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STIMULANTS AND INHIBITORS OF PSILOSTACHYINOLIDE PRODUCTION IN CALLUS CULTURE

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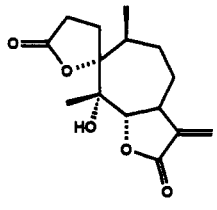
ABSTRACT.—Inhibitors of the primary metabolism of terpenes (KCN, chloramphenicol, cycloheximide, and ferulic acid) added to callus culture of *Ambrosia tenuifolia* completely inhibited sesquiterpene lactone production. Nitrogen sources (adenine, asparagine, arginine and leucine) promoted sesquiterpene lactone biosynthesis. Possible precursors of sesquiterpene lactones from this species (coronopilin [**4**], dihydroparthenolide [**5**] and peruvinic acid [**6**]) when added to the medium resulted in an enhancement of sesquiterpene lactone content in calli, except when incubated with dihydroparthenolide [**5**], where only traces of these products were found.

The plant in vitro culture technique has been successfully used for source material to obtain purified enzymes (1) and secondary metabolites (2–4), with some currently employed as medicines (3), to study biotransformations of supplied metabolites (5,6), and also to elucidate mechanisms that regulate the biosynthesis of terpenoids (7,8).

Due to the significant pharmacological activities displayed by sesquiterpene lactones (9), many attempts have been made to establish in vitro cultures for their production (4). Previous studies have demonstrated that callus cultures of *Ambrosia tenuifolia* Spreng (Compositae), accumulated sesquiterpene lactones in higher levels than the whole plant (10). It was also shown that a variety of factors modified those levels by interfering with sesquiterpene lactone biosynthesis and/or accumulation (3,7), as well as with calli growth (10–12).

A knowledge of product accumulation regulation may be of importance for such methods as genetic engineering to improve secondary metabolite production (3). This paper evaluates the effects of inhibitors, nitrogen sources, and compounds potentially related to the biosynthesis of sesquiterpene lactones from *A. tenuifolia* on the production of psilostachyin [**1**], and psilostachyins B [**2**] and C [**3**] (13).



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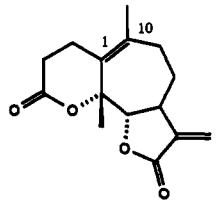

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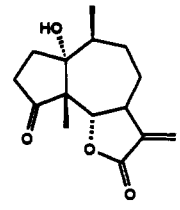

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RESULTS AND DISCUSSION

Callus culture of *A. tenuifolia* treated with inhibitors of the metabolism of terpenes (KCN, chloramphenicol, cycloheximide, and ferulic acid) added in a range of non-toxic concentrations for the tissue, produced total inhibition of sesquiterpene lactone production after 10 days of treatment. The same effect has been observed in tissue cultures of *Rosa damascena* (7), *Jasminum officinale* (14) and *Gardenia jasminoide* (14). The results suggest that primary metabolic processes such as the production of proteins, ATP and NADPH, affected by KCN and ferulic acid (7), decreased the accumulation of sesquiterpene lactones. Inhibitors of cytoplasmatic and chloroplastic protein biosynthesis (cycloheximide and chloramphenicol) may affect the accumulation of sesquiterpene lactones by interfering with the synthesis of enzymes involved in their production (15).

The concentration of sesquiterpene lactones in calli increased in treatments in which a nitrogen source was supplied (Table 1). Illuminated calli containing asparagine (225 mM), arginine (125 mM), leucine (175 mM), or adenine (2.5 mM) produced the highest concentrations of psilostachyin [**1**] (13). The effectiveness of arginine as a stimulant in the production of **1** was related to the presence of light. When treated in the dark, the best results were observed in cultures containing asparagine (125 mM) and adenine (2.5 mM).

The production of psilostachyins B [**2**] and C [**3**] (13) was higher in illuminated calli when arginine and asparagine were supplied (Table 1). Cultures added with arginine (225 mM) yielded mainly **2** and **3**, with traces of **1**. Similar concentrations of **1–3** were found either in the light or in the dark when leucine and adenine were supplied.

Results showed that the biosynthesis of sesquiterpene lactones is stimulated in calli grown under the influence of various nitrogen sources. Amino acids supplied to the medium under conditions that are favorable for the biosynthesis of proteins (16) also favor the production of psilostachyinolides. Sesquiterpene lactone accumulation was also increased by the addition of adenine, a purine reported as an effective nitrogen source

TABLE 1. Effect of Different Nitrogen Sources on Psilostachyinolide Production in Tissue Culture of *Ambrosia tenuifolia*.

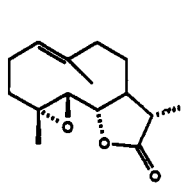
Treatment	Sesquiterpene Lactones (mg/100 g dry wt)			
	Psilostachyin [**1**]		Psilostachyins B [**2**] and C [**3**]	
	A[a]	B[a]	A[a]	B[a]
Control	44±1	74±1	10±2	31±2
Asparagine (mM)				
125	323±2	76±2	306±1	475±1
175	270±1	139±2	139±2	167±1
225	16±1	558±1	154±1	486±2
Arginine (mM)				
125	—	472±1	—	760±1
175	158±1	289±2	501±1	830±1
225	traces	310±1	561±1	398±1
Leucine (mM)				
125	119±1	90±2	300±1	270±1
175	119±1	120±1	270±1	190±1
225	90±1	80±1	9±0	80±1
Adenine (mM)				
1.5	192±2	170±2	478±1	450±1
2.0	289±1	250±1	131±1	120±2
2.5	313±1	300±2	816±1	790±1

[a]The calli were incubated in the dark (A) or in the light (B). Standard errors are recorded.

(17,18), to the Murashige-Skoog media. In fact, dark-grown cultures containing 2.5 mM adenine produced the highest levels of the psilostachyinolides **1–3** (Table 1); hence this may be the best nitrogen source for the production of sesquiterpene lactones.

Compounds related to the biosynthesis of sesquiterpene lactones in *A. tenuifolia* such as coronopilin [**4**] (19), dihydroparthenolide [**5**] (20), and peruvinic acid [**6**] (13) were supplied to the medium to show their effect on the levels of sesquiterpene lactones produced, as well as the ability of the calli to transform them.

Coronopilin [**4**] has been proposed as a likely precursor of psilostachyinolides, because simple reactions may lead to these compounds (21). Compound **4** was added at various concentrations to the Murashige-Skoog medium and incubated during 5 days (treatment A), and 10 days (treatment B) (Table 2). In treatment A an increase of psilostachyinolide concentration was observed when **4** was added at 5 mg/liter, while higher concentrations of substrate inhibited their biosynthesis. In treatment B the opposite effect was observed; a substrate concentration of 15 mg/liter induced the highest accumulation of psilostachyinolides, their concentration being significantly higher than in controls (Table 2).



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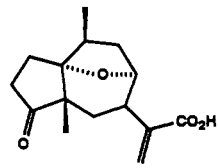

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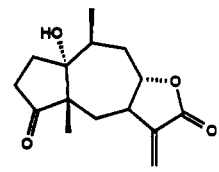

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Previous reports have shown that older calli had lower levels of sesquiterpene lactones (1), as observed by comparing treatments A and B supplied with coronopilin [**4**] (5 mg/liter). Nevertheless, a higher concentration of **4** (15 mg/liter) produced a significant accumulation of sesquiterpene lactones in older calli. These results suggest that coronopilin [**4**] may be incorporated and transformed into different psilostachyinolides. However, further studies must be performed to determine the actual mechanism of action of **4**.

TABLE 2. Effect of Coronopilin [**4**] and Peruvinic Acid [**6**] on Psilostachyinolide Production in Tissue Culture of *Ambrosia tenuifolia*.

Treatment	Sesquiterpene Lactones (mg/100 g dry wt)			
	Psilostachyin [**1**]		Psilostachyins B [**2**] and C [**3**]	
	A[a]	B[a]	A[a]	B[a]
Control	44±1	28±2	10±2	26±2
Coronopilin (mg/liter)				
5	286±1	—	102±1	—
10	34±1	40±1	—	118±2
15	4±2	801±2	3±1	901±1
Peruvinic Acid (mg/liter)				
5	19±1	27±2	45±1	30±2
10	279±1	75±1	389±1	298±1
15	68±1	1±0	246±1	15±1

[a]Content of psilostachyinolides after 5 days (A) and 10 days (B) of incubation at 25° in the dark. Standard errors are recorded.

Dihydroparthenolide [**5**] is thought to be involved in the earlier steps of the biogenesis of sesquiterpene lactones (20). Added to the culture media it exhibited an inhibitory effect and only trace levels of psilostachyinolides, if any, were found in both periods of incubation.

Peruvinic acid [**6**] and its lactone peruvin [**7**] have been isolated from some collections of *A. tenuifolia* (13). The acid **6** was supplied to the culture medium to screen the ability of calli to convert **6** into **7** (13). However, no peruvin [**7**] could be detected in the analyzed extracts after the treatments. Nevertheless, higher levels of psilostachyinolides were found when **6** was added at 10 mg/liter to the culture medium. Additional studies on this or similar model systems may lead to further understanding of biotransformations and the biosynthesis of sesquiterpene lactones.

EXPERIMENTAL

PLANT MATERIAL.—*Ambrosia tenuifolia* Spreng was collected in Reserva Suquía, Córdoba, Argentina in 1988, by N. von Müller, and identified by Dr. L. Ariza. The specimens have been deposited in the Museo Botánico Córdoba [CORD], Córdoba, Argentina.

CALLUS CULTURE.—Explants were excised from subapical leaves and sterilized in 70% EtOH for 3 min and in NaOCl (1% active chloride) for an additional 3 min. They were then placed on sterilized Murashige-Skoog (22) with 1.3% agar medium containing 10 μM of kinetin, 1 μM of 2,4-D, and 10 μM of ascorbic acid and cysteine, and incubated for 2 months in the dark at 25°. Callus tissue was subcultured every 10 days to establish a continuous line of calli (up to 6 to 10 passages). A group of calli was then grown in the dark at 25° for 10 days in Murashige-Skoog medium containing each of the following inhibitors: cycloheximide (5, 10, and 15 mg/liter), chloramphenicol (200, 300, and 400 mg/liter), KCN (2.5, 5, and 7.5 mg/liter) and ferulic acid (2.5, 5, and 7.5 mg/liter). Another set of calli was grown in medium supplied with asparagine, arginine, and leucine (125, 175, and 225 mM) and adenine (1.5, 2, and 2.5 mM). Growth proceeded for 11 weeks at 25°, in the dark or under 12 Wm^{-2} light intensity from Sylvania 400 W mercury lamps. A third group of calli was grown in medium supplied with coronopilin [**4**], peruvinic acid [**6**] (13), and dihydroparthenolide [**5**] (21) (5, 10, and 15 mg/liter) during 5 and 10 days in the dark at 25°. Compounds **5** and **6** were obtained from *A. tenuifolia* according to the given references.

SESQUITERPENE LACTONE ANALYSIS.—Dry calli were extracted with $CHCl_3$, and the solvent was removed under a vacuum at 40° to give a pale yellow oil. Waxes were precipitated by addition of MeOH, extracts were then filtered (Millipore 0.45 μm) and the filtrate directly analyzed by reversed-phase hplc (23).

The sesquiterpene lactone content of the crude $CHCl_3$ extract was determined by peak integration of the hplc elution trace by employing ludartin (24) as an external standard. The analyses were performed in a Konik 500-A liquid chromatograph, detector uv-vis KNK 029-757, $\lambda = 225$ nm; isocratic conditions were set with MeOH-H_2O (1:1), employing a Spherisorb S5 ODS column, 250×4.6 mm plus pre-column 40×4 mm, at a flow rate of 1 ml/min.

Retention times (R_fs) and response factors (R_fs) were determined with standard solutions of authentic samples of sesquiterpene lactones purified from *A. tenuifolia*. Psilostachyin [**1**], and psilostachyins B [**2**] and C [**3**] were indentified in calli extracts by comparing their R_fs with those from the standard solutions and by the enhancements produced in peak heights of the chromatogram after addition of authentic samples to a portion of the analyzed extracts. Compounds **2** and **3** were quantified as mixtures since they could not be resolved by our hplc system. Quantitation was achieved by integration of peak areas and related to the R_fs. The minimum detectable concentration was determined as 3×10^{-6} mg/ml.

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