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ORIGIN OF BISTRAMIDE A IDENTIFIED IN *LISSOCLINUM BISTRATUM* (UROCHORDATA): POSSIBLE INVOLVEMENT OF SYMBIOTIC PROCHLOROPHYTA

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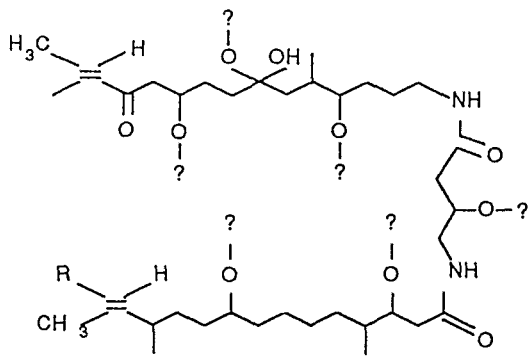
An attempt was made to determine whether bistramide A (=bistratene A), a polycycloether with biological activity isolated from *Lissoclinum bistratum* Sluiter, originates from the ascidian itself or from its symbiont Prochlorophyta. A high-pressure liquid chromatography assay carried out on the whole ascidian, the ascidian without a portion of its *Prochloron* and the *Prochloron* themselves gave concentrations respectively of 0·182, 0·166 and 0·850%. In view of these results, as well as of the capacity of *Prochloron* to synthesize complex nitrogenous products and the cytotoxicity of these products, it is hypothesized that bistramide A originates exclusively in *Prochloron.*

INTRODUCTION

Bistramide A, a diamidopolycycloether isolated from a New Caledonian ascidian, *Lissoclinum bistratum* Sluiter (Gouiffès *et al.*, 1988a; Gouiffès, 1988), is noted for its cytotoxic and antifungal properties (Gouiffès *et al.*, 1988b), its capacity as a cell-differentiation agent (Roussakis *et al.*, 1989) and its inhibitory effect on the sodium channels of skeletal and heart muscle (Verbist *et al.*, 1989).

A different designation (bistratene A) has been proposed for the same substance found recently in a sample of *L. bistratum* from Australia (Degnan *et al.*, 1989). In both cases, the structures described do not seem absolutely certain, and only study by X-ray crystallography is likely to resolve the problem of the positioning of the three ether linkages that cyclicize the molecule (Figure 1).

The difficulty of obtaining the raw material required for extraction of this substance, as well as its structural complexity which makes synthesis uncertain, are current limitations on complete pharmacological and toxicological evaluation. However, the fact that *L. bistratum* is a species harbouring a large quantity of symbiont Prochlorophyta (*Prochloron*) raises the question of the biosynthetic origin of bistramide A. In the event that these microorganisms are the originators of the substance, it may be possible to produce it by *in vitro* culture. Accordingly, we sought to determine in which prochlorophyte and/or ascidian organism bistramide A was present, and at what concentration.



R = CH_3- CHOH - Bistramide A

R = CH_3- CO - Bistramide C

Figure 1. Bistramide A and bistramide C.

MATERIAL AND METHODS

Obtaining the raw material

L. bistratum samples gathered in March 1989 at Nouméa, New Caledonia, were gently squeezed to obtain a portion of the *Prochloron* present. The isolated mass was suspended in buffer (filtered sea water + Hépès 1·2%, to neutralize the acidity excreted by the ascidian when *Prochloron* are released; pH obtained: 7·10) and centrifuged several times (2 min, 800 g) to eliminate animal cells of ascidian origin. These procedures, carried out on the site where samples were gathered, provided for three groups: intact ascidians (IA), ascidians without a portion of their *Prochloron* (SA) and isolated *Prochloron* (P). The three groups were immediately lyophilized and then extracted by dichloromethane, giving the following yields: IA 0·8%, SA 0·9% and P 10·4% (dry weight). At the same time, fraction P was examined under the microscope to be sure that no animal cells were mixed with the *Prochloron*.

Screening and identification of bistramide A

Bistramide A screening was performed on the three extracts by reference to a sample of the pure substance (Gouiffès *et al.*, 1988a), using the following chromatographic systems: (1) thin-layer chromatography (TLC) on silica with ethyl acetate:2-propanol (90:10, v/v) as solvent and 1% vanillin in H_2SO_4 as spray reagent, (2) high-pressure liquid chromatography (HPLC) on Lichrosorb C18 Merck with methanol:water (90:10, v/v) as solvent and (3) HPLC on Lichrosorb Diol Merck with cyclohexane:ethanol (95:5, v/v) as solvent.

Around 3·0 mg of bistramide A were obtained from each extract by semi-preparative HPLC on R Sil C18 Alltech with methanol:water (90:10, v/v) as solvent to determine the infrared (IR) spectra, molecular weight and mass spectra.

Bistramide A assay

Bistramide A was assayed in the three extracts by HPLC using a Kontron 420 pump with a Rhéodyne 7125 injector (10 µl loop), a Merck Hibar Lichrosorb Diol column (5 µm, 250 x 4 mm; cyclohexane:ethanol 95:5, 1·5 ml min^{-1}) and a Kontron 735 LC UV detector (254 nm) coupled to a Kontron recorder-integrator. The reproducibility study of injections showed a standard deviation of 0·84% (N=20) for a mean retention time of 8·8 min and a standard deviation of 1·93% (N=8) for peak areas obtained from the same injected mass.

A standard curve was determined from 10 concentrations of control bistramide, 2 injections per concentration, *i.e.* injected masses of 10-100 ng of bistramide. Processing of the peak areas obtained as a function of the injected mass gave a standard curve determined by linear regression, with a correlation coefficient of 0·999.

The three extracts were injected at concentrations of 10 (IA), 15 (SA) and 25 (P) mg ml^{-1}. The results obtained correspond to the mean of two injections.

Extract cytotoxicity

The cytotoxic activity of extracts (IA, SA, P) and bistramide A was determined on human carcinoma KB cells cultured in 96-hole microplates. Cells (2500) were dropped into each hole containing 0·1 ml of BME medium (Gifco) to which foetal calf serum was added at day 0. Dissolved drugs (0·05 ml in medium with 4% ethanol) were added at day 1 in decreasing concentrations (2 holes for each concentration). Cell growth was estimated by a colorimetric test at 96 h (day 4): MTT [3-(4,5-dimethyltriazol-2-yl)-2,5-diphenyltetrazolium bromide] 0·01 ml was added, and after four hours dark-blue crystals (formed by reduction of MTT through the action of dehydrogenases within the mitochondria of the living cells) were rendered soluble by isopropanol acid. Microplates were read using an ELISA multiskan equipped with a 570 nm filter. Determination of the optical density of each hole then enabled a dose/effect curve to be plotted indicating IC_{50} (inhibiting concentration for 50% of cell growth) for each product relative to controls (8 holes containing 2500 cells in 0·15 ml of medium). The positive control compound was 6-mercaptopurine which had IC_{50} limits of 0·06 to 0·1 µg ml^{-1}.

RESULTS

Identification of bistramide A

Products isolated from extracts IA and SA (ascidians) had the same Rf (TLC, system 1) and retention times (Rt, HPLC, system 3) as reference bistramide A. Co-chromatography of these extracts in the presence of an excess of bistramide A confirmed that the peak studied was actually that of bistramide A previously isolated from this species.

Identical results (Rf and Rt) were obtained with the product isolated from extract P (*Prochloron*). The identity of this product with bistramide A was confirmed by IR spectrometry, mass spectrometry and determination of molecular weights (bistramide A: 704,4989, $C_{40}H_{68}N_2O_8$; product isolated from P: 704,4981, $C_{40}H_{68}N_2O_8$).

Bistramide C was also detected in the three extracts. This product, which had a lower concentration than that of bistramide A, was not measured. It has an IR spectrum very similar to that of bistramide A (Gouiffès, 1988) but a different chromatographic behaviour (analytical selectivity α=1·60 on Diol).

Bistramide A concentration and cytotoxic activity

Bistramide A concentrations relative to the dry weight of the three organisms were 0·182% (IA), 0·166% (SA) and 0·850% (P). The concentration for *Prochloron* was thus 4.6 times as high as that for the intact ascidian (IA).

Bistramide A concentration of the three extracts was 22·4% (IA), 18·4% (SA) and 8·2% (P) and cytotoxic activity (IC_{50} values) were respectively 0·42, 0·51 and 1·20 $\mu g\ ml^{-1}$. The IC_{50} values were thus in relation to bistramide A concentrations and to reference bistramide A activity (IC_{50}: 0·10 $\mu g\ ml^{-1}$).

DISCUSSION

The organisms from which bistramide A has been isolated both in New Caledonia and Australia are symbiotic associations between an ascidian (*Lissoclinum bistratum*) and Prochlorophyta (*Prochloron*). *Prochloron* are chlorophyll-containing prokaryotic organisms which are symbionts of several families of ascidians, particularly the *Didemnidae* (Kott, 1984). They form a new natural division, the Prochlorophyta, created in 1976 (Lewin, 1976) and have recently been the subject of a review (Lewin & Cheng, 1989). Four different types of *Prochloron* have now been described, one of which is supposed to be common to various species of the *Diplosoma*, *Tridemnum* and *Lissoclinum* (*L. bistratum*, *L. patella* and *L. voeltzkowi*) genera (Kott *et al.*, 1984).

In this association, *Prochloron* use the ammonia secreted by the ascidian as a source of nitrogen. In return, a portion (estimated as 20%) of the metabolites synthesized by the *Prochloron* is transferred to the ascidian, which thus benefits from photosynthetic products and a source of nitrogenous organic products (Griffiths & Thinth, 1987; Parry, 1988).

Since it seemed that both the ascidian and *Prochloron* have the capacity to synthesize nitrogenous metabolites and that neither can be excluded *a priori* as the source of bistramide A, it was our intention to screen for this toxin and assay it in both organisms. However, it proved materially impossible to obtain ascidians free of *Prochloron*, although it was possible to identify bistramide A in *Prochloron* and demonstrate that the concentration was markedly higher (4·6 times) than in the ascidian-*Prochloron* association. Our results differ from those of Degnan *et al.* (1989) who failed to find the toxin in isolated *Prochloron* but determined that it was present in the ascidian-*Prochloron* association. They concluded that bistramide A was synthesized by the ascidian alone. It is unlikely that any hypothesis concerning the origin of bistramide A can satisfy these divergent results.

Only the following possibilities seem compatible with our results: either synthesis of bistramide A by the ascidian, with total or partial transfer to *Prochloron*; or synthesis by *Prochloron*, with no or partial transfer to the ascidian.

Several arguments seem to favour synthesis by *Prochloron*, although none is conclusive:

1. There are structural analogies between bistramide A and certain metabolites from other prokaryotes such as cyanophytes and bacteria (Moore *et al.*, 1986; Mundt & Teuscher, 1988; Kogure *et al.*, 1989).

2. Bistramide A is toxic for various types of eukaryotic cells in culture and for several species of invertebrates but innocuous for prokaryotic microorganisms (Gouiffès *et al.*, 1988b). In this respect, it would seem unlikely that *L. bistratum* could tolerate a high concentration of bistramide in its own tissues. Otherwise, it would be necessary to suppose that the ascidian synthesizes and excretes the toxin by specialized cell constituents that prevent any intracellular diffusion, or that the product is synthesized in the form of an inert precursor which only releases the bistramide to *Prochloron*.

3. A simple calculation shows that the presence of 21% of *Prochloron* is sufficient to account for the bistramide A concentration found in the ascidian (dry weights). This level is compatible with that determined by Müller *et al.* (1984) for *Didemnum molle* (24%).

Moreover, the possibility of a transfer of bistramide A from *Prochloron* to the ascidian is questionable since the toxicity of the product (noted above) as well as its steric volume (molecular weight 704) make uptake by the ascidian unlikely after release into the external environment.

Our conclusion is that *Prochloron* are the source of bistramide A and that the product remains in these same cells after elaboration. Confirmation of this hypothesis would require screening for bistramide A in *in vitro* cultures of *Prochloron* from *L. bistratum*. Such cultures are difficult to control as demonstrated by failures in earlier trials with other species (Lewin & Cheng, 1989). Moreover, a successful attempt would probably require the presence of the ascidian itself, or ascidian extracts, since bistramide A production seems specific to the association of *Prochloron* with *L. bistratum*. In fact, the product has not been found either in *L. patella* or *L. voeltzkowi*, both of which harbour *Prochloron* considered identical to those of *L. bistratum*. If such cultures were possible, bistramide A production by this means could then be envisaged.

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