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Short Communication

Determination of caulerpenyne, a toxin from the green alga *Caulerpa taxifolia* (Caulerpaceae)

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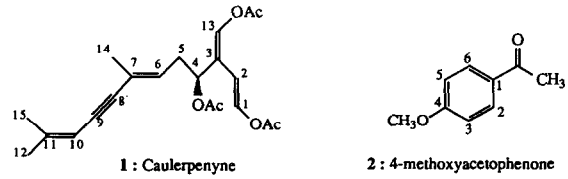
Abstract

A high-performance liquid chromatographic method is described for the determination of caulerpenyne, a neurotoxic sesquiterpenoid isolated from the green alga *Caulerpa taxifolia*. UV and refractometric detection were compared in order to determine the amounts of this toxin in the alga and the alimentary chain. UV detection at 252 nm was more sensitive and could be applied to the determination of very low concentrations of caulerpenyne (the detection limit per gram of dried seaweed was 27 μg/g). The chromatographic conditions adopted were a 5-μm silica column with isocratic binary elution with ethyl acetate–hexane (23:77, v/v) at a flow-rate of 1 ml/min.

1. Introduction

The genus *Caulerpa* has been widely studied and the structures of many new compounds have been described [1–12]. Secondary metabolites isolated from the lipid extract are deterrent or toxic towards fish, sea urchin larvae, bacteria and fungi, and are probably implicated in the defence of algae against predators in tropical regions [1,13]. Amico *et al.* [9] first reported the structure of caulerpenyne (**1**) (Fig. 1) isolated from *Caulerpa prolifera*. This neurotoxic sesquiterpenoid is also present, with some new derivatives, in the tropical alga *C. taxifolia* (Vahl) C. Agardh [1], recently introduced in the Mediterranean Sea [14]. In this work, high-performance liquid chromatography (HPLC) was used in order to prepare and purify **1** and its derivatives (it must be noted that **1** was the major compound of the lipid extract from *C. taxifolia* [1]). However, to our knowledge there has been no attempt to determine precisely the amount of **1** in the alga. Such an investigation could be useful in evaluating the toxicity of this



1 : Caulerpenyne **2** : 4-methoxyacetophenone

Fig. 1. Structures of compounds **1** and **2**.

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species and its variability according to the season of collection and the geographical location. Moreover, the yield and the accumulation of the toxin **1** in the alimentary chain could be quantified.

In this paper, we report a method for the determination of caulerpenyne (**1**) from green seaweed belonging to the Caulerpaceae family using normal-phase HPLC. This work, which can be applied to related compounds characterized by similar UV absorption, completes our previous study on the determination of sterols and diterpenoids from brown algae [15] in the field of sesquiterpenoids from green seaweed.

2. Experimental

2.1. Instrumentation

Separation and purification were carried out on a Model L6000 liquid chromatograph equipped with a mono-pump system (Merck, Darmstadt, Germany). A Merck L4250 variable-wavelength UV–Vis detector set at 252 nm, with a 15-μl flow cell, was used for the analysis of samples with low concentrations of caulerpenyne, whereas a Merck L8810 differential refractometer was used for the detection of samples with high concentrations. Retention times and peak areas were obtained with a Shimadzu (Kyoto, Japan) Chromatopac C-R5A integrator. The column (Interchrom, 250 × 4 mm I.D.) was packed with 5-μm silica (Intersphere ID; Interchim, Montluçon, France) and used at a temperature of 22°C. The flow-rate was 1 ml/min and the injection volume was 20 μl.

2.2. Mobile phase

The solvents were freshly distilled and then filtered and degassed *in vacuo* through a sintered-glass filter. The composition of mobile phase was ethyl acetate–hexane (23:77, v/v).

2.3. Standards

Reference samples for the standardization were commercial 4-methoxyacetophenone (**2**) (Carlo Erba, Milan, Italy), used as an internal standard for both UV and refractive index (RI) detection, and caulerpenyne (**1**), which was isolated directly from a diethyl ether extract of *C. taxifolia*.

2.4. Sample preparation for HPLC analysis

Caulerpenyne (**1**) was extracted and purified form a diethyl ether extract of *C. taxifolia* by normal-phase semi-preparative HPLC using a 5-μm silica column (Interchrom, 250 × 10 mm I.D.) with ethyl acetate–hexane (25:75, v/v) as the mobile phase at a flow-rate of 3 ml/min. The testing of purity was performed using NMR and analytical HPLC. Reference mixtures of caulerpenyne were obtained form stock standard solutions of each standard: 6.35–25.38 mg/l in ethyl acetate for UV detection and about 1000 times more concentrated (2.86–11.44 mg/ml) for RI detection. Calibration was achieved using 4-methoxyacetophenone (**2**) as the internal standard with a stock standard solution of 16.83 mg/l for UV detection and 10.10 mg/ml for RI detection. For the preparation of the reference mixtures, 100 μl of internal standard solution were added to give volumes of each standard solution and diluted to 200 μl with ethyl acetate–hexane (23:77, v/v). Under identical conditions, a diethyl ether extract of the alga [stock standard solution of 106.00 mg of extract per litre and 22.38 mg/l (UV detection) or 11.34 mg/ml (RI detection)] was mixed with 100 μl of internal standard solution and diluted with ethyl acetate–hexane (23:77, v/v). This solution was injected directly into the HPLC apparatus.

2.5. Standardization

Calibration graphs $m_i/m_{is} = f(A_i/A_{is})$, where m_i/m_{is} = sample mass per unit mass of internal standard weight and A_i/A_{is} = sample peak area per unit internal standard peak area, were straight lines (regression lines were obtained from four points). Equations and correlation coefficients (r) are given in Table 1 for UV (252 nm) and RI detection; the internal standard was 4-methoxyacetophenone for both detection methods.

3. Results and discussion

Caulerpenyne (1) was determined by normal-phase HPLC with isocratic binary elution with ethyl acetate–hexane (23:77, v/v). Its retention time is given in Table 1.

Under these conditions, the capacity factor (k') for **1** is 1.53 and the resolution (R_s) between **1** and **2** is 2.53, showing that 4-methoxyacetophenone (**2**) could be used as an internal standard if no secondary compounds with similar retention times (t_R) are present in the studied extracts. Moreover, **2** with UV spectroscopic data ($\lambda_{max} = 262$ nm; molar absorptivity $\varepsilon = 19\,500$) near to those of **1** ($\lambda_{max} = 252$, 265 and 280 nm; $\varepsilon = 33\,100$, 27 900 and 17 000), is suitable as internal standard not only with RI but also with UV detection.

After several preliminary experiments, including gradient elution in combination with UV detection, we selected the binary eluant system ethyl acetate-hexane (23:77, v/v), allowing an optimum separation of the analyte compounds from *Caulerpa taxifolia*. After many experiments at different wavelengths, the wavelength chosen for UV detection was 252 nm. UV detection, which is generally more sensitive than RI detection, was found to be about 500 times more sensitive for **1**. It could be used for the determination of very small amounts of **1** (the detection limit per gram of dried seaweed was 27 μg/g), whereas under the same conditions RI detection could only be used for samples containing high concentrations of **1** (the detection limit value per gram of dried seaweed was 16.6 mg/g).

The standardization of **1** was achieved under these conditions, with **2** as an internal standard for both UV (252 nm) and RI detection. Equations and correlation coefficients for the calibration graphs obtained with standard caulerpenyne are given in Table 1. The standard deviation of residues (R.S.D.) from the regression line of this standard is also given (based on four points).

In the determination of **1**, the accuracy of the method is calculated from difference ($d = m_i - m_i$) between a known mass of the sample studied and the mean of the calculated values from the calibration graph (Table 2). The value of the experimental coefficient ($t_{exp.}$) is lower than the corresponding Fischer coefficient (t) evaluated with a confidence level of 99%. On this basis, the method is not burdened with a systematic error.

Fig. 2 and Table 3 show that the diethyl ether extracts from *C. taxifolia* with a low concentration of **1** can only be analysed using UV detection. The precision given in Table 3 as a percentage with a confidence level of 99% was obtained from four measurements on the same sample.

4. Conclusions

This work was undertaken with two purposes: to determine the seasonal variation of the pro-

Table 1
Retention times and equations of calibration graphs for standard caulerpenyne

Detection method	Retention time (min)		Equation[a]	Correlation coefficient (r)	R.S.D.[b] (%)
	1	2			
UV	9.36	10.23	$y = 1.677\,x - 0.006$	0.999	0.0019
RI	9.36	10.23	$y = 1.863\,x + 0.001$	0.999	0.0016

HPLC with a normal-phase silica (5 μm) column (250 × 4 mm I.D.), eluted with ethyl acetate–hexane (23:77, v/v) at a flow-rate of 1 ml/min and with UV (252 nm) and RI detection. Internal standard, 4-methoxyacetophenone.

[a] $y = m_i/m_{is}$ (sample mass per unit mass of internal standard); $x = A_i/A_{is}$ (sample peak area per unit internal standard peak area). Number of points on the calibration graphs: four ($y = 0.38$, 0.75, 1.13, 1.51 and $x = 0.23$, 0.45, 0.68, 0.90 for UV detection; $y = 0.,28$, 0.57, 0.85, 1.13 and $x = 0.15$, 0.30, 0.46, 0.61 for RI detection). Number of points at each level: four.

[b] Relative standard deviation of the residues.

Table 2
Comparison of a known mass of standard with the calculated value

Detection method	$\bar{m}_i$ (mg)	s (mg)	$d = m_i - \bar{m}_i$	$t_{exp.}$[a]
UV (252 nm)	1.27	0.003	0.004	2.60
	1.90	0.005	0.003	1.20
	2.54	0.005	0.006	2.40
RI	5.74	0.165	0.024	0.29
	8.58	0.159	0.007	0.09
	11.43	0.131	0.011	0.17

$\bar{m}_i$ = Mean mass of sample calculated fro calibration graph; s = standard deviation from four measurements (n); m_i = known mass of sample; $t_{exp.} = d\sqrt{n}/s$; t = corresponding Fischer coefficient for a confidence level of 99%.
[a] Fischer coefficient for a confidence level of 99% *is* $t = 5.84$.

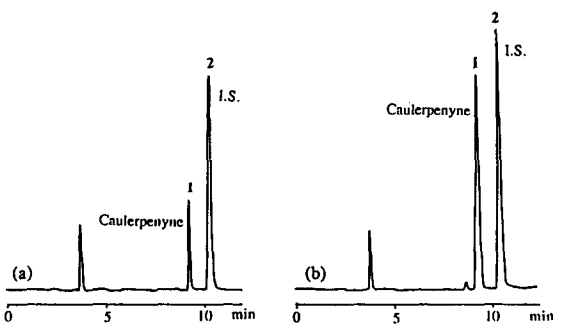

Fig. 2. Examples of HPLC analyses with UV detection of diethyl ether extracts from *C. taxifolia*: (a) extract with a low concentration of **1**; (b) extract with a high concentration of **1**. For experimental conditions, see Table 1.

duction of toxin **1** from *C. taxifolia* recently introduced in the Mediterranean Sea and to follow it in the alimentary chain. A rapid, simple and reproducible method was developed that could be applied to the determination of other sesquiterpenoids from green algae, structurally related to **1**. Moreover, this method is able to analyse precisely either very low-concentration extracts using UV detection (*e.g.*, to determine **1** in the alimentary chain), or high-concentration samples using UV or RI detection (*e.g.*, for seaweed extract analysis).

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Table 3
Composition of extracts originating from the green alga *C. taxifolia*

Detection method	Low concentration of **1** (mg/g)	Precision (%)[a]	High concentration of **1** (mg/g)	Precision (%)[a]
UV (252 nm)	1.06	1.9	20.7	1.5
RI	–[b]	–[b]	18.9	10.0

Concentration of caulerpenyne (**1**) in diethyl ether extracts from *Caulerpa taxifolia*. For both extracts, alga was collected at a 5-m depth at Cap Martin (French Mediterranean coast) in February and May 1993. Results are given in mg/g of dried seaweed. Analyses were carried out in quadruplicate on the same sample.
[a] Precision for a confidence level of 99%.
[b] Not detected.

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