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Caulerpenyne from *Caulerpa taxifolia*: A comparative study between CPC and classical chromatographic techniques

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ABSTRACT

Caulerpenyne (Cyn) is a cytotoxic compound firstly isolated in 1978 from *Caulerpa prolifera*. This metabolite, constituted by a highly reactive diacetoxybutadiene moiety, exhibited a wide range of biological properties with mainly antibacterial properties and antitumoral activities. Few structure–activity relationships (SAR) are available to design more potent bioactive derivatives by pharmacomodulation. Cyn can be produced by total synthesis or extracted from natural sources in particular the green alga *Caulerpa taxifolia*. Since conventional chromatographic procedures to isolate Cyn from *C. taxifolia* are time- and solvent-consuming, it was crucial to find a more efficient process to obtain pure Cyn. In our study, Cyn has been purified from *C. taxifolia* with two different techniques: Centrifugal partition chromatography (CPC) and a classical chromatographic process. The comparative study showed that CPC constitutes a very simple and efficient process to access Cyn.

1. Introduction

Several seaweeds, in particular green algae, represent a rich source of molecules (Andrade et al., 2013). They contain a high concentration of marine salts, pigments such as chlorophyll or carotenoids (Dambeck and Sandmann, 2014), lipids, sugars (Athukorala and Yuan, 2013), and in some extent mid-polar to apolar bioactive secondary metabolites.

The green alga *Caulerpa taxifolia* (Vahl) C. Agardh has been of a great research interest since its accidental introduction in the Mediterranean Sea in 1984 (Meinesz and Hesse, 1991). The rapid and important proliferation of *C. taxifolia* was attributed to its high temperature tolerance, asexual reproduction, and chemical defenses (Meinesz and Hesse, 1991). Its efficient mechanism of chemical defense relies on the production of several terpenoids (Guerriero et al., 1995) in particular caulerpenyne (Cyn) (Fig. 1). Cyn, a sesquiterpenoid constituted by a reactive diacetoxybutadiene moiety, was shown to be the most abundant metabolite of *C. taxifolia* (Amico et al., 1978), acting as a grazer repulsive (Paul and Fenical, 1986) but also as a wound closure in case of cell disruption (Adolph et al., 2005).

Cyn exhibit a wide range of biological properties with mainly antitumoral activities by inhibiting the growth of several human cancer cell lines (Fischel et al., 1995) and antibacterial properties (Hodgson, 1984). To date, few structure–activity relationships (SAR) studies were performed on Cyn to yield by pharmacomodulation more potent bioactive derivatives for therapeutic purposes. In 2006, Commeiras and co-workers have shown on few Cyn derivatives a tied link between the acetoxybutadiene moiety and their ability to inhibit tubulin assembly (Commeiras et al., 2006). In 2014, Richter and co-workers have shown on Cyn and related *bis*-enols esters that the butadiene moiety was essential for the inhibition of human 5-lipoxygenase (Richter et al., 2014). Further SAR studies are still needed to better understand Cyn and Cyn derivatives biological mode of action which imply an easy access to Cyn in enough amounts.

Cyn was obtained by synthesis or directly from *C. taxifolia.* A few total syntheses of Cyn have been reported (Commeiras et al., 2001, 2003). Unfortunately, they are costly effective as several steps are

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necessary and they lack enantioselectivity. Since *C. taxifolia* is a cultivable green alga which is not an endangered species in the Mediterranean Sea, the best way to obtain enantiopure Cyn remains the direct purification from the species.

In the literature, Cyn was extracted and isolated in one or several steps by using different approaches based mainly on a silica gel (Valls et al., 1994; Guerriero and D'Ambrosio, 1999; Cengiz et al., 2011; McConnell et al., 1982) and size exclusion chromatographies (Amade et al., 2001). These conventional chromatographic methods exhibited several disadvantages such as large solvent volumes and time-consuming procedures. Furthermore, the alga is also composed by several polymers (cellulose, lignin, proteins, and starch), water-soluble monosaccharides (Rabemanolontsoa and Saka, 2013), fatty acids (Ivesa et al., 2004), and pigments such as chlorophyll (Aguilar-Santos, 1970) which often stain irreversibly on the chromatographic support. Moreover, *C. taxifolia* produces in a less extent few others bioactive secondary metabolites such as caulerpin (Aguilar-Santos, 1970) which exhibit a close polarity with Cyn. Chlorophyll and caulerpin often interfere with the isolation of Cyn in a pure state. Finally, Cyn is also a sensitive secondary metabolite which can be easily and rapidly degraded in presence of water (Amade et al., 2001). All these considerations prompted us to develop a more efficient procedure for extraction and isolation of Cyn from natural raw materials.

Centrifugal partition chromatography (CPC) represents a very good alternative to conventional processes despite the instrumental higher costs. CPC is a separation process that relies on a two-phase solvent system made from a pair of immiscible solvents, one used as the stationary phase and the other as the mobile phase (Marchal et al., 2003). This technique is a liquid-liquid partition chromatography that uses the centrifugal force for the retention of the stationary phase (Chollet et al., 2015). CPC exhibits several advantages, compared to classical liquid chromatography techniques using solid supports, such as a higher sample loading capacity and the possibility to inject a relatively crude extracts, a very low solvent consumption, the absence of irreversible adsorption, and a total recovery of the sample without any deterioration. Furthermore, CPC can easily be scaled-up from analytical to preparative scale in rapid and convenient way (Yuan et al., 2008). Several studies have shown that CPC represents a suitable method to obtain pure natural products from terrestrial and marine complex extracts such as essential oils (Wei et al., 2012), lichens (Roullier et al., 2009), and algae (Hwang et al., 2012; Becerra et al., 2015; Lee et al., 2016).

In this paper, we describe a simple, efficient, and scalable CPC method to isolate pure Cyn from *C. taxifolia*.

2. Materials and methods

2.1. Reagents

Cyclohexane (*c*-Hex), *n*-heptane (Hept), ethyl acetate (EtOAc), methanol (MeOH), and dichloromethane (DCM) used for extraction and CPC were purchased from VWR (Rectapur grade). Acetonitrile (MeCN) and water (H_2O) used for high performance liquid chromatography (HPLC) analyses were analytical grade and purchased from VWR (Chromanorm grade). Sephadex LH-20 (GE Healthcare) and Diol-functionalised silica gel (75–200 μm) used for open column chromatography were purchased from Sigma–Aldrich. Thin-layer chromatography (TLC) analyses were performed with silica gel 60 plates (Merck, Silica gel F254).

Fresh *C. taxifolia* were collected from Villefranche-sur-Mer (France) in September 2013 and kept frozen until extraction.

2.2. Instruments

HPLC analyses were performed with a Waters Alliance 2695 HPLC system (Waters Corporation, Milford, MA) coupled with a Waters 996 photodiode array (PDA) detector. Analyses were performed with a bifunctional Macherey-Nagel NUCLEODUR® Sphinx RP column (5 μm, 250 mm × 4.6 mm) consisting of a balanced ratio of propylphenyl and C18 ligands, at room temperature.

Purifications by CPC were performed on a SCPC-250 CPC system (Armen instrument, France) fitted with one rotor containing 800 twin-cells of 300 μL (total volume: 250 mL). The CPC was connected to a preparative HPLC system (Spot prep 2, Armen Instrument France) equipped with a quaternary gradient pump 50 mL/min, an automated loop injection and backflush valves, a UV/Vis PDA detector and a fraction collector, all controlled by the Armen Glider CPC software. The CPC system configuration allows to purify from 0.05 g up to 6 g.

NMR (^{1}H and ^{13}C) analyses were performed in deuterated MeOH (CD_3OD) on a Bruker Avance 500 MHz spectrometer.

2.3. Extraction of C. taxifolia and Cyn quantification

C. taxifolia was freeze-dried before extraction. The dried fronds and stolons (8 g) were crushed and then extracted six times with 200 mL of MeOH–DCM (1:1, v/v). The combined fractions were evaporated under reduced pressure to yield the crude organic extract (1.7 g).

2.4. Cyn isolation by conventional chromatography

The crude extract (1.7 g) was suspended in MeOH and extracted several times with *c*-Hex. The *c*-Hex fractions were evaporated under reduced pressure to yield 128 mg of the Cyn fraction which was subjected to a Sephadex LH-20 size exclusion chromatography (ID 1.6 cm; *h* 32 cm) in MeOH to afford 6 fractions (fr. 1–6). Fraction 4 was further chromatographed on Diol-functionalised silica gel with a gradient of AcOEt–MeOH from 100:0 to 0:100 to give 40 sub-fractions (subfr. 1–40). Subfr. 8 yielded pure caulerpenyne (0.4 mg). The remaining sub-fractions were discarded as Cyn purity by HPLC was less than 50%.

2.5. Cyn isolation by CPC

2.5.1. Selection of the two-phase solvent system

Shake flask experiments were performed to determine the partition coefficient K_{Cyn} of caulerpenyne (Cyn) between the two-phase solvent systems tested to determine a well-suited ($K_{Cyn} \sim 1$) biphasic solvent mixture for separation by CPC. The solvent systems from the Arizona family, in particular Arizona A, N, U, and Z, were screened. They were prepared by mixing at room temperature heptane, water, ethyl acetate, and methanol in different proportions (Foucault and Chevolot, 1998; Berthod et al., 2005). K_{Cyn} was determined by dissolving a suitable amount of the crude extract in 2 mL of the upper (or lower) phase. The solution was shaken with an equal volume of lower (or upper) phase until complete dissolution of the extract. After decantation, the two phases were then separated and the Cyn concentration in each phase was first estimated by TLC and then accurately quantified by HPLC. For each system, K_{Cyn} was calculated by using the following equation:

$$K_{Cyn} = \frac{[C_{Cyn}]_{stat.}}{[C_{Cyn}]_{mob.}} = \frac{A_{stat.}}{A_{mob.}}$$

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