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Short communication

Cynthichlorine: a bioactive alkaloid from the tunicate *Cynthia savignyi*

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Abstract

From ether extracts of the tunicate *Cynthia savignyi*, collected in Morocco, a new alkaloid – cynthichlorine – has been isolated. The structure of cynthichlorine has been characterized by extensive 2D-NMR data. Cynthichlorine possesses antifungal activity against two tomato pathogenic fungi: *Botrytis cinerea* and *Verticillium albo atrum* and antibacterial activity against *Agrobacterium radiobacter*, *Escherichia coli* and *Pseudomonas aeruginosa* and cytotoxicity against *Artemia salina* larvae.

Keywords: Cynthichlorine; *Cynthia savignyi* antibacterial; antifungal; cytotoxicity

1. Introduction

Marine tunicates are a very rich source of nitrogenous secondary metabolites, and particularly of peptides and alkaloids [1–3].

In the course of our continuing investigation of tunicates for the presence of bioactives metabolites, we observed that the crude ether extract of the tunicate *Cynthia savignyi* possesses antifungal activity against two tomato pathogenic fungi: *Botrytis cinerea* and *Verticillium albo atrum*, antibacterial activity against some gram (+) and gram (−) bacteria and cytotoxicity against *Artemia salina* larva.

Since preliminary experiments indicated that most of the cytotoxicity was associated with fractions containing alkaloid derivatives, these were examined in detail and a new natural alkaloid was isolated.

2. Experimental

2.1. General procedures

NMR spectra were recorded on a JEOL EX 400 spectrometer. IR spectra were recorded on Perkin–Elmer FT-IR spectrometer.

2.2. Collection, extraction and separation

The tunicate *Cynthia savignyi* was collected between 0 and 40 m deep by SCUBA diving or by dredging in the Roscoff area (Atlantic sea, Aïn sbaâ, Casablanca, Morocco).

The specimens, cut into small pieces, were placed in $CHCl_3$/EtOH (5/5) for extraction. The $CHCl_3$/EtOH extracts of freshly collected tunicates were filtered and the resulting filtrates were concentrated by evaporation under reduced pressure. The residual solution was then extracted successively with hexane, ether, dichloromethane and methanol.

Chromatography of the ether extract (7 g) on silica-gel 60 Merck (0.063–0.200 mm) with a mixture of ether/methanol (80/20) gave a fraction (838 mg) containing cynthichlorine. This was purified by HPLC (Waters) on

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RP18 column with a mixture of Acetonitrile/Water (60/40), Cynthichlorine represent 2.1% of ether extract.

2.2.1. Cynthichlorine

Pale yellow solid; UV (MeOH) λ_{max} (log ε) 224 (4.13), 262 (3.64), 316 (3.06) nm; IR ($CHCl_3$) λ_{max} 3435, 3208, 3109, 2951, 2852, 1763, 1744, 1620, 1590/cm; ^1H ET ^{13}C-NMR data, in $CDCl_3$ see Table 1; ^1H NMR data in C_3D_6O: δ 3.45 (1H, d, $J = 17$ Hz, H-8a), 3.57 (3H, s, H-10), 3.71 (1H, d, $J = 17$ Hz, H-8b), 7.46 (1H, d, $J = 2$ Hz, H-4), 7.64 (1H, d, $J = 2$ Hz, H-6), 10.70 (1H, bs, NH); HREIMS m/z 306.9594 [M^+], Calc. for $C_{11}H_8NO_3^{35}Cl_3$ 306.9570; EI MS m/z (rel. int.) 311 (18), 309 (58), 307 (59), 272 (16), 264 (29), 262 (24), 250 (26), 248 (28), 232 (55), 230 (100), 215 (60), 213 (98), 187 (50), 185 (79), 152 (22), 150 (67), 125 (29), 123 (87).

2.3. Antibacterial test

Antibacterial assays were conducted using the standard disc-diffusion assay [4]. Product was applied to 6 mm sterile discs in aliquots of 30 µl of solvent, allowed to dry at room temperature, and placed on agar plates seeded with microorganisms. The bacteria were maintained on nutrient agar plates and incubated at 37 °C for 24 h. Zones of growth inhibition, if any, were measured following incubation. Product was assayed twice at a concentration of 30 µg/disc.

2.4. Antifungal test

2.4.1. Mould species

The strain *Botrytis cinerea* was isolated from infected stalk attacked plants (tomato) harvested in the region of Casablanca (Morocco). *Fusarium* and *Verticillium* strains were isolated from soil collected around the roots of attacked plants. All the isolates were grown on PDA or on Czapeck-Dox broth with nitrate. Incubation was done at 25 °C in dark for 29 days.

Table 1
NMR spectral data of cynthichlorine

No.	δ^1H (m, J in Hz)	$\delta^{13}C$ [a]	HMBC correlations
1	8.24 (br.s, 1H)		C-2
2		173.6	
3		60.4	
3a		131.8	
4	7.22 (d, 1H, 2)	123.3	C-3, C-5, C-6, C-7a
5		129.0	
6	7.26 (d, 1H,2)	130.5	C-4, C-5, C-7, C-7a
7		116.4	
7a		137.8	
8a	3.33 (d, 1H, 17)	42.7	C-2, C-3, C-9
8b	3.43 (d, 1H, 17)		C-2, C-3, C-3a, C-9
9		168.6	
10	3.50 (s, 3H)	52.6	C-9

[a] Assignments were made by DEPT and HMQC.

2.4.2. In vitro inhibition assays

The in vitro assays to study the inhibition were carried out according to the method described by Benhammou [5]. Fifty microgram of extracts were applied to 5 mm sterile discs, incorporated in the culture media and poured in petri plates. Concentrations of 0.1, 0.5, 1, 1.5 and 2 mg/ml were used. The poured plates were allowed to solidify and spot inoculated with the mould strains to be tested. The inhibition was evaluated by the mycelium reduction around the spot inoculation.

2.5. Cytotoxicity test

Samples were prepared by dissolving extracts in DMSO. Brine shrimp eggs were hatched in a shallow rectangular dish filled with artificial sea water which was prepared with a commercial salt mixture and double-distilled water. After 24 h the phototropic nauplii were collected by pipette from the lighted side, having been separated by the divider from their shells. Ten shrimps were transferred to each sample vial. The nauplii can be counted macroscopically in the stem of the pipette against a lighted back-ground.

The vials were maintained under illumination. Survivors were counted, after 24 h, and the percent deaths at each dose and control (solvent) were determined [6]. LD_{50}'s was determined from the 24 h counts. The LD_{50} was derived from the best fit line obtained by linear regression analysis, after transforming dose–response data into a straight line by means of a logarithmic transformation.

3. Results and discussion

Cynthichlorine was isolated as a pale yellow. The IR spectrum showed absorption bands characteristic of cyclic amide and ester functional groups (3435, 3208, 1763 and 1744/cm). UV absorption maxima at 241, 262 and 317 were compatible with an oxindole chromophore [7]. The EI MS spectrum displayed a typical isotopic cluster at m/z 311 (18), 309 (58) and 307 (59) pointed to the presence of 3 chloride atoms in the molecule. This was supported by HREIMS analysis which provided the molecular formula $C_{11}H_8NO_3Cl_3$, indicative of 7° of insaturation Fig. 1.

The ^1H NMR spectrum showed the following significant signals (Table 1): two aliphatic methylene protons (AB system) at δ 3.33 and 3.43 ppm, one methyl group at δ 3.50 ppm, two doublet aromatic protons characteristic of a 1,2,3,6-tetrasubstituted benzene ring at δ 7.22 and 7.26 ppm ($J = 2$ Hz) and one exchangeable NH proton of the amide group at δ 8.24

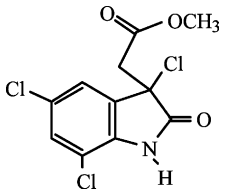

Fig. 1. Structure of cynthichlorine.

ppm. A combination of ^{13}C and DEPT-NMR spectra showed eleven carbon signals including one methyl, one methylene, two methine and seven quaternary carbons.

The ^{13}C chemical shifts and HMQC data confirmed the presence of a tetrasubstituted benzene ring including two methine at δ 123.3 (C-4) and 130.5 (C-6) and four quaternary sp^2 carbons at δ 131.8 (C-3a), 129.0 (C-5), 116.4 (C-7) and 137.8 (C-7a). The upfield chemical shift of C-7 was consistent with the allocation of an azote atom in β-position. Consequently, Cynthichlorine was determined to be an oxindole derivative. It was confirmed by the observation of a long-range correlation between aromatic proton at δ 7.22 (H-4) and sp^3 quaternary carbon at δ 60.4 (C-3) in the HMBC spectrum.

To account for the molecular formula $C_{11}H_8NO_3Cl_3$ of cynthichlorine together with its ^{13}C data the oxindole moiety should be substituted by three chlorine atoms positioned at C-5 and C-7 sp^2 quaternary carbons of the benzene ring and at C-3 sp^3 carbon of the pentacyclic amide. Heteronuclear long-range correlations indicated that the three-proton singlet at δ 3.50 ppm and the ^{13}C signals at δ 168.6 and 52.6 ppm were attributable to a carbomethoxy group, in agreement with the ester absorption bands observed in the IR spectrum and the presence in the mass spectrum of a fragment ion at *m/z* 248 corresponding to the loss of CO_2CH_3 radical from the molecular ion at *m/z* 307. Furthermore, HMBC correlations between the non-equivalent aliphatic methylene protons attached on C-8 (HSQC) and carbons at δ 168.6, 60.4 and 131.8 ppm resulted in the assignment of a carbomethoxymethyl group linked to sp^3 quaternary carbon C-3 of the oxindole moiety.

On the basis of the above data, the structure of cynthichlorine was established as 3,5,7,-trichloro-3-carbomethoxymethyloxindole.

The structure was confirmed by comparison of ^1H NMR data of **1** in acetone-d_6 with those reported for the synthetic product obtained from the chlorination of 3-methylindolyl methylester [8].

3.1. Antibacterial assays

Antibacterial activity of cynthichlorine was measured as the radius of the zone of inhibition around the disc (Table 2).

Table 2
Antibacterial activity of cynthichlorine

Bacteria	Zone of inhibition (mm)
A. radiobacter	8
E. coli	10
S. aureus	0
P. aeruginosa	7

In the same way, we have evaluated tetracycline activity against these bacteria. Inhibition zones are, 21, 13, 20 and 17 mm, respectively against *Agrobacterium radiobacter*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*.

3.2. Antifungal test

The in vitro inhibition assays on solid media by incorporating cynthichlorine in the medium with moulds isolates showed a zone of inhibition around the spot inoculation. This zone was measured after 2 weeks incubation on five different sides.

Results reported in Table 3 showed an inhibitory action on the mycelium development of the strains.

Differences in the action on the three mould isolates were observed. The most sensitive strains was *Verticillium albo atrum*.

3.3. Cytotoxicity test

Cynthichlorine has been tested at 10, 30, 50 and 100 μg/ml. LD_{50} value is 48.5 μg/ml (Table 4).

Cynthichlorine is cytotoxic against *Artemia salina* larva, but less cytotoxic than podophyllotoxin (LD_{50} =

Table 3
Zone of inhibition after 15 days of inoculation

Moulds	Zone of inhibition (mm)
Botrytis cinerea	6
Fusarium oxysporum	0
Verticillium albo atrum	7
White temoin	0
Solvent	0

Table 4
Cytotoxicity of cynthichlorine against *Artemia salina* larva

[*C*] (μg/ml)	% of death
10	10
30	40
50	50
100	64

2.4 μg/ml) and more toxic than digitalin ($LD_{50} = 151$ μg/ml) and caffeine ($LD_{50} = 306$ μg/ml).

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