Phytochemistry, Vol. 28, No. 8, pp. 2093–2096, 1989.
Printed in Great Britain.

0031–9422/89 $3.00 + 0.00

STEREOSTRUCTURES OF TWO BIOLOGICALLY ACTIVE SESQUITERPENE LACTONES FROM *INULA RACEMOSA*

P. S. KALSI,* RITA GOYAL, K. K. TALWAR† and B. R. CHHABRA

Department of Chemistry, Punjab Agricultural University, Ludhiana, India

(*Received in revised form* 12 *December* 1988)

Key Word Index—*Inula racemosa*; Compositae; alantodiene; isoalantodiene; sesquiterpene lactones; eudesmanolides; alantolides; plant growth regulators.

Abstract—Two new sesquiterpene lactones, alantodiene and isoalantodiene, have been isolated from *Inula racemosa*. Both lactones display biological activity as plant growth regulators. Their structures have been elucidated using spectral data and chemical correlation. The most active plant growth regulator yet isolated from this source is isoalantodiene which is a potent root initiator with hypocotyl cuttings of *Vigna radiata* and it also increases the nitrate reductase activity in this plant.

INTRODUCTION

In our search for new plant growth regulators, particularly sesquiterpenes with an α-methylene-γ-lactone moiety, extensive investigations have continued on the hexane extracts from the powdered roots of *Inula racemosa*. We have isolated alantolactone and isoalantolactone [1, 2] as the major crystalline constituents, and also inunal, isoalloalantolactone, isoinunal [3, 4] and several biologically inactive epoxy alantolides [4].

Here we report the stereostructure elucidation of two dienes with alantolide structural features and of these isoalantodiene (**1**) is a particularly potent plant growth regulator, which not only initiates adventitious root formation in the stem cuttings of *Vigna radiata*, but also increases the nitrate reductase activity in this plant.

RESULTS AND DISCUSSION

The hexane extracts from the powdered roots of *I. racemosa* on cooling afforded the known major crystalline alantolides, alantolactone and isoalantolactone. The mother liquor on extensive chromatography yielded biologically active compounds inunal and isoinunal in addition to several other oxygenated alantolides [4]. Further chromatography of the appropriate fractions led to the isolation of two dienes, the most biologically active is the sesquiterpene lactone **1** which we propose to name isoalantodiene.

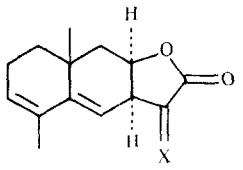
Isoalantodiene, $C_{15}H_{18}O_2$, mp 82° showed characteristic absorption bands at 1760 (γ-lactone), 1650 (trisubstituted double bond) and a strong band at 810 cm^{-1} typical of an α-methylene-γ-lactone moiety [5]. The ^1H NMR spectral features of this diene are in part similar to those of alantolactone (Table 1) but for the fact that the diene displayed a broad three proton singlet at δ1.75 (vinylic methyl) and an additional one proton multiplet at 5.60 (C-3 olefinic proton). These data suggested structure **1** for this compound. Isoalantodiene reacted with diazomethane to give an isomeric mixture of pyrazolines separated by chromatography. The pyrazoline, mp 118°, is assigned stereostructure **4** on the basis of deshielding of 8α-H (δ5.53) compared to **1** where it resonates at δ4.85, because of the *cis*-placement of 8α-H with the –N=N– grouping of the pyrazoline moiety while it is *trans*-placed in pyrazoline (**5**), mp 134°, as no deshielding of 8α-H is observed (Table 1).

Reference to the literature shows that isoalantodiene has an identical stereostructure with 3-dehydroalantolactone [6]. The ^1H NMR spectral data and UV spectra (λ_{max}^{EtOH}, 227, ε7240; lit [6] λ_{max}^{MeOH} 227, ε7245) are also identical. The significant difference is that compound **1** in our hands is a crystalline solid mp 82° while 3-dehydroalantolactone is reported as a liquid. Isoalantodiene was reacted with sodium borohydride to afford dihydroisoalantodiene, mp 51°. The ^1H NMR spectral features of this product were in complete accord with structure **3**, but the mp again differs from that of dihydroisoalantodiene (lit. mp 81°) [7].

Further fractions from the chromatography yielded another liquid sesquiterpene lactone ($C_{15}H_{18}O_2$) named alantodiene. The IR spectral features indicated the presence of an α-methylene-γ-lactone (1760, 1660 and 810 cm^{-1}). Its alantolide nature was established from the typical signal of 8α-H which was displayed as a multiplet at δ4.80. The ^1H NMR spectrum further revealed the presence of a pair of doublets at δ5.77 and 6.43 (1H each, $J = 2.5$ Hz) characteristic of a methylenic group conjugated with the lactonic carbonyl group. These observations coupled with the presence of a sharp three proton singlet at δ0.98 (angular methyl) a three proton broad singlet at 1.83 (vinylic methyl) and a one proton multiplet at 5.83 (characteristic of an olefinic proton on a trisubstituted double bond) indicated that alantodiene is represented by structure **2**. In keeping with the fact that the diene system in alantodiene is in conjugation with the α-methylene-γ-lactone moiety, it showed a red shift in its

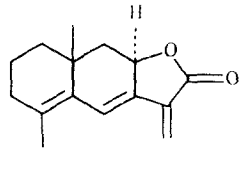
*Part 26 in the series, 'Terpenoid Plant Growth Regulators'.

†Department of Chemistry, Punjab Agricultural University, Ludhiana, India.

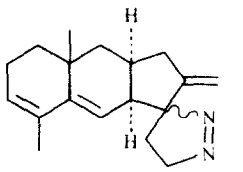


1 X = CH_2

3 X = Me

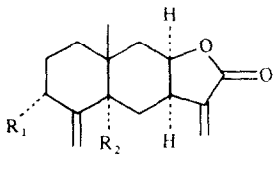


2



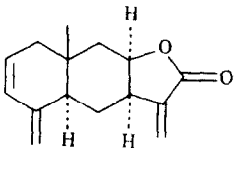
4 α, C-N

5 β, C-N

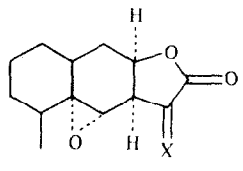


6 R^1 = OH; R^2 = H

7 R^1 = H, R^2 = OH

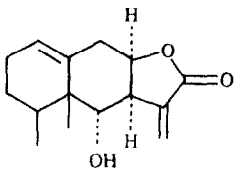


8

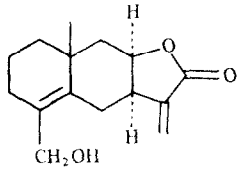


9 X = Me

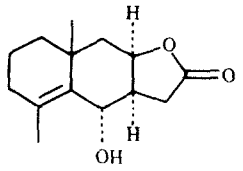
10 X = CH_2



11



12



13

UV absorption (λ_{max}^{EtOH} 265 nm, ε 10,840) compared to that in isoalantodiene (**1**) (λ_{max}^{EtOH} 227 nm).

As these naturally occurring alantodienes are found to be biologically active, it was thought worthwhile to prepare such dienes from isotelekin (**6**) by a dehydration reaction which should be very facile since the C-3 hydroxyl is axial. Isotelekin (**6**) was reacted with *p*-toluene sulphonic acid in benzene when instead of yielding the expected diene (**8**) it gave a mixture of isoalantodiene and alantodiene separated by chromatography and found identical in all respects with the natural products (**1** and **2**). The formation of these dienes from isotelekin (**6**) could be explained by involving anionotropic shifts via the intermediate formation of tertiary and primary allylic alcohols and of these **12** has been isolated earlier from *I. racemosa* [4]. The dehydration of tertiary allylic alcohols would give rise to these dienes. Telekin (**7**) also yielded the same mixture of dienes under similar reaction conditions, and this suggests its involvement in the proposed biogenetic pathway. Dihydroepoxyalantolactone (**9**) under acidic conditions affords dihydroisoalantodiene (**3**) and an eremophilane derivative [7]. The 5α,6α-epoxyalantolactone (**10**) on treatment with aqueous hydrochloric acid yielded isoalantodiene (**1**) alongwith an eremophilane derivative (**11**) [8]. The coexistence of these dienes alongwith the naturally occurring epoxide **10** [4] can be best explained biogenetically by postulating the anionotropic shifts from allylic alcohol **13** which itself arises from the acid catalysed epoxide ring opening.

Nitrate reductase is a rate limiting step between nitrate and amino acids and significant correlation between nitrate reductase activity and plant growth has been observed [9]. Growth regulators, GA, cytokinins and IAA have been shown to stimulate nitrate reductase activity [10–13].

Isoalantodiene (**1**) increases the nitrate reductase activity in intact seedlings (Table 2). Higher nitrate reductase activity was observed in the root than in the shoot which is due to the fact that roots are the primary site of nitrate reduction. An increase in the concentration of isoalantodiene leads to increased enzyme activity. At 40 μg/ml the nitrate reductase activity in root was increased to 75% and that of shoot to 57% over the control value.

An interesting observation is that a dramatic increase (185%) in nitrate reductase activity occurred in excised shoot (Table 3), which was 128% higher than in intact shoot, at 40 μg/ml of isoalantodiene. In sunflower seedlings root excision is known [11] to decrease the nitrate reductase activity and addition of GA_3 to such seedlings stimulated the nitrate reductase activity. The present results indicate that isoalantodiene can simulate the hormonal effects in the root as well as in the shoot to stimulate or induce nitrate reductase activity.

Moreover, nitrate reductase is an -SH containing molecule [13] and isoalantodiene with an α-methylene-γ-lactone moiety can attract such biological nucleophiles. Thus the possibility of involvement of an -SH group in the regulation of enzyme activity by isoalantodiene cannot be ruled out.

Work from our laboratory has shown that several naturally occurring terpenoid α-methylene-γ-lactones display root initiation properties in hypocotyl cuttings of mung beans and this activity is further enhanced on several chemical and stereochemical modifications [14].

Both the alantolides are significantly more active in stimulating rooting in the hypocotyl cuttings of *Vigna radiata* compared to the parent compound alantolactone (Table 4). Significantly, the pyrazoline derivatives in which the α-methylene-γ-lactone moiety has reacted, still show significant activity over the control. This activity may be due to the conjugated diene system which may be binding the biological nucleophiles containing -SH groups in a manner similar to an α-methylene-γ-lactone moiety [15].

Table 1. ^{1}H NMR spectral data for alantolides

Compound	H-3	H-6	H-8	H-13	H-14	H-15	H-16
1	5.60 (1H, *m*)	5.31 (*d*, 1H, $J=4$ Hz)	4.85 (1H, *m*)	5.60, 6.22 (1H each, *br s*)	1.05 (3H, *s*)	1.75 (3H, *br s*)	—
2	—	5.83 (1H, *m*)	4.80 (1H, *m*)	5.77, 6.43 (1H each, *br s*)	0.98 (3H, *s*)	1.83 (3H, *br s*)	—
3	5.76 (1H, *m*)	5.50 (1H, *d*, $J=4$ Hz)	4.93 (1H, *m*)	1.31 (3H, *d*, $J=7$ Hz)	1.17 (3H, *s*)	1.86 (3H, *d*, $J=1.5$ Hz)	—
4	5.66 (1H, *m*)	5.17 (1H, *d*, $J=4$ Hz)	5.53 (1H, *m*)	—	1.13 (3H, *s*)	1.80 (3H, *br s*)	4.73 (2H, *m*)
5	5.73 (1H, *m*)	5.70 (1H, *m*)	4.77 (1H, *m*)	—	1.03 (3H, *s*)	1.70 (3H, *br s*)	4.77 (2H, *m*)

Table 2. The effect of isoalantodiene (**1**) on nitrate reductase activity in six-day-old *Vigna radiata* seedlings grown in water and light*

Plant part	Isoalantodiene concentration (μg/ml)	Nitrate reductase activity†	Increase over control
Root	20	0.23 ± 0.01	144
	40	0.28 ± 0.02	175
	control	0.16 ± 0.01	100
Shoot	20	0.09 ± 0.01	128
	40	0.11 ± 0.01	157
	Control	0.07 ± 0.02	100

*Values are means of three replications ± s.d.
†Expressed as μmol of nitrite produced/hr/g fresh weight.

Table 3. The effect of isoalantodiene (**1**) on nitrate reductase activity in excised seedlings of *Vigna radiata**

Tissue	Isoalantodiene concentration (μg/ml)	Nitrate reductase activity†	Increase over control
Shoot (excised)	20	0.14 ± 0.02	200
	40	0.20 ± 0.01	285
	Control	0.07 ± 0.01	100

*Values are means of three replications ± s.d.
†Expressed as μmol of nitrite produced/hr/g fresh weight.

It may be mentioned that some pyrazoline derivatives derived from α-methylene-γ-lactones displayed a higher activity compared to their parent compounds and this may be due to the biotransformation of these pyrazolines to active compounds in the plant system [16].

EXPERIMENTAL

All the compounds gave a satisfactory C and H analysis. IR: Nujol; ^{1}H NMR: $CDCl_3$, TMS as int. standard. All the chromatographic separations were performed on silica gel and silica gel impregnated with $AgNO_3$ (10%).

Isolation of alantodiene (**2**) *and isoalantodiene* (**1**). Powdered *I. racemosa* roots (40 kg) were extracted with petrol (40–60°) at room temp. and concd under red. pres. to yield an extract (1.5 kg) which on storing at 0° for 4 days gave solid alantolactone and isoalantolactone as major components (1 kg) and a mother liquor (0.45 kg). The mother liquor (10 g) was chromatographed extensively on silica gel $AgNO_3$ (10%) and was made free from alantolactone and isoalantolactone by eluting with petrol–Et_2O (19:1). The fraction (3 g) eluted with petrol–Et_2O (1:1) was chromatographed over silica gel–$AgNO_3$ (10%) (300 g). Elution with petrol–Et_2O (1:1) yielded a pure liquid compound **2** (0.15 g). (Found 78.19; H, 7.84, $C_{15}H_{18}O_2$ requires: C, 78.23; H, 7.88%). Further elution of the column with the same solvent yielded a solid crystalline compound (**1**, 0.2 g), mp 82°. (Found: C, 78.27; H, 7.82, $C_{15}H_{18}O_2$ requires: C, 78.23; H, 7.88%).

Table 4. Effect of alantolides on the number of roots per rooted segments produced by hypocotyl cuttings of *Vigna radiata* after seven days

Compound	Number of roots* [(mean ± s.d. (30)]			
	5	10	15	20
Alantolactone	4.8 ± 0.4	5.4 ± 0.4	7.8 ± 0.9	5.3 ± 0.2
1	7.7 ± 0.8	10.8 ± 1.2	13.2 ± 1.1	27.8 ± 1.4
2	8.3 ± 0.2	10.6 ± 1.5	10.9 ± 1.7	13.5 ± 0.9
4	6.3 ± 0.4	6.1 ± 0.9	6.5 ± 1.3	10.5 ± 0.5
5	7.6 ± 1.2	8.8 ± 0.5	8.6 ± 2.0	9.4 ± 0.4

*Control experiment, water: 4.5 ± 0.8 (mean no. of roots ± s.d.).

Reaction of **1** *with diazomethane.* A soln of isoalantodiene (**1** 0.2 g) on reaction with CH_2N_2 yielded a mixture of two pyrazoline derivatives which were separated by CC. Elution with petrol–Et_2O (9:1) afforded two crystalline compounds. Compound **4** (0.12 g), mp 118°. (Found: N, 10.20, $C_{16}H_{20}O_2N_2$ requires: N, 10.29%). Compound **5** (0.05 g) mp 134°. (Found: N 10.25, $C_{16}H_{20}O_2N_2$ requires: N, 10.29%).

Reaction of isotelekin with p-TsOH. A soln of isotelekin (**6**, 1.0 g) in C_6H_6 (100 ml) was refluxed with *p*-TsOH (20 mg) for 6 hr. The product mixture of two components was chromatographed and elution with petrol–Et_2O (9:1) yielded alantodiene (**2**, 0.3 g) and isoalantodiene (**1**, 0.6 g) mp 82°.

Reduction of isoalantodiene with $NaBH_4$. Isoalantodiene (**1**, 0.1 g) on reduction with $NaBH_4$ (0.05 g) in MeOH yielded **3** (0.07 g) $C_{15}H_{20}O_2$, mp 51° (Found: C, 77.53; H, 8.70. $C_{15}H_{20}O_2$ requires: C, 77.55; H, 8.68%).

Reaction of compound **10** *with* HCl/THF. To a soln of compound **10** (0.5 g) in THF (5 ml) at room temp. was added conc

HCl (37%, 0.5 ml). After 2 hr, the viscous liquid product (0.45 g) was chromatographed. Elution with petrol–Et_2O (9:1) gave (**1**, 0.2 g) mp and mmp with an authentic sample 82°; elution with petrol–Et_2O (4:1) yielded (**11**, 0.2 g) identified as an eremophilanolide derivative.

Nitrate reductase activity in root and shoot. Seeds of mung bean were surface sterilized in 1% NaOCl and germinated in glass trays on filter papers. The seedlings were grown in dist. H_2O for 6 days under continuous illumination at $27 \pm 2°$ and thereafter treated with isoalantodiene (**1**) in nitrate free Hoagland's soln for 24 hr. In all, two concns of isoalantodiene (20 and 40 μg/ml) along with nitrate free Hoagland's soln (without any chemical) as control were tested. For each treatment 40 plants were cultured in 50 ml of soln. Treatment was continued for 24 hr. Before nitrate reductase assay, seedlings were washed thoroughly in dist. H_2O and separated into root and shoot portions.

Effect of root excision. Roots were excised 5 cm below the cotyledonary node and thereafter cuttings were treated with isoalantodiene in nitrate free Hoagland's soln at two concns (20 and 40 μg/ml) along with control (without isoalantodiene).

Extraction and assay of enzyme. Nitrate reductase activity was assayed according to Jaworski [18]. Shoots or roots weighing 200 mg were cut into small pieces and suspended in a screw cap vial containing 5 ml of the medium, consisting 0.1 M Pi buffer (pH 7.5), 0.02 M $NaNO_3$, and 1% *n*-PrOH. The vial was flushed with N_2, sealed and incubated at 30° in the dark for 1 hr. An aliquot (1 ml) was removed for determining release of nitrite into the medium.

Rooting in hypocotyl cuttings of Vigna radiata *seedlings.* This was done by the established method [14].

Acknowledgements—The authors thank the Indian Council of Agricultural Research, New Delhi for financial support. One of us (R. G.) is thankful to BARC, DAE, Trombay, for the award of Junior Research Fellowship.

REFERENCES

1. Miller, R. B. and Nash, R. D. (1974) *Tetrahedron* **30**, 2961.
2. Tsuda, K., Tanaba, K., Iwai, I. and Funakoshi, K. (1957) *J. Am. Chem. Soc.* **79**, 5721.
3. Kaur, B. and Kalsi, P. S. (1985) *Phytochemistry* **24**, 2007.
4. Kalsi, P. S., Goyal, R., Talwar, K. K. and Chhabra, B. R. (1988) *Phytochemistry* **27**, 2079.
5. Horak and Pliva (1960) *Chem. Ind.* 102.
6. Perezc, A. L., Letucia, N. M. and Alfonso, R. D. V. (1987). *Phytochemistry* **26**, 765.
7. Kitagawa, I., Shibuya, H., Takeno, H., Nishino, T. and Yosioka, I. (1976) *Chem. Pharm. Bull.* **24**, 56.
8. Kilagawa, I., Shibuyam, H. and Kawaai, M. (1977) *Chem. Pharm. Bull.* **25**, 2638.
9. Srivastva, H. S. (1980) *Phytochemistry* **19**, 725.
10. Roth-Bejerano, N. and Lips, H. (1970). *New Phytol.* **69**, 165.
11. Sekhon, B. S., Dass, G. and Bhatia, I. S. (1983) *Proc. Indian Natl Sci. Acad.* B **49**, 495.
12. Lips, H. and Roth-Bejerano, N. (1969) *Science* **166**, 109.
13. Beevers, L. and Hageman, R. H. (1969) *Annu. Rev. Plant Physiol.* **20**, 495.
14. Kalsi, P. S., Sood, V. B., Masih, A. B., Gupta, D. and Talwar, K. K. (1983) *Phytochemistry* **22**, 1387.
15. Shibaoka, H., Shimokoriyama, M., Irichijima, S. and Tamura, S. (1967) *Plant Cell Physiol.* **8**, 297.
16. Talwar, K. K. (1988) Presented at International Congress of Pl. Physiol. Feb. 17, New Delhi, India.
17. Jaworski, E. G. (1971) *Biochem. Biophys. Res. Commun.* **43**, 1274.