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# In vitro antiviral activity on dengue virus of marine natural products

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**Abstract.** Metabolites isolated from marine invertebrates, callipeltin A **1**, crambescidin **2**, ptilomycalin A **3**, celeromycalin **4**, gymnochrome B **5**, gymnochrome D **6** and isogymnochrome D **7** previously shown bioactive on either herpes simplex virus 1 (**2**, **3**, **4**) or human immunodeficiency virus (**1**, **5**, **6**, **7**), were tested on a new in vitro bioassay using the dengue virus 1. Only gymnochrome D and isogymnochrome D isolated from the living fossil crinoid *Gymnocrinus richeri* are highly potent dengue antiviral agents.

**Key words.** Marine natural products; porifera; echinodermata; antiviral; dengue.

Dengue fever is a human mosquito-born flavivirus infection spread all over the tropical zone. Dengue virus is a member of the Flaviviridae family. There are four distinct antigenetically related serotypes of dengue virus. It is one of the most important viral diseases transmitted by *Aedes aegypti* mosquitoes. Severe forms such as dengue haemorrhagic fever and dengue shock syndrome appeared and were responsible for many lethal cases [1–4].

Unfortunately, there is at present neither prophylaxis nor specific treatment to cure the disease. Thus, we extended our antiviral screening of marine natural products to a new in vitro dengue antiviral bioassay.

The bioactivity of antiviral pure compounds [on human immunodeficiency virus (HIV) or herpes simplex virus (HSV)] isolated from marine invertebrates from New Caledonia was further investigated on dengue viruses: callipeltin A **1**, a new cyclopeptide recently isolated from a lithistid sponge *Callipelta* sp. [5]; crambescidin **2**, ptilomycalin A **3**, celeromycalin **4** – identified as the bioactive components of starfishes *Celerina heffernani* and *Fromia monilis* [6, 7]; gymnochromes **5**–**7**, isolated from the living fossil crinoid *Gymnocrinus richeri* [8] and chemically related to hypericins [9], were previously studied for their antiviral activity in an HIV bioassay (A. Bousseau, unpublished results).

This is, to our knowledge, the first report of the search for bioactive metabolites against dengue viruses.

## Materials and methods

### Dengue virus bioassay

**Extracts and purified compounds.** Purified compounds were obtained from L. Minale (Università degli studi di Napoli Frederico II, Dipartimento di chimica delle sostanze naturali, Napoli) [5–8].

Test solution samples were dissolved in DMSO up to 1% maximum final concentration, and solutions were diluted with culture medium as a stock solution (1 mg/ml).

**Dengue 1 viruses.** The prototype dengue virus serotype 1 strain (Hawaï/1944) was used in this study. Dengue

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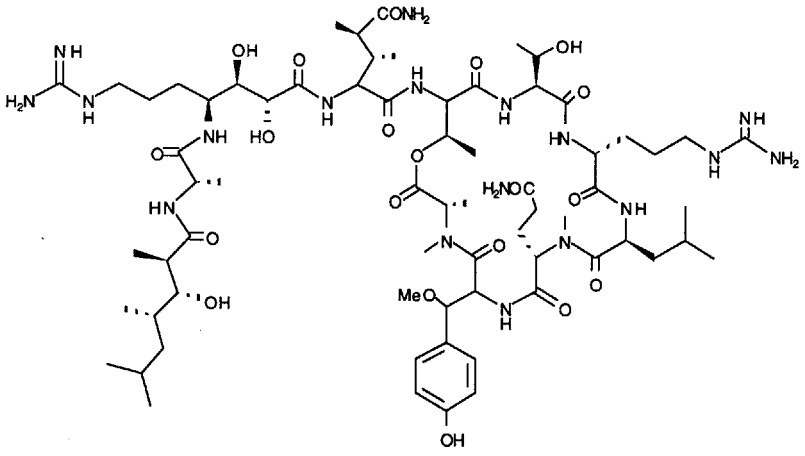
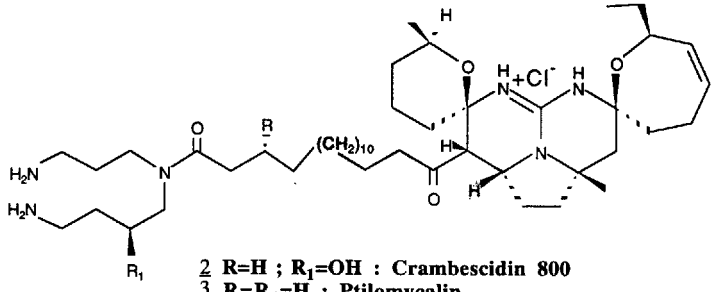
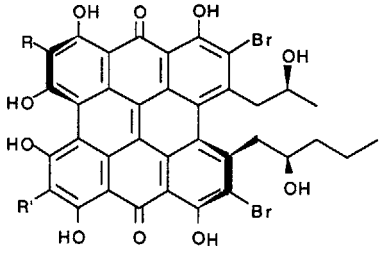


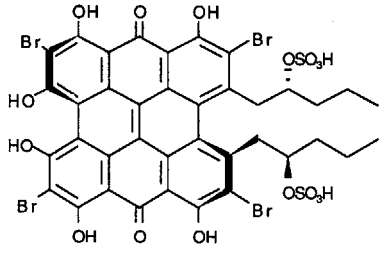
**1 Callipeltin A**



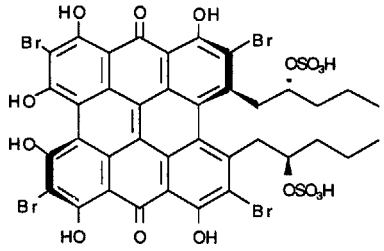
**2 R=H ; $R_1$=OH : Crambescidin 800**
**3 R=$R_1$=H : Ptilomycalin**
**4 R=OH ; $R_1$=H : Celeromycalin**



**5 R=H, R'=Br or R=Br, R'=H**
**Gymnochrome B**



**6 Gymnochrome D**



**7 Isogymnochrome D**

suspensions were prepared in sucking mouse brain (20%, w/v) as antigens, stored at −80 °C until used and titrated as described by Depres et al. [10], but on porcine PS cells propagated at 37 °C in Leibovitz L15 (Gibco) growth medium supplemented with 5% fetal calf serum (FCS). Virus titres are expressed in focus-forming units per ml (FFU/ml) of inoculum.

**Antiviral assay.** A plaque reduction assay on porcine PS cells was done to evaluate antiviral activity from crude and purified extracts [11–13]. The test was performed using 24-well plates (Nunclon, 2 $cm^2$). Confluent monolayer cells were infected with dengue viruses at a multiplicity of infection (MOF) of 0.05 FFU per cell for 1-h incubation at 37 °C allowing viral adsorp-

Table 1. Activity of marine natural products on dengue virus-infected cells.

| | Molecular weight | DV $RF_{50}$%, μg/ml |
|---|---|---|
| Callipeltin A | 1504 | cytotoxic |
| Crambescidine | 800 | inactive |
| Ptilomycaline | 784 | inactive |
| Celeromycaline | 800 | inactive |
| *G. richeri*, crude extract | - | 17 |
| Gymnochrome B | 856 | 1 |
| Gymnochrome D | 1123 | <1 |
| Isogymnochrome D | 1123 | <1 |

tion. Monolayers are then overlaid with a 1.5% carboxymethylcellulose-containing Leibovitz L15 medium supplemented with 3% FCS without (control) or with extracts (test) at different concentrations in quadruplicate cultures. After a 5-day incubation period at 37 °C in the dark, foci of infected cells were visualized by immunological staining according to Okuno et al. [14]. After removal of the medium, cells were briefly washed three times with phosphate-buffered saline (PBS). They were then fixed with 3% paraformaldehyde in PBS for 30 min at room temperature and permeabilized with 0.5% Triton X-100 in PBS for 4 min at room temperature. Enzyme-linked immunosorbent assay (ELISA) was performed using a 1:200 dilution of a hyperimmune mouse ascitic fluid directed against dengue 1 virus for 30 min at 37 °C followed by peroxydase-conjugated goat antimouse immunoglobulin (Biosys) at a 1:200 dilution for 1 h at 37 °C. The peroxydase substrate used was 3,3′-diaminobenzidine tetrahydrochloride (0.05%) in a buffer containing 0.1 M tris-HC1, pH 7.4, and 0.03% hydrogen peroxide. The reaction was stopped by adding 1 vol of 2 N $H_2SO_4$ for 30 min at room temperature. The antiviral activity of the extracts was determined by the reduction of foci (RF) formed by dengue virus 1 compared with controls as follows:

$$RF\ (\%) = (C - T) \times 100/T$$

where C is virus control and T indicates treated with extract.

**Virucid effect (extracellular activity).** When a compound exhibited an antiviral activity ($RF_{50}$), the extracellular activity was performed. In Eppendorf tubes, equal volumes of dengue virus stock (50–100 FFU) and extract solutions with increased concentrations were mixed and incubated at 37 °C for different time laps (30 min, 1 h and 2 h). Confluent monolayers were challenged with these suspensions. Infectious titres were determined, then compared with the control [10–13].

## Results

Among the seven pure compounds tested in this bioassay, only the gymnochromes showed significant activity. Callipeltine was shown to be highly cytotoxic against the PS cells, and the purified starfish compounds were shown to be inactive at doses as low as 10 μg/ml. The gymnochromes inhibited dengue virus–inducted cell lysis, and moreover they were not cytotoxic to the host cells. None showed any virucid effect. Both isomers gymnochrome D and isogymnochrome D exhibited relevant activity (table 1) with a foci-reducing effect at doses lower then 1 μg/ml (<0.89 nM).

## Discussion

Only three compounds of the seven molecules tested – gymnochromes B and D, and isogymnochrome D – showed an inhibition effect of the replication of dengue viruses at a very low concentration, lower for both gymnochrome D and isogymnochrome D. Their antiviral interest is enhanced by their lack of cytotoxicity. Gymnochrome D and isogymnochrome D differ only by their ellipticity, and both compounds bear two sulphated groups, unlike gymnochrome B, whose antiviral activity is lower. It would appear to be worth testing hypericins as well in this bioassay to check the effect of bromine on antiviral activity.

The activity of these gymnochromes enhances the interest of sulphated metabolites isolated from marine organisms that are a rich source of various sulphated antiviral agents [15–17], and are, in our research, the first reported source of antiviral agents against dengue viruses.

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