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Il Farmaco 55 (2000) 492–494

IL FARMACO

Short Communication

Isolation and bioactivities of epidioxysterol from the tunicate *Cynthia savignyi*

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Received 20 September 1999; accepted 28 March 2000

Abstract

From a hexane extract of the tunicate *Cynthia savignyi*, collected in Morocco, epidioxysterol or 5,8-α-epidioxy-5α-cholest-6-en-3β-ol has been isolated. This is the first example of epidioxysterol found in the tunicate *C. savignyi*. The structure of epidioxysterol has been characterised by NMR data (^{1}H, ^{13}C and 2D). Epidioxysterol possesses antifungal activity against three tomato pathogenic fungi: *Botrytis cinerea*, *Fusarium oxysporum* and *Verticillium albo atrum* and antibacterial activity against *Agrobacterium tumefaciens*, *Escherichia coli*, *Staphylococcus faecalis*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* and cytotoxicity against *Artemia salina* larvae.

Keywords: *Cynthia savignyi*; Epidioxysterol; Antibacterial; Antifungal; Cytotoxicity

1. Introduction

Several species of the tunicates are widespread in the Atlantic and Mediterranean sea, but very few of them have been reported to contain antifungal active compounds [1–3].

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

Since preliminary experiments indicated that most of the activity was associated with fractions containing steroid derivatives, these were examined in detail and an antimicrobial compound epidioxysterol was isolated.

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2. Experimental

2.1. General procedures

NMR spectra were recorded on a Jeol EX 400 spectrometer.

2.2. Collection, extraction and separation

The tunicate *C. 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 hexane extract (5 g) on silica gel 60 Merck (0.063–0.200 mm) with a mixture of

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PII: S0014-827X(00)00040-9

dichloromethane–methanol (95/5) gave a fraction (102 mg) containing epidioxysterol. This was purified by thick layer chromatography with silica gel (1 mm thickness) and mixture of isooctane–ethyl acetate (8/2), epidioxysterol represent 1.3% of hexane extract.

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 $disc^{-1}$.

2.4. Antifungal test

2.4.1. Mould species

The strain *B. cinerea* was isolated from infected stalk attaked plants (tomato) harvested in the region of Casablanca (Morocco). *Fusarium* and *Verticillium* strains were isolated from soil collected around the roots of attaked 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.

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]. Extracts (50 μg) 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. Toxicity test

Samples were prepared by dissolving extracts in DMSO. Brine shrimp eggs were hatched in a shallow rectangular dish filled with artificial seawater, 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} values were 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.

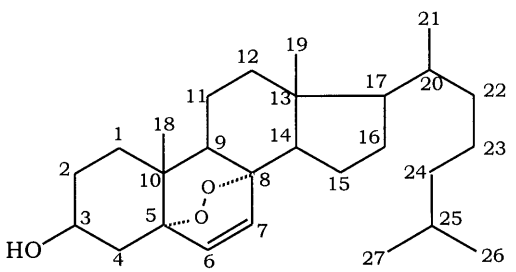

Fig. 1. Structure of epidioxysterol.

3. Results and discussion

The 400 MHz NMR spectra of the natural epidioxysterol was very informative and compatible with the proposed structure. The ^{13}C NMR chemical shift values of epidioxysterol, except for the $C_{11,}$ C_{14} and C_{15} carbons and the B-ring moitey, are almost superimposable with those of the authentic product, the isolation and

Table 1
^{1}H and ^{13}C data of epidioxysterol

Atom no.	^{13}C NMR	^{1}H NMR		
	δ (ppm)	δ (ppm)	Multiplicity	J (Hz)
1	34.81	1.66	m	
2	30.31	1.53	m	
3	66.61	3.96	m	
4	37.07	1.88	m	
5	82.31	–		
6	135.51	6.21	d	8.5
7	130.91	6.46	d	8.5
8	79.56	–		
9	51.28	1.45	m	
10	37.07	–		
11	23.51	1.49	m	
12	39.51	1.96	m	
13	44.86	–		
14	51.75	1.53	m	
15	20.71	1.39	m	
16	28.31	1.31	m	
17	56.61	1.15	m	
18	12.71	0.77	s	
19	18.31	0.85	s	
20	35.31	1.32	m	
21	18.71	0.88	d	6.5
22	36.07	1.28	m	
23	23.91	1.33	m	
24	39.51	1.09	m	
25	28.11	1.48	m	
26	22.61	0.84	d	6.6
27	22.91	0.84	d	6.6

Table 2
Antibacterial activity of epidioxysterol sterol

Bacteria	Zone of inhibition (mm)
Agrobacterium tumefaciens	18
Escherichia coli	5
Staphylococcus faecalis	6
Staphylococcus aureus	12
Pseudomonas aeruginosa	13

Table 3
Zone of inhibition after 15 days of inoculation

Moulds	Zone of inhibition (mm)
Botrytis cinerea	9
Fusarium oxysporum	15
Verticillium albo atrum	7

Table 4
Toxicity of epidioxysterol against *Artemia salina* larva

[C] μg/ml	% of death
10	20
30	25
50	42
100	63

the structure elucidation of which have already been reported in the preceding papers [7] (Fig. 1).

The chemical shifts of carbons in positions 6 and 7, which appears at δ 135.51 and 130.91, respectively are inverted. As well as which are in position α of the double bond (C_5 and C_8). The same notice was observed for C_{11}/C_{15} and C_9/C_{14} carbons.

In fact, the use of 2D NMR (HMQC and HMBC) experiments as well as the high resolution (400 MHz), which had not used in anterior studies [7], have allowed to attribute different protons and carbons of epidioxysterol structure without any ambiguity (Table 1).

3.1. Antibacterial assays

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

In the same way, we have evaluated tetracycline activity against these bacteria. Inhibition zones are 21, 13, 20 and 17 mm, respectively against *A. tumefaciens*, *E. coli*, *P. aeruginosa* and *S. aureus*.

3.2. Antifungal test

The in vitro inhibition assays on solid media by incorporating the epidioxysterol 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 strain was *B. cinerea*, which showed higher zones than the other strains.

3.3. Toxicity test

Epidioxysterol has been tested at 10, 30, 50 and 100 μg/ml. The LD_{50} value is 71.49 μg/ml (Table 4).

Epidioxysterol is toxic against *A. salina* larva, but less toxic than podophyllotoxin ($LD_{50} = 2.4$ μg/ml) and more toxic than digitalin ($LD_{50} = 151$ μg/ml) and caffeine ($LD_{50} = 306$ μg/ml).

Acknowledgements

Financial support of this work by the Centre National de la Coordination et de la Planification de la Recherche Scientifique et Technique (CNCPRST) is gratefully acknowledged. We thank Mr M. CHLAIDA (Departement of Biology) for the identification of *Cynthia savignyi*.

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