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Note

Isolation of an antimicrobial bromoditerpene from a marine alga aided by improved bioautography

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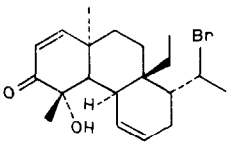
Bioautography is a technique familiar to microbiologists in the search for antibiotics from microorganisms and different procedures have been used to improve its performance[1–3]. Most of the published procedures are based on the "contact" technique where the antibacterial compound is transferred from the chromatographic layer to an inoculated agar plate through a diffusion process. These procedures suffer several disadvantages such as low sensitivity, spurious inhibition of the contact area and are not suitable for water-insoluble compounds. Recently, a "direct" bioautographic detection on the chromatographic layer which makes use of an appropriate device for spreading the agar on the plate has been published[4].

We describe here a simple "contact" bioautography assay to isolate easily a strong antimicrobial bromoditerpene, sphaerococcenol A, from the marine red alga *Sphaerococcus coronopifolius:*



I

Although the compound and the extract were water-insoluble, it was possible to perform bioautography by improving the diffusion process that takes place through the contact of the chromatographic layer with the inoculated agar plate.

Compound I was isolated from material collected in Spain[5] and Yugoslavia[6] but not from *S. coronopifolius* collected in the bay of Naples and Sicily from which other diterpenoids were isolated[7,8]. However, it was reported that a lipophilic extract of this species collected along the eastern coast of Sicily exhibited good *in vitro* antimicrobial activity[9]. We show here, through its isolation aided by bioautography, that compound I is the major component responsible for that activity.

In the disc-diffusion agar test, sphaerococcenol A exhibited a strong antibacterial activity against *Bacillus subtilis* and *Escherichia coli* and against the fungus *Phoma tracheiphila* which causes a destructive disease of *Citrus* trees in the Mediterranean region.

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EXPERIMENTAL

TLC plates and conditions

Commercially available 20 cm × 20 cm thin-layer chromatographic (TLC) aluminium sheets (layer thickness 0.2 mm) precoated with silica gel 60 were obtained from Merck (Darmstadt, F.R.G.). They were divided into sizes of 10 cm × 5 cm. Before use, the plates were washed with the elution solvent in the developing tank. About 20 or 50 μg of methanolic solution containing 1 mg/ml of compound I or the extract were applied to the TLC plates to form spots. Camag Microcap micropipettes were used for sample application. The spot size was minimized by applying the sample volume in small increments on top of each other, with complete drying of the solvent after each application. Chromatography was effected by using a 10 cm × 10 cm twin-trough chamber (Camag, Muttenz, Switzerland), preequilibrating the layer with the vapours of the elution solvent and then developing up to 9 cm. The plates were thoroughly dried in a stream of air.

Preparative TLC was performed on 20 cm × 20 cm glass supported plates (thickness 0.5 mm, Merck) precoated with silica gel 60. The extract was applied as an acetone solution using a laboratory-made 2-ml dispenser. The chromatography was performed in 20 cm × 20 cm twin-trough Camag chambers with the same precautions as in analytical TLC. All solvents were of analytical grade and mixtures were made up on a v/v basis.

Chemical visualization

A solution of 1% ceric sulphate in 1 *M* H_2SO_4 was used. Intensification of the different colours of the spots was obtained by heating in an oven at 115°C for 10 min. Visualization on the preparative TLC plates was done only at an edge, the remaining silica layer being protected with a clean glass plate.

Culture medium and microorganisms

For bioautography, antibiotic medium No. 1 (Difco, Detroit, MI, U.S.A.) was dissolved in a phosphate saline buffer at pH 7.3 (ref. 10) and sterilized by autoclaving at 121°C for 15 min. After cooling at 45°C, 1 ml of *Bacillus subtilis* ATCC 6633 spore suspension (Difco) or *Escherichia coli* strain B ATCC 11303 inoculum (Sigma, St. Louis, MO, U.S.A.) was added to 200 ml culture medium. Before congealing, 15 ml of the inoculated agar were added to sterile 15-cm glass Petri plates, swirling carefully.

In addition to the above bacteria, the fungus *Phoma tracheiphila* was used in the disc-diffusion agar plate tests. The microorganism was obtained from the Institute of Plant Pathology of the University of Catania as a 12-days-old pycnidiospores suspension.

Plant material

S. coronopifolius (fresh weight 2 kg) was collected at a depth of 4–5 m at Castelluccio, eastern Sicily in September 1988. A voucher specimen is deposited in the Herbarium of the Algology Laboratory of the Institute of Botany of the University of Catania.

RESULTS AND DISCUSSION

The fresh alga was immediately soaked in isopropanol. The solution was concentrated to give a residue that was partitioned between a 1 *M* sodium nitrate solution and diethyl ether. The ether layer yielded, after desiccation and concentration, 1.91 g of deep green semisolid extract which was strongly active against *B. subtilis* and *E. coli* in the disc-diffusion agar plate test.

Silica gel TLC [dichloromethane–light petroleum (b.p. 40–70°C), 6:4] of the extract showed, after visualization, about 15 differently coloured spots. However, a much simpler pattern was observed by bioautographic detection. Only the spots at R_F 0.32 and 0.44 were antimicrobially active as well as the origin spot. This selectivity was a guide for the preparative TLC.

However, practical difficulties were encountered using the classical bioautographic procedure. The use of glass-supported TLC plates is not advisable since air-bubbles form easily. The interposition of a sheet of lens-tissue paper, as is usual, prevents the bubbles and avoids the detachment of a portion of the silica layer when the TLC plate is removed from the agar, but strongly decreases the sensitivity. Thus, the use of aliminium-supported plates is crucial.

These plates can be slightly bent longitudinally in the middle and placed carefully on the agar layer in the Petri dish. In this way, wetting of the silica gel layer proceeds homogeneously in 20–30 s from the middle to the edge of the plate, avoiding trapping of air-bubbles between the agar surface and the thin-layer plate.

Antibiotic medium No. 1 was the culture agar. However, it was prepared in a phosphate saline buffer instead of water. This detail prevents the growth-inhibiting effect of residual solvent or acidic silica sites that causes an initial diffuse inhibition of the contact trace left by the plate on the agar layer. The TLC plate is kept at 4°C for 1 h to allow diffusion from the silica gel into the inoculated agar. It is then gently removed and the bioautographic Petri plate is incubated at 25°C for 14–18 h in sealed plastic bags. Antimicrobial substances are visible on the plates as clear zones without growth of the microorganism.

Although the spaerococcenol A and the extract are insoluble in water, clear inhibition spots have been obtained using moistured agar plates. These, prepared in a few days in advance and stored at 2–8°C, were reequilibrated at 25–30°C to improve their wettability before using for bioautography. In this case an efficient diffusion process takes place from the TLC plate to the agar layer.

The information gained by bioautography led us to a preparative TLC dedicated to the isolation of the active compounds from the extract, avoiding collection of the inactive compounds. Thus, 450 mg of the extract were distributed in four silica gel plates and eluted with the same solvent. The band at R_F 0.32 when scraped off and eluted with diethyl ether gave, after crystallization from *n*-hexane, 35 mg of sphaerococcenol A identified by comparison of its physical (m.p.) and spectral (infrared, mass and ^{1}H nuclear magnetic resonance) properties with those reported in the literature[5,6]. The TLC band at R_F 0.44 gave instead a mixture (8 mg) of two inseparable brominated compounds with molecular weight 462.

Sphaerococcenol A is still detectable by bioautography when 10 μg of it are spotted and eluted on the TLC plate. Its activity in the diffusion test from a 6-mm antibiotics disc to a Müller-Hinton agar plate is (μg applied), mm zone of inhibition:

against *B.* subtilis, (10) 9; streptomycin sulphate as control, (0.15) 15; against *E. coli*, (10) 8; streptomycin sulphate as control, (1.5) 12; against *P. tracheiphila*, (10) 9; filipin as control, (20) 11.

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