Phytochemistry, 1977, Vol. 16, pp. 1913–1917. Pergamon Press. Printed in England.

METABOLISM OF KAURENOIDS BY *GIBBERELLA FUJIKUROI* IN THE PRESENCE OF THE PLANT GROWTH RETARDANT, *N,N,N*-TRIMETHYL-1-METHYL-(2′,6′,6′-TRIMETHYLCYCLOHEX-2′-EN-1′-YL)PROP-2-ENYLAMMONIUM IODIDE*

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(*Received* 20 *April* 1977)

Key Word Index—Gibberellin biosynthesis; chemical inhibition; substrate metabolism; mevalonate; *ent*-kaur-16-ene; kaur-16-ene; 13β-kaur-16-ene; *ent*-kaur-15-ene.

Abstract—The plant growth retardant, *N,N,N*-trimethyl-1-methyl-(2′,6′,6′-trimethylcyclohex-2′-en-1′-yl)prop-2-enylammonium iodide, is shown to block gibberellin biosynthesis in *Gibberella fujikuroi* between mevalonate and *ent*-kaur-16-ene, probably by inhibiting *ent*-kaur-16-ene synthetase A-activity. In the presence of the plant growth retardant, cultures of the fungus incorporate (26.5%) added *ent*-[^{14}C]-kaur-16-ene into gibberellin A_3. Under the same conditions kaur-16-ene, 13β-kaur-16-ene, and *ent*-kaur-15-ene are not metabolised to gibberellin analogues.

INTRODUCTION

Mutants of *Gibberella fujikuroi*, blocked for GA†-biosynthesis, provide a convenient means of studying both the GA-pathways [1–3] in the fungus and the microbiological transformation [4–6] of analogues of the intermediates which normally occur beyond the block. GA-biosynthesis however, can also be blocked chemically. Synthetic plant growth retardants, such as AMO-1618, have been shown [7] to inhibit GA-biosynthesis in cultures of *G. fujikuroi*. Surprisingly there are no reports of the use of these chemically blocked cultures in the study of the microbiological transformation of diterpenes related to GA-biosynthesis.

The report by Haruta *et al.* [8] that *N,N,N*-trimethyl-1-methyl-3-(2′,6′,6′-trimethylcyclohex-2′-en-1′-yl)prop-2-enylammonium iodide (**1**) is a potent plant growth retardant prompted us to investigate this readily accessible compound as an inhibitor of GA-biosynthesis. In this paper we show that compound **1** is a powerful inhibitor of GA-biosynthesis in the fungus *G. fujikuroi* and illustrate the use of these GA-blocked cultures in the study of the metabolism of analogues of *ent*-kaur-16-ene.

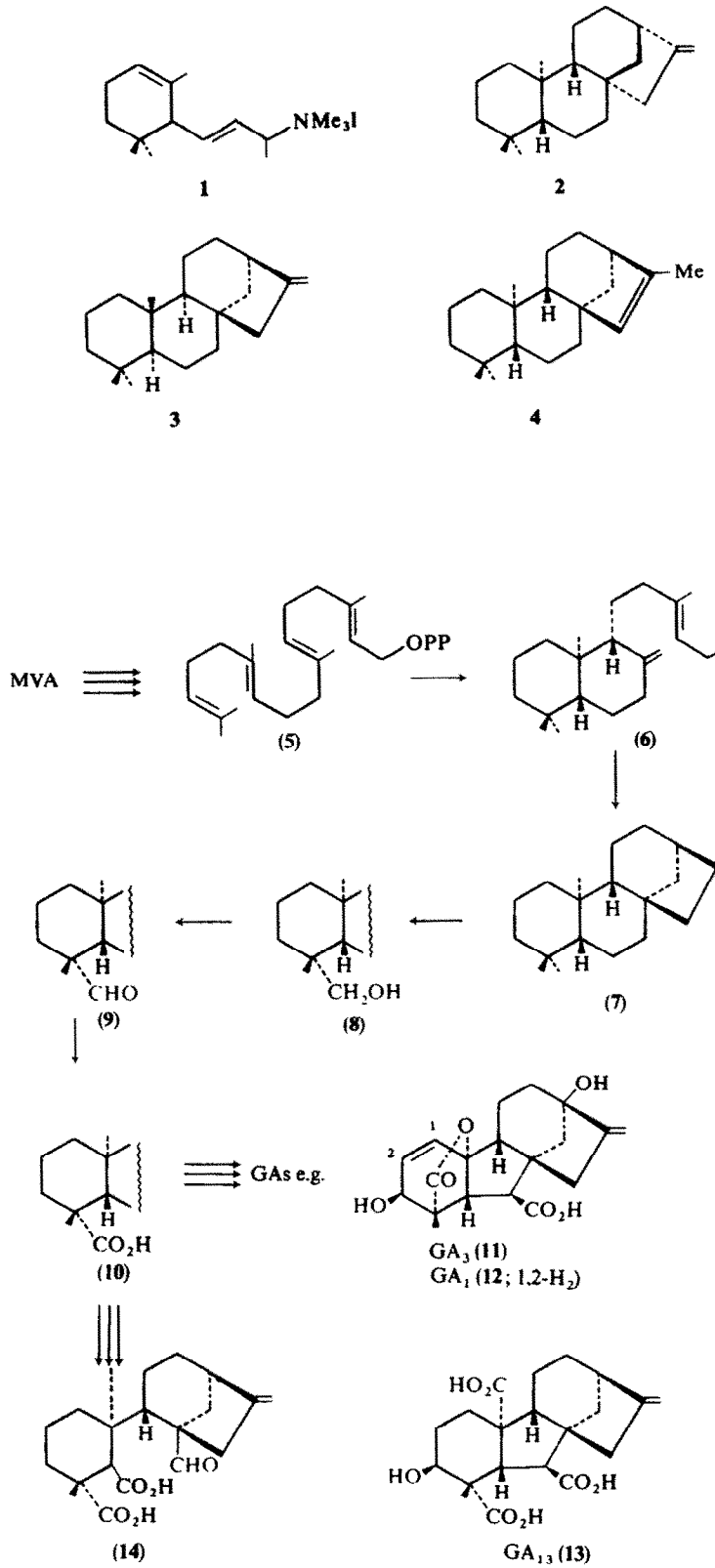
RESULTS AND DISCUSSION

The wild-type strain GF-1a of *G. fujikuroi* was used since it has been shown [9] to be a relatively high producer of a wide range of GAs. In exploratory experiments, this strain was grown for three days on a synthetic medium containing 0, 1, 10 and 100 mg/l. of compound **1**. The pigmented mycelium was then resuspended in a nitrogen-free medium containing the same original concentration of compound **1**. In addition the mycelium from untreated cultures were resuspended in the same range of concentrations of compound **1**. These resuspension cultures were grown in shake flasks for a further three days. The acidic metabolites were isolated from the medium, derivatised, and analysed by GLC and GC–MS. Untreated cultures gave the usual range of GAs (**11**, *iso*-**11**, **12**, **13** and **14**). The growth retardant (**1**) inhibited GA production at the three concentrations, without reducing the mycelial weight. The inhibition appeared to be *ca* 95% complete at the two higher concentrations both when growth retardant was present from the beginning or added only to the resuspension medium. In subsequent experiments therefore the growth retardant was usually added to the resuspension medium only and, to ensure maximal inhibition of GA-production, the highest concentration was used.

To obtain a quantitative measure of inhibition, the percentage incorporation of MVL-[2-^{14}C] into GA_3 (**11**), a terminal GA in the fungal pathway (Scheme 1), was determined in the absence and presence of compound **1**. In these experiments the total acidic metabolites from 50 ml resuspension cultures were diluted with a large excess of GA_3 and the GA_3-[^{14}C] was crystallized to constant specific radioactivity. The incorporation from (3*R*)- MVL-[2-^{14}C] into GA_3 was 13.0% in the control cultures and 0.29% in the presence of the growth retardant. Thus the conversion of MVL into GA_3 (**11**) was inhibited to the extent of 97.8% by compound **1**. The radio-chemical purity of the GA_3-[^{14}C] from these experiments was tested by methylation. After four recrystallizations, the specific activities of the GA_3-[^{14}C] methyl esters correspond to 97.5% inhibition of GA_3-production by compound **1**. During recrystallization of GA_3-[^{14}C] methyl ester the measured specific activity

*Part 19 in the series 'Fungal Products'. For part 18 see Lunnon, M.W. and MacMillan, J. (1976) *J. Chem. Soc. Perkin I* 184.

†Abbreviations: GA = gibberellin; AMO-1618 = 2′-isopropyl-4′-(trimethylammonium chloride)-5′-methylphenylpiperidine-1-carboxylate; MVL = mevalonolactone; MVA = mevalonic acid; GGPP = geranylgeranyl pyrophosphate; CPP = copalylpyrophosphate.



Scheme 1. Partial GA biosynthetic pathway in *Gibberella fujikuroi*.

varied with the solvent system used and with the conditions under which the crystals were dried. This variability, presumably due to tenaciously held solvent of crystallization, was standardized by crystallizations from C_6H_6–MeOH and drying at 140° for 6 hr.

In qualitative experiments *ent*-kaurene (7), *ent*-kaurenol (8), and *ent*-kaurenoic acid (10), known intermediates in the normal GA-pathway (Scheme 1), were shown to be incorporated into GAs by resuspension cultures of GF-1a in the presence of the plant growth retardant (1). The methylated products from each of these substrates were shown by GC–MS to be the same as those from untreated cultures (11, *iso*-11, 12, 13, 14). To obtain quantitative data *ent*-kaurene-[^{14}C] was prepared by incubation of MVL-[2-^{14}C] with a cell-free preparation from *G. fujikuroi* and its incorporation into GA_3-[^{14}C] by resuspension cultures was compared in the presence and absence of compound 1. The total products extracted from the culture filtrate were mixed with 20 mg unlabelled GA_3 (11), the GA_3 was then isolated by TLC of the acidic fraction and crystallized to constant specific radioactivity. The measured incorporations were 23 ± 1% and 26.5 ± 1% for the untreated and treated cultures. The slightly lower value for the control culture is presumably caused by dilution of the GA_3-[^{14}C] derived from *ent*-kaurene-[^{14}C] by the GA_3 produced from the endogenous pools of unlabelled precursors. This value can be corrected (28.5 ± 1%) assuming that the amount of unlabelled GA_3 produced by the control culture in the present experiment was the same (*ca* 5 mg) as that previously found by Bearder *et al.* [2]. Whatever the precise percentage incorporations, these results show that *ent*-kaurene is an efficient precursor of GA_3 both in the absence and presence of compound 1.

The above experiments show that the plant growth retardant 1 inhibits GA-biosynthesis between MVA and *ent*-kaurene (7). To determine if compound 1 inhibits the cyclization of GGPP (5) to *ent*-kaur-16-ene (7), GGPP (5) and CPP (6) were separately incubated with soluble cell-free preparations from *G. fujikuroi* [10] and *Marah* (*Echincystis*) *macrocarpus* [11] in the absence or presence of compound 1 and AMO-1618 at 10 μM concentrations. The results are shown in Table 1. The incorporation of the substrates into *ent*-kaurene (7) was very low, especially from GGPP, but the results indicate that compound 1 inhibits the cyclization of GGPP to CPP (A-activity of *ent*-kaurene synthetase) and that it is at least as effective as AMO-1618 [12, 13]. The data indicate that compound 1 also inhibits the cyclization (B-activity of *ent*-kaurene synthetase) of CPP (6) to *ent*-kaurene (7), but to a much smaller extent than for the A-activity.

Cultures of *G. fujikuroi* in which GA-biosynthesis is blocked provide a simple and convenient method for the study of the microbiological conversion of analogues of the normal intermediates. In the absence of the normal intermediates beyond the block, the metabolism of these analogues can be determined without recourse to labelled substrates. Using the mutant B1-41a, the position of the block between *ent*-kaurenal (9) and *ent*-kaurenoic acid (10) has limited our studies to analogues of *ent*-kaurenoic acid (10) and later intermediates. The effective inhibition of GA-biosynthesis by the plant growth retardant 1 extends this approach to analogues of *ent*-kaurene (7) and later intermediates. The method is illustrated with reference to (a) kaur-16-ene [(+)-kaurene] (2) which is the enantiomer of the natural intermediate *ent*-kaur-16-ene [(−)-kaurene] (7); (b) 13β-kaur-16-ene (phyllocladene) (3); and (c) *ent*-kaur-15-ene (isokaurene) (4).

Resuspension cultures were grown in the presence of each of these substrates (40–80 mg/l.) and the plant growth retardant (100 mg/l.). For comparison, control cultures were grown in the absence of these substrates with and without compound 1. GLC traces from the derivatised acidic products from the culture medium containing each of the substrates were identical to those obtained from the control cultures containing the inhibitor while those from control cultures without inhibitor showed the usual range of products. GLC and GC–MS of the mycelial extract showed the presence of unmetabolised kaurene (2), 13β-kaurene (3), and *ent*-kaur-15-ene (4). These results show that kaurene (2), 13β-kaurene (3), and *ent*-kaur-15-ene (4) are not metabolised to GA-analogues by cultures of GF-1a, the former result confirming the previous finding by Cross *et al.* [14] that kaur-16-ene-[17-^{14}C] (2) was not converted into the enantiomer of GA_3(11).

EXPERIMENTAL

Culture conditions. Strain GF-1a of *Gibberella fujikuroi*, maintained on potato–dextrose-agar slants and stored at −5°, was sub-cultured in the synthetic medium ICI 40%-N [15] in shake-flasks. After 3 days at 25°, 1 ml aliquots were transferred to either 500 ml conical flasks containing 50 or 500 ml, or 100 ml conical flasks containing 10 or 25 ml, of ICI 40%-N medium and grown as shake cultures until the onset of pigmentation (3–4 days). The mycelium was collected by filtration, washed ×3 with nitrogen-free medium (ICI 0%-N), and transferred either to ICI 0%-N medium or to ICI 0%-N medium containing 100 mg/l. of compound 1. The volume of resuspension medium was equal to that of the original cultures from which the mycelium was obtained. Compound 1 was usually added in MeOH soln to hot ICI 0%-N medium prior to the addition of the mycelium.

Extraction of resuspension cultures. The mycelium was collected by filtration and extracted with MeOH (25 ml) which was evapd to dryness to give the mycelial extract. The culture filtrate was adjusted to pH 8 with satd. $NaHCO_3$ soln and extracted with EtOAc (3 × 50 ml); recovery from the EtOAc gave the neutral-basic fraction. The aq. layer was adjusted to pH 2.5 with 2M HCl

Table 1. Incorporation of radio-activity into *ent*-kaur-16-ene from GGPP-[^{14}C] and CPP-[^{3}H] in the presence of 10 μM concentrations of compound 1 and AMO-1618

Substrate	Dpm added	System	Incorporation (dpm) into *ent*-kaur-16-ene		
			Control	Compound 1	AMO-1618
GCPP-[^{14}C]	22 400	*G. fujikuroi*	65	1	0
CPP-[^{3}H]	36 960	*G. fujikuroi*	4308	2370	4105
GCPP-[^{14}C]	22 400	*M. macrocarpus*	537	33	201
CPP-[^{3}H]	36 960	*M. macrocarpus*	6668	4788	6343

and extracted (3 × 30 ml); recovery from the EtOAc gave the acidic fraction..

GLC conditions. Me derivatives were prepared in MeOH with CH_2N_2 in Et_2O; MeTMS derivatives were prepared in C_5H_5N in sealed tubes with $(Me_3Si)_2NH$–Me_3SiCl–C_5H_5N (2:1:1). Silanized glass columns (152.5 × 0.64 cm) were packed with 2% QF-1 (A) or 2% SE-33 (B) on 80–100 mesh demineralized and silanized Gas-Chrom A; N_2-flow, 75 ml/min.

GC-MS. As in ref. [1] except for GLC columns which were silanized glass packed with 2% SE-33 (172 × 0.32 cm) (C), and 2% QF-1 (152 × 0.32 cm) (D) on demineralized and silanized Gas Chrom A.

Effect of compound **1** *on GA-production.* The mycelium from 4 × 10 ml cultures of *G. fujikuroi*, strain GF-1a, grown on ICI 40%-N medium, was resuspended in 10 ml ICI 0%-N medium containing 0, 0.01, 0.1 and 1.0 mg compound **1**. After 3 days' incubation, the cultures were extracted as described above. The acidic fractions were analysed for GAs as the Me and MeTMS derivatives by GLC on columns A and B and by GC-MS on column C. In a second experiment, the mycelium from 10 ml cultures of GF-1a, grown in 0, 0.01, 0.1 and 1.0 mg compound **1** was resuspended in 10 ml ICI 0%-N medium containing the same concn of compound **1**. After 3 days culture similar results were obtained.

Feeding of unlabelled substrates. Substrates (1 or 2 mg) were added in MeOH or EtOAc (100–200 µl) to 100 ml conical flasks containing 5 ml H_2O immediately after autoclaving. After cooling 20 ml of resuspension culture was added to each flask which was then incubated at 25° for 5 days on a reciprocal shaker. The mycelium and culture filtrates were extracted as described above. Aliquots (1:500) of the mycelial extract were analysed by GLC on column B. Aliquots (1:100 or 1:200) of the neutral, basic and acidic fractions were analysed as the Me and MeTMS derivatives by GLC on columns A and B and, where appropriate, by GC-MS on column C or D. In all experiments the GLC and, where appropriate, the GC-MS results were compared with two control resuspension cultures, one without substrate and the other without substrate and compound **1**.

MVL-[2-^{14}C] incubation. 3(*RS*)-MVL-[2-^{14}C] (4.55 × 10^6 dpm; 10.9 mCi/mmol) in C_6H_6 (40 µl) was added to ICI 0%-N medium (50 ml) immediately after autoclaving. This medium was divided between two 100 ml conical flasks each containing the pigmented mycelium resuspended in 25 ml of ICI 0%-N medium. In addition one of the flasks contained compound **1** (5 mg). After 3 days incubation at 25° on a reciprocal shaker, the filtrate from each flask was acidified to pH 3.0 with M HCl and extracted with EtOAc (3 × 50 ml). The gummy residues, recovered from the EtOAc extracts of the control and treated cultures, were diluted with 125 and 130 mg respectively of unlabelled GA_3. Each extract was dissolved in satd $NaHCO_3$ soln (5 ml) which was extracted with EtOAc (3 × 10 ml) and acidified to pH 2.5 with 2M HCl. Extraction with EtOAc (4 × 10 ml) gave GA_3 which was crystallized from Me_2CO-petrol. After 4 crystallizations the sp. act. (dpm/mg) was constant at 2280 [13% incorporation from (3*R*)-MVL] in the control culture and 52 (0.288%) in the culture treated with compound **1**. Methylation (CH_2N_2) gave GA_3-[^{14}C] Me ester which was recrystallized (× 2) from EtOAc–petrol and dried at 120° for 36 hr: control, 1839 dpm/mg; treated, 49 dpm/mg. Further recrystallization (× 2) from C_6H_6–MeOH and drying at 140° for 6 hr gave constant sp. act.: control, 2017 dpm/mg (11.5% incorporation); treated, 52 dpm/mg (0.28%).

Preparation of cell-free extracts. (a) *G. fujikuroi.* A cell-free extract was prepared from the strain M419 [13]. The mycelium was collected by filtration and homogenised in a Sagers press [10] and the homogenate was resuspended in 0.1 M tricine buffer at pH 8 containing 0.3 M sucrose and 10 mM mercaptoethanol (80 ml buffer for 30 g wet mycelium). The resuspension was centrifuged at 15 000 g for 20 min at 0° and the supernatant was re-centrifuged at 120 000 g for 2 hr. The resulting supernatant was used for the experiments described later. (b) *Marah macrocarpus.* Cell-free extracts were prepared from the endosperm of immature seed as previously described [11]. The 120 000 *g* supernatant fraction was used in the experiments described later.

Preparation of ent-*kaur*-16-*ene*-[^{14}*C*]. 3(*RS*)-MVA-[2-^{14}C] (5 µCi/µmol; 5 µmol) was incubated at 30° with the cell-free system from *G. fujikuroi* (25 ml) containing ATP (5 mM), $MgCl_2$ (5 mM), $MnCl_2$ (1 mM) and K-Pi buffer at pH 7.5 (83 mM) in a total vol. of 30 ml. After 2 hr, Me_2CO (25 ml) was added and the mixture was extracted with EtOAc (3 × 25 ml). The dried extract was subjected to TLC on Si gel impregnated with 5% $AgNO_3$ in C_6H_6–*iso*-Pr_2O–EtOAc (2:1:1). The radioactive bands were detected by radio-scanning and the *ent*-kaur-16-ene-[^{14}C] at R_f *ca* 0.7 was eluted and further purified by rechromatography on $AgNO_3$-Si gel in C_6H_6. The yield of *ent*-kaur-16-ene-[^{14}C] (20 µCi/µmol) was 1.54 × 10^6 dpm or 34.4 nmol.

Preparation of geranylgeranyl-[^{14}*C*] *pyrophosphate and copalyl-*[1-$^3H_2$] *pyrophosphate.* Gernaylgeranyl-[^{14}C] pyrophosphate (20 µCi/µmol) was prepared biochemically from 3(*RS*)-MVA-[2-^{14}C] (5 µCi/µmol) using the *M. macrocarpus* cell-free system as described by Oster and West [16]. Copalol-[1-$^3H_2$] obtained from Professor C. A. West, was pyrophosphorylated according to the method of Cramer and Bohm [17].

ent-*Kaur*-16-*ene*-[^{14}*C*] *incubation.* Mycelium from a culture grown in 50 ml ICI 40%-N medium was resuspended in 50 ml ICI 0%-N medium in a 500 ml conical flask. Mycelium from a similar culture but grown in the presence of compound **1** (5.0 mg) was similarly resuspended in medium containing compound **1** (5.0 mg). The control and treated resuspension cultures were each treated with *ent*-kaur-16-ene-[^{14}C] (5.31 × 10^6 dpm) in Me_2CO (20 µl) and incubated at 25° for 5 days on a reciprocal shaker. The culture filtrates were extracted as described for the MVL-[2-^{14}C] incubation. To the total crude extract from each culture, unlabelled GA_3 (20 mg) was added, followed by EtOAc (10 ml) and the soln was extracted with 0.5 M K_2HPO_4 (3 × 10 ml). The latter extract was acidified to pH 2.5 with M HCl then extracted with EtOAc (3 × 30 ml). Recovery from the EtOAc gave 53.2 mg from the control and 32.5 mg from the treated cultures. These residues were fractionated by PLC on SiO_2 gel G, developed with $CHCl_3$–EtOAc–HOAc (5:15:1). The major radioactive zone at R_f *ca* 0.4 was eluted with H_2O-satd EtOAc to give 38.0 mg (control) and 24.4 mg (treated) from which GA_3 was recrystallized (× 4) using Me_2CO–petrol. After the first crystallization the sp. act. was constant within experimental error. The mean dpm/mg (± 3%) from the last 3 crystallizations was 5825 (control) and 6769 (treated) corresponding to 22.7 and 26.4% incorporation of *ent*-kaur-16-ene-[^{14}C] into GA_3-[^{14}C].

Incubation of geranylgeranyl pyrophosphate and copalyl pyrophosphate with cell-free systems from G. fujikuroi *and* M. macrocarpus *in the absence and presence of growth retardant* **1**. (a) *G. fujikuroi* system. Geranylgeranyl-[^{14}C] pyrophosphate (0.5 nmol; 10 nCi) or copalyl-[1-$^3H_2$] pyrophosphate (0.5 nmol; 16.5 nCi) were incubated at 30° with the fungal cell-free system (0.75 ml) containing $MgCl_2$ (5 mM) and K-Pi buffer at pH 7.6 (50 mM) in a total vol. of 1 ml. Three incubations for each substrate were made; one control with no added growth retardant, one containing AMO 1618 (10 µM) and the third containing compound **1** (10 µM). The incubations were stopped after 2 hr by the addition of Me_2CO (1 ml) containing unlabelled *ent*-kaur-16-ene (10 µg). After extraction with EtOAc (3 × 1 ml), the extracts were applied as spots to an Si gel plate and developed in petrol. The *ent*-kaur-16-ene was visualized with I_2 vapour and the Si gel in this region was removed and counted by liquid scintillation. (b) *M. macrocarpus.* The above procedure was repeated using the *M. macrocarpus* cell-free system (0.75 ml) containing $MgCl_2$ (5 mM) and K-Pi buffer at pH 7 (10 mM).

Mycelial dry weight. Using 10 ml resuspension cultures the following mycelium dry weights were obtained after 3 days incubation: control culture (148 mg); cultures treated with 1.0 mg compound **1** from the beginning (152 mg); and cultures treated with 1.0 mg compound **1** after resuspension (133 mg). Using 100 ml resuspension cultures grown for 7 days after

resuspension, the following results were obtained: control, wet wt 3.8 g (dry wt 1.0 g); treated, (10 mg inhibitor), wet wt 3.5 g (dry wt 0.9 g).

Acknowledgements—We thank the ARC, NATO and NSF for financial support. We also express our appreciation of generous gifts of compounds from the following: Professor S. Tamura (University of Tokyo) for the plant growth retardant (**1**); Professor R. C. Cambie (University of Auckland) for kaur-16-ene and phyllocladene; and Professor C. A. West (U.C.L.A.) for copalol-[1-$^3H_2$]. We also thank Mr. P. Gaskin for GC–MS determinations.

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