCOR, DEPT) (Table 1) showed the presence of isopropyl, benzyl, tetra-substituted benzene groups, two tetra-substituted double bonds, and one carbonyl group. The carbonyl group was presumed to be in a conjugated lactone judging from the degree of unsaturation and number of oxygen atoms. While there were several possibilities to connect these

moieties, the final proof of the structure was given by X-ray crystallography.

Preliminary X-ray photographs showed orthorhombic symmetry. Accurate lattice constants of a=14.526(6), b=12.530(7) and c=20.581(16) were obtained from diffractometer measured 2θ-values, and systematic extinctions and density measurements were uniquely accommodated by space group Pbca with one molecule of $C_{21}H_{18}Cl_2O_3$ forming the asymmetric unit. All unique diffraction maxima with 2θ<116° were collected on a computer controlled four circle diffractometer with CuKα radiation and variable speed 2θ-θ scans. After correction for Lorentz, polarization and background effects, 2105 (82 %) reflections were judged observed ($|F_o| \geq 4.0\sigma(F_o)$). The structure was solved by standard heavy atom techniques, and full-matrix least-squares refinements with anisotropic heavy atoms and riding hydrogens have converged to a standard crystallographic residual of 4.65 % for the observed reflections.[4]

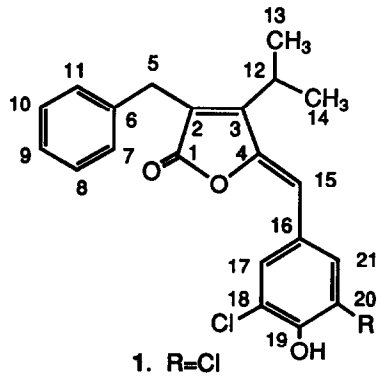
The computer generated perspective of the resulting structure of **1** is shown in Fig. 1. The right-hand half of the molecule is planar, and the left-hand phenyl ring is rotated out of this plane, i.e. the C1-C2-C5-C6 torsional angle is -90° The C4-C15 double bond has the Z configuration - the configuration that minimizes the interaction between the isopropyl and dichlorophenol fragments - while the C4-C15-C16 angle opens to 130° to relieve crowding. The basic skeleton, which can be divided into two phenylpropanoid moieties and one isoprene unit, is probably biosynthesized by the condensation of phenylalanine, tyrosine and mevalonate or an amino acid such as valine.

Table 1. ^1H and ^{13}CNMR Data of Nostoclide I, **1** and Nostoclide II, **2** (δ in CD_2Cl_2).

	H/C #	1	2
^1H	5	3.80 (*s*)	3.79 (*s*)
	7-11	7.21-7.32 (*m*)	7.18-7.32 (*m*)
	12	3.14 (*sept*, J=7.2)	3.14 (*sept.*,J=7.1)
	13, 14	1.30 (*d*, J=7.2)	1.31 (*d*, J=7.1)
	15	6.02 (*s*)	6.09 (*s*)
	17, 21	7.73 (*s*)	7.83 (*d*, J=2.1)
	20		7.03 (*d*, J=8.5)
	21		7.61 (*d,d*, J=8.5, 2.1)
^{13}C	1	170.4	170.7
	2	148.1	147.3
	3	138.7	138.8
	4	148.5	152.2
	5	30.2	30.1
	6	130.4	131.3
	7-11	127.0-129.1	127.0-129.1
	12	27.0	27.0
	13, 14	21.8	21.8
	15	107.6	108.9
	16	126.4	127
	17	130.4	131.3
	18	122	121
	19	158.1	158.3
	20	122	117
	21	130.4	125.7

The compound **2** forms yellow prisms, mp.132-133° , from MeOH. It has a molecular formula, $C_{21}H_{19}ClO_3$ (obsd. m/z 354.1031; calcd. m/z 354.1023), and UV absorption maxima, λ_{max} (MeOH)

nm (ε): 241.3 (12,255), 355.4 (28,595), which shift to 258.7 (14,216) and 418.5 (31,863) upon addition of NaOH. The ^{1}H- and ^{13}H-NMR spectra (Table 1) are very similar to those of **1**, except for the signals for *p*-hydroxyphenyl moiety, which show the typical proton coupling pattern of 1,2,4-tri-substituted benzene. The chemical shifts of C-20 and C-21 are also shifted to higher field. Thus the compound was determined as the 20-dechloro analogue of **1**.



1. R=Cl
2. R=H

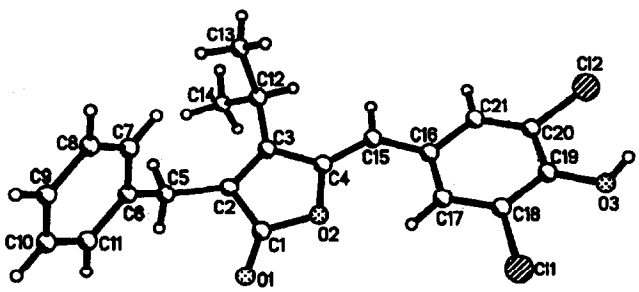


Figure 1. A computer generated perspective drawing of the final X-ray model of **1**.

Both compounds, **1** and **2** showed moderate cytotoxicity (LC_{50}: 10 μg/ml) against the cell lines, Neuro-2a CCL 131 and KB CCL17. Although other biological actions of these compounds are still under investigation, the compounds seem to be allelopathic agents. In fact, this organism was first noticed to sustain unusually clean, contamination-free culture. Hapaloindoles[5,6] from *Haplosiphon fontinalis* and cyanobacterin[7,8] from *Scytonema hofmanni* are noted as allelochemicals in blue-greens. Especially, cyanobacterin has a structural resemblance to nostoclides. What is special about nostoclides is that they are extra-cellular metabolites, which are excreted into the medium in enormous quantities.

There are a number of reports on the symbiosis and metabolism of *Peltigera canina*.[9] However, only common lichen substances such as gyrophoric acid and tenuiorin were isolated from the thalli of the lichen,[10] and no substance, which resembles nostoclides, has been reported.

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