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REGULATION OF ISOPENTENOID BIOSYNTHESIS BY PLANT GROWTH RETARDANTS IN *NICOTIANA TABACUM*

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Key Word Index—*Nicotiana tabacum*; Solanaceae; isopentenoid biosynthesis; growth retardants; sterols; gibberellins; 2,3-oxidosqualene.

Abstract—The effects of AMO-1618, AY-9944 and SKF-7997 on the growth of *Nicotiana tabacum* and on the biosynthesis of acidic and neutral isopentenoids in the seedlings were examined. All three compounds significantly retarded stem elongation but not dry wt of the plants. Incorporation of radioactivity from [2-^{14}C]-mevalonate into acidic and neutral isopentenoids showed a direct relationship between the radioactivity in the two fractions and stem growth. All three compounds inhibited steps before as well as after squalene formation, but the acylic and polycyclic isopentenoids which accumulated as a result of the inhibition were not necessarily the same in each of the treatment groups. This indicates that stem growth is probably influenced not by a single product of isopentenoid biosynthesis, but rather by several products, which may even act independently in their effects on developmental processes.

INTRODUCTION

The utility of quaternary ammonium carbamates (e.g. AMO-1618) and synthetic amines (e.g. SKF-7997 and AY-9944) in studies of sterol biosynthesis has been demonstrated in plant[1–10] as well as animal [11–15] systems. The effects of these and related synthetic compounds (e.g. pyrimidine analogs) on the biosynthesis of sesquiterpenes and diterpenes in tracheophytes and fungi has also been examined [16–28]. There is general agreement that application of these compounds to seedlings inhibits stem growth without necessarily affecting other developmental processes [4, 10, 27, 29], but their mode of action on isopentenoid metabolism is not clear. Thus some authors believe that AMO-1618 specifically inhibits the cyclization of geranylgeranyl pyrophosphate to copalyl pyrophosphate [24, 26, 29] in plants, and there is good evidence for this from *in vitro* [18–20, 25, 26, 28] and *in vivo* [18, 24] experiments. However, there is also good evidence that AMO-1618 inhibits sterol synthesis *in vitro* [6, 7] and *in vitro* [4, 10]. Thus, AMO-1618 and analogous growth retardants may not act by specifically inhibiting a single step in the isopentenoid pathway, as previously suggested [29].

Independently, our two groups have used synthetic bioregulators to control flowering [1, 17] and stem growth [4, 10] of tracheophytes, and also to examine their effects on sterol biosynthesis [1, 4, 6, 7, 10]. The purpose of the present study was to relate the growth effects of a typical plant bioregulator (AMO-1618), which is also known to inhibit sterol synthesis in animals [14], and of two common mammalian hypocholesterolemic agents (SKF-7997 and AY-9944) to the biosynthesis of various isopentenoids in tobacco seedlings.

RESULTS

Changes in weight and height of 21-day-old tobacco seedlings were determined on the third day after application of AMO-1618, SKF-7997 and AY-9944 to the stem apices and compared with control plants. Although there were no significant differences between the mean (10 replications) dry weights of plants that had undergone these treatments (control, 42.1 ± 6.1 mg; AMO-1618, 35 ± 3.7 mg; SKF-7997, 34.0 ± 3.2 mg; AY-9944, 39.4 ± 3.7 mg), significant differences were observed between the mean heights of the control group and of the plant groups three days after treatment. All of the treatments significantly retarded stem growth. Thus, relative to the mean height of the control plants, AMO-1618 induced 61.4%; SKF-7997, 53.8% and AY-9944, 29.0% inhibition of stem growth.

The neutral lipid fraction obtained from cellulase hydrolysis of lyophilized plant material was first separated into four major classes of isopentenoids by Al_2O_3 CC: I, hydrocarbons; II, steryl esters and 2,3-oxidosqualene; III, 4-mono- and 4,4-dimethyl sterols; IV, desmethyl sterols. The hydrocarbon fraction (I) of the control plants, when further fractionated by TLC, gave essentially three radioactive zones: squalene, kaurene and unidentified material more polar than kaurene (Table 1). Whereas all the radioactivity in Fraction I from the control plants is accounted for by these three fractions, significant radioactivity in Fraction I from the treated plants was

Table 1. Distribution of the radioactivity (dpm) in the hydrocarbon fraction after TLC separation*

Treatment	Total activity of the fraction	Recovered activity		
		Squalene	Kaurene	Unknown
Control	1380	320	100	1011
AMO-1618	820	70	0	—
SKF-7997	4290	130	60	2198
AY-9944	6970	110	48	240

*TLC system: Si gel G–*n*-hexane. R_f values: squalene, 0.74; kaurene, 0.64; unknown (0.25).

observed at an R_f less than that for squalene (0.74), e.g. at R_f 0.36 in SKF-7997-treated plants.

Fraction II of the control plants showed only one radioactive zone, corresponding to steryl esters and 2,3-oxidosqualene which are not separated on a thin-layer chromatogram, developed with C_6H_6–Et_2O (9:1). The radioactive zone was eluted from the plate and hydrolysed with 20% ethanolic KOH. Following hydrolysis, the radioscan showed a single peak corresponding in its position to free sterols. No radioactivity was found in the area of the chromatogram where unhydrolysed steryl esters and 2,3-oxidosqualene would be located. Fraction II from the plants treated with AMO-1618 showed a different picture. The unhydrolysed material again gave a single peak due to the mixture of steryl esters and 2,3-oxidosqualene, but after hydrolysis, two peaks were observed, one corresponding to free sterols (R_f 0.28) and the other one (R_f 0.96) to 2,3-oxidosqualene. Although neither of the two zones represents pure compounds, the presence of 2,3-oxidosqualene was demonstrated in the less polar zone by GLC and MS and the presence of sterols in the more polar zone by GLC. Both zones, when eluted from the plate and examined by GLC, showed peaks with retention times identical to the respective standards. The mass spectrum of the less polar material showed the presence of peaks corresponding to 2,3-oxidosqualene (*m/z* 426 [M^+], 3%), as well as peaks due to the loss of 1–3 isoprene units (*m/z* 357, 289 and 221), and to numerous long-chain fatty alcohols (M^+ at *m/z* 560 with additional peaks representing the C_nH_{2n+1} and C_nH_{2n} ion series). The absence of peaks above 560 m.u. demonstrates the absence of fatty acid esters of sterols and indicates complete hydrolysis.

Table 2 summarizes the distribution of radioactivity among the neutral lipid fractions of each treatment group. When the radioactivity in the neutral lipid fractions is summed up, it is found that the rate of incorporation for the various treatment groups roughly parallels their growth rate, or that the per cent inhibition of incorporation (last column of Table 2) approximately parallels the growth inhibition.

The composition of the sterol fraction in the control plants did not differ significantly from that in plants treated with either AY-9944 or SKF-7997 (Table 3). The amount of material from the AMO-1618 treatment and in the ester fraction from the SKF-7997 treatment was insufficient for analysis.

Table 2. Incorporation of radioactivity (dpm) from [2-^{14}C]mevalonic acid into neutral lipid fractions by tobacco seedlings subjected to various treatments

Treatment	Squalene*	2,3-Oxidosqualene†	Esterified sterols‡	4-Mono- and 4,4-dimethyl sterols§	4-Desmethyl sterols§	Total	% Inhibition‖
Control	320	463	47764	22900	106920	178367	0
AMO-1618	70	1333	8119	16240	9080	34842	80.5
SKF-7997	130	633	8502	18480	70220	97965	55.1
AY-9944	110	305	13308	33800	63990	111593	37.5

*Purified by TLC, following Al_2O_3 chromatography.
†Purified by TLC, following Al_2O_3 chromatography and saponification.
‡Liberated from steryl esters by saponification.
§Isolated by Al_2O_3 chromatography.
‖Total incorporation as % control group.

Table 3. Comparison of the relative amount of sterols, in % total sterols, in control and treated plants

Sterols	Control		AY-9944		SKF-7997
	Free	Esterified	Free	Esterified	Free
Sitosterol	47	32	54	39	47
Stigmasterol	26	17	25	27	25
Campesterol	11	17	11	18	10
Cholesterol	16	34	9	15	13
Unknown	—	—	1	1	—
24-Alkylsterol to Cholesterol ratio	5.25:1	1.94:1	10.00:1	5.60:1	6.31:1

However, differences were observed between the 24-alkyl sterol to cholesterol ratio between the control and treatment groups that were analysed. Thus, under the influence of AY-9944 and SKF-7997, less cholesterol was synthesized relative to the alkylated sterols when the sterol profiles were examined by GLC. It was not possible to ascertain the effect of AMO-1618 on this ratio. Examination of the effect of the three bioregulators on the synthesis of steroid intermediates showed that SKF-7997 and AMO-1618 altered the synthesis of 4-monomethyl and 4,4-dimethyl sterols profoundly, while AY-9944 apparently did not (Table 4).

The total radioactivity of the acid and neutral lipid fractions is compared for all experimental groups in Table 5. The greatest inhibition in the incorporation occurred in both acidic and neutral fractions in plants treated with SKF-7997. In every treatment group the decrease in radioactivity incorporated into acidic lipids was paralleled by a decrease in the neutral lipids. Incorporation rates were always greater in the acidic lipid fraction than in the neutral lipids. However, some of the radioactivity of the acidic lipid fraction may be due to neutral lipids that remained in the aqueous phase after extraction with hexane.

When the acidic lipid fraction was derivatized and chromatographed by HPLC [30], radioactivity was found in many fractions. Large amounts of irrelevant UV-absorbing material in the plant extracts made the UV detector useless.

Table 6 shows the distribution of radioactivity among the eluate fractions. Only one radioactive peak corresponded to gibberellins available to us as reference standards. The material in this peak was further analysed by TLC. Each of the four groups yielded the same radioactive compound, having an R_f of 0.23 on Si gel G, developed with CCl_4–Me_2CO (3:1), and having an R_f of 0.25 on a Si gel G plate impregnated with 10% silver nitrate and developed with the same solvent system [31]. None of the gibberellins available to us as reference material behaved like this in either TLC system. In HPLC, the unidentified material behaved like GA_{20}, but GA_{20} is much less polar (R_f 0.51 on $AgNO_3$-impregnated Si gel).

Table 5. Total radioactivity ($dpm \times 10^{-4}$) of the lipid fractions in control and treated plants

Treatment	Lipid fraction		Total	% Inhibition
	Acidic	Neutral		
Control	63.0	31.3	94.3	—
AMO-1618	29.0	16.7	45.9	51.3
SKF-7997	17.3	9.5	26.8	71.6
AY-9944	52.8	20.4	73.2	22,4

DISCUSSION

This investigation represents the first unequivocal demonstration of the synthesis of 2,3-oxidosqualene in tracheophytes. Moreover, the present study confirms and extends our earlier observations of the inhibition of sterol synthesis in plants by AMO-1618, SKF-7997 and AY-9944 [1, 4, 6, 7, 10]. Obviously, the inhibitory action on sterol production is not confined to animals, but what is different is the nature of the polycyclic and acyclic isopentenoids which accumulate as the result of the inhibition in plants and animals. For instance, in mammals $\Delta^{5,7}$-sterols accumulate in the presence of AY-9944 [11–13, 15], while in algae and tracheophyte suspension cultures Δ^{8}-sterols accumulate [3, 8]. We did not find any significant changes in the desmethyl sterol com-

Table 4. Comparison of relative amounts of free 4-mono- and 4,4-dimethyl sterols in control and treated plants

Steroid intermediate	Treatment			
	Control	SKF-7997	AY-9944	AMO-1618
Cycloartenol	16	28	14	6
24-Methylenecycloartenol	24	—	32	29
24-Methylenelophenol	60	—	54	—
Unknown		72	—	65

Table 6. Radioactivity (dpm × 10^{-3}) in acidic lipids fractionated by HPLC

Elution vol. (ml)	Control	AMO-1618	SKF-7997	AY-9944	Reference gibberellins
0–102	tr*	tr	tr	tr	
102–126	23.4	13.2	20.8	31.6	
126–150	15.3	10.0	1.4	14.3	
150–180	91.9	18.9	58.5	52.7	
180–210	12.5	7.0	4.7	28.5	
210–492	tr	tr	tr	tr	
492–552	tr	6.7	tr	tr	
552–762	tr	tr	tr	tr	
762–792	tr	3.1	tr	14.7	
792–897	tr	tr	tr	tr	
897–972	6.3	11.0	3.8	25.0	20
972–1407	tr	tr	tr	tr	5,7,4,13,14
1407–1467	tr	3.9	tr	1.9	
1467–1800	tr	tr	tr	tr	1,3
Total	149.4	73.8	89.2	168.7	

*tr, traces.

position (Table 3), but Δ^8-sterols may have accumulated in the 4-mono- and 4,4-dimethyl fraction, which we have labelled 'unknown' in Table 4. The changes in the 24-alkyl sterol to cholesterol ratio (Table 3) suggest that AY-9944 and SKF-7997 may regulate the C-24 reductase and C-24 transmethylase.

Earlier investigators [24, 27, 29] have assumed that sterol synthesis is unaffected by the presence of plant growth retardants in concentrations that inhibit gibberellin production, but the present study does not support this view. Rather, the data presented in Tables 1, 2 and 6 show that several products of the isopentenoid pathway before and after squalene formation are affected by applying AMO-1618, SKF-7997 and AY-9944 to tobacco seedlings. For instance, while AY-9944 administration had a small effect on the total radioactivity incorporated into squalene metabolites (Table 2), a significant change in the radioactivity of the hydrocarbon fraction relative to the control was apparent (Table 1). Our results with the AMO-1618 treatment contradict findings that it inhibits kaurene synthesis without affecting squalene [26] or sterol [24] synthesis.

If it were true that the mode of action of these bioregulators is to block a specific step in the isopentenoid pathway (e.g. from geranylgeranyl pyrophosphate to kaurene) and the blocked formation of some product (e.g. gibberellins) is responsible for growth retardation, we would expect only a single branch of the pathway to be affected. However, when the various branches of the isopentenoid pathway were examined, this was not found to be true. Because earlier studies have failed to examine more than one or two sequences in the isopentenoid pathway, e.g. farnesyl pyrophosphate → petasin [23], farnesylpyrophosphate → geranylgeranyl pyrophosphate → kaurene → gibberellins [18–22, 25, 26, 28], farnesyl pyrophosphate → squalene → 2,3-oxidosqualene [2, 7] → sterols [1–15, 24, 27] or farnesyl pyrophosphate → squalene → pentacyclic triterpenoids [7], their results and conclusions concerning the mechanisms of action of plant growth retardants are not comparable with ours nor necessarily with those of each other.

A possible explanation for the mode of action of the bioregulators used in our experiments is that they disrupt the functional integrity of the endoplasmic reticulum or that they are differentially bound to isopentenoid carrier proteins. Either possibility conceivably would have concomitant effects on the formation of several isopentenoids. Since both sterols and gibberellins can independently reverse the retardant action on stem growth [4, 10], we suggest that several end-products of the isopentenoid pathway may act independently on developmental processes, but produce the same end response.

EXPERIMENTAL

Plants. Nicotiana tabacum (cv. Turkish Samson) seeds were germinated and grown as described earlier [4]. Groups of ten 21-day-old seedlings were measured (from base of cotyledons to tip of apex) and then treated with 300 μg of AMO-1618, SKF-7997 or AY-9944 per plant, supplied as single drops (10 μl of a 30 μg/ml soln in 0.05% Tween 20) to the stem apices. Control seedlings were supplied with only Tween 20. When the applied soln had been absorbed (6–8 hr), [2-^{14}C]mevalonic acid (0.5 μCi/plant) was applied (10 μl of a 50 μCi/ml soln in 0.05% Tween 20) to each stem apex. Then, 24 and 72 hr after [2-^{14}C] MVA application, stem heights were again recorded and, after the second measurement, the above-ground parts of the plant were cut off, weighed, placed immediately into liquid N_2, lyophilized, and reweighed.

Extraction. To weighed samples of the lyophilized seedlings, 5 ml 0.2 M acetate buffer, pH 4.6, 10 mg cellulase (Calbiochem, San Diego, CA) per 100 mg plant tissue was added. The stoppered vessels were kept at 30° for 10 days, and were shaken once a day for 10 min. Following the incubation, the tissue suspension was diluted with 50 ml 0.5 M Pi buffer, pH 9.0, and extracted with three portions of

50 ml hexane. The pooled hexane extract was dried over Na_2SO_4 and then concentrated under red. pres. This represents the glycoside-free neutral lipid fraction.

The aq. layer was acidified to pH 2.5 by addition of 3 N HCl and then extracted with three portions of 50 ml EtOAc. The EtOAc extracts were pooled, dried over Na_2SO_4 and concentrated under red. pres. to yield the acidic lipid fraction.

Chromatography. The neutral lipid fraction was first fractionated by adsorption chromatography on a 105 × 10 mm column of 10 g neutral alumina, activity grade II (Woelm, Eschwege, W. Germany) with 2-ml portions of eluents as follows: Fractions 1–5, hexane; 6–10, 10% Et_2O in hexane; 11–15, 20%; 16–20, 30%; 21–25, 40% Et_2O in hexane; 26–30, Et_2O; 31–33, MeOH.

Fractions 1–5, containing the hydrocarbons, were pooled and further fractionated by TLC. An aliquot, along with suitable reference standards, was applied to a 5 × 20-cm Si gel G plate (250 μm layer, Analtech, Newark, DE). The chromatogram was developed with hexane and scanned in a radiochromatogram scanner. Zones were located with respect to standard compounds by spraying the chromatogram with 50% aq. H_2SO_4 and heating it.

Steryl esters and 2,3-oxidosqualene, which are eluted in Fractions 6–10, are not separable by this method. Therefore, these fractions were pooled and evaporated. The residue was saponified by refluxing it in 20% KOH in EtOH for 20 min. The hydrolysate was diluted to 50 ml with H_2O and extracted with three portions of 50 ml hexane. The pooled hexane extract was dried and evaporated as before, and an aliquot was subjected to TLC fractionation as described above, except the mobile phase was C_6H_6–Et_2O (9:1). In this solvent system 2,3-oxidosqualene and steryl esters have an R_f of about 0.95; 4,4-dimethyl sterols, R_f 0.36; 4-monomethyl sterols, R_f 0.30; and 4-desmethyl sterols, R_f 0.26. Radioactive zones were eluted from the plates with Et_2O, and their radioactivity was determined by evaporating the solvent in liquid scintillation vials, dissolving the residue in 5 ml PPO–POPOP cocktail, and counting.

Fractions 16–20, containing the sterols, were further fractionated by HPLC with a modification of the method of Hunter *et al.* [32]. A 300 × 4.6 mm i.d. column, packed with Zorbax ODS, was eluted with 0.5% 2-propanol in hexane at a rate of 0.5 ml/min. The sterols, which were detected by their absorption at 205 nm, were eluted between 45 and 65 min after injection. The sterol-containing fractions were pooled, and aliquots were analysed qualitatively as well as quantitatively by GLC using a 6 ft × 10-mm i.d. glass column, packed with either 2% OV-17 or SE-30 on Gas-Chrom Q, operated at 280° or 235°, respectively. The carrier gas was He at a flow-rate of 30 ml/min.

The acidic lipid fraction was esterified to obtain the *p*-nitrobenzyl esters of carboxylic acids. Aliquots of the esterified lipids were combined with 10-μg samples of similarly prepared *p*-nitrobenzyl esters of gibberellins and fractionated by HPLC [30]. The radioactive fractions from these chromatograms were further examined by TLC [31]. Reference standards for this included the *p*-nitrobenzyl esters of gibberellins and of oleanolic acid. After development, the chromatograms were scanned, as detailed above.

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