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CIS-9,10-DIHYDROCAPSENONE: A POSSIBLE CATABOLITE OF CAPSIDIOL FROM CELL SUSPENSION CULTURES OF *CAPSICUM ANNUUM*

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Key Word Index—*Capsicum annuum*; Solanaceae; cell suspension culture; phytoalexin; catabolism; sesquiterpenoid; capsidiol; capsenone; *cis*-9,10-dihydrocapsenone; *trans*-9,10-dihydrocapsenone.

Abstract—A new eudesmane sesquiterpenoid, *cis*-9,10-dihydrocapsenone, has been isolated in small amounts from unelicited cell suspension cultures of *Capsicum annuum* during the stationary phase of the growth cycle. It is proposed that the new sesquiterpenoid is an intermediate on the initial part of the route by which endogenous capsidiol is catabolized in pepper cultures.

INTRODUCTION

In tissue cultures of pepper, the sesquiterpenoid phytoalexin capsidiol (1) accumulates to high levels in response to a number of elicitor preparations including commercially obtained cellulase and pectinase [1]. Minor congeners of capsidiol have been isolated from both elicitor-treated tissue cultures [1] and whole fruits [2] and the partial structures of several of these compounds have been elucidated [2]. However, 13-hydroxycapsidiol (2), the principle metabolite formed by pepper cultures and fruits treated with exogenously supplied capsidiol [3], has not been detected in either healthy or elicitor-treated cultures [1, 2]. We report the isolation and characterization of a new eudesmane sesquiterpenoid, *cis*-9,10-dihydrocapsenone (4) from unelicited pepper cultures which had reached the stationary phase of the growth cycle.

RESULTS AND DISCUSSION

The new eudesmane 4 was first detected by TLC (blue colour with vanillin–H_2SO_4) and GC analysis of Et_2O extracts of media taken from tissue cultures in the stationary phase of the growth cycle. Its IR spectrum indicated the presence of an unconjugated ketone [1719 (*s*) cm^{-1}], a hydroxyl group [3400 (*br*) cm^{-1}] and a terminal methylene group [888 (*s*), 3080 (*w*) and 1647 (*w*) cm^{-1}]. The MS indicated a molecular mass of 236 ($[M]^+$) consistent with the molecular formula $C_{15}H_{24}O_2$ and confirmed the presence of one hydroxyl group (*m/z* $[M-18]^+$). Its ^1H NMR spectrum had features indicative of the eudesmane system. However since only a very small amount of material was available and the spectrum indicated significant contamination with other (hydrocarbon) material, spectra were obtained for two related compounds, capsenone (3) and *trans*-9,10-dihydrocapsenone (5). These compounds were obtainable by straightforward biochemical and chemical procedures in sufficient quantity and purity so as to give almost full spectral details (Table 1). This assisted the interpretation of the spectra of 4 and indicated the likely conformation of all three species 3–5.

The general spectral features of this class of compound have been reported previously [4], and in particular data for 3 have been determined at 100 MHz. The present assignments differ slightly from those reported earlier [4]. The *trans*-decalin system can exist in only one conformation at the ring junction and the ketonic ring (ring A) in 3 and 5 adopts a normal chair form as confirmed by the values of ^3J (H-2ax, H-3) (8.8 Hz) and ^4J (H-2eq, H-4) (1.0 Hz). The 3-OH and 4-Me groups are equatorial and axial, respectively, as is implicit in data reported for similiar systems [4].

Ring B in capsenone (3) adopts the envelope conformation which relieves the steric interaction of 5-Me and 7-isopropenyl groups but this interaction in compound 5 leads to a boat (or twist–boat) conformation for ring B, confirmed by couplings to H-7 (two $\sim$4.5 Hz, two $\sim$8.9 Hz), and a ^4J = 0.4 Hz between H-6eq and H-8eq. All shift differences between 3 and 5 (Table 1) are in keeping with the depicted conformations. The *cis*-decalin system proposed for 4 is normally conformationally labile, existing in principle in two forms. This would lead to a complex ^1H spectrum but examination of Dreiding molecular models shows that in this case both the chair–chair forms experience unfavourable 1,3-interactions for the 4- or 5-Me groups. In only one form can these be reduced by the adoption of a boat (or twist–boat) conformation and it is likely that the conformation shown for 4 (or a corresponding twist–boat form) is the sole contributing structure for *cis*-9,10-dihydrocapsenone. The discernible features of the ^1H spectrum are in keeping with this structure i.e. the shifts for the remote isopropenyl group are nearly the same as those for 5; an upfield shift of 0.7 ppm for H-3 is due to a change to a shielded (by 4-Me) equatorial position from a deshielded (by 5-Me) axial position in 5; the 14-Me and 15-Me protons are downfield in 4 relative to 5 since both groups are effectively equatorial to both rings; protons H-2ax, H-2eq and H-7 are all upfield as expected for *cis*-linked rings. The IR and mass spectra of 4 and 5 were similiar and suggest that these two compounds are isomers.

The discovery of small amounts (*ca* 1 μg/g fr. wt) of *cis*-9,10-dihydrocapsenone (4) in unelicited cell suspension

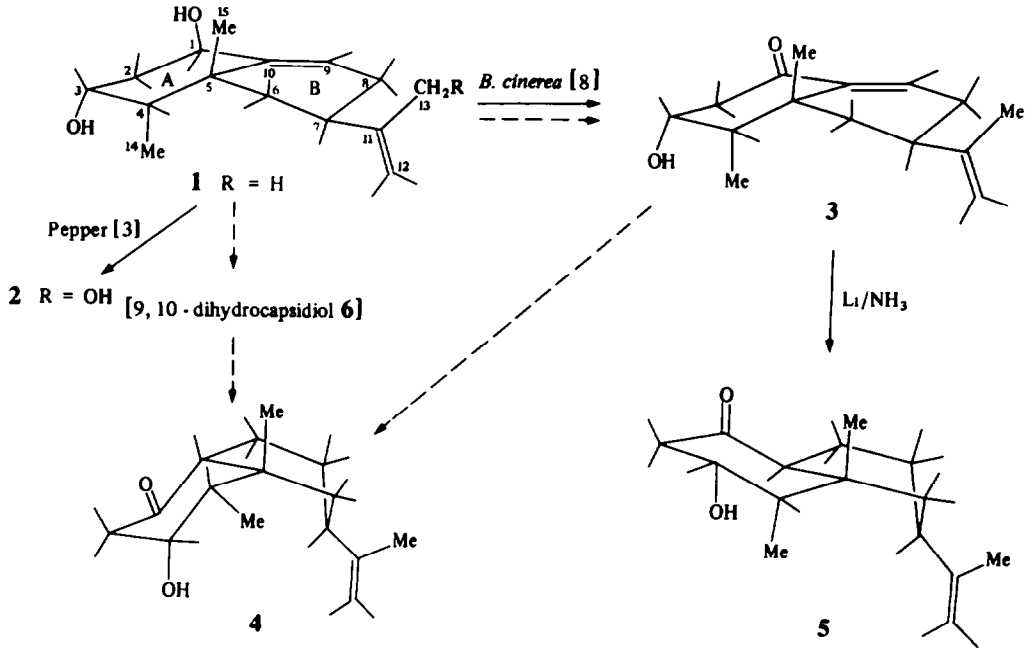

Table 1. ^{1}H NMR data for capsenone (3) and isomeric dihydroderivatives (360 and 400 MHz, $CDCl_3$, TMS as int. standard)

H	3	4	5	
2	ax 2.38	ax ~2.25	ax 2.67	$J_{2,3}$ = 8.8 Hz, $J_{2,2}$ = 14.0 Hz
	eq 2.74	eq ~2.4	eq 2.52	$J_{2,3}$ = 4.5 Hz, $J_{2,4}$ = 1.0 Hz
3	4.50	3.7	4.39	$J_{3,4}$ = 4.5 Hz
4	1.92		1.85	
6	*psax 1.39		psax 1.32	$J_{6,7}$ = 8.9 Hz, $J_{6,7}$ = 14.4 Hz
	*pseq 2.02		pseq 2.03	$J_{6,7}$ = 4.9 Hz, $J_{6,8}$ ~ 0.4 Hz
7	2.22	~2.0	2.37	
8	psax 1.95		psax 1.42	$J_{7,8}$ ~ 8.8 Hz
	pseq 2.33		pseq 1.85	$J_{7,8}$ ~ 4.5 Hz
9	6.75		psax 1.85	$J_{9,10}$ = 8.7 Hz
			pseq 1.75	$J_{9,10}$ = 5.0 Hz
10			2.07	$J_{2eq,10}$ = ~0.5 Hz
12	4.75	4.69	4.74	
13	2.2	1.70	1.74	
14	1.05	1.42	1.26	
15	1.15	1.27	1.08	

*psax and pseq indicate a pseudo-axial and pseudo-equatorial position, respectively.

cultures of *C. annuum* may have revealed part of the initial route by which endogenous capsidiol is catabolized by such cells. Under normal circumstances, unelicited cell suspension cultures do not accumulate phytoalexins. However, it is widely accepted that conditions of stress often induce the accumulation of phytoalexins [5] and it may well be that shearing forces within the agitated cultures over a long period of time and at high cell densities caused damage to some cells with the consequent elicitation of very low levels of stress metabolites. In view of the structure of the new compound it seems likely that it is formed from capsidiol, possibly via capsenone, although neither of these two compounds could be detected at the time of harvesting the pepper cultures and there is no direct evidence to support this proposed route. The currently postulated biosynthetic pathway leading to capsidiol formation envisages the direct precursor of capsidiol to be a *cis*-eudesmanoid intermediate which is converted to capsidiol by a concerted mechanism involving both a methyl migration (from C-10 to C-5) [6] and a hydride shift (from C-5 to C-4) [7] with the consequent formation of a double bond between carbons 9 and 10 of the molecule. The reduction of this double bond must therefore occur at a stage subsequent to capsidiol forma-

tion and since capsenone (3) can be formed from capsidiol (1) (albeit by fungal agency [8]) it seems plausible to propose the biochemical sequence 1 → 3 → 4 exists in *C. annuum*. An alternative route is that 4 could arise from 1 via 9,10-dihydrocapsidiol (6) although this compound has yet to be isolated from biological sources.

EXPERIMENTAL

Plant tissue cultures. Callus cultures of *Capsicum annuum* L. var. New Ace were initiated from excised hypocotyl sections of seedlings germinated at 24° in the dark under sterile conditions on agar plates of Gamborg's B5 medium [9] containing 2,4-D (1 mg/l.), kinetin (0.1 mg/l.) and sucrose (30 g/l.) and maintained by sub-culture every 4 weeks. Cell suspension cultures were established by transfer of callus pieces to liquid media (100 ml in 250 ml conical flasks) of the same composition. Cultures were grown in the dark at 25° in an orbital incubator at 110 rpm and maintained by sub-culture every 2 weeks.

Fungal cultures. Botrytis cinerea (BC3) (from our departmental collection) was grown and maintained on potato dextrose agar (Oxoid). Liquid cultures were established by inoculating 2 l. flasks containing 666 ml of a synthetic medium [8] with agar squares cut from the growing edge of the mycelial mat and grown in shake culture (140 rpm) at 25° in the dark for 64 hr.

Isolation of cis-9,10-*dihydrocapsenone* [(1R,4R,5S,6R,8R) 5,6-*dimethyl*-4-*hydroxy*-8-(2-*propenyl*)*bicyclo*[4.4.0]*decan*-2-*one*] (4). Ten flasks of *C. annuum* cell suspension culture were allowed to grow for a period of 5 weeks without sub-culture. The plant material (*ca* 430 g fr. wt) was harvested by filtration through Miracloth and then steeped in $CHCl_3$–MeOH (2:1) for 6 hr. After filtering off the cell debris, the solvent was removed *in vacuo* and the residue added to the filtered culture media. The combined material was partitioned against Et_2O (3 × 0.5 vol) and after reducing the pooled Et_2O extracts to dryness *in vacuo*, the residue was purified by TLC (0.5 mm rhodamine 6G-impregnated silica gel, EtOAc–cyclohexane 1:1). The dihydro compound (R_f 0.36) was visualized as a light pink band under UV light (254 nm) (R_f 0.56 EtOAc–*iso*-PrOH, 9:1) and gave a bright blue colour on spraying with vanillin–H_2SO_4 [10]. After elution from the gel with Et_2O the compound (*ca* 400 μg) gave the following spectral data. MS *m/z* (rel. int.): 236 $[M]^+$ (16), 218 $[M-H_2O]^+$ (8), 203 (5), 193 (50), 187 (6), 175 (33), 161 (25), 147 (51), 135 (61), 119 (51), 107 (84), 93 (100), 79 (92), 67 (80), 55 (66) and 44 (99); IR ν_{max}^{KBr} cm^{-1}: 3400, 3080, 2940, 2870, 1719, 1647, 1455, 1378, 1046, 1034 and 888; ^1H NMR: see Table 1.

Preparation of trans-9,10-*dihydrocapsenone* [(1S,4R,5S,6R,8R) 5,6-*dimethyl*-4-*hydroxy*-8-(2-*propenyl*)*bicyclo*[4.4.0]*decan*-2-*one*] (5). This compound was prepared from capsidiol (1) via capsenone (3). (a) *Capsidiol* (1). After removing the uppermost section of 100 unripe capsicum fruits (of an unknown Spanish variety purchased from a local wholesaler) with a knife, the fruits were arranged upright in trays and the exposed cavities filled with a soln of commercial cellulase (*ex Trichoderma viride*, 400 mg/l.) in a method adapted from that of Brooks *et al.* [1]. The fruits were incubated at 17° for 72 hr after which time the diffusate was collected and the inner cavities of the fruits rinsed with H_2O. The combined aq. soln (diffusate and washings) totalling *ca* 5.5 l. was partitioned extensively against half vols of Et_2O. The Et_2O extracts were combined, reduced to dryness *in vacuo* and the resulting material purified by PTLC (0.75 mm plates as above developed with EtOAc–*iso*-PrOH, 9:1) to give 145 mg crude capsidiol (R_f 0.50). The capsidiol was further purified by recrystallization (× 2) from Et_2O–petrol and had physical (mp, ORD) and spectral (MS, IR) properties identical to those reported [4]. (b) *Capsenone* (3) was prepared by a method adapted from [8]. Equal aliquots of a soln of 1 (45 mg in 20 ml EtOH) were added to 3 flasks of *B. cinerea* in shake culture prepared as above. After 5 hr the mycelium was filtered through four layers of muslin and rinsed with H_2O. The combined media and washings were worked up as above and after PTLC (as above but with EtOAc–cyclohexane, 1:1) yielded unmetabolized capsidiol (12 mg, R_f 0.12) and capsenone (18.5 mg, R_f 0.41, 41% overall yield). (c) *trans-9,10-Dihydrocapsenone* (5). To metallic Li (3 mg) in dry liquid NH_3 (3 ml) at −68° there was added with vigorous stirring 17 mg 3 in dry Et_2O (4 ml) over a 10 min period. After a further 10 min stirring unreacted Li was destroyed by the addition of 1 ml of *tert*-BuOH–Et_2O (1:1). After allowing the mixture to warm to room temp. over 2 hr, H_2O (5 ml) was added and the aq. phase extracted (× 2) with equal vols of E_2O. The combined Et_2O extracts were back-washed (× 2) with equal vols of H_2O and reduced to dryness *in vacuo*. TLC (as above) with EtOAc–cyclohexane (1:1) gave unreacted 3 as a UV quenching band (purple) and the product as a light pink band (under UV light, 254 nm) at R_f 0.47. After elution from the gel with Et_2O the product (5.1 mg, 30% yield) gave the following spectral data. MS, *m/z* (rel. int.): 236 $[M]^+$ (6), 218 $[M-H_2O]^+$ (7), 203 (6), 185 (4), 175 (13), 161 (8), 147 (8), 135 (87), 123 (29), 107 (31), 93 (58), 81 (33), 67 (48), 55 (65) and 43 (100); IR ν_{max}^{KBr} cm^{-1} 3400, 3090, 2970, 2940, 2870, 1705, 1645, 1440, 1386, 1025 and 886 cm^{-1}; ^1H NMR: see Table 1.

Analytical methods. The conditions used for GC analysis have been described previously [11]. The *RR*, values (Me stearate = 1) were as follows: capsidiol, 2.29; *cis*-9,10-dihydrocapsenone, 2.51; *trans*-9,10-dihydrocapsenone 3.07; capsenone, 3.74.

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